Identification of taxa-specific responses to bioremediation treatments in hydrocarbon-contaminated Arctic soils

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August 2012

A thesis submitted to McGill University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Abstract

A warming climate and improved technology have allowed northern countries to more thoroughly explore and exploit Arctic resources. This increased activity has led to an elevated risk of petroleum contamination, and consequently, there is a need to develop strategies to effectively and efficiently degrade these contaminants on site. While many Arctic soil microorganisms are known to naturally metabolize petroleum hydrocarbons in contaminated sites, a process known as bioremediation, treatments directed at stimulating the hydrocarbon-degrading activity of these microbes (e.g. nutrient amendments) have varied in effectiveness.

The objective of this study was to determine whether microbial taxa respond equally to disturbances of the soil environment by hydrocarbon contaminants and nutrient amendments, and whether the most efficient hydrocarbon degraders are naturally stimulated. To determine whether the bacteria inhabiting contaminated Arctic soils assimilate added nitrogen equally, a novel 15N-stable isotope probing approach was developed. After a month of *in situ* incubation, it was determined that many hydrocarbon-degrading bacteria had incorporated the added nitrogen, but to varying extents. The *Alphaproteobacteria* most effectively used the added nitrogen, as determined by both 16S rRNA and *alkB* gene enrichment, and this was noteworthy given that they were not expected to be the most effective hydrocarbon-degrading group.

To assess whether the relative abundance of bacterial taxa in hydrocarbon-contaminated soils was determined by soil characteristics as opposed to hydrocarbon-degrading ability, 18 soils from across the Arctic were collected and treated with diesel and monoammonium phosphate. Bacterial diversity and community composition were

determined through 16S rRNA gene sequencing on the Ion Torrent platform, while hydrocarbon degradation was measured using gas chromatography. It was found that *Actinobacteria* dominated soils with low organic matter, while *Proteobacteria* dominated those with high organic matter. In addition, the extent of bacterial diversity and the relative abundance of specific assemblages of *Betaproteobacteria* in uncontaminated soils were predictive of hydrocarbon degradation with and without nutrient amendments, respectively. Relative abundance of *Betaproteobacteria* was associated with efficient hydrocarbon degradation in the presence of added nutrients, suggesting that this may be an important group to target.

Finally, to determine whether modifying the microbial community within a given soil would impact rates of hydrocarbon degradation, gentamicin and vancomycin were used to inhibit specific portions of the bacterial community. Bacterial 16S rRNA gene diversity and community composition were again determined using the Ion Torrent platform, qPCR was used to quantify bacterial and fungal populations within each treatment, and GC analysis was used to determine hydrocarbon degradation. Bacterial 16S rRNA gene abundance declined in soils treated with gentamicin, but diesel degradation was highest in the presence of both gentamicin and vancomycin. Bacterial community composition shifted under all treatments, and *Xanthomonadaceae* (*Gammaproteobacteria*) and *Micrococcaceae* (*Actinobacteria*) dominated soils treated with both antibiotics. Diesel degradation was much less effective when nutrients were also added to soils treated with gentamicin and vancomycin, possibly due to competition from a larger fungal population.

Overall, these results suggest that more effective *in situ* treatments of hydrocarbon-contaminated Arctic soils are possible through selective targeting of efficient hydrocarbon-degrading consortia. Future research should aim to understand which soil microorganisms most quickly degrade various contaminants *in situ*, as well as the main biotic and abiotic factors that limit their activity.

Résumé

Le réchauffement climatique et l'amélioration de la technologie ont permis aux pays situés au nord du globe d'exploiter les ressources de l'Arctique comme jamais auparavant. L'accroissement de l'activité humaine augmente le risque de contamination par des produits pétroliers, d'où la nécessité de développer des stratégies afin d'être en mesure de dégrader ces contaminants de façon rapide et efficace. Bien que plusieurs des microorganismes qui peuplent le sol de l'Arctique possèdent la capacité de métaboliser des hydrocarbures, les traitements utilisés afin de stimuler l'activité de ces bactéries (ex. ajouts de nutriments) n'ont pas tous été aussi efficaces que souhaité.

L'objectif de cette étude était de déterminer si les microorganismes réagissent de la même façon aux perturbations causées par les hydrocarbures et les ajouts de nutriments, et si les espèces efficaces dans la dégradation des hydrocarbures sont naturellement stimulées. Afin de déterminer si le taux d'assimilation de l'azote est le même pour toutes les bactéries qui vivent dans les sols contaminés de l'Arctique, une nouvelle technique de sondage à l'aide de l'isotope stable 15N a été développée. Après un mois d'incubation, nous avons déterminé que plusieurs groupes de bactéries ont incorporé l'azote, mais à des degrés divers. Les *Alphaproteobacteria* ont été les plus efficaces dans l'utilisation de l'azote, tel que démontré par l'enrichissement des gènes de l'ARNr 16S et *alkB*, ce qui constitue un fait intéressant étant donné qu'elles n'étaient pas considérées comme le groupe de bactéries le plus efficace dans la dégradation des hydrocarbures.

Afin d'évaluer si l'abondance des espèces bactériennes est influencée plutôt par les caractéristiques du sol que par leurs capacités de dégradation, 18 sols prélevés dans différentes régions de l'Arctique ont été traités avec du diésel et des nutriments. La

diversité et la composition des communautés microbiennes ont été déterminées par séquençage sur la plateforme Ion Torrent, alors que la chromatographie en phase gazeuse a permis de mesurer la dégradation des hydrocarbures. Le groupe des *Actinobacteria* prédominait dans les sols à faible teneur en substances organiques (<10%), tandis que le groupe des *Proteobacteria* prédominait dans les sols à haute teneur en substances organiques. De plus, la diversité bactérienne et l'abondance relative de certains groupes de *Betaproteobacteria* constituent un facteur prédictif de la dégradation efficace des hydrocarbures. L'abondance relative de *Betaproteobacteria* est associée à une dégradation efficace d'hydrocarbures en présence de nutriments, ce qui suggère qu'il s'agirait d'un bon groupe à cibler durant la biorémédiation.

Enfin, dans le but de déterminer si une modification de la communauté microbienne influence la dégradation, des fractions de la communauté microbienne ont été inhibées à l'aide de la gentamicine et de la vancomycine. La dégradation du diésel donnait les meilleurs résultats en présence à la fois de la gentamicine et de la vancomycine. Tous les traitements utilisés ont provoqué des changements dans la composition de la communauté microbienne des sols et les groupes des *Xanthomonadaceae* (*Gammaproteobacteria*) et des *Micrococcaceae* (*Actinobacteria*) prédominaient dans les sols traités avec les deux antibiotiques. La dégradation du diésel était moins efficace lorsque des nutriments étaient également ajoutés en même temps que la gentamicine et la vancomycine, possiblement à cause de la compétition d'une population fongique plus importante.

Ces résultats suggèrent qu'il est possible d'améliorer l'efficacité des traitements pour les sols arctiques contaminés par des hydrocarbures. Dans le futur, les recherches

devraient se concentrer sur l'identification des microorganismes du sol qui dégradent le plus rapidement les divers contaminants associés à l'exploitation des hydrocarbures, ainsi que sur la compréhension des facteurs qui peuvent limiter leur activité.

Contributions to knowledge

1. A new stable isotope probing method was developed that allows the identification of substrate-incorporating microorganisms through large-scale sequencing. This technique may aid in the conception of high-throughput technologies for assessing microbial function *in situ*.

2. For the first time, the specific bacteria involved in nitrogen incorporation in a contaminated Arctic soil were identified directly. This was also the first time that a ^{15}N-SIP experiment has been successfully conducted in a soil environment using a non-gas substrate.

3. This was the first study to use deep 16S rRNA gene sequencing to examine the influence of hydrocarbons and nutrient amendments across a range of Arctic soils, without the confounding influence of varied contaminant age, concentration, and composition.

4. *Actinobacteria* and *Proteobacteria* were found to dominate low and high organic matter soils respectively, especially in the presence of diesel and nutrients, a relationship that shows that environmental factors, as opposed to the hydrocarbon-degrading abilities of various taxa, initially determine the dominant microorganisms in hydrocarbon-contaminated Arctic soils.

5. The relative abundance of specific assemblages of the main *Betaproteobacteria* families in uncontaminated soils was predictive of unstimulated diesel degradation, while bacterial diversity in uncontaminated soils was predictive of total diesel degradation with monoammonium phosphate added. Such measures can be used to determine the hydrocarbon-degrading capacity of Arctic soils *a priori*, and may assist in determining whether biostimulation is a viable option for treating specific diesel-contaminated soils.

6. The relative abundance of *Betaproteobacteria* in diesel-contaminated soils treated with monoammonium phosphate was positively correlated with hydrocarbon degradation, indicating that this may be an effective group to stimulate.

7. The addition of antibiotics to contaminated soils can actually increase rates of hydrocarbon degradation. While the widespread application of antibiotics in Arctic soils is neither recommended nor practical, this demonstrates in principle that degradation can be enhanced through modification of the microbial community, possibly by reducing competitive interspecies relationships.

Acknowledgements

Thank you first to my supervisor, Dr. Charles Greer. The freedom that you gave me was frightening at first, but made me independent in ways that I hadn't been before. Thank you for supporting me, giving me the chance to fulfill a dream by traveling to the Arctic, and helping to shape even my strange ideas. Your positive and approachable nature is a huge bonus, and your door was always open when I needed input. Thanks also to my co-supervisor Dr. Lyle Whyte for valuable input, and for providing finances that allowed me to attend a conference outside of Montreal.

Thanks to Dr. Étienne Yergeau for lessons in statistics, bioinformatics, and creating scientific papers. Thank you also for your help with networking, building my CV, and helping me to find a way to continue my career in Montreal. It was a great privilege to work with one of the rising stars in microbial ecology.

Thank you to the staff and visitors of CFS-Alert, and to Drew and Andrew from CFS-Trenton. Not only did my trips to the Arctic change my perspective on my project and on Arctic biology, but they were some of the most entertaining weeks of my life. Thank you to the alpha wolf of 2010 for not stealing my coat or my life.

The weight of directing such a long and independent project was lightened by the technical expertise offered by the other members of the Greer lab. I received exceptional knowledge and assistance in both the lab and field from Danielle Beaumier, Julie Champagne, Martin Désilets, Karine Drouin, Miria Elias, Nathalie Fortin, Jennifer Holdner, Dr. David Juck, Dr. Louis Jugnia, Diane Labbé, Suzanne Labelle, Christine Martineau, Claude Masson, Christine Maynard, Danielle Ouellette, Sylvie Sanschagrin, Josée Sirois, and Olivia Wojciechowicz. I also received technical assistance from other

BRI employees, including Chantale Beaulieu, Stéphane Deschamps, Fréderique Lebrun, and Punita Mehta.

Thank you to all of my lab friends. Because of you, I was eventually OK with the fact that I worked in a gray-tone industrial park across from Dollarama headquarters. It is quite possibly the ugliest square kilometre in Montreal. Thank you to Paola, Valentina, Lia, Katrina, Joanne, Christine Martineau, Christine Maynard, Julie, Miria, Geneviève, Olivia, William, Marc, Dave, and Étienne. Thibault and Leandro, I really wish that you had stayed longer. Al, it was great talking hockey and belittling the Leafs together.

I would not have made it without the encouragement and perspective that I received from my family, my friends, and my M.Sc. supervisor Dr. Hugh Henry. A huge thanks to Arun Kumar, Russell Cattus, and Bili, the NRC's bilingual raccoon, as life is nothing without humour.

More than anything, I thank my wife Emily for taking a chance on me. Without you, all the friends and encouragement in the world could not have kept me going.

I would also like to thank the following agencies for their financial and technical support: Association of Polar Early Career Scientists (APECS), Natural Sciences and Engineering Research Council of Canada (NSERC), the NRCan PERD Program, the International Arctic Science Committee (IASC), Centre d'Études Nordiques (CEN), the Northern Scientific Training Program (NSTP) and McGill University.

This work is dedicated to my wife, Emily Bell, for going out of her mind and making the decision to follow me to Montreal two months after we first met.

I couldn't possibly be here otherwise.

Contribution of authors

The candidate wrote all content for Chapters 1, 2, 3, and 7, with helpful suggestions provided by Dr. Charles W. Greer and Dr. Lyle G. Whyte. For chapters 4,5, and 6, the candidate designed and conducted experiments, analyzed the data, and wrote the manuscripts, with helpful comments provided by Dr. David Juck, Dr. Charles W. Greer and Dr. Lyle G. Whyte. Dr. David Juck also assisted with the fieldwork for all studies. Dr. Christine Martineau assisted with the experimental design of the study in Chapter 4, Christine Maynard conducted the majority of sequencing for the study in Chapter 5, while Dr. Etienne Yergeau was of invaluable help in the design, direction, and analysis of the studies in Chapters 4-6, and also provided helpful suggestions during the writing of these manuscripts.

Table of contents

Chapter 7: General discussion and conclusions

List of tables

List of figures

Key abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid
alkB	alkane monooxygenase B
ANOVA	analysis of variance
BLAST	basic local alignment search tool
C	carbon
^{13}C	heavy stable isotope of carbon
CFS-Alert	Canadian Forces Station Alert, Nunavut
CsCl	cesium chloride
DNA	deoxyribonucleic acid
EPS	exopolysaccharide
GC	gas chromatography
HGT	horizontal gene transfer
JP-5	jet propellant 5
MAP	monoammonium phosphate
MID	multiplex identifier
N	nitrogen
^{15}N	heavy stable isotope of nitrogen
NH_4^+	ammonium
NO_3^-	nitrate
NMDS	nonmetric multidimensional scaling
NRC	National Research Council Canada
OTU	operational taxonomic unit
PAH	polyaromatic hydrocarbons
PCB	polychlorinated biphenyl
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
RDP	Ribosomal Database Project
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase polymerase chain reaction
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
SIP	stable isotope probing

1

General introduction

As human influence in the Arctic increases due to a combination of improved technology and a general desire by northern countries to exploit newly available resources, there is a heightened risk of petroleum contamination in Arctic soils. It has long been known that certain microorganisms are capable of metabolizing petroleum contaminants, as petroleum can be used as both a source of energy and carbon, and many such microbes inhabit the Arctic. This biological degradation of contaminants is known as bioremediation, and occurs naturally at many contaminated sites across the Arctic.

Air temperatures in the high Arctic may only rise above freezing for ~2 months each year, and this affects rates of bioremediation since substantial hydrocarbon degradation is only known to occur above 0°C (Walworth et al 2001). As a result, human intervention is often advised in order to maximize contaminant degradation over the short Arctic summer. This intervention generally involves stimulating the indigenous microbial community in affected soils, often via the addition of growth-limiting nutrients. Nevertheless, the success of such treatments has varied between contaminated sites (Powell et al 2006a, Delille and Coulon 2008, Yergeau et al 2009), suggesting that blanket treatments that target the microbial community as a whole may not promote optimal hydrocarbon degradation.

In addition to an ability to metabolize petroleum and tolerate its toxicity, the microorganisms that inhabit hydrocarbon-contaminated soils must be adapted to the specific soil environment in which they find themselves, and must also be able to compete for nutrients, water, and space with other contaminant-tolerant microbes. The superior competitor in a given environment is not necessarily the most effective hydrocarbon degrader, so it is important to understand how various taxa contribute to hydrocarbon degradation, as well as how individual groups and interspecific interactions are affected by current bioremediation treatments.

In this thesis, I aim to understand how microbial taxa respond to nutrient amendments in hydrocarbon-contaminated Arctic soils, and also to determine whether more efficient bioremediation might be possible through the stimulation of specific taxonomic groups.

1.1 Objectives

1. Determine which microorganisms incorporate added nitrogen in hydrocarbon-contaminated Arctic soils.
2. Identify the primary physicochemical factors affecting the formation of microbial communities in Arctic soils following hydrocarbon contamination and nutrient amendments, to determine whether disturbance or the initial soil environment is more influential in shaping community composition.
3. Link specific taxonomic groups to efficient remediation of hydrocarbon contaminants.

4. Establish whether altering relative taxonomic abundance within a given environment can influence rates of bioremediation.

1.2 Thesis outline

This thesis includes a general introduction, a literature review, four manuscripts that are either published (Chapter 4), in revisions (Chapters 5 and 6), or submitted for publication (Chapter 3), and a discussion/conclusions section. Chapter 2 provides background and justification for this project, while Chapter 3, submitted to *FEMS Microbiology Ecology*, is a more in-depth review of why the identification of efficient hydrocarbon-degrading microorganisms *in situ* is so important. Chapter 4, published in *Applied and Environmental Microbiology*, presents a novel [^{15}N]DNA-SIP technique that was used to identify nitrogen-assimilating bacteria in petroleum-contaminated Arctic soils following the addition of monoammonium phosphate, a nutrient amendment that has been used successfully in the bioremediation of hydrocarbon-contaminated Arctic soils. Chapter 5, in revisions for *The ISME Journal*, describes a study in which Arctic soils with varied physicochemical characteristics were contaminated with diesel. Shifts in the bacterial community and rates of diesel degradation were quantified both with and without the addition of monoammonium phosphate, and several bacterial taxa that were associated with efficient bioremediation were identified. In Chapter 6, in revisions for *FEMS Microbiology Ecology*, antibiotics that targeted different portions of the microbial community were added to diesel-contaminated soils to determine whether bioremediation would be affected by shifts in relative taxonomic abundance within a common soil

environment. Chapter 7 presents a general discussion and conclusions in relation to the stated objectives, as well as potential future directions for this work.

2

Literature review

Human activity in the Arctic is increasing rapidly, as improved technology and retreating ice sheets make this region more accessible (Harsem et al 2011). Northern countries are scrambling to lay claims to Arctic land, sea and ice (Young 2009, Harsem et al 2011), as this biome is thought to contain a wealth of resources, including fish stocks, minerals, and petroleum. Since low temperatures in the Arctic limit the growth and reproduction of many organisms (e.g. Sand-Jensen et al 1999, Fraser et al 2007), and because this biome has historically experienced only minimal human influence (Sanderson et al 2002), the effects of human disturbance may be magnified.

2.1. Petroleum contaminants in the Arctic

2.1.1. Risk of petroleum contaminants

Unlike other environmental contaminants that are distributed widely across the Arctic, sometimes far from their point of origin, petroleum hydrocarbon contaminants tend to be primarily localized near areas of human activity (Thomas et al 1992). Low levels of natural hydrocarbons occur across the Arctic, as a result of petroleum deposits or decaying biomass (AMAP 2007). Although natural seeps are the main source of hydrocarbon input to the terrestrial Arctic environment, with seeps contributing almost 10

times more than human activities, the most substantial human sources lead to severe local contamination (AMAP 2007). The Arctic is thought to contain considerable reserves of petroleum (an estimated 30% of the world's undiscovered gas and 13% of its undiscovered oil), and this represents a major financial opportunity for northern countries (Gautier et al 2009). The increased human activity that will result from petroleum exploitation will create the potential for more contamination events across the Arctic. In addition, the spill volume can be enormous: the infamous Exxon Valdez spill discharged over 10 million gallons (Paine et al 1996), and the Usinsk, Russia pipeline spill of 1994 released more than 30 million gallons (Etkin 1999). The environment within the spill zone is then forced to contend with the effects of highly concentrated hydrocarbon compounds.

There are thousands of known petroleum compounds, but most are classified as aliphatic (e.g. alkanes, cycloalkanes) or aromatic, with a variety of branched, unsaturated, and sulfur-containing derivatives (AMAP 2007). Many petroleum compounds negatively affect both ecosystem and human health. Arctic petroleum spills have directly hindered the growth and survival of many species of birds (Piatt et al 1990), plants (Wein and Bliss 1973, Atlas et al 1976), and even large mammals (Atlas et al 1976, Hurst and Oritsland 1982). The impact varies seasonally, dependent on species migrations and life cycles (AMAP 2007). In humans, petroleum compounds target the lungs, skin, and nervous system, pose chronic exposure risks (some are known carcinogens), and can cause harm through inhalation, ingestion, and contact (AMAP 2007). In the Arctic, hydrocarbon contaminants are thought to mostly pose risks at high concentrations, but the effects on Arctic inhabitants have not been extensively studied due to difficulties in acquiring

baseline data, and because long-term damage may not be immediately visible (AMAP 2007).

2.1.2. Treatment challenges

Petroleum contamination is already a major concern in the Arctic, as sites can remain polluted decades after the initial spill (Pelley 2001, Neff et al 2011). Arctic machinery and communities are frequently powered by petroleum fuel (e.g. Arnow 2010), and the extraction and shipping of petroleum through extensive pipelines also pose great spill risks (Pelley 2001). While periodic contamination is an inevitable consequence of petroleum use at any latitude (AMAP 2007), the technical challenges associated with petroleum-related activities are much greater in the Arctic, as a result of extreme cold temperatures, slumping permafrost, and the remoteness of many Arctic operations (Hnatiuk 1983, Burgess and Smith 2003). In addition, petroleum contaminants may be more prone to migrating off-site in the Arctic, as they hit the relatively impermeable permafrost layer and disperse (Barnes and Chuvilin 2009).

Predictably, the treatment of hydrocarbon-contaminated Arctic sites is equally challenging. The relocation of large amounts of contaminated soil to treatment facilities is not feasible, owing to technical and financial limitations (Greer 2009). The removal of large amounts of soil may also lead to permafrost thaw and slumping, which can trigger the collapse of nearby buildings (Johnston 1963). Fortunately, on-site treatment is possible, as a variety of Arctic microorganisms are known to degrade petroleum hydrocarbons (Greer 2009, Greer et al 2010). Unlike synthetic compounds such as PCBs and chlorinated solvents, hydrocarbons can be found almost ubiquitously across natural

environments, so many microbial taxa have evolved pathways that can be used to exploit hydrocarbon contaminants for growth-supporting carbon and energy, ultimately leading to the catabolism of the pollutants. Harnessing this activity is more challenging in the Arctic than it is at lower latitudes, as hydrocarbon degradation by microorganisms is negligible below 0°C (Walworth et al 2001), and the nutrient and water content of the soil may also be low (Greer 2009). The degradation or detoxification of contaminating substances such as petroleum by living organisms is known as bioremediation, and is the preferred approach in the cleanup of hydrocarbon-contaminated Arctic soils. Such activity must necessarily be maximized during the short Arctic summer, when microbial populations are capable of efficiently degrading hydrocarbons.

2.1.3. CFS-Alert

Canadian Forces Station Alert (CFS-Alert) is the northernmost permanent human settlement on Earth. It is located at the northern tip of Ellesmere Island, Nunavut, Canada (82°28'N, 62°30'W), and has been active as a weather and communications site since 1950 (Gray 1997). Annual precipitation averages 153.8 mm, while the average daily temperature is -18.0°C. The average annual daily maximum is -14.7°C, and the average annual daily minimum is -21.3°C (http://www.climate.weatheroffice.ec.gc.ca/index.html). The permafrost layer is at most ~1 m deep at most sampled locations (personal communication, Dr. David Juck), and reaches a depth of at least 480 m (Gray 1997). The population at the station fluctuates from about 50 people in the winter, to over a hundred during the summer. The nearest Inuit community is 800 km south at Grise Fjord (Souliere 1997).

In 2009, the station consumed 1.78 million litres of JP-8 jet fuel for electricity and heating (Arnow 2010). While there are many spot contaminations of hydrocarbons around CFS-Alert, the four sites that are of greatest concern contained an estimated 17 575 m^3 of soil contaminated with C6 to C50 hydrocarbons as of 2009, with concentrations exceeding 25 000 mg/kg in some locations (personal communication, Dr. David Juck). Alert is accessed solely by air, so the relocation of this expanse of contaminated soil is unrealistic. Through an NRCan PERD program, our lab's research has focused on determining cost- and time-effective *in situ* treatments for these contaminated sites. The development of efficient on-site treatments for Alert and similar Arctic communities will be essential to the reduction of contaminants at historical and future spill sites.

2.2. Microbial life in the Arctic

2.2.1. Adaptations to a cold existence

The lower temperature limit for microbial activity on Earth is not known; however, microbial respiration has been recorded at temperatures as low as -15°C (Steven et al 2007). The vast majority (> 80%) of the Earth's surface experiences average temperatures of 15°C or lower (Rodrigues and Tiedje 2008). Microorganisms that are active below this threshold are termed psychrophilic, those that function optimally at cold temperatures, or psychrotolerant, those that are active at cold temperatures but operate optimally at higher temperatures. Many psychrotolerant species have evolved flexible metabolic pathways, and can alter gene expression depending on external temperatures (Trevors et al 2012). This is not necessarily the case for obligate psychrophiles; for example, *Exiguobacterium sibiricum*, a bacterium isolated from the Siberian permafrost,

showed only minimal changes in RNA expression when temperatures were raised from 4°C to 28°C, suggesting that it has adapted to a life of permanent cold (Trevors et al 2012). Interestingly, there was no differential expression of genes related to temperature stress in incubations conducted at 4°C and 10°C (Trevors et al 2012).

In Arctic soils, microbial communities face a number of challenges that distinguish them from communities at lower latitudes, including low to extreme subzero temperatures, freeze-thaw cycling, and low water and nutrient availability (Margesin and Miteva 2011). As a result of lower chemical reaction rates, cold-tolerant microorganisms often modify enzymes to reduce non-covalent molecular interactions, thus making the enzymes more flexible (Piette et al 2011). A comparative study of archaea showed that hydrophobic amino acids such as leucine tend to decrease, while polar amino acids such as glutamine and threonine increase in cold-adapted homologues of enzymes, although this is not universal among cold-adapted proteins, and many other amino acid substitutions may occur to modify flexibility and substrate specificity (Siddiqui and Cavicchioli 2006). Hydrophobicity tends to decrease in enzyme cores and increase on enzyme surfaces, in order to reduce stabilizing van der Waals forces, and increase enzyme flexibility (Siddiqui and Cavicchioli 2006).

In addition, cell membrane fluidity is maintained by introducing branched and unsaturated lipids, and by reducing mean lipid chain length (Piette et al 2011). Protein folding machinery has also been shown to be largely upregulated at cold temperatures, and this may be a limiting step in low temperature enzyme reactions (Piette et al 2010). The protection of cellular machinery from freezing or extreme drops in temperature is also necessary, so microorganisms produce antifreeze, ice-nucleating, and other cold

acclimation proteins, and may alter the ionic composition of the cytoplasm to lower its freezing point (Casanueva et al 2010). Under the extreme cold of the Arctic winter, some microbial groups are completely inactive (McMahon et al 2009), and may simply remain in a dormant or vitrified state that maintains their viability until soils thaw each summer.

2.2.2. Arctic microbial diversity

While specific adaptations are clearly required for life in the cold, these adaptations are apparently commonly derived and/or shared by microorganisms. In fact, it has been found that many mesophilic enzymes are easily adapted to cold environments with only small structural modifications (D'Amico et al 2002). Unlike other so-called extreme environments that are low in microbial diversity, such as hot springs, sea ice, and barren rock walls (Lozupone and Knight 2007), Arctic soils are host to a wide array of microbial species. In general, biodiversity is thought to decrease towards the poles (Willig et al 2003), but recent studies have shown that microbial operational taxonomic unit (OTU) richness within a gram of soil in the Arctic is as high if not higher than what is found at lower latitudes (Figure 2.1.; Neufeld and Mohn 2005), and that the taxonomic composition of Arctic microbial communities, at least on a coarse scale, is similar to that of many environments across the globe (Chu et al 2010). This may reflect the fact that, during summer, Arctic temperatures are relatively mild and constant, which may create soil environments that are similar to those observed at lower latitudes.

Most microbial activity is likely to occur in the active layer (suprapermafrost layer) if Arctic soils, when temperatures rise above 0°C, yet the number of

microorganisms identified in a high Arctic permafrost soil, as determined by qPCR, was only one level of magnitude lower than the unfrozen layer above (Yergeau et al 2010a).

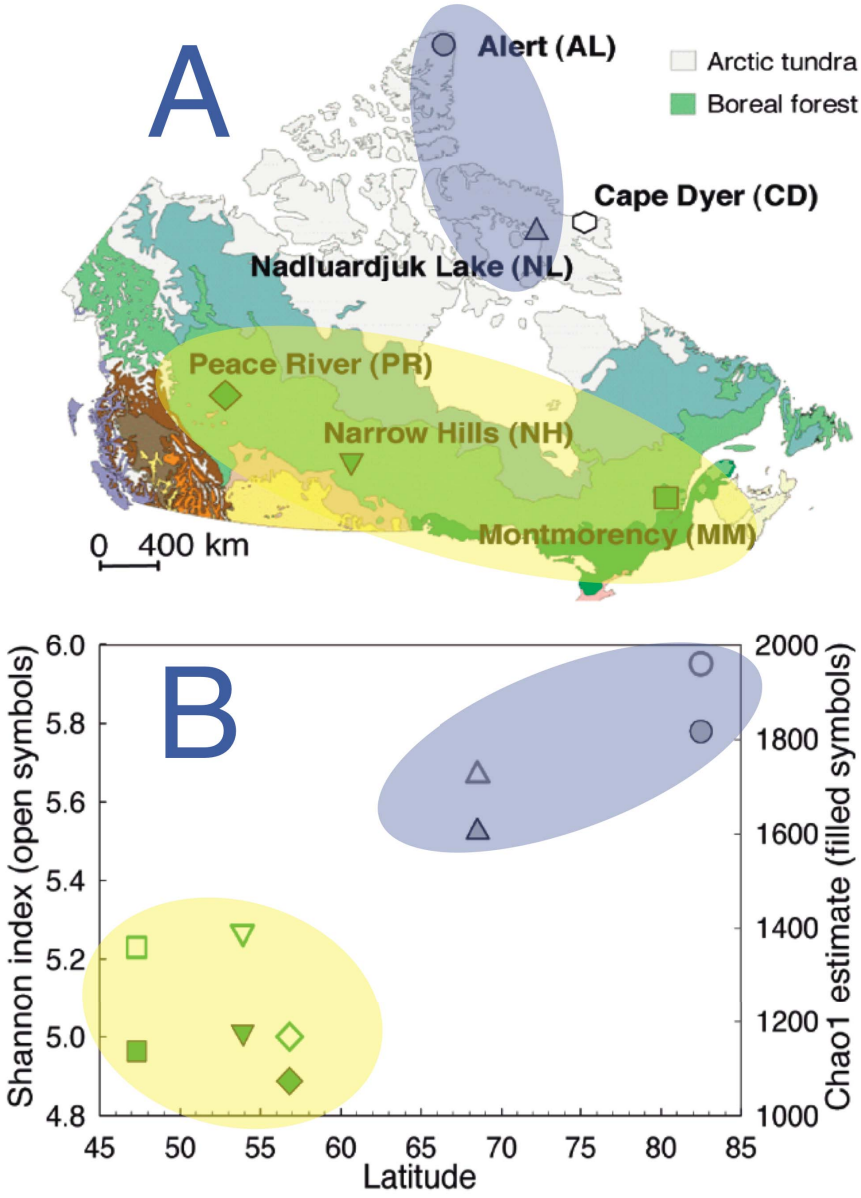

Figure 2.1. Assessment of ribosomal sequence tags shows that Arctic soils can be at least as rich in microbial species as lower latitude soils. A) Sampling locations; B) relationship between Shannon/Chao indices and latitude (adapted from Neufeld and Mohn 2005).

This permafrost soil also supported a wide diversity of microorganisms (Yergeau et al 2010a), and may contribute more to biogeochemical processes in the near future as the climate warms, and permafrost thaw extends the depth of the active layer. The composition of active layer communities can also be expected to shift in response to future climate change, but predictions are mixed on whether this will occur in the short or long-term, and whether this is related to direct effects of warming on microbial physiology, or indirect effects, such as alterations in plant biomass and species composition (Rinnan et al 2007, Yergeau et al 2012a). Other climate factors may also affect microbial diversity in Arctic soils, as freeze-thaw cycles in the Antarctic have been shown to alter the abundance of fungi, as well as a variety of functional genes (Yergeau and Kowalchuk 2008).

Species richness and diversity are generally thought to have a positive influence on ecosystem function, although results have been mixed (Bell et al 2005, Fargione et al 2007, Jiang 2007, Wertz et al 2007, Haddad et al 2008, Gravel et al 2011, Peter et al 2011, Hooper et al 2012). While certain microorganisms may be functionally redundant with respect to specific biogeochemical processes, a review of the literature showed that changes in community composition are frequently associated with long-term shifts in function, suggesting that many taxa are not easily replaced (Allison and Martiny 2008). Horizontal gene transfer (HGT) may lead to functional redundancy between some closely related microbial species, but the natural extent of this mode of genetic recombination is unknown, and there are likely some barriers to HGT at higher phylogenetic levels in order to allow microbial species to exploit specific niches in the environment (Kurland et al 2003). In Arctic soils, fungi and bacteria are known to partition carbon substrates

(Rinnan and Bååth 2009) and are also in strong competition for nitrogen (Siciliano et al 2009), although this competition may actually limit the full extent of some biogeochemical processes.

2.3. Petroleum degradation by Arctic microorganisms

2.3.1. Main catabolic pathways

Because of the similarities between Arctic and lower latitude microbial communities, the main pathways for hydrocarbon catabolism are homologous. In the case of both aliphatic and aromatic hydrocarbons, most compounds must first be activated by the introduction of oxygen. When molecular oxygen is available, alkane-degrading microorganisms can incorporate it into alkanes directly, while compounds such as fumarate are substituted under anaerobic conditions (Rojo 2009). The best-known alkane monooxygenases are *alkB* and cytochrome P450 in bacteria (van Beilen et al 2006), while an alternative NADH-dependent cytochrome P450 is used in the activation of alkanes in fungi (Yadav et al 2003). Oxidation of alkanes occurs either terminally or subterminally, and both strategies can be used by the same bacterial strain, possibly as an adaptation for metabolizing alkanes of varying chain length (Whyte et al 1998). Several *alkB* genes have been identified in both Gram-negative and Gram-positive Arctic bacteria, and were initially found in isolates of *Pseudomonas* and *Rhodococcus* (Whyte et al 1996, van Beilen et al 2002). An Arctic strain of *Rhodococcus* was found to contain four distinct *alkB* homologs, possibly to enable growth on different alkane substrates (Whyte et al 2002b). It is still unknown how many uncultured hydrocarbon degraders contain a diverse complement of alkane monooxygenase genes, although it is now possible to

assess the diversity of these genes in the natural environment using PCR-based methods (Kloos et al 2006, van Beilen et al 2006).

Aromatics, especially polyaromatics, are considered to be more recalcitrant to biodegradation than alkanes, since a greater input of energy by microorganisms is required initially to overcome the resonance energy of these compounds. Aromatic degradation is mainly initiated by Rieske non-heme iron oxygenases that separate into four major enzyme families. These mainly group by substrate specificity: the Toluene/Biphenyl family, the Naphthalene family, the Benzoate family, and the Phthalate family, with several enzymes that are outliers to these groups (Gibson and Parales 2000). When oxygen is available, complex aromatics are converted by microorganisms to common intermediates such as protocatechuate and catechol, and are eventually cleaved by various dioxygenases (Fuchs et al 2011). These intermediate compounds can be metabolized by many microbial species (El Azhari et al 2007, Kasuga et al 2007). Under anaerobic conditions, aromatics are generally converted to benzoyl-CoA, which can be further metabolized by the addition of fumarate, through O_2-independent hydroxylation, or by carboxylation (Fuchs et al 2011).

Arctic *Pseudomonas* strains have been found to carry pathways for the degradation of both alkanes and aromatics (Whyte et al 1997), and can therefore select the most efficient or available source. However, many bacterial isolates have only been observed to carry specific pathways (Foght et al 1990), and it is unknown how widely HGT is used to share catabolic plasmids following soil contamination. Many genes involved in hydrocarbon catabolism are carried on plasmids, and even when growth is

possible in their absence, possession of additional plasmids may enhance the breakdown of hydrocarbons, or increase tolerance to toxicity (Whyte et al 1998).

2.3.2. Petroleum tolerance

Arctic microorganisms must be well adapted to cold temperatures, but those living in hydrocarbon-contaminated soils must also adapt to tolerate high hydrocarbon concentrations. While petroleum may be a source of carbon and energy for many microorganisms, it is also toxic when concentrated, mainly due to negative interactions with cell walls (Ramos et al 2002, Kang and Park 2010). Tolerance to diesel in a strain of *Acinetobacter* was enhanced in the presence of increased exopolysaccharide (EPS), possibly by adsorption of the contaminants or as a barrier to diffusion, which would maintain separation between hydrocarbons and the cell wall (Kang and Park 2010). An Arctic strain of *Rhodococcus* has been shown to produce a biosurfactant at the cell surface, which increases hydrophobicity, and facilitates incorporation and subsequent degradation of insoluble hydrocarbons (Whyte et al 1999). This strain was also shown to modify its EPS depending on the carbon substrate that was present (Whyte et al 1999). Other suggested mechanisms of tolerance include alterations of cell membrane structure, creation of vesicles to compartmentalize hydrocarbons, and energy-requiring efflux pumps that are used to expel toxic compounds into the extracellular medium (Ramos et al 2002). Strains of *Pseudomonas* are known to be particularly tolerant to a variety of toxic compounds as a result of their many efflux pumps (Ramos et al 2002), and this may help explain their dominance in many hydrocarbon-contaminated soils (Aislabie et al 2006, Yergeau et al 2012c).

All of the adaptations mentioned previously demand inputs of energy and resources, requiring trade-offs in active microorganisms. For example, an Arctic *Rhodococcus* strain was shown to decrease fatty acid saturation at cold temperatures, but this adaptation was reduced in the presence of hydrocarbons, suggesting a possible tradeoff in tolerance to cold and hydrocarbon toxicity (Whyte et al 1999). Nevertheless, it is important to keep in mind that effective tolerance to contaminants does not ensure efficient contaminant degradation.

2.3.3. Treatment of contaminated soils

Because most hydrocarbon degradation occurs above 0°C (Walworth et al 2001), there is very minimal bioremediation occurring throughout much of the Arctic year. As a result, it is important to optimize microbial activity during the few months in which temperatures are relatively mild. A number of *in situ* approaches have been used to increase rates of hydrocarbon degradation. Bioaugmentation involves the inoculation of soil with exogenous hydrocarbon-degrading microorganisms, or the addition of hydrocarbon degraders that have been concentrated from the local environment, but to date, this approach has had limited success (Thomassin-Lacroix et al 2002, Thompson et al 2005, van Herwijnen et al 2006). The modification of soil properties such as chemical composition (Ferguson et al 2004), salinity (Rhykerd et al 1995), temperature (Zhou and Crawford 1995, Mohn et al 2001), and oxygen (Zhou and Crawford 1995) has been attempted, but nutrient amendments have generally seen the most success, and are commonly used to treat hydrocarbon-contaminated Arctic soils (Mohn et al 2001, Greer 2009, Yergeau et al 2009). The addition of appropriate nitrogen-based nutrient

amendments is generally thought to increase the potential for microbial growth and activity, especially in the presence of a rich carbon source such as petroleum (Walworth et al 1997), and these nutrients may also provide terminal electron acceptors to catabolic reactions under anaerobic conditions (Powell et al 2006a).

2.3.4. Key hydrocarbon-degrading taxa

Members of each of the three domains of life are known to degrade hydrocarbons (Silva et al 2009, Greer et al 2010, Prince et al 2010, Tapilatu et al 2010). Bacteria are the most frequently studied hydrocarbon degraders, and isolates of several key bacterial genera from contaminated polar soils have been shown to grow on a variety of hydrocarbon substrates in culture. Because most bacteria are not amenable to culturing (Amann et al 1995), these studies have been weighted towards strains of the easily-cultured *Pseudomonas*, *Rhodococcus*, and *Sphingomonas* (Table 2.1.).

It is only with improved technologies for the culture-independent analysis of microbial communities that the true diversity of hydrocarbon-contaminated soils has become known (Yergeau et al 2009, Iwai et al 2010, Liang et al 2011, de Menezes et al 2012, Yergeau et al 2012c). These studies have shown that microbial communities in some soils reorganize substantially in response to contamination, while others are less responsive (Yergeau et al 2009), that oxygenases related to the degradation of both alkanes and aromatics are far more diverse than imagined (Iwai et al 2010, Liang et al 2011), and that many genes involved in both hydrocarbon degradation and general cell metabolism increase in abundance or expression following hydrocarbon contamination and subsequent bioremediation treatments (de Menezes et al 2012, Yergeau et al 2012c).

Table 2.1. Bacterial strains that have been cultured from hydrocarbon-contaminated polar soils, and their hydrocarbon growth substrates (adapted from Whyte et al 1996, Aislabie et al 2006). Despite several new isolates of marine and ice-inhabiting hydrocarbon degraders in polar environments, a thorough search of the literature did not reveal new hydrocarbon-degrading soil isolates after 2006.

Bacterial strains	*Hydrocarbon growth substrates*
Alkane degraders	
Acinetobacter ADH-1	Crude oil, aromatic gas-oil, hydrogenated gas oil, kerosene, dodecane, hexadecane, cyclohexane
Arthrobacter protophormiae MTCC 688	Hexadecane
Pseudomonas DhA-91	Jet A-1 fuel, octane, dodecane
Pseudomonas Ps 8	Jet A-1 fuel, hexadecane, pristane
Pseudomonas 5B	JP8 jet fuel, hexane
Rhodococcus 5/1, 5/14 and 7/1	JP8 jet fuel, C_6-C_{20} *n*-alkanes, pristane
Rhodococcus Rho10	Jet A-1 fuel, dodecane
Rhodococcus 43/02	JP5 jet fuel, dodecane, hexadecane, pristane
Sphingomonas DhA-95	Jet A-1 fuel, dodecane, pristane
Alkane and aromatic degraders	
Pseudomonas B17 and B18	C_5-C_{12} *n*-alkanes, toluene, naphthalene
Pseudomonas DhA-91	Jet A-1 fuel, octane, dodecane
Pseudomonas PK4	Pyrene, dodecane, hexadecane
Pseudomonas 30-3	JP8 jet fuel, C_8-C_{13} *n*-alkanes, toluene, m- and p-xylene, 1,2,4-trimethyl benzene
Aromatic degraders	
Pseudomonas Cam-1 and Sag-50G	Biphenyl
Pseudomonas IpA-92	Toluene
Pseudomonas IpA-93	Toluene, benzene
Pseudomonas Ant 5	JP8 jet fuel, NAH, 2MNAH
Pseudomonas Ant 7	JP8 jet fuel, p-xylene, 1,2,4-trimethyl benzene naphthalene, 1-methyl naphthalene, 2-methyl naphthalene
Pseudomonas 7/22	JP8 jet fuel, toluene, m- and p-xylene, 1,2,4-trimethyl benzene
Pseudomonas K319	Pyrene
Pseudomonas 5A	JP8 jet fuel, benzene, toluene, m-xylene
Sphingomonas Ant 17	JP8 jet fuel, m-xylene, 1-methyl naphthalene, 2-methyl naphthalene, dimethylnaphthalene, 2-ethylnaphthalene, fluorene, phenanthrene
Sphingomonas Ant 20	JP8 jet fuel, 1-methyl naphthalene, phenanthrene
Sphingomonas 43/03	Phenanthrene
Sphingomonas 43/04	Phenanthrene

In addition to the well-known cultured hydrocarbon degraders, these studies identified increases in various microbial taxa in response to hydrocarbon contaminants, including *Mycobacterium*, *Streptomyces*, *Nocardia*, *Caulobacter*, *Arthrobacter*,

Burkholderia, *Comamonas*, *Acidovorax*, *Sordariomycetes*, *Thaumarcheaota*, and a number of still uncultured microorganisms (de Menezes et al 2012, Yergeau et al 2012c).

Aside from the fact that culturing excludes many potential hydrocarbon degraders, the *in situ* hydrocarbon-degrading capacity of microorganisms that interact with many co-occurring species cannot be quantified in isolation. A mixed culture study showed that 70% of a diesel-degrading bacterial community was not involved in actual diesel degradation (Hesselsoe et al 2008), and even this represented a vastly simplified community. These non-degrading bacteria were still responsible for consuming nutrients and producing waste products, and potentially limited the effectiveness of the more productive strains. On the other hand, the activity of some microorganisms may rely on cometabolism to effectively catabolize hydrocarbons (Beam and Perry 1974), and will this be less efficient in the absence of their metabolic partners. Many other complex community interactions can shape the abundance and activity of hydrocarbon degraders, including predation, parasitism, the production of required metabolites, and the scavenging of compounds that are either detrimental or essential to the activity and survival of these microorganisms (Little et al 2008).

The hydrocarbon-degrading efficiency of cultured microorganisms may also increase or decrease in the absence of the physical structure and challenges presented by the soil environment. The fact that bioaugmentation has been generally ineffective as an *in situ* bioremediation strategy (Thompson et al 2005) shows that efficient hydrocarbon degradation in culture is not sufficient to ensure survival and dominance in a complex soil environment. Factors that may limit the activity of hydrocarbon-degrading microorganisms *in situ* include pollutant tolerance (Ramos et al 2002, Kang and Park

2010), water availability (Greer 2009), nutrient concentrations (Boopathy 2000, Chaîneau et al 2005), antibiotics (Stapleton et al 2000a, Kaszab et al 2010), substrate colonization (Pandey and Jain 2002), pH optima (Margesin and Schinner 2001), and predation and parasitism (Kota et al 1999, Little et al 2008).

2.4. Effects of nitrogen amendments on microorganisms

2.4.1. Nitrogen limitation and availability

Nitrogen is the major limiting nutrient to biomass production in many terrestrial ecosystems (Vitousek et al 1997), and as mentioned earlier, this property can often be exploited to increase rates of hydrocarbon degradation at polluted sites. On the other hand, while nitrogen may be limiting to the overall biomass, many microbial taxa are adapted to specific forms and concentrations of this nutrient, and will not necessarily become more abundant or active following its addition to soil. This may be the result of direct negative effects of nitrogen on cell function, or of indirect effects, such as an increase in the abundance and activity of a competitor that thrives under high nitrogen conditions. Nitrogen form and concentration have been shown to affect the relative abundance of microbial taxa in hydrocarbon-contaminated soils (Chaîneau et al 2005, Powell et al 2010). Some authors have mentioned that an understanding of the specific dynamics of nitrogen use within microbial communities would help in optimizing the bioremediation of hydrocarbons (Breedveld and Sparrevik 2000, Powell et al 2006b); however, this has not been thoroughly explored.

With the exception of atmospheric nitrogen fixers, most microorganisms obtain nitrogen in bioavailable forms such as NH_4^+ and NO_3^-. Certain species preferentially

assimilate NH_4^+ as a nutrient source over NO_3^-, while some are entirely unable to assimilate NO_3^- (Imsenecki et al 1976, Rice and Tiedje 1989, Recous et al 1990). The mechanism behind this physiological difference is not entirely clear, but may be related to the additional energy that is required to convert NO_3^- into an amino group that can be incorporated into glutamine, which is the main starting point for protein biosynthesis (Tempest et al 1970).

Highly active nitrifying and denitrifying bacteria have been detected in hydrocarbon-contaminated soils (Deni and Penninckx 1999, Powell et al 2006a), and the conversion of bioavailable nitrogen species to N_2 and N_2O by nitrogen-cycling bacteria likely limits the supply of nitrogen to other active microorganisms (Powell et al 2006b). Denitrifiers sequentially reduce NO_3^- to N_2 gas (Zumft 1997), whereas nitrifiers can release N_2O either as a by-product of nitrification, or through nitrifier denitrification, whereby NO_2^- is fully reduced to N_2 (Wrage et al 2004), and have been shown to be the primary source of N_2O release in fertilized Arctic soil without a corresponding increase in population size (Siciliano et al 2009). While denitrifiers that have been identified from hydrocarbon-contaminated soils (e.g. *Pseudomonas*) are primarily heterotrophs that contribute to contaminant reduction (Powell et al 2006b), nitrifiers are autotrophic, and may oxidize large quantities of NH_3 and NO_2^- to meet basic energy requirements (Ferguson et al 2007), resulting in a subsequent loss of available nitrogen for other species. Nitrogen, along with other nutrients, may also be lost from Arctic soil systems abiotically, especially during spring thaw in the presence of heavy melt-water runoff (Herrmann and Witter 2002).

2.4.2. Stimulant or inhibitor?

Whether nitrogen stimulates or inhibits specific microorganisms depends heavily on the type, concentration, and context. Although the addition of nitrogen can stimulate microbial communities under certain conditions, added nitrogen can also be seen as a disturbance to soil ecosystems, and has been shown to reduce microbial respiration, biomass, and diversity (Allison et al 2007, Ramirez et al 2012). Nitrogen inputs have had mixed effects on fungal:bacterial ratios (de Vries et al 2006, Bell et al 2010, Stark et al 2012), and this could have fairly important implications in terrestrial Arctic ecosystems, considering that fungi are also the major providers of plant nitrogen in Arctic tundra soils (Hobbie and Hobbie 2006). Understanding the effect of nutrients on various microbial taxa will be critical in designing more targeted and efficient bioremediation treatments.

Nitrogen addition has had fairly consistent effects on soil bacterial communities from a wide range of pristine North American ecosystems. In general, an increase in *Actinobacteria* and *Firmicutes*, and a decrease in *Acidobacteria* and *Verrucomicrobia* is expected (Ramirez et al 2012). In addition, *Alphaproteobacteria*, *Gammaproteobacteria*, and *Bacteroidetes* have been shown to respond well to nitrogen fertilization at high concentrations or following long-term fertilization (Campbell et al 2010, Fierer et al 2012). A major hydrocarbon-degrading family of the *Gammaproteobacteria*, the *Xanthomonadales*, showed an especially dramatic increase following extended nitrogen fertilization (Campbell et al 2010). However, nitrogen inputs have not had consistent effects on microbial Shannon diversity and OTU richness estimates (Campbell et al 2010, Ramirez et al 2010), suggesting that other factors may affect the relative success of

minority species. Nitrogen fertilization has also been shown to overwhelm the effects of temperature on decomposition in soils, and has shifted community substrate preference towards more labile carbon sources (Ramirez et al 2012).

2.5. Bioremediation ecology

2.5.1. Improved sequencing techniques

The advent of phylogenetic comparison using 16S rRNA gene sequences (Woese and Fox 1977) led to huge leaps forward in the field of microbial ecology. Numerous community fingerprinting techniques have been used over the past few decades, including Denaturing Gradient Gel Electrophoresis (DGGE; Muyzer et al 1993), Terminal Restriction Fragment Length Polymorphism (T-RFLP; Liu et al 1997), Automated rRNA Intergenic Spacer Analysis (ARISA; Cardinale et al 2004), clone library analysis (Olsen et al 1986), and phylogenetic microarrays (Brodie et al 2006), and these have demonstrated how the taxonomic structure of microbial communities can change under different environmental and treatment conditions. Many of these techniques have also been adapted to explore the DNA and RNA diversity of key functional genes, but are limited in their ability to assess the true genetic diversity of environmental samples, as they provide little resolution beyond the few dominant taxa or genes, and traditional Sanger sequencing of even a few hundred cloned DNA fragments requires a large investment of time and money.

Next-generation sequencing technologies now allow for high-throughput comparisons of many samples, at only a fraction of the cost and effort in the lab, and have opened the door to far more extensive phylogenetic comparisons of microbial habitats

(Tringe and Hugenholtz 2008). Bioinformatic analyses and data interpretation are now frequently the bottlenecks to extensive ecological studies (Bell et al 2012). While many of the early studies that have incorporated these technologies have focused on the microbial diversity of pristine environmental samples (Shokralla et al 2012), next-generation sequencing is also being used to study contaminated environments, and assess bioremediation effectiveness (Bell et al 2012). Several metagenomic approaches have been used to determine which microorganisms inhabit contaminated environments, what catabolic genes are involved in degradation, and what other genetic information is modified when compared with reference environments (Figure 2.2.).

2.5.2. Ecology of hydrocarbon-contaminated soils

Despite these advances, many uncultured microbial species are still known only from 16S rRNA gene barcodes. High-throughput culture-independent approaches for assessing microbial function are still in the development stage (Chen and Murrell 2010, Lidstrom and Konopka 2010, Pan et al 2011), so relatively little is known about the actual ecology of soil microorganisms in pristine environments, while even less is known about the ecology of the diverse microorganisms that survive in contaminated soils.

Are many of these species functionally redundant? Does species competition limit rates of hydrocarbon degradation? Do keystone species exist, as is the case in systems of higher organisms? The current state of microbial ecology in contaminated soils and some possible future directions are discussed in the next chapter.

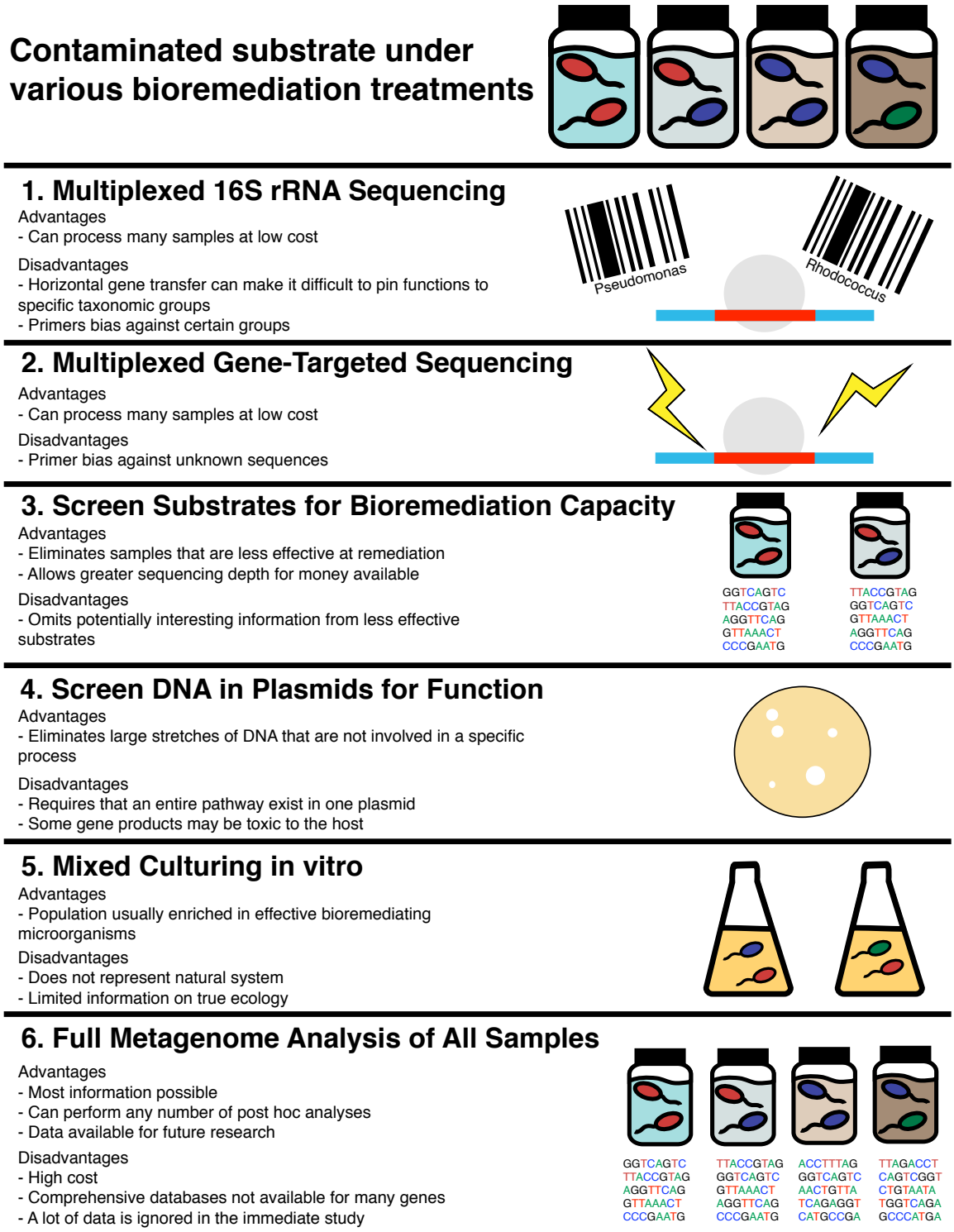

Figure 2.2. Methods for integrating metagenomics into bioremediation studies. This figure is reproduced from Figure 1 of Bell et al. (2012) with kind permission of Springer Science and Business Media.

Connecting Text

The fact that microorganisms can degrade hydrocarbons has long been known, and this ability has been harnessed in the treatment of contaminated Arctic soils. Nevertheless, bioremediation treatments have not been equally effective across environments. The likelihood of contamination, especially in remote and environmentally challenging locations such as the Arctic, is only going to increase in the coming years, and will require effective bioremediation strategies. As presented in the previous chapter, a number of treatments are available for the stimulation of hydrocarbon-degrading activity *in situ*, but the complexity of microbial communities, and the fact that high-throughput sequencing technologies have only recently become available, mean that we are still left with a very basic understanding of microbial activity and interactions within hydrocarbon-contaminated soils. There are many microorganisms that can contribute to the bioremediation of petroleum contaminants, but given that interspecific competition may limit the effectiveness of certain taxa, should they all be stimulated? The next chapter explores the current state of knowledge as it pertains to the microbial ecology of hydrocarbon-contaminated soils, as well as future research directions that could help to identify the contributions of individual taxa. Such knowledge will be critical in designing more targeted bioremediation treatments.

3

Towards identifying the contributions of microbial taxa to bioremediation in hydrocarbon-contaminated soils

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Submitted to *FEMS Microbiology Ecology*.

This chapter was written by the candidate, with helpful comments provided by Dr. Charles W. Greer and Dr. Lyle G. Whyte.

3.1. Abstract

The ability to degrade petroleum hydrocarbons is widespread amongst soil microorganisms. Although the initial transformation of certain hydrocarbon compounds may require specialist microorganisms, the degradation of many common intermediates is likely performed most rapidly by a few generalist species. Nevertheless, greater interspecies competition is also likely to be attracted by these intermediates, potentially limiting rates of bioremediation. This review discusses some of the mechanisms by which the abundance and activity of efficient hydrocarbon degraders may be limited in contaminated soils, and how the relative importance of individual taxa to the breakdown

of hydrocarbons can be assessed in complex soil communities. Such studies will ultimately help in developing more targeted and effective bioremediation treatments.

3.2. Introduction

Microorganisms are capable of metabolizing many toxic compounds, as they will essentially "eat or breathe anything" given a sufficient difference in voltage potential between electron donors and acceptors (Nealson 2003). The bioremediation of contaminated environments using indigenous microorganisms is frequently exploited to treat many types of contamination, and the removal or detoxification of most pollutants depends on the presence of a few specialized microorganisms (Löffler and Edwards 2006). However, a range of hydrocarbon compounds are found in abundance naturally, as they originate from environmental as well as anthropogenic sources, and phylogenetically diverse taxa are capable of exploiting them for growth-supporting energy and carbon in most environments (Greer et al 2010). As a result, human intervention is not always required for substantial mineralization of contaminating hydrocarbons to occur, and concentrations may simply be monitored as they diminish over time through natural attenuation (Stapleton et al 2000b).

In many cases, however, faster and more complete remediation is made possible by manipulating contaminated soils. Microbial growth and activity in soil can be limited by a shortage of essential nutrients or oxygen (Boopathy 2000, Meidute et al 2008), so the nutrient-amendment and/or aeration of hydrocarbon-contaminated soils may promote bioremediation activity (Delille and Coulon 2008, Yergeau et al 2009). Hydrocarbon-contaminated soils have also been treated by using surfactants (Ron and Rosenberg 2002,

Lai et al 2009), increasing salinity (Rhykerd et al 1995), increasing temperature (Zhou and Crawford 1995, Delille et al 2007), introducing target plant species (Phillips et al 2008, Lefrançois et al 2010), and have been inoculated with cultures of known hydrocarbon-degrading microorganisms, a process known as bioaugmentation (Thomassin-Lacroix et al 2002, Cunliffe et al 2006, van Herwijnen et al 2006). The objective of each of these treatments is to increase the growth and/or activity rates of hydrocarbon-degrading microorganisms, but it is mostly unknown how individual taxa and their interactions are affected.

Such an understanding is crucial, as the effectiveness of standard bioremediation treatments has varied between sites. For instance, nitrogen addition has often stimulated *in situ* bioremediation, but the extent of this effect differs across seemingly similar soil environments (Powell et al 2006a, Delille and Coulon 2008, Yergeau et al 2009). Soil microbial communities are also altered differentially depending on the amount of nitrogen added (Braddock et al 1997, Chaîneau et al 2005), and nitrogen is assimilated with varying efficiency by hydrocarbon-degrading taxa within a single community (Chapter 4; Bell et al 2011). Since many microorganisms are adapted to metabolizing hydrocarbons, it is by no means obvious that the most efficient hydrocarbon degraders should be promoted in the event of a contamination, or in response to bioremediation treatments. Dejonghe et al. (2001) proposed purposefully manipulating microbial species abundance within contaminated soils, but little is known about which taxa should be promoted and when. Most microbial species have yet to be cultured (Amann et al 1995), meaning that realistic functional studies of hydrocarbon-degrading communities must be conducted in the field or in soil microcosms. Mass-sequencing technologies are enjoying a

breakthrough period, which allows for quick characterization of contaminated soil communities, but high-throughput techniques for assessing the function of microbial taxa *in situ* are still mostly in the developmental stage (Chen and Murrell 2010, Lidstrom and Konopka 2010, Pan et al 2011, Konopka and Wilkins 2012).

Does the ecology of microbial communities mirror that of larger multicellular organisms? Single-celled organisms are not nearly as physically complex as multicellular organisms, but are extremely diverse taxonomically and metabolically. Although contaminating hydrocarbons consist of many unique compounds, potentially allowing for niche differentiation across this energy/carbon source, many distantly related microbial taxa possess closely related pathways for hydrocarbon catabolism (Prince et al 2010). In addition, there is strong evidence that horizontal gene transfer (HGT) is used to transfer key hydrocarbon-degrading genes within microbial communities (Wilson et al 2003, Ma et al 2006, Yergeau et al 2012c), but it is unknown how widely this occurs, and whether possession of these genes alone is sufficient for rapid use of hydrocarbon substrates. Other unknowns include the extent of functional redundancy in hydrocarbon degradation, whether specific 'keystone species' exist, and whether the thousands of minority taxa that live in contaminated soils play any role in remediation. An improved understanding of the roles of individual taxa in contaminated soils could lead to the design of more effective bioremediation treatments.

3.3. Use of hydrocarbon contaminants by soil microbial communities

Many reviews have focused on the variety of hydrocarbon degradation pathways that have been identified in microorganisms (e.g. Peng et al 2008, Rojo 2009, Fuchs et al

2011), so the main strategies are only briefly summarized here. Specialized pathways may be important in the initial breakdown of large or complex molecules (Peng et al 2008), but it is likely that common intermediates can be metabolized by a large number of taxa, increasing the role of interspecies competition on rates of bioremediation. Gravel et al. (2011) showed that microbial species richness was positively correlated with the extent of carbon use in communities of specialist bacteria, but was less important in groups of generalists. On the other hand, the generalist assemblages were more productive overall, suggesting that it is important to understand what types of assemblages dominate hydrocarbon degradation in soils.

3.3.1. Degradation pathways

Petroleum contaminants can be comprised of thousands if not tens of thousands of distinct compounds (Frysinger et al 2003, Marshall and Rodgers 2004). These compounds split into two major categories, which are the aliphatic (alkanes) and aromatic hydrocarbons. The number of potential molecules increases dramatically as a result of heteroatom substitutions (e.g. N, O, and S), differing degrees of hydrogen saturation, non-aromatic ring structures, and side branching (Seo et al 2009, Wilkes and Schwarzbauer 2010, Konopka and Wilkins 2012). Complex high-molecular weight compounds such as resins and asphaltenes also exist in crude oil, but knowledge of the structure and degradation pathways of these compounds remains limited (Murgich et al 1999, Sabbah et al 2011, Tavassoli et al 2012). The initial transformation of these compounds can require a diverse array of peripheral or channeling pathways (Fuchs et al 2011). It is likely that the most readily degraded compounds will be those that are most similar to

compounds that already exist in the local soil environment, since the extant microbial community should already be adapted to exploiting those resources.

Alkane degradation is common among bacteria, as well as fungi (Watkinson and Morgan 1990, Rojo 2009). Under oxic conditions, microbes mostly initiate attack on alkanes using enzymes related to methane monooxygenase, alkane hydroxylase, and cytochrome oxygenase, to introduce reactivity into these inert compounds (Rojo 2009), and this eventually produces end-products that can readily be assimilated into the tricarboxylic acid cycle. Under anaerobic conditions, fumarate is added instead of oxygen to destabilize alkanes, with nitrite and sulfite used as terminal electron acceptors (Rojo 2009). The degradation of aromatic hydrocarbons tends to be more energy-intensive initially, as the resonance energy of the molecule must be overcome. Aerobic degradation of aromatics is generally initiated by oxygenases, converting compounds to common intermediates like protocatechuate and catechol, which are eventually cleaved by dioxygenases (Fuchs et al 2011). These intermediates can be processed by a wide range of microbial species (El Azhari et al 2007, Kasuga et al 2007). Anaerobic pathways usually convert aromatics to benzoyl-CoA, which is then reduced to simple straight-chain compounds via fumarate addition, O_2-independent hydroxylation, and carboxylation (Fuchs et al 2011). Many bacteria are capable of degrading simpler aromatics, but high molecular weight polycyclic aromatic hydrocarbon degradation is mostly performed by fungi (Peng et al 2008).

3.3.2. Are specialists necessary?

The extent to which specialist microorganisms are required in hydrocarbon degradation is unknown. Despite the potential for genetic exchange of key enzymes involved in hydrocarbon degradation, some cellular machinery may not be widely transferred, since taxa survive in multispecies communities by exploiting specific specialist or generalist niches. A study of isolates from diesel-contaminated water samples showed that particular species of *Pseudomonas* and *Psychrobacter* were capable of using naphthalene and aliphatic hydrocarbons with carbon chain lengths ranging from C13-C21 as sole carbon sources, while other bacteria could only survive on some of these compounds (Ciric et al 2010). Similarly, species of *Pseudomonas*, *Flavobacterium*, and *Rhodococcus* that were isolated from gasoline-contaminated soils could each degrade a wide array of hydrocarbon contaminants, but were most efficient as a consortium (Lu et al 2006). The extent of specialization among uncultured hydrocarbon-degrading microorganisms has not been determined; however, multiple taxa have been identified in stable isotope probing studies that have used a single labeled compound as a growth substrate, demonstrating that the partitioning of certain compounds does occur (Madsen 2006). Although certain large hydrocarbon molecules are quite complex, interspecies competition likely plays a larger role once compounds have been reduced to intermediates of common pathways (Figure 3.1.). Other non-degrading species can also obtain hydrocarbon-derived carbon through cross-feeding of produced metabolites or grazing on microbial biomass (Pfeiffer and Bonhoeffer 2004, DeRito et al 2005, Lueders et al 2006), thus sequestering resources and contributing to competition without being directly involved in hydrocarbon degradation.

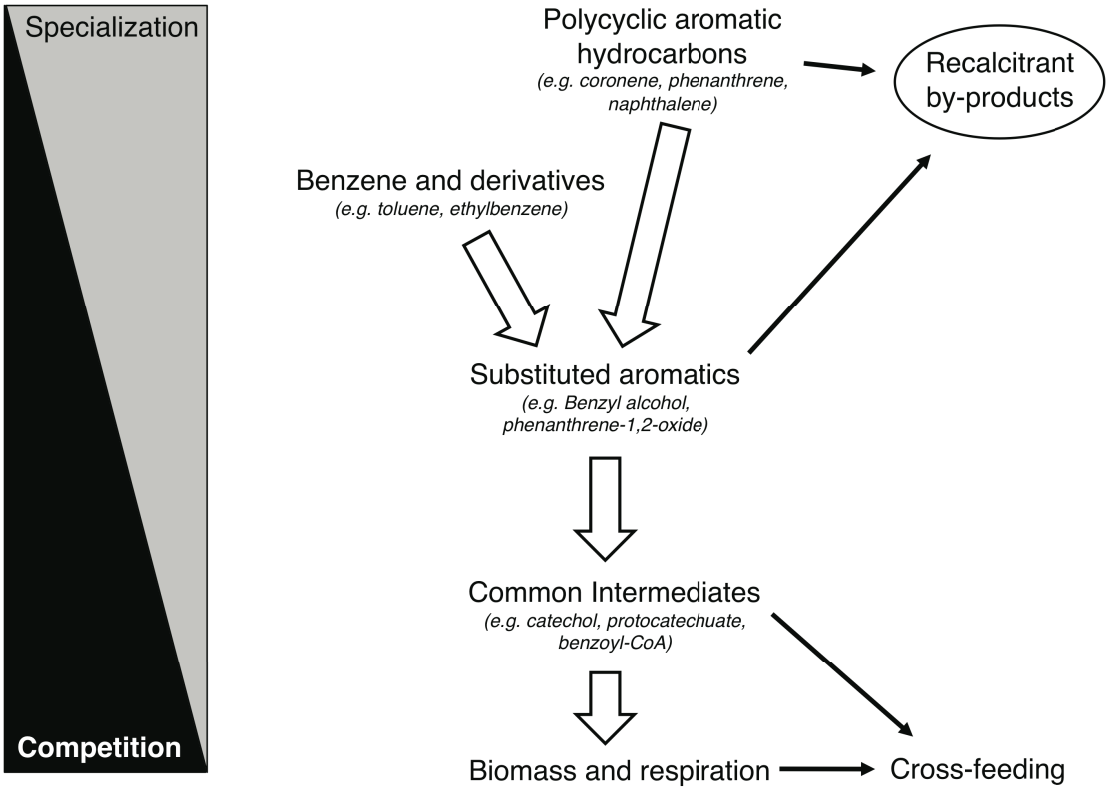

Figure 3.1. Specialized microorganisms are required to initiate the catabolism of complex hydrocarbons. As they are converted into intermediates of common pathways they become more accessible to a wider range of taxa, thus increasing the potential for competition. Specialized microorganisms would also be required to degrade recalcitrant by-products, whereas a wide variety of microorganisms, able to obtain carbon secondarily from metabolites produced by hydrocarbon-degrading microorganisms, would increase the potential for interspecies competition.

Hydrocarbon degraders may also separate and specialize temporally, as the composition and concentration of the contaminant changes. Succession of dominant genera has been shown to occur during the remediation of a diesel-contaminated Arctic soil, as *Gammaproteobacteria* dominated during the early, efficient period of hydrocarbon degradation, but were eventually replaced by *Actinobacteria* and *Alphaproteobacteria* (Yergeau et al 2012c). Similarly, a TRF study of a California soil contaminated with weathered hydrocarbons showed early success by *Pseudomonas* and

Flavobacterium during the period of rapid hydrocarbon depletion, followed by the replacement of these phylotypes by four other unidentified groups (Kaplan and Kitts 2004). It is thought that this separation is primarily driven by a shift towards lower concentrations of hydrocarbon contaminants that are dominated by recalcitrant compounds; however, other soil parameters were also modified over the course of the remediation process (Kaplan and Kitts 2004, Yergeau et al 2012c), and may have played a role in species succession.

Spatial separation can also promote niche differentiation. Soil tilling has frequently been associated with decreased microbial biomass, and has had mixed effects on diversity (Lupwayi et al 2001, Cookson et al 2008, Helgason et al 2010), and this could be due to a reduction in the number of available niches as soil is homogenized. In undisturbed soils, microbial density can vary dramatically on the millimetre scale (Nunan et al 2003), and different soil compartments have been shown to harbour distinct groups of alkane degraders (Schulz et al 2012). Fine-scale variation in soil pH also occurs, especially in the rhizosphere (Hinsinger et al 2003), and these microniches may harbour distinct groups of acidophilic, alkaliphilic, and neutrophilic microorganisms. Hydrocarbon solubility is affected by pH (Shin et al 2004), and increases from acidic to neutral pH have led to a doubling in the rate of hydrocarbon degradation, but the pH optima for hydrocarbon degradation have been shown to be compound-dependent (Leahy and Colwell 1990). Current bioremediation treatments such as soil turning and blanket nutrient amendments promote homogenization, potentially reducing microbial diversity, and promoting specific generalists that are adapted to the averaged conditions of the soil.

3.3.3. Differing abilities and functional redundancy

Even when several taxa are capable of degrading a particular compound, the rate at which each processes the compound often differs (Sorkhoh et al 1990, Whyte et al 1998, Obuekwe et al 2009, Ciric et al 2010), but it is not necessarily the most efficient hydrocarbon degraders that will be promoted naturally *in situ*, or that will be stimulated by bioremediation treatments, as microorganisms must compete with co-occurring taxa for space, water, and nutrients. Shifts in the dominant microorganisms in a contaminated soil may have limited effects on rates of bioremediation if these taxa are functionally redundant with respect to the degradation of a particular compound. For instance, the addition of root exudates to phenanthrene-contaminated soil led to *Pseudomonas* and *Arthrobacter* replacing *Pseudoxanthomonas* and *Microbacterium* as the dominant genera in the community, with no effect on rates of phenanthrene breakdown (Cébron et al 2011). On the other hand, microbial communities transplanted from one environment to another were shown to be less effective by up to 20% at carbon processing in their new environment (Strickland et al 2009), while similar transplant experiments have also shown that function is dependent on the existence of particular microbial communities (Keiser et al 2011).

Regardless of a species' potential for hydrocarbon degradation, observed activity can be severely limited by competition for space and resources in the soil environment. In a soil contaminated with the pesticide 2,4-dichlorophenoxyacetic acid (2,4-D), the addition of the known 2,4-D-degrading bacterium *Alcaligenes xylosoxidans* to undisturbed soil only marginally increased rates of degradation. However, when soils were inoculated with *A. xylosoxidans* 15 days after chloroform fumigation, 51.2% of 2,4-

D in the soils was degraded, as compared with 4.5% in the undisturbed but inoculated soils (Gunalan and Fournier 1993). Chloroform fumigation has been shown to alter community composition and reduce bacterial taxonomic and functional diversity (Degens 1998, Bonkowski and Roy 2005), and in this case, the number of colony-forming bacteria decreased by an order of magnitude (Gunalan and Fournier 1993). This demonstrates that interspecies competition may be a huge influential force on bioremediation, and that it is crucial to understand the contributions of individual taxa to hydrocarbon degradation and community dynamics.

3.4. Factors affecting the relative abundance of hydrocarbon degraders

Many studies have surveyed the diversity of hydrocarbon degraders in contaminated soils and have identified the most numerous taxa (e.g. Saul et al 2005, Labbé et al 2007, Pérez-de-Mora et al 2011), while others have identified active degraders through functional approaches such as stable isotope probing (Madsen 2006). It has been suggested that during bioremediation, as few as 20% of the species present may be responsible for 80% of the energy flux (Dejonghe et al 2001), but is that energy flux maximized when so many competing taxa are partitioning resources and space?

Although many studies have assayed functional genes in isolation (e.g. Wasmund et al 2009, Iwai et al 2010, Schulz et al 2010), the extent of horizontal gene transfer in hydrocarbon-contaminated soils is unknown, and the phenotype of microorganisms that carry these genes may be at least as important as the density and diversity of genes. Although the ability to laterally transfer genetic information distinguishes prokaryotes from most higher organisms, recent metagenomic studies suggest that discrete bacterial

species do exist (Caro-Quintero and Konstantinidis 2012), meaning that each must maintain a distinct phenotype that allows the occupation of a specific niche in a complex multi-species environment. In diesel-contaminated Arctic soils, bioremediation capacity was predictable by the relative abundance of certain bacterial taxa, and as in uncontaminated environments, it was the soil environment that selected for microbial dominance as opposed to innate hydrocarbon-degrading ability (Chapter 5; Bell et al submitted). This suggests that at least in some environments, there is potential for optimizing bioremediation if the most effective hydrocarbon degraders can be identified and their activity promoted.

Aside from key environmental parameters that are known to shape soil microbial communities (Chu et al 2010, Ramirez et al 2012, Chapter 5; Bell et al submitted), several factors may allow less effective hydrocarbon degraders to dominate soils following disturbance by hydrocarbons and subsequent bioremediation treatments. Some of these potential bottlenecks are discussed below.

3.4.1. Nutrient acquisition and use

The ability to efficiently acquire and use nutrients in the soil is critical, as nutrient availability is considered to be one of the most important factors that limits rates of bioremediation (Boopathy 2000). In culture, bacteria can adapt to many resource regimes without a decrease in fitness (Velicer and Lenski 1999), but this is unlikely to be the case in most natural environments, since each microbial species contends with so many competitors. Varying the concentration of nitrogen added to a soil contaminated with crude oil altered which microorganisms dominated, as well as the extent of aromatic and

aliphatic degradation (Chaîneau et al 2005). Similarly, the addition of nitrogen and phosphorous to a creosote-contaminated soil increased the degradation of 4-ring but not 3-ring aromatic compounds, although the microbial community was not examined in this study (Breedveld and Sparrevik 2000). Nitrogen concentration and the dominant form of nitrogen present correlated with the structure of the alkane-degrading community in hydrocarbon-contaminated Antarctic soils (Powell et al 2010), while the lowest of three nutrient concentrations was the most effective at promoting microbial activity in a soil contaminated with JP-5 fuel (Braddock et al 1997). Differential nitrogen assimilation by bacteria in hydrocarbon-contaminated soils has also been shown directly in a hydrocarbon-contaminated high Arctic soil (Chapter 4; Bell et al 2011). These results suggest that although certain consortia may be more effective at degrading certain hydrocarbon compounds, their relative abundance is likely governed by specific nutrient regimes. Nutrient addition may promote a higher abundance of hydrocarbon degraders, but the dominant microbial groups may not always be those that are most effective for the desired application.

The optimal resource conditions for a specific microorganism in a hydrocarbon-contaminated soil are likely to reflect its niche prior to the hydrocarbon disturbance. For instance, in aquatic systems, bacterial carbon processing was up to five times higher when carbon was presented in pulses as opposed to continuously (Lennon and Cottingham 2008). The authors suggested that many biotic systems have evolved to respond to feast-famine regimes, and that the pulsing of resources encourages faster rates of mineralization in bacteria. Ramirez et al. (2012) suggest that copiotrophic microorganisms that primarily use labile carbon sources are likely to rapidly and

positively respond to nutrient inputs, however, the copiotrophic strategy may be adopted by different taxa depending on the environment (Chapter 5; Bell et al submitted). A common garden soil experiment showed variation in organic matter decomposition between microbial communities depending on historical resource exposure, despite long periods of growth in common environments (Keiser et al 2011). How closely hydrocarbon contaminants resemble carbon sources that naturally occur in the soil, and whether those sources are present chronically or periodically, may affect the bioremediation capacity of locally adapted microorganisms. Soils in which local organisms are poorly adapted to the specific contaminants may be appropriate targets for bioaugmentation approaches.

3.4.2. Access to bioavailable hydrocarbon substrates

The relative success of hydrocarbon degraders also depends heavily on access to the hydrocarbon substrate. Early colonization of newly formed niches is one of the major methods employed by microorganisms to competitively exclude co-occurring species from essential resources (Hibbing et al 2010). High initial growth rate can affect which microbial species thrive and establish following disturbance (Haddad et al 2008), and initial colonization may be a difficult obstacle to overcome. In a study of two *Streptococcus* species that inhabit human oral cavities, early colonization of an area by one species was shown to exclude the other, while areas could be inhabited by both species when co-colonization occurred (Kreth et al 2005). Early colonization of growth substrates by fungi has been shown to diminish the ability of bacteria to compete over time (Mille-Lindblom et al 2006). At the same time, the fungi that grew most

successfully in the absence of bacteria were those that were most suppressed when faced with direct competition (Mille-Lindblom et al 2006), suggesting that competitive interactions can severely limit the realized niche of specific taxa.

In soils, not all hydrocarbons will necessarily be bioavailable to hydrocarbon-degrading microorganisms, as these contaminants may bind to organic matter and fine particles within the soil matrix (Loser et al 1999). Increasing the bioavailable surface area of hydrocarbons for colonization by microorganisms has been shown to increase rates of degradation (Ron and Rosenberg 2002). In addition, soil microorganisms are known to produce and transfer bioemulsifiers (Ron and Rosenberg 2002), suggesting that substrate accessibility may be a major limiting factor in the growth and activity of efficient hydrocarbon degraders. Chemotactic sensing of hydrocarbon contaminants has been identified in several bacterial species (Pandey and Jain 2002, Lanfranconi et al 2003, Lacal et al 2011), and although chemotaxis is widespread amongst microorganisms in some soils (Buchan et al 2010), it is unknown whether chemotactic sensing is used to aid certain microorganisms in quickly colonizing key hydrocarbon surfaces. With sufficient access to hydrocarbon substrates, cell-level solubilization and uptake of contaminants are likely rate limiting to rates of hydrocarbon degradation (Sekelsky and Shreve 1999), meaning that a microorganism that colonizes quickly but is inefficient at assimilating hydrocarbon compounds may represent a serious obstacle to effective bioremediation.

3.4.3. Antimicrobial production and resistance

To combat other disadvantages, microorganisms can produce antimicrobials or invest in antimicrobial resistance. Microbial warfare is known to occur commonly in a

variety of natural environments (Hibbing et al 2010), and competition can intensify following disturbance (Violle et al 2010), meaning that relatively higher resource investment in antimicrobial production and resistance may be required in hydrocarbon-contaminated soils. Analysis of isolates cultured from hydrocarbon-contaminated environments has shown that select bacterial strains, including dominant hydrocarbon degrading genera such as *Pseudomonas*, *Burkholderia*, and *Sphingomonas*, are resistant to a variety of antimicrobial compounds (Stapleton et al 2000a, Kaszab et al 2010). In environments contaminated with heavy metals, an increase in antibiotic resistance relative to uncontaminated equivalents has been observed (Alonso et al 2001); however, the extent of this activity in hydrocarbon-contaminated soils has not been assessed *in situ*.

Shifts in antimicrobial resistance gene expression can be assessed using microarrays and metatranscriptomic analysis (Yergeau et al 2010b, Yergeau et al 2012d), and could be used to determine how much microbial communities invest in competition following the addition of hydrocarbons and various bioremediation treatments. These data can also be linked with shifts in species abundance and other functional genes (Yergeau et al 2012d) in order to determine which microorganisms are most successful in competition. Genetic investment in antimicrobial resistance can also be assessed by quantifying resistance-carrying mobile elements (Hardwick et al 2008, Byrne-Bailey et al 2011), which would provide an estimate of how widely these adaptations are shared in contaminated soils. Soil antibiotic concentrations can also be quantified directly (e.g. Wu et al 2010), and potentially correlated with the presence and absence of specific microbial groups.

3.4.4. Differential selection by plants

The presence of plants in hydrocarbon-contaminated soils can affect the relative abundance of soil hydrocarbon degraders, due to their various influences on the soil rhizosphere. The presence of plants alters soil parameters when compared with unplanted bulk soil, including soil pH, organic matter content, and nutrient concentrations (Raynaud et al 2008, Lefrançois et al 2010). Root exudates likely play an important role in shaping soil microbial communities, although other forms of plant-derived carbon substrates may be equally important (Dennis et al 2010). Although phytoremediation is a popular bioremediation strategy for treating hydrocarbon-contaminated soils, its effects on degradation have been mixed (Gerhardt et al 2009).

In addition to modifying soil properties, the presence of plants adds a layer of structural complexity that can enhance environmental heterogeneity within a limited area. The concentration of alkane monooxygenase and naphthalene dioxygenase genes was shown to be 2 to 4 times higher in plant roots than in the surrounding soil in the presence of petroleum contaminants (Siciliano et al 2001). Similarly, other studies have shown that rhizosphere microbial communities in petroleum-contaminated soils are distinct from those in adjacent bulk soil (Kirk et al 2005, Lefrançois et al 2010). Although microbial community composition is plant-dependent, a higher diversity of plants tends to promote a higher diversity of microorganisms (Grüter et al 2006, Thion et al 2012).

The effects of plants on hydrocarbon degradation are varied. Phenanthrene degradation by *Pseudomonas putida* is reduced by the addition of plant root exudate compounds, although increased densities of *Pseudomonas* may still lead to increased rates of overall hydrocarbon degradation in the presence of plant exudates (Rentz et al 2004).

Nevertheless, hydrocarbon degradation rates in intact soil microbial communities have been inhibited by the addition of plant root exudates, with the extent of inhibition varying based on the plant species that produced the exudates (Phillips et al 2012). The microbial communities harboured by different plant species can vary in their potential for degrading aliphatic and aromatic hydrocarbon components (Phillips et al 2008). To further complicate matters, the microbial species that are able to colonize plant rhizospheres can vary in different soil types (Afzal et al 2011).

3.4.5. Identifying efficient hydrocarbon degraders

Assessing the hydrocarbon-degrading potential of microbial strains in culture is relatively straightforward, but determination of the most efficient hydrocarbon degraders *in situ* is far more challenging. This is due to a combination of technological limitations, and the many environmental and interspecies interactions that limit the function of individual microbial taxa in natural soil environments.

Stable isotope probing (SIP) is one of the more wide-spread techniques for assessing substrate assimilation at the community level, and has been used in a variety of bioremediation studies (Madsen 2006). The combination of SIP and metagenomics has the potential to reveal rare but active taxa, as well as functional genes from active microorganisms (Chen and Murrell 2010). One SIP study showed that dominant *Geobacter* species in a contaminated aquifer were not relevant to toluene degradation *in situ* (Pilloni et al 2011). SIP has also been used semi-quantitatively to determine the relative incorporation of added nitrogen by bacterial groups in hydrocarbon-contaminated soils following biostimulation. In this study, it was determined that *Alphaproteobacteria*

most effectively incorporated added nitrogen, despite the fact that they were not presumed to be the most effective hydrocarbon-degrading group at this site (Chapter 4; Bell et al 2011).

Several techniques are also available to evaluate substrate incorporation at the single cell level, once potentially relevant microbial groups have been identified. Combined microautoradiography and fluorescence *in situ* hybridization (MAR-FISH) uses radioactively-labeled substrates and fluorescent-labeled taxonomic probes to visualize substrate incorporation by target taxonomic groups. This technique has been successfully used to show that roughly 70% of the biomass in a diesel-degrading culture was not directly relevant to degradation, and that the less common *Alpha-* and *Betaproteobacteria* rather than the dominant *Gammaproteobacteria* contributed most to diesel catabolism (Hesselsoe et al 2008). It has also been used to show partitioning of hydrocarbon substrates between groups of *Rhodocyclales* in activated sludge (Hesselsoe et al 2009). Raman-FISH replaces radioactive substrates with stable isotopes and has been used in contaminated groundwater to observe naphthalene assimilation by *Pseudomonas* populations at the single-cell level (Huang et al 2007), and has been combined with RNA-SIP to show that an unculturable *Acidovorax* sp. assimilated naphthalene at concentrations that *Pseudomonas* isolates did not (Huang et al 2009). Innovations on these techniques are emerging, including the extremely high-resolution nanoSIMS technique (Musat et al 2012). Combining these techniques with manipulations of contaminated soil environments should help in distinguishing dominant from efficient hydrocarbon degraders.

3.5. Actual vs. potential hydrocarbon-degrading activity

If efficient hydrocarbon degraders are identified in a contaminated soil, potential bioremediation may be high, while observed activity in culture or in soil may be substantially lower (Figure 3.2.). In the soil environment especially, microbial species are likely to incur damage to cellular components and nucleic acids as a result of interspecies interactions and abiotic stressors (Konopka and Wilkins 2012), leading to trade-offs in resource allocation. Induction of hydrocarbon-degrading pathways may depend on specific environmental signals, and the breakdown of some compounds may require cometabolic associations with other organisms. When compared with efficient hydrocarbon degraders, co-occurring microorganisms will either be mutualists that contribute to their success, commensals that co-exist but have limited influence on their activity, or competitors, predators or parasites that inhibit their effectiveness (Little et al 2008).

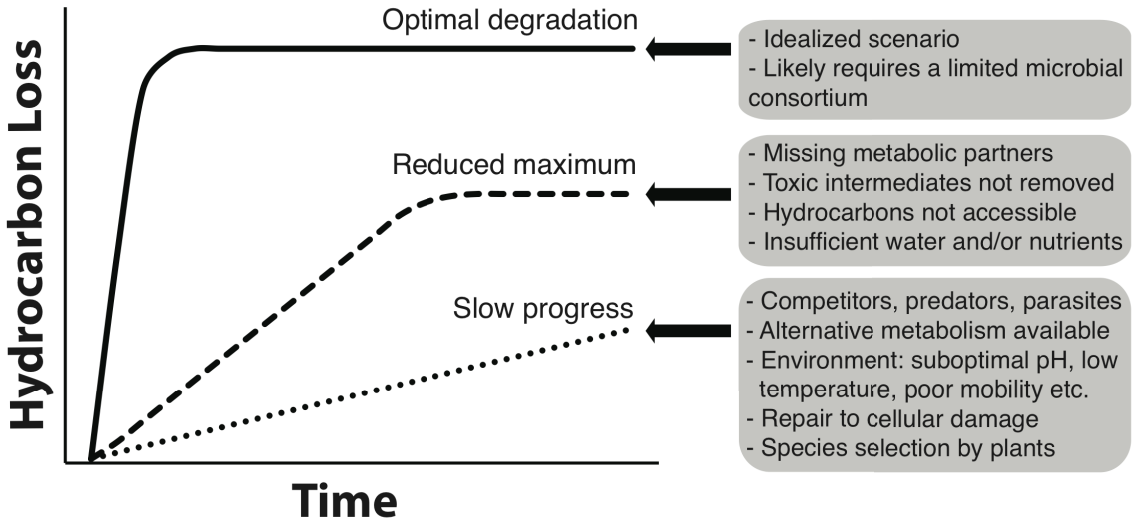

Figure 3.2. Hypothetical hydrocarbon degradation by a soil microbial community under ideal conditions, or under two scenarios that may occur both in culture and natural soil environments: 1) reduced maximum degradation, and 2) slow progress. Factors that can contribute to these scenarios are shown in the right-hand boxes, and are potential targets for optimizing bioremediation treatments.

Remaining active in hydrocarbon-contaminated soils does not necessitate using hydrocarbons as an energy source. Many of the varied forms of metabolism that supported microbial life prior to the hydrocarbon disturbance may persist in contaminated soils, diverting microbial activity and resources. This would be especially true in soils that are chronically exposed to substantial amounts of natural hydrocarbons, as they are likely to be occupied solely by microorganisms that are adapted to hydrocarbon toxicity, and possibly to using alternative energy sources in the presence of hydrocarbons. Hydrocarbon toxicity tolerance mostly requires avoiding negative interactions between these contaminants and the cell wall (Ramos et al 2002, Kang and Park 2010). Suggested mechanisms of survival include producing exopolysaccharides to adsorb contaminants, altering cell membrane structure, compartmentalization of hydrocarbons in vesicles, and creation of energy-requiring efflux pumps to expel unwanted compounds from the cytosol (Ramos et al 2002, Kang and Park 2010). Such adaptations must be widespread given the diversity of microorganisms that have been identified in hydrocarbon-contaminated soils (Chapter 4; Bell et al 2011, Chapter 5; Bell et al submitted).

3.5.1. Other forms of metabolism

Several studies have identified microorganisms in hydrocarbon-contaminated environments that actively metabolize compounds other than hydrocarbon contaminants. Autotrophic nitrifiers actively oxidized ammonia in the presence of hydrocarbons, despite potential competitive inhibition by hydrocarbons (Deni and Penninckx 1999). Other autotrophs have also been identified, as active cyanobacterial mats can harbour oil-degrading bacteria on top of marine oil slicks (Sorkhoh et al 1995), while soil microalgae

have been shown to grow actively in the presence of hydrocarbon contaminants, with the growth of some species stimulated at certain concentrations (Megharaj et al 2000). Anammox bacteria have been detected in a hydrocarbon-contaminated estuary (Hu et al 2012), but have yet to be identified in hydrocarbon-contaminated soils.

Despite having the capacity to degrade hydrocarbons, heterotrophs may opt for other soil carbon sources. Bacterial diversity declined following biostimulation in low organic matter soils, but remained high in soils containing more than 10% organic matter (Chapter 5; Bell et al submitted), suggesting that certain species that would otherwise be outcompeted are able to continue to survive on these varied carbon substrates. This decline in diversity was not observed in the absence of added nutrients, suggesting that the effect is not attributable to reduced toxicity in high organic matter soils as a result of increased contaminant sorption. The inhibition of hydrocarbon-degrading activity by adding plant root exudates indicates that some rhizosphere-associated bacteria may focus on these alternative carbon sources (Rentz et al 2004, Phillips et al 2012). Since microbial communities have evolved to use carbon substrates that are consistently available in their local environment, energy and carbon extraction from these sources may be more energetically favourable, thus promoting their use if they are present. Substantial carbon cross-feeding from hydrocarbon degraders has been observed (DeRito et al 2005), possibly limiting rates of bioremediation if the cross-feeding population sequesters resources that could benefit active degraders. On the other hand, heterotrophs that do not primarily degrade contaminants may benefit bioremediation if they can quickly process toxic metabolite intermediates that would otherwise limit reaction rates (Pelz et al 1999).

3.5.2. Predators and parasites

Microbial predators can affect rates of hydrocarbon degradation, although most studies have been conducted in culture or aquatic systems. However, the soil flagellate *Heteromita globosa* has been shown to be an important regulator of degradation in batch culture (Mattison and Harayama 2001, Mattison et al 2005), and a handful of studies have demonstrated that protists can be present in equal or greater numbers in soils following contamination (Stoeck and Edgcomb 2010). Predation in hydrocarbon-degrading communities has actually been shown to promote hydrocarbon mineralization in some cases (Rogerson and Berger 1983, Mattison and Harayama 2001, Mattison et al 2005, Tso and Taghon 2006). Predators may alter species composition by selectively consuming certain microbial species (Tso and Taghon 2006, Worsfold et al 2009), and have even been shown to increase overall bacterial biomass (Worsfold et al 2009), which may augment or limit hydrocarbon-degrading activity depending on which microorganisms are targeted. Selection of prey species in an aromatic-degrading community was dependent mainly on cell size, protein content, and hydrophobicity (Tso and Taghon 1999). Predation can also stimulate microbial growth and activity rates (Kinner et al 2002, Mattison et al 2005).

Parasitic biological agents may also grow at the expense of efficient hydrocarbon degraders. Phage are among the most common biological units on Earth (Fuhrman 1999), and have been shown to suppress microbial biomass and activity in an Arctic soil (Allen et al 2010a). Phage are known to rapidly coevolve with soil bacterial populations (Gómez and Buckling 2011), suggesting that they could continue to influence microbial communities following species reorganizations induced by hydrocarbon disturbance. On

the other hand, high phage density in a bioreactor treating wastewater from crude oil extraction was thought to have played an important role in high rates of carbon mineralization (Rosenberg et al 2010), possibly due to the mechanisms discussed previously for predators. Bacteria have also been shown to parasitize co-occurring microorganisms. The predatory bacterium *Bacteriovorax* spp. has been identified in hydrocarbon-contaminated marine samples (Gertler et al 2009), while a number of soil bacteria have been shown to grow using only arbuscular mycorrhizal fungal mycelia as a food source (Lecomte et al 2011). Some microorganisms may also remain active by "cheating", assimilating the end-products of degradation without investing degradative enzymes of their own. Cheating has been demonstrated in groups of toxin-producing bacteria (Jousset et al 2009), and is also predicted to exist in the breakdown of complex hydrocarbons (Allison 2005, Modak et al 2007, Schuster et al 2010). In the case of hydrocarbon degradation, this may mostly represent cross-feeders that use secondary metabolites (DeRito et al 2005). Such cheating activity may limit the activity of hydrocarbon degraders, as they do not receive the full energetic payoff from their investment in breaking down complex hydrocarbons.

3.5.3. Quantifying hydrocarbon-degrading activity

Quantification of the active investment in hydrocarbon degradation by microbial communities *in situ* may help in determining the extent to which bioremediation can be enhanced. Several studies have used RT-qPCR to quantify transcript levels of oxygenases that are known to be involved in the initial stages of hydrocarbon catabolism (Cunliffe et al 2006, Yergeau et al 2009, Yergeau et al 2012c). Similarly, a recent

metatranscriptomic analysis of a soil contaminated with phenanthrene showed an increase in transcripts coding for a variety of enzymes involved in the degradation of polycyclic aromatics relative to the uncontaminated control (de Menezes et al 2012). The metatranscriptome also showed that most of these transcripts could be linked to *Actinobacteria* (de Menezes et al 2012), and future studies combining metatranscriptomics and metagenomics will be able to determine whether transcript:gene ratios are equivalent across potential hyxdrocarbon-degrading groups. The advent of high-throughput SIP-proteomic technologies will allow comparisons between transcript and protein abundance (Pan et al 2011).

The environmental factors that affect the transcriptional regulation of key genes have not been thoroughly studied *in situ*. The regulation of transcription is likely complex, as environmental microorganisms integrate a wide array of signals in order to adapt to highly specific niches (Cases and de Lorenzo 2005). It is known, however, that the presence of certain labile carbons can inhibit the promoters that affect the expression of genes involved in the degradation of more recalcitrant compounds, and that both oxygen concentration and microbial growth rate play roles in promoter repression (Cases and de Lorenzo 2005). The presence of co-contaminants may also affect microbial expression, either through direct interference with expression, or by posing further challenges to which microorganisms must adapt. Cadmium and mercury had no effect on polycyclic aromatic hydrocarbon degradation by soil microbial communities, but did inhibit the production of ligninolytic enzymes by fungi (Baldrian et al 2000). Catabolic gene expression of various microorganisms may be differentially promoted and repressed

depending on environmental conditions, so it is important to identify which taxa should ideally be most active.

3.6. Promotion and suppression of taxonomic groups

With the exception of studies that have tested bioaugmentation as a bioremediation strategy, the direct manipulation of microbial species composition in hydrocarbon-contaminated soils has not been thoroughly investigated. Since soil physico-chemical parameters appear to influence microbial species dominance more than hydrocarbon-degrading ability (Chapter 5; Bell et al submitted), appropriate rearrangement of the relative abundance of species is likely to increase rates of bioremediation (Dejonghe et al 2001). Some microbial ecology studies have directly manipulated species diversity and relative abundance, and this has been shown to have direct impacts on community function and productivity (Table 3.1.).

Table 3.1. Studies in which microbial diversity and/or composition was directly manipulated.

Manipulation	*Factors altered*	*Experimental matrix*	*Reference*
Added various numbers of bacteria cultured from tree rainpools	Community diversity and respiration	Phosphate buffer + sterile beech leaf microcosms	Bell et al. 2005
Dilution to extinction	16S rRNA diversity, chitinase diversity, chitin and cellulose degradation	Lake water microcosms	Peter et al. 2011
Added varying concentrations of fungi and bacteria	Fungal:Bacterial ratio and biomass	Liquid media	Mille-Lindblom et al. 2006
Selective introduction of cultured micropredators	Species composition and microbial biomass	Liquid media	Worsfold et al. 2009

Selective introduction of bacterial isolates and a micropredator	Bacterial density, micropredator abundance, decomposition of organic matter	Liquid media	Jiang 2007
Selective introduction of bacterial isolates	N_2O and CO_2 production	Liquid media	Salles et al. 2009
Added enrichments of PAH-degrading bacteria	Initial counts of added species but not final counts	Liquid media	van Herwijnen et al. 2006
Added various soil inocula to different types of sterile leaf litter	Community composition and CO_2 production	Sterile leaf litter	Strickland et al. 2009
Added enrichments of indigenous bacteria as a bioaugmentation treatment	Initial counts of added species, but not final counts	Soil microcosms and *in situ* biopiles	Thomassin-Lacroix et al. 2002
Used chloramphenicol and cycloheximide to suppress bacteria and fungi respectively	NO_3^- and NH_4^+ assimilation	Soil slurries	Myrold and Posavatz 2007
Used cycloheximide to suppress fungi	N_2O production and soil NO_3^- availability	Soil microcosms	Siciliano et al. 2009
Chloroform fumigation	PLFA diversity, respiration, straw mineralisation	Soil microcosms	Degens 1998
Chloroform fumigation	PLFA and CLPP diversity, CFUs, DGGE banding, microfauna diversity, microbial biomass, N mineralization, thymidine and leucine incorporation, respiration, ryegrass decomposition, stability following Cu addition	Soil microcosms	Griffiths et al. 2000
Chloroform fumigation	DGGE of DNA and RNA, functional stability of decomposition following heat and Cu addition	Soil microcosms	Griffiths et al. 2004
Dilution to extinction	*nirA* and *norG* DGGE banding, denitrification and NO_2^- oxidation after heat disturbance	Soil microcosms	Wertz et al. 2007
Soil fumigation and filtering	Plant biomass production, microbial biomass, respiration, CLPP diversity		Bonowski and Roy 2005
Added AMV (amoxicillin, metronidazole, bismuth) or cefoperazone to suppress gut community	16S rRNA diversity	Murine gut	Antonopoulos et al. 2009
Added streptomycin or vancomycin to suppress gut community	FISH and plating diversity, susceptibility to infection	Murine gut	Sekirov et al. 2008
Added vancomycin to suppress gut community	16S rRNA diversity	Murine gut	Robinson and Young 2010

The effects of biodiversity on ecosystem productivity are variable (Bullock et al 2001, Fargione et al 2007, Doherty et al 2011), and several studies on microbial communities have shown that the presence or absence of specific phylotypes is more important than the overall number of species in determining functional capacity (Salles et al 2009, Strickland et al 2009, Peter et al 2011). The selective introduction of cultured isolates has been used to assess the importance of microbial diversity and of specific strains (Bell et al 2005, Jiang 2007, Salles et al 2009, Worsfold et al 2009), but this inevitably excludes many potentially important and as yet unculturable species. Instead, direct manipulation of taxonomic abundance within soil may lead to a better understanding of the interactions between key microbial groups.

3.6.1. Promotion of species

Although bioaugmentation has briefly increased the density and activity of specific hydrocarbon degraders, it has ultimately had little effect on bioremediation over longer time scales (Thomassin-Lacroix et al 2002, van Herwijnen et al 2006). The careful selection of strains suited to particular environments may improve the survival of introduced microorganisms (Thompson et al 2005), but the fact that hydrocarbon degraders are so widespread (Greer et al 2010), and that similar bacterial communities appear in geographically disparate but physically comparable environments (Chu et al 2010, Ramirez et al 2012, Chapter 5; Bell et al submitted) suggests that dispersal does not limit the presence of many microbial taxa. On the other hand, fungi and protists are thought to be more limited in their dispersal abilities than bacteria (Foissner 2006), so the

introduction of key eukaryotic groups may have a more lasting influence on bioremediation.

The conditions under which bioaugmented strains are cultivated may also impact their eventual success. The use of complex media in the pre-culturing of microorganisms has increased hydrocarbon-degrading activity in the soil environment (Cunliffe et al 2006). Similarly, pre-exposure to site environmental conditions may allow better establishment when strains are added to soil. The inoculation of contaminated soils with soils engaged in active bioremediation may help in quickly establishing an efficient bioremediating community, but it is unknown whether this would affect ultimate rates of hydrocarbon breakdown.

3.6.2. Suppressing competitors

Among higher organisms, the loss of key species that maintain ecological equilibrium has led to a rapid depletion of ecological resources (Beschta and Ripple 2009), which is precisely the goal in bioremediation. As mentioned earlier, the chloroform fumigation of soils allowed introduced *Alcaligenes* to more efficiently degrade 2,4-D, possibly due to the removal of competitors (Gunalan and Fournier 1993). Other studies have used varying degrees of chloroform fumigation and filtration to eliminate susceptible groups within the microbial community (Degens 1998, Griffiths et al 2000, Griffiths et al 2004, Bonkowski and Roy 2005), or have used various dilutions of the initial microbial community to reinoculate sterile soil, leading to chance extinctions of microbial taxa at the lowest dilutions (Wertz et al 2007, Peter et al 2011). These manipulations have had mixed effects on microbial function. Since next-generation

sequencing now enables thorough community profiling for limited cost, similar experiments could now be used to correlate specific soil microbial taxa with the efficient degradation of hydrocarbon compounds.

Other strategies for more targeted alterations of microbial abundance and activity have recently been explored. Many microorganisms possess the ability to combat competing species by producing anti-microbial compounds (Little et al 2008, Allen et al 2010b, Hibbing et al 2010), and the addition of group-specific antimicrobials could allow reproducible and targeted experiments that assess the hydrocarbon-degrading capacity of distinct taxa. A recent gut microbiology study used vancomycin, an antibiotic that largely targets Gram-positive bacteria, to alter community diversity without significantly decreasing microbial biomass (Robinson and Young 2010). Vancomycin has been used to the same end in other studies, along with streptomycin, AMV, and cefaperazone, with comparable results (Sekirov et al 2008, Antonopoulos et al 2009). Fungi and bacteria have often been shown to engage in antagonistic relationships (Mille-Lindblom et al 2006, Meidute et al 2008, Lecomte et al 2011), and cycloheximide and chloramphenicol have also been used in soil to inhibit fungi and bacteria, respectively (Myrold and Posavatz 2007, Siciliano et al 2009). Although it is unlikely that any one antibiotic will inhibit every member of a taxonomic group, major shifts in taxonomic abundance can still help to reveal the effects of individual taxa on hydrocarbon degradation.

As opposed to the direct suppression of microbial growth, the suppression of specific activities may help in quantifying the contributions of metabolic pathways to bioremediation. This has been used previously to determine the effects of nitrification (Bremner et al 1986, Powell and Prosser 1986, Deni and Penninckx 1999), nitrogen

assimilation (Myrold and Posavatz 2007), denitrification (Bremner et al 1986, Bremner and Yeomans 1986, Yeomans and Bremner 1986), and sulfate reduction (Winfrey and Ward 1983) on the nutrient dynamics in soils and sediments. The inhibition of methanogenesis, methane oxidation, and various monooxygenases involved in hydrocarbon degradation has also been demonstrated (Hamamura et al 1999, Chan and Parkin 2000, Keener et al 2001, Mahendra and Alvarez-Cohen 2006). In the future, more specific gene inactivation may be possible, as RNA external guided sequences have been used in culture to inhibit the expression of targeted mRNA sequences (Shen et al 2009), and may eventually be adapted for use in natural environments.

3.7. Conclusions

To date, much of the work on the characterization of microbial communities in hydrocarbon-contaminated environments has focused on the identification and quantification of hydrocarbon-degrading microorganisms and genes. However, the complexity of soil environments may uncouple potential hydrocarbon-degrading activity from the actual *in situ* function of individual microorganisms. Furthermore, the vast majority of soil microorganisms are known only from sequence data, and many of the complex interactions between species are difficult to replicate in culture. Determining the contributions of individual taxa through the use of techniques that assess functional contributions of microbial groups and individual cells *in situ*, and by promoting or inhibiting specific microorganisms, will eventually pinpoint which key taxa should be stimulated by bioremediation treatments.

3.8. Acknowledgements

This work is supported by a National Sciences and Engineering Research Council of Canada grant to TH Bell.

Connecting Text

In Chapter 3, the potentially complex ecology of microorganisms in hydrocarbon-contaminated soils was outlined. Within this section, it was concluded that competition for space and resources could limit the activity of the most efficient hydrocarbon-degrading microorganisms. One main resource is nutrients, and in the subsequent chapter, a study is described in which a novel stable isotope probing approach was used to determine whether bacteria vary in how effectively they assimilate added nitrogen in uncontaminated and hydrocarbon-contaminated soils from CFS-Alert, Nunavut.

4

Identification of nitrogen-incorporating bacteria in petroleum-contaminated Arctic soils by using [^{15}N]DNA-based stable isotope probing and pyrosequencing

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Published in: *Applied and Environmental Microbiology*, June 2011. **77**: 4163-4171, doi: 10.1128/ AEM.00172-11.

+ selected for cover photo of following issue

This chapter was written by the candidate, with helpful comments provided by all co-authors. The candidate also designed and executed the experiment, and performed the data analysis. Dr. Christine Martineau and Dr. Etienne Yergeau were invaluable in experimental design and troubleshooting, while Dr. Yergeau also provided helpful direction for data analysis.

4.1. Abstract

Arctic soils are increasingly susceptible to petroleum hydrocarbon contamination, as exploration and exploitation of the Arctic increase. Bioremediation in these soils is

challenging due to logistical constraints and because soil temperatures only rise above 0°C for ~2 months each year. Nitrogen is often added to contaminated soil *in situ* to stimulate the existing microbial community, but little is known about how the added nutrients are used by these microorganisms. Microbes vary widely in their ability to metabolize petroleum hydrocarbons, so the question becomes: which hydrocarbon-degrading microorganisms most effectively use this added nitrogen for growth? Using [^{15}N]DNA-based stable isotope probing, we determined which taxonomic groups most readily incorporated nitrogen from the monoammonium phosphate added to contaminated and uncontaminated soil in Canadian Forces Station-Alert, Nunavut, Canada. Fractions from each sample were amplified with bacterial 16S rRNA and alkane monooxygenase B (*alkB*) gene-specific primers and then sequenced using large-scale parallel-pyrosequencing. Sequence data was combined with 16S rRNA and *alkB* gene quantitative PCR data to measure the presence of various phylogenetic groups in fractions at different buoyant densities. Several families of *Proteobacteria* and *Actinobacteria* that are directly involved in petroleum degradation incorporated the added nitrogen in contaminated soils, but it was the DNA of *Sphingomonadaceae* that was most enriched in ^{15}N. Bacterial growth in uncontaminated soils was not stimulated by nutrient amendment. Our results suggest that nitrogen uptake efficiency differs between bacterial groups in contaminated soils. A better understanding of how groups of hydrocarbon-degraders contribute to the catabolism of petroleum will facilitate the design of more targeted bioremediation treatments.

4.2. Introduction

Soils in the Arctic are increasingly vulnerable to contamination by hydrocarbons as exploration and exploitation of Arctic resources becomes more technically feasible. While hydrocarbon spills are commonly degraded year-round by naturally occurring soil bacteria at lower latitudes, the remediation of high Arctic soils is limited to about 2 months of summer where soil temperatures rise above 0°C. Certain psychrophilic bacteria remain at least somewhat active over parts of the winter, since microbial respiration has been recorded at temperatures as low as -15°C (Steven et al 2007), and yet most significant hydrocarbon degradation is known to occur above 0°C (Walworth et al 2001). As a result, bioremediation treatments that make optimum use of the short summer are highly desirable. In addition, logistical constraints generally limit the treatment of contaminated Arctic soils to what can be easily transported or found on site. An *in situ* treatment that is commonly applied to hydrocarbon-contaminated polar soils is biostimulation (Aislabie et al 2006), the addition of nutrients to soil in an attempt to promote the growth and activity of nutrient-limited bacteria. Although this generally increases the rates of hydrocarbon breakdown, the degree to which this occurs varies between sites (Powell et al 2006a, Delille and Coulon 2008, Yergeau et al 2009). An understanding of how these nutrients are used within the soil microbial community will help in developing treatments that optimize hydrocarbon bioremediation over the Arctic summer.

The nitrogen content of Arctic soils is generally low (Shaver and Chapin 1980, Martineau et al 2010), and nitrogen has been shown to be a major limiting factor in Arctic bioremediation (Mohn and Stewart 2000), but biostimulation studies have not specifically

examined which organisms are active in incorporating added nutrients. Nitrogen-based fertilizers are applied as blanket treatments to contaminated soil, but not all microorganisms have the same capacity for nutrient assimilation and subsequent growth. This may be due to differences in nitrogen uptake mechanisms or to competition from co-occurring species (Leigh and Dodsworth 2007, Hibbing et al 2010). Bacteria also vary in the rate and efficiency with which they degrade alkane substrates (Sorkhoh et al 1990, Whyte et al 1998, Obuekwe et al 2009), so an understanding of which species are most effectively incorporating added nutrients in contaminated Arctic soils will help in determining whether these treatments can be further optimized for more rapid mineralization of hydrocarbons.

Stable isotope probing (SIP) is a popular approach for characterizing microbial activity *in situ*. While large-scale sequencing has provided an unprecedented view of microbial diversity, other approaches are still required to determine which microorganisms are active under various environmental conditions. Many studies have used DNA-SIP to examine uptake of carbon sources in environmental samples (e.g. Antony et al 2010, Martineau et al 2010, Winderl et al 2010), and a few have used it to look at nitrogen uptake (Buckley et al 2007b, Buckley et al 2008, Wawrik et al 2009). A recent review (Chen and Murrell 2010) recommends the use of large-scale sequencing of SIP fractions to determine the activity of less-abundant species, since traditional SIP approaches using denaturing gradient gel electrophoresis and clone libraries tend to only identify the major substrate consumers (Antony et al 2010, Winderl et al 2010). While [^{15}N]DNA-based SIP (^{15}N-SIP) is often complicated by a small degree of DNA separation between labeled and unlabeled DNA molecules (0.016 g ml^{-1} for 100% ^{15}N-labeled DNA

compared to 0.036 g ml^{-1} for 100% ^{13}C-labeled DNA (Buckley et al 2007a)), mass sequencing provides large amounts of sequence data that make enrichment visible even in the presence of high levels of background DNA.

In the present study, we added ^{14}N- and ^{15}N-labeled monoammonium phosphate (MAP) to hydrocarbon-contaminated and uncontaminated Arctic soils, separated DNA by weight in CsCl gradients, and amplified 16S rRNA and *alkB* genes from DNA isolated from several different buoyant density ranges. These amplicons were sequenced, and the ^{14}N- and ^{15}N-amended samples were compared among CsCl fractions with equal densities to determine the degree of ^{15}N enrichment for various phylogenetic groups. While cross-feeding is considered a drawback in studies that look only to identify a primary consumer (Neufeld et al 2007), we were interested in identifying all bacteria that used added nitrogen to facilitate growth during the incubation. We found that a wide diversity of bacteria incorporated nitrogen in the contaminated soils, but the degree of ^{15}N enrichment varied greatly between taxonomic groups, suggesting that some bacteria are benefiting more from the addition of nitrogen than others.

4.3. Methods

4.3.1. Site description

Samples were treated and incubated on site at Canadian Forces Station (CFS)-Alert, on the northern tip of Ellesmere Island, Nunavut, in the Canadian High Arctic. CFS-Alert (82°31'N, 62°17'W) is the northernmost permanent human settlement in the world and is located at an elevation of 30.5 m. The annual precipitation averages 153.8 mm, while the average daily temperature is -18.0°C. The average annual daily maximum

is -14.7°C, and the average annual daily minimum is -21.3°C (http://www.climate.weatheroffice.ec.gc.ca/index.html). The contaminated soil that was used in the present study experienced a large JP-8 fuel spill in 1999 from a ruptured pipeline. JP-8 fuel has a maximum allowance of 25% aromatic compounds by volume, while the remainder consists of aliphatic hydrocarbons (https://assist.daps.dla.mil/quicksearch/basic_profile.cfm?ident_number_33505). The soil in the area is naturally unvegetated, contains many colluvial blocks and rubble, and is dominated mainly by shale. Uncontaminated soil was taken from an adjacent area outside of the spill site that was similarly unvegetated.

4.3.2. Collection of samples, *in situ* incubation, and soil analyses

A composite soil sample was collected from multiple locations within the delineated fuel spill, and a second sample was collected from the adjacent uncontaminated soil. Each sample was separated into three: one portion was left untreated, and the other two were amended with either ^{14}N-labeled (Tri-County Agromart, Trenton, Ontario, Canada) or ^{15}N-labeled (Icon Services, Inc., Mt. Marion, NY) MAP at a concentration of 250 mg/kg of soil. Our lab previously found that MAP was the most effective nitrogen-based fertilizer for promoting bioremediation at our site (Greer 2009). Each treatment was separated into three 50-g subsamples, which were placed inside 50 ml Falcon (BD, Franklin Lakes, NJ) tubes. These were incubated at ambient conditions *in situ* with open tops between 14 July and 17 August 2009. The soil temperature was measured throughout this time period with an iButton temperature probe (Maxim, Sunnyvale, CA). After the incubation, the Falcon tubes were sealed and returned to the

lab on ice. The soil water content was determined by drying soil overnight at 100°C, while the total soil carbon was determined by mass loss after combustion at 600°C. Samples incubated in parallel were sent to Maxxam Analytics (Montreal, Quebec, Canada), where soil was analyzed for pH, total N, and F1-F4 hydrocarbons according to the protocol set forth by The Canadian Council of Ministers of the Environment (http://www.maxxam.ca/solutions/sol_env_CCME_Petr_Hydroc_0805.pdf).

4.3.3. DNA extraction and ultracentrifugation in CsCl gradients

Prior to extraction, soil was prewashed based on the protocol of Fortin et al. (2004). Total soil DNA was then extracted from 10 g subsamples from each replicate by using MoBio DNA PowerSoil isolation kits (MoBio Laboratories, Carlsbad, CA). CsCl gradient fractionation, DNA precipitation, and DNA quantitation were carried out as described previously (Buckley et al 2007b, Buckley et al 2007a). In brief, equal amounts of DNA from each replicate were combined within each treatment (a total of 4 μg for each contaminated soil treatment and 2 μg for each uncontaminated soil treatment), and the volume was adjusted to 0.45 ml with TE buffer (50 mM TRIS-HCl, 15 mM EDTA [pH 8.0]) and added to 13-by-51-mm polyallomer Quick-Seal centrifuge tubes (Beckman, Fullerton, CA), along with 4.3 ml of 1.762 g of CsCl ml^{-1} in gradient buffer (15 mM TRIS-HCl, 15 mM KCl, 15 mM EDTA [pH 8.0]). The tubes were spun for 65 h at 46,000 rpm (168,500 x *g* maximum) in a Vti80 rotor (Beckman), and the resulting gradient was collected in 100 μl fractions from the bottom of each tube. The buoyant density of each fraction was determined by measuring 15 μl volumes from each sample on a Reichert AR200 refractometer (Depew, NY). Buckley et al. (2007a) used an

electrical tape mask to measure 5 µl volumes on this model, and our trials showed that even without a mask, volumes as small as 5 µl produced refractive index values that were equivalent to those produced by 100 µl volumes when samples were placed centrally on the refractometer prism. Each fraction was ethanol precipitated, and DNA-density profiles were calculated for each treatment by using the PicoGreen dsDNA quantitation assay (Invitrogen, Carlsbad, CA).

4.3.4. PCR of selected density ranges and amplicon pyrosequencing

Based on the PicoGreen DNA profiles (Figure 4.1.), equal density ranges of 0.006 to 0.010 g ml^{-1} were selected from ^{14}N- and ^{15}N-incubated samples (i.e., samples that had been incubated with ^{14}N and ^{15}N, respectively). As mentioned above, 100% ^{15}N-labeled DNA would have a buoyant density 0.016 g ml^{-1} more than identical but unlabeled DNA. Each density range was amplified separately with both 16S rRNA and *alkB* gene primers containing unique multiplex identifier (MID) tags. We used MID-1 through MID-34 from the extended MID set recommended by Roche Diagnostics (Roche 2009). Each forward primer began at the 5' end with the Primer A-key (5'-CGTATCGCCTCCCTCGCGCCA-3'), followed by the library key sequence (5'-TCAG-3'), the appropriate MID sequence, and finally the template-specific sequence. Reverse primers were similarly designed, except that the Primer-A key was replaced with the Primer B-key (5'-CTATGCGCCTTGCCAGCCCGC-3'), and no MID sequences were added. The template-specific sequences used in 16S rRNA gene amplification were the forward primer Univ-9F (5'-GAGTTTGATYMTGGCTC-3') and the reverse primer BR534/18 (5'-ATTACCGCGGCTGCTGGC-3') (Wilmotte et al 1993), while the *alkB*

genes were amplified by using the degenerate primer set of *alkB*-1f (5'-AAYACNGCNCAYGARCTNGGNCAYAA-3') and *alkB*-1r (5'-GCRTGRTGRTCNGARTGNCGYTG-3') developed by Kloos et al. (2006). Reactions were carried out in 50-µl volumes containing 1 µl of template DNA, either 40 pmol of each 16S rRNA gene or 80 pmol of each *alkB* primer, 8 µl of 1.25 mM deoxynucleoside triphosphates, 1 mM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase in 5 µl of the 10x *Taq* DNA polymerase buffer provided (GE Healthcare, Baie d'Urfe´, Canada). Cycling conditions for 16S rRNA gene amplifications involved an initial 5 min denaturing step at 95°C, followed by 35 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and a final elongation step of 15 min at 72°C. Cycling conditions for the *alkB* primers were identical, except that the annealing temperature began at 65°C, and was lowered by 1°C in each of the first 10 cycles. PCR products were pooled in an equimolar ratio, except that 10 times less product was added from each *alkB* gene amplification than was added from each 16S rRNA gene amplification. The pooled sample was then analyzed on a Roche/454 GS FLX sequencer using Titanium chemistry at the DNA Sequencing Facility at the University of Pennsylvania. Only 1/8 of a plate was used, since this was sufficient for the desired sequencing depth.

4.3.5. qPCR

For each density range, quantitative real-time PCR (qPCR) was performed to determine relative 16S rRNA and *alkB* gene copy numbers. These values were combined with sequencing results to yield relative numbers for each taxonomic group. All qPCRs were performed in 20-µl volumes using the SYBR green QuantiTect PCR mix (Qiagen,

Mississauga, Ontario, Canada) in a Rotor-Gene 3000 apparatus (Corbett Life Science, Sydney, New South Wales, Australia). Bacterial 16S rRNA gene copy numbers were assessed with the Eub338/Eub518 primer set described by Fierer et al. (2005), and conditions were as described in Yergeau et al. (2010a). Real-time qPCR of *alkB* genes was performed with the primers described above but did not contain the additional adaptor, library, or MID sequences, and cycling conditions were as described above, with the addition of 15 cycles. Standards were made from 10-fold dilutions of linearized plasmids containing the gene fragment of interest that was cloned from amplified pure culture DNA (16S rRNA gene, $R^2 = 0.992$; *alkB*, $R^2 = 0.994$).

4.3.6. Analysis

Differences in mean soil carbon and extracted DNA were statistically analyzed by one-way analysis of variance and Tukey's *post hoc* test. Sequence data were analyzed mainly by using the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/). The sequences were then deconvoluted and binned according to their MID tags, and the MID and forward primer were trimmed by using the Pipeline Initial Process tool. This resulted in 32 distinct datasets (16 16S rRNA gene datasets and 16 *alkB* datasets, each with 4 density ranges x 2 isotope labels [^{14}N and ^{15}N] x 2 soil types [contaminated and uncontaminated]). Datasets containing MID sequences associated with the 16S rRNA gene amplifications were individually classified using the RDP Classifier tool with an 80% bootstrap cutoff. Datasets derived from *alkB* gene amplifications were compared to a BLAST database made from all of the *alkB* sequences contained in GenBank (613

sequences as of November 2010) using blastall. The best hit from a blastx search was then used to determine class-level phylogeny.

To calculate overall diversity in each of the two soil types, the relative number of copies from each taxonomic group was calculated for each fraction, and the copy numbers were combined from all ^{14}N and ^{15}N fractions within each soil type. To calculate the relative enrichment of each 16S rRNA gene defined taxonomic group within the ^{15}N-treated soils, the proportion of 16S rRNA gene sequences for each group was determined within the defined CsCl density ranges. The proportion of sequences representing a taxonomic group within a given density range was then multiplied by the number of 16S rRNA gene copies within that same range (as defined by 16S rRNA gene qPCR) to give the relative number of copies for each taxonomic group. Relative copy numbers within the ^{15}N-incubated soils were then corrected for slight differences in total DNA and overall phylogenetic composition between the ^{14}N- and ^{15}N-incubated soils. Enrichment of a specific taxonomic group was determined by dividing its relative copy numbers from a ^{15}N density range by its relative copy numbers from the corresponding ^{14}N density range. Since the calculation of enrichment involves the combination of sequence and qPCR data, enrichment can occur even when the percentages of taxonomic groups remain similar (i.e., 20% of 109 sequences is more than 20% of 108 sequences). Each group is compared to itself within a given density range rather than to the other members of the community, so deficits are expected to occur for each enriched group at a lighter density range, rather than a given group within each range. The same calculation was performed with the *alkB* gene sequences but was instead based on the number of *alkB* gene copies as determined by qPCR.

4.3.7. Sequence accession numbers

The sequence data generated in the present study were deposited in GenBank and are accessible through the accession numbers JF358018 to JF409700.

4.4. Results

4.4.1. Soil characteristics

Over the course of the incubation, the mean soil temperature was 7.4°C ± 3.0°C (as measured by the iButton temperature probe), while the mean air temperature was 4.5°C ± 3.6°C, and the total precipitation was 1.1 mm (http://www.climate.weatheroffice.ec.gc.ca/index.html). The addition of MAP was an effective biostimulation treatment at our site and led to an ~4-fold decrease in the F2 hydrocarbon fraction (C10 to C16) by the end of the incubation (Table 1). A summary of other soil characteristics can be found in Table 4.1.

Table 4.1. Characteristics from petroleum hydrocarbon-contaminated and uncontaminated Alert soils taken for *in situ* incubations.

		Water Content				Total Petroleum Hydrocarbons (ppm)			
Treatment	pH	% Start	% End	% Total C[a]	DNA (ng/g soil)[bc]	F1	F2	F3	F4
Contaminated									
No MAP	7.36	18.23	4.6	5.85±0.53 A	1.12±0.42 C	ND	4300	15	<10
250 mg/kg MAP	6.85	18.23	4.7	5.56±0.13 A	6.24±0.99 A	27	1000	31	<10
Uncontaminated									
No MAP	7.63	19.95	3.5	5.55±0.47 A	4.59±0.55 AB	<10	<10	<10	<10
250 mg/kg MAP	7.52	19.95	4.7	5.59±0.79 A	2.85±0.39 BC	<10	<10	<10	<10

Abbreviations: MAP, monoammonium phosphate; ND, not determined

[a]No significant differences according to one-way ANOVA ($P = 0.695$).

[b]Different letters indicate significantly ($P < 0.05$) different averages according to Tukey's HSD test. One-way ANOVA was significant ($P = 0.0024$).

[c]Indicates amount of extractable DNA from 10 g (dry equivalent) soil.

4.4.2. Ultracentrifugation and DNA quantification

Total DNA recovered in the gradients was 3,407 and 3,861 ng from the ^{14}N- and ^{15}N-incubated contaminated samples, respectively, while 1,615 and 1,493 ng were recovered from the ^{14}N- and ^{15}N-incubated uncontaminated samples, respectively. Quantification of DNA within fractions following centrifugation showed a strong shift in the DNA profile from the ^{15}N-incubated contaminated soil toward the heavier densities within the gradient (Figure 4.1.a), while there was no obvious shift in the DNA profile in the ^{15}N-incubated uncontaminated soil (Figure 4.1.b). This is not entirely surprising, since MAP addition led to a significant increase in extractable DNA in the contaminated soil, likely a reflection of newly formed DNA in these samples, but a slight decrease in extractable DNA in the uncontaminated soil (Table 4.1.).

4.4.3. General community composition within MAP-treated soils

We received a total of 45,984 classifiable 16S rRNA gene sequences and 5,698 classifiable *alkB* sequences, with an average of 3,577 16S rRNA gene and 394 *alkB* sequences per contaminated density range, and 2,171 16S rRNA gene and 318 *alkB* sequences per uncontaminated density range. The average read length was 271 bases. When all density ranges of the ^{14}N- and ^{15}N-treated contaminated soil were combined and adjusted for relative 16S rRNA gene copy numbers, the majority of the sequences retrieved belonged to the *Actinobacteria* and *Proteobacteria*, while nine different phyla each made up at least 1% of the sequences from the combined density ranges of the uncontaminated soil (Figure 4.2.). The *alkB* sequences were dominated by the *Actinobacteria*, as well as the *Gammaproteobacteria*, while the *Betaproteobacteria* and

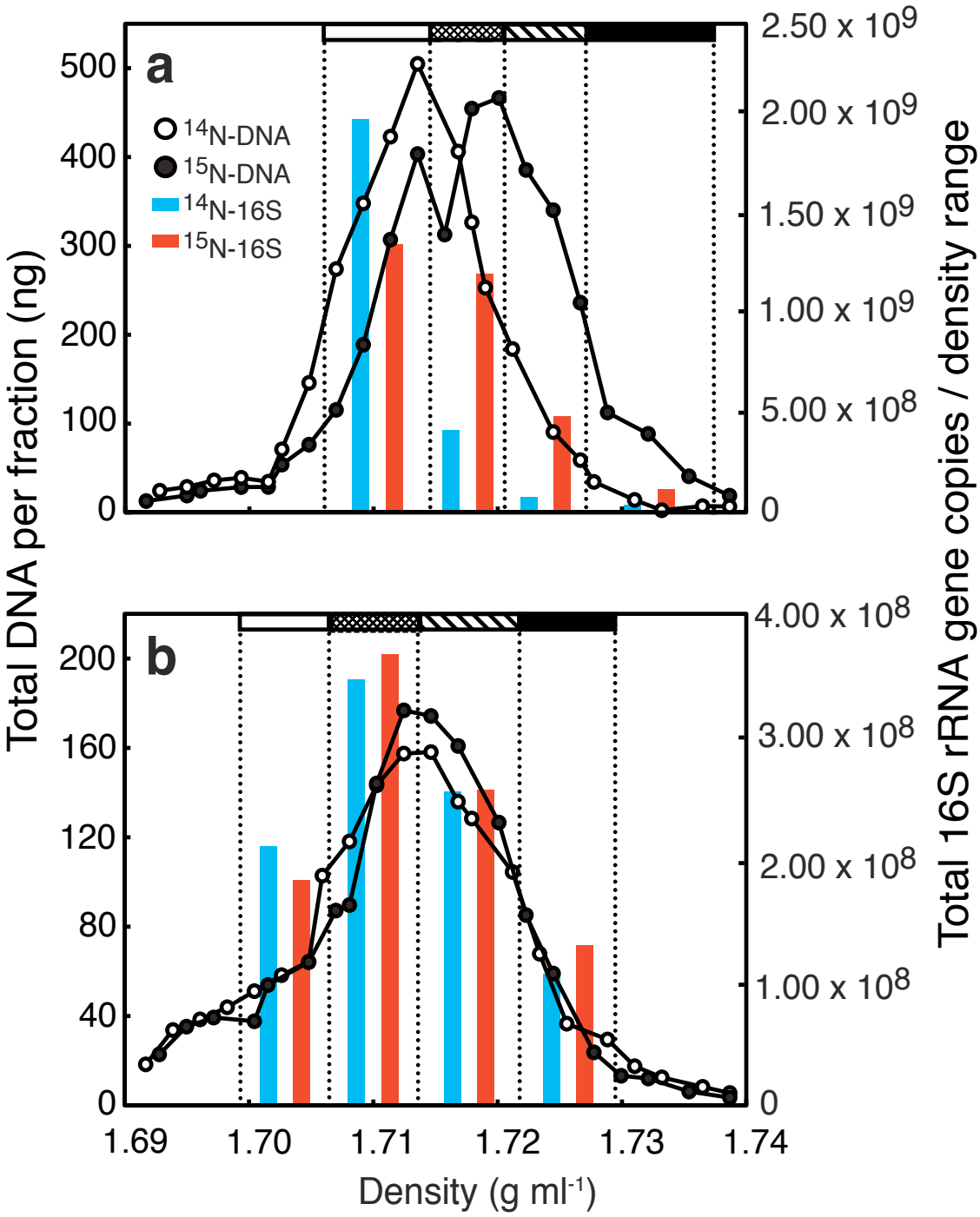

Figure 4.1. Quantitative profiles of DNA distribution in CsCl SIP gradients, as assessed by PicoGreen, in both hydrocarbon-contaminated (a) and uncontaminated (b) Arctic soils. Density ranges that were amplified for pyrosequencing occur between the dotted vertical lines and are indicated by shaded bars at the top of each graph. 16S rRNA gene copies as determined by qPCR are also shown for each density range.

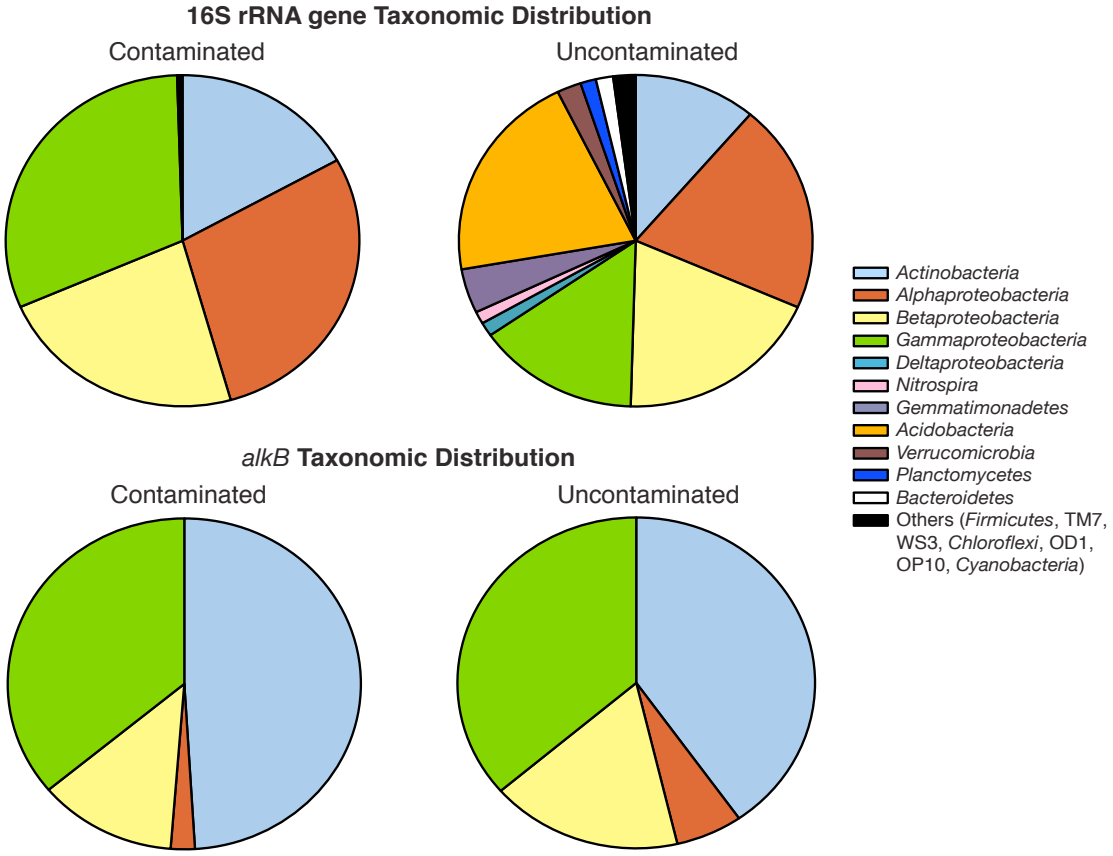

Figure 4.2. Overall phylogenetic diversity of bacteria from the combination of all fractions within the contaminated and uncontaminated soils amended with MAP. The top panel depicts 16S rRNA gene diversity, while the bottom panel depicts the diversity of *alkB* sequences across all fractions.

Alphaproteobacteria dropped in relative abundance compared to the 16S rRNA gene sequence data (Figure 4.2.).

There were 10 bacterial families that each made up at least 1% of the community in the contaminated soil, compared to 9 in the uncontaminated soil. The *Sphingomonadaceae* dominated the contaminated soil and were also the second-most represented family in the uncontaminated soil, although they represented only ca. 5% of

the total bacterial community in the uncontaminated soil as opposed to just 20% in the contaminated soil (Table 4.2.). The *Xanthomonadaceae* were the most represented family in the uncontaminated soil and represented a similar percentage of the total community in the contaminated soil (Table 4.2.).

Table 4.2. Bacterial families representing approximately 1% or more of the overall community in their respective treatments, along with the most commonly identified genera from within these families.

Family	Affiliation	Main genera	% Population[a]
Contaminated			
Sphingomonadaceae	*Alphaproteobacteria*		**20.33**
		Sphingobium	8.81
		Sphingomonas	0.28
Pseudomonadaceae	*Gammaproteobacteria*		**17.69**
		Pseudomonas	5.85
		Azomonas	2.68
Comamonadaceae	*Betaproteobacteria*		**13.08**
		Polaromonas	3.79
		Variovorax	3.05
		Hydrogenophaga	0.71
Alcaligenaceae	*Betaproteobacteria*		**7.57**
		Pusillimonas	<0.01
		Achromobacter	<0.01
		Pigmentiphaga	<0.01
Xanthomonadaceae	*Gammaproteobacteria*		**5.11**
		Pseudoxanthomonas	0.63
		Thermomonas	0.39
		Lysobacter	0.12
Nocardiaceae	*Actinobacteria*		**4.13**
		Rhodococcus	3.36
Caulobacteraceae	*Alphaproteobacteria*		**3.04**
		Brevundimonas	2.34
		Phenylobacterium	0.34
		Caulobacter	0.16
Microbacteriaceae	*Actinobacteria*		**2.82**
		Salinibacterium	0.13
		Agrococcus	0.03
Nocardioidaceae	*Actinobacteria*		**1.72**
		Pimelobacter	0.78
		Aeromicrobium	0.17
		Nocardioides	0.12

Sinobacteraceae	*Gammaproteobacteria*		**1.32**
		Nevskia	1.30
		Steroidobacter	0.02
Uncontaminated			
Xanthomonadaceae	*Gammaproteobacteria*		**5.46**
		Lysobacter	0.81
		Thermomonas	0.52
Sphingomonadaceae	*Alphaproteobacteria*		**5.39**
		Sphingomonas	0.19
		Sphingobium	0.18
Sinobacteraceae	*Gammaproteobacteria*		**3.53**
		Nevskia	3.38
		Steroidobacter	0.15
Gemmatimonadaceae	*Gemmatimonadetes*		**3.42**
		Gemmatimonas	3.42
Comamonadaceae	*Betaproteobacteria*		**2.45**
		Polaromonas	0.57
		Variovorax	0.26
		Ramlibacter	0.25
Burkholderiales	*Betaproteobacteria*		**1.87**
		Methylibium	0.66
Oxalobacteraceae	*Betaproteobacteria*		**1.79**
		Duganella	0.42
		Janthinobacterium	0.18
		Massilia	0.11
Planctomycetaceae	*Planctomycetes*		**1.13**
		Pirellula	0.23
		Zavarzinella	0.11
Caulobacteraceae	*Alphaproteobacteria*		**1.04**
		Brevundimonas	0.68
		Caulobacter	0.25
		Phenylobacterium	0.07
Nitrospiraceae[a]	*Nitrospira*		**0.95**
		Nitrospira	0.95

[a]Family percent values shown in bold.

4.4.4. Enriched groups within ^{15}N fractions

It was expected that organisms that incorporated the ^{15}N label would be enriched in the ^{15}N sample at the higher densities but at a deficit in the lighter density range. The 16S rRNA gene sequence data suggested that most phylogenetic groups were enriched to some degree in the ^{15}N-incubated contaminated sample; however, the degree to which

they were enriched varied widely, with the *Alphaproteobacteria* demonstrating the highest level of ^{15}N enrichment (Figure 4.3.). Overall, the *Alphaproteobacteria* made up a much smaller proportion of the *alkB* gene sequences retrieved (Figure 4.2.), but they were still identified as the most highly enriched phylogenetic group. They were at a slight ^{15}N deficit in the heaviest density range, but this may simply be due to a small number of sequences migrating to lower densities than were included in our analysis, while the bulk of enrichment occurred in DNA that had a higher natural buoyant density. The *alkB* gene sequence data also showed the *Actinobacteria* to be enriched in the heavy ^{15}N fractions, while the *Betaproteobacteria* and *Gammaproteobacteria* were not enriched to any great degree (Figure 4.3.). No clear enrichment was seen in the ^{15}N-treated uncontaminated soil.

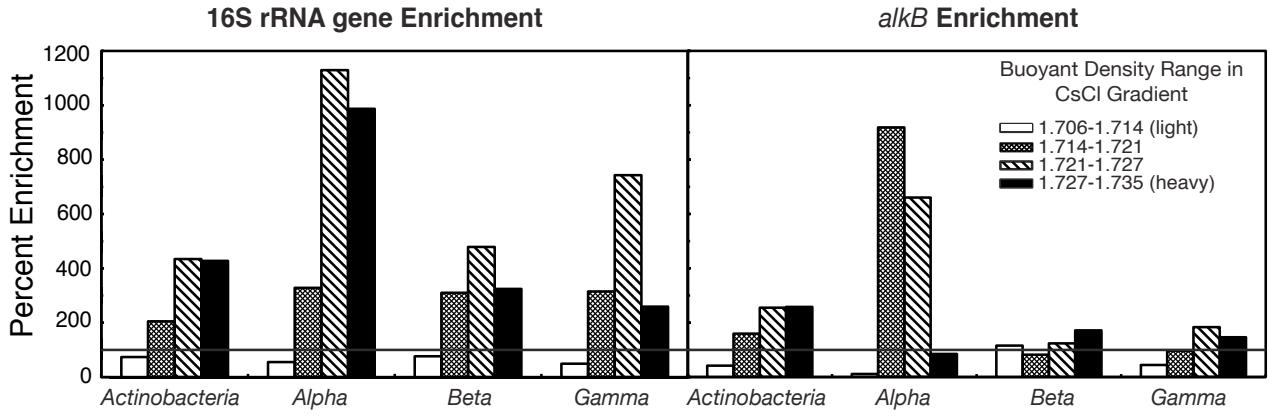

Figure 4.3. Percent enrichment of major bacterial groups in the ^{15}N-amended contaminated soil compared to the ^{14}N-amended control. The black horizontal line occurs at 100% which indicates no enrichment, while everything above the line is enriched in the ^{15}N treatment, and everything below is at a deficit compared to the ^{14}N control.

Each of the major phylogenetic groups that were identified in the contaminated soil were dominated by a few major families, and the degree of enrichment for each

family representing at least 1% of the total bacterial community is shown in Figure 4.4. In addition to being the most highly represented family, the *Sphingomonadaceae*, along with the *Caulobacteraceae*, were the most highly ^{15}N-enriched groups in the heavier fractions according to the 16S rRNA gene data (Figure 4.4.). The *Sinobacteraceae* were ^{15}N enriched to a much lower extent than the other two major *Gammaproteobacteria* families (*Xanthomonadaceae* and *Pseudomonadaceae*), while the *Nocardiaceae* were much less efficient at ^{15}N incorporation than the other major families of *Actinobacteria* (*Nocardioidaceae* and *Microbacteriaceae*) (Figure 4.4.).

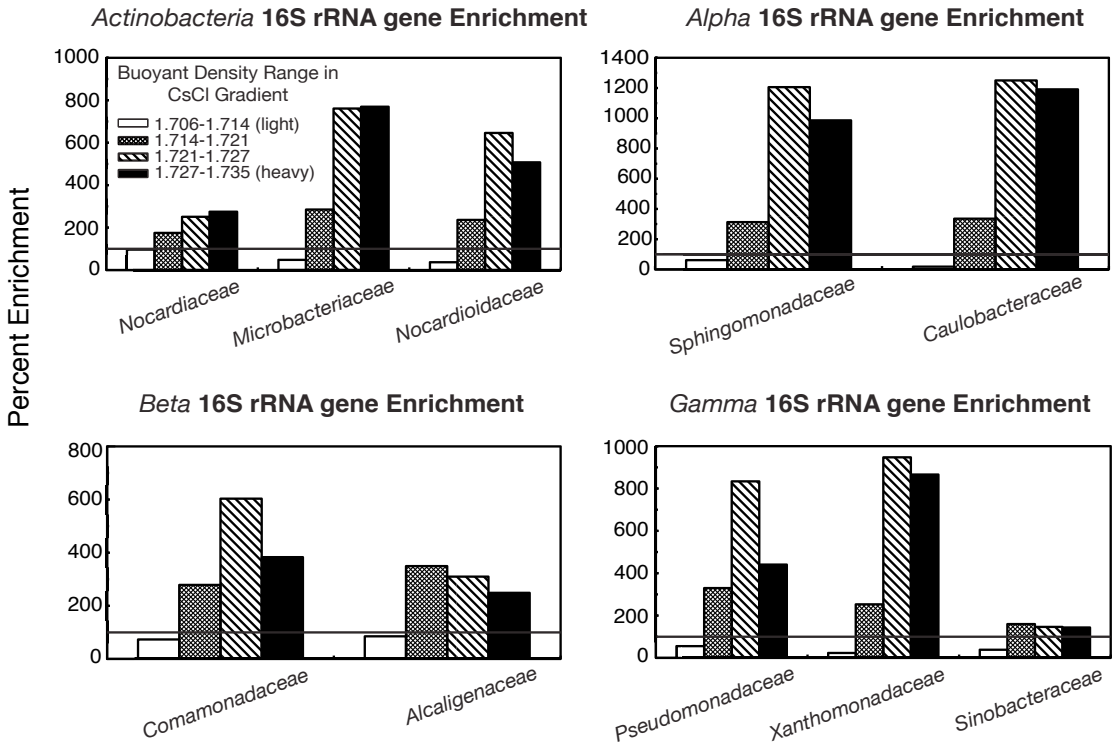

Figure 4.4. Percent enrichment of the bacterial families that made up at least 1% of the community in the ^{15}N-amended contaminated soil compared to the ^{14}N-amended control. The black horizontal line occurs at 100% which indicates no enrichment, while everything above the line is enriched in the ^{15}N treatment, and everything below is at a deficit compared to the ^{14}N control.

4.5. Discussion

Phylogenetic distribution varied widely between the MAP-treated contaminated and uncontaminated soils, as has been observed previously in other Arctic and alpine environments (Atlas et al 1976, Labbé et al 2007). The vast majority of 16S rRNA gene sequences retrieved from the contaminated soil identified most closely with groups of *Proteobacteria* and *Actinobacteria* that are known to be involved in the degradation of hydrocarbons. In addition, the 4-fold reduction of hydrocarbons in the sample was similar to that observed by Yergeau *et al*. (2009) and suggests that the biostimulation treatment was quite effective despite the limited time of the incubation. A much more diverse bacterial community was seen in the MAP-treated uncontaminated soils, where the *Acidobacteria* appeared as the dominant phylum and *Xanthomonadaceae* appeared as the dominant family. Both groups have represented large portions of the microbial community in other Arctic soils treated with nitrogen-based fertilizer (Neufeld and Mohn 2005, Campbell et al 2010). The proportion of *Gemmatimonadetes*, *Alphaproteobacteria*, *Actinobacteria*, and *Verrucomicrobia* in the uncontaminated soil was similar to that observed in other Arctic and lower-latitude soils, while *Betaproteobacteria* and *Gammaproteobacteria* were more highly represented in our soils (Chu et al 2010).

Sequence data from the ^{14}N- and ^{15}N-incubated soils were compared within each density range and expressed as a percentage to demonstrate the relative amount of ^{15}N enrichment between taxonomic groups. Because of the labour-intensive nature of SIP experiments, we chose to combine replicates at the CsCl gradient stage and were therefore unable to perform statistical analyses on the resulting enrichment data. Although extrapolation of the results must be done with caution, the effects that we

observed were surprisingly strong. The 16S rRNA gene enrichment data showed that the *Actinobacteria*, along with the *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*, were overrepresented in the heavier fractions of the ^{15}N-treated contaminated soil. Although all of these major groups were enriched in the heaviest ^{15}N density ranges, it was the *Alphaproteobacteria* that stood out as being enriched 10- to 11-fold relative to controls. *Alphaproteobacteria* were also confirmed as being the most ^{15}N-enriched group according to the *alkB* gene data. A higher percentage of enrichment relative to other taxonomic groups indicates increased cell replication due to the addition of fertilizer. The prevalence of *Alphaproteobacteria* and *Gammaproteobacteria* was greater in other nitrogen-treated Arctic soils compared to controls (Campbell et al 2010).

No bacterial groups were clearly enriched in the uncontaminated soils. It may be that bacteria are not capable of assimilating the added fertilizer in the absence of sufficient amounts of carbon to permit growth. A previous study showed that the addition of a carbon source increased extractable DNA from 1.8 to 12.9 μg of DNA g^{-1} soil, while also largely increasing the incorporation of added ^{15}N, as demonstrated by DNA quantification profiles (Buckley et al 2008). It may also be that a longer incubation period is required to detect ^{15}N enrichment in the uncontaminated soils, since another ^{15}N-SIP study allowed soils to incubate with ^{15}N substrates for 2 months prior to sampling (Buckley et al 2008).

The *Sphingomonadaceae* represented roughly 20% of the bacterial community in the MAP-treated hydrocarbon-contaminated soils and were also among the most heavily ^{15}N-enriched bacterial groups. Members of this family have previously been detected in contaminated polar soils and are generally associated with a high capacity for

polyaromatic hydrocarbon (PAH) degradation (Aislabie et al 2006). Sequences from *Comamonadaceae* represented just 13% of the contaminated soil community, and they have also been identified as degraders of aromatics (Master and Mohn 1998, Liang et al 2011). The dominance of these groups was somewhat surprising, since previous work on hydrocarbon-contaminated CFS-Alert soils showed that untreated soils contained a high ratio of alkane to PAH-degrading genes (Yergeau et al 2009). The limited label incorporation in the *alkB* gene sequences of both the *Betaproteobacteria* and *Gammaproteobacteria* also suggests that alkane degradation was not the dominant form of metabolism in these soils at the time of sampling. One possibility is that the aliphatic hydrocarbons were degraded very quickly during the incubation and that nitrogen was acquired secondarily by PAH-degrading bacteria during community succession, since microbial communities and their associated nutrients have the potential to turnover on the scale of days to months (Schmidt et al 2007). The potential of cross-feeding to cause such high specific SIP labeling has not previously been shown; however, strong secondary label acquisition has been observed after only a week as a result of what was presumed to be micropredation (Lueders et al 2006). Future ^{15}N-SIP studies using a time course design (as in (Lueders et al 2006)) will yield more detailed information on the order of nitrogen incorporation and species succession within biostimulated soils.

It has been suggested that PAH-degrading bacteria in soils do not exhibit much *in situ* growth (Johnsen et al 2005), but the extremely high level of ^{15}N incorporation by the *Sphingomonadaceae* suggests otherwise. Strains of *Sphingomonas* have been shown to effectively degrade PAHs in the presence of both high and low concentrations of nitrogen (Leys et al 2005), indicating that they are a diversely adapted group. *Sphingmonadaceae*

also seem to be well adapted to this environment in the absence of contamination, since they were the second-most represented family in the uncontaminated soil. In studies of other contaminated soils at CFS-Alert, it was the pseudomonads that dominated the soil microbial communities for the first year following aeration and the addition of MAP, at which point the sphingomonads replaced them as the most common microorganisms (Yergeau et al 2012c). The density of pseudomonads is suspected to be a major factor in determining the rate of bioremediation in contaminated Arctic soils (Yergeau et al 2012c). The *Pseudomonadaceae*, the second-most represented family in our contaminated soil sequences, were likely responsible for the dominance of *Gammaproteobacteria* within our *alkB* sequences and may also have contributed to PAH degradation, since they have been shown to possess both aliphatic and aromatic degrading genes in these High Arctic soils (Whyte et al 1997). The successful growth of *Caulobacteraceae* in this and other contaminated CFS-Alert soils (Yergeau et al 2012c) is unexpected, since it has not previously been identified as a hydrocarbon degrader in the Arctic, being more commonly linked with heavy metal resistance (Braz and Marques 2005, Hu et al 2005). However, concentrations of arsenic and nickel are naturally high in the soils surrounding CFS-Alert (unpublished results), perhaps leading the *Caulobacteraceae* to be uniquely adapted to this region. More detailed studies of these organisms may help to clarify whether they actually do play an important role in Arctic bioremediation.

One of the major advantages of applying SIP to bioremediation studies is that it is relatively easy to maintain *in situ* conditions. Our samples were left to incubate under on-site temperatures and precipitation and were treated similarly to other biostimulated soils

at CFS-Alert. Some major concerns in previous SIP studies were the addition of unrealistic amounts of substrate, cross-feeding, and disturbance of the soil in order to apply the substrate (Madsen 2006, Neufeld et al 2007). Because we were simulating an *in situ* remediation treatment, soil disturbance and nutrient addition were considered to be part of the "natural" environment. We were also not concerned with cross-feeding, since we were interested in identifying all active bacteria that accessed and incorporated added nitrogen through any route during the incubation and to then determine which bacteria had most successfully incorporated the nitrogen amendment into their DNA. All previous SIP studies of hydrocarbon-contaminated soils have used ^{13}C-labeled substrates (Madsen 2006, Chen et al 2010), but it was unknown whether the organisms degrading these substrates were the same ones that most successfully incorporated other macronutrients in the soil.

To our knowledge, this is the first study to combine DNA-SIP and large-scale sequencing. It is often difficult to determine the heavy fraction within a CsCl gradient (Cupples et al 2007), and recent SIP experiments have selected specific small density ranges that the authors define as "heavy" and "light" from which to construct clone libraries (Buckley et al 2007b, Buckley et al 2008, Antony et al 2010, Winderl et al 2010). Previous ^{15}N studies have used a secondary gradient that allows wider separation of labeled and unlabeled DNA (Buckley et al 2007a), but this can only be easily used to target a few specific fractions of the initial gradient at a time. The benefit of large-scale sequencing is that enrichment can be observed even in the presence of a high background of unlabeled DNA, without prior knowledge of which fractions will contain the labeled organisms. High-throughput analysis of environmental samples is a major hurdle in SIP

studies (Chen and Murrell 2010), and an unbiased, rapid method of fraction screening could allow automation of future SIP experiments. Although this method certainly benefits ^{15}N-SIP studies due to the lower proportion of nitrogen than carbon in DNA, it could also benefit ^{13}C-SIP studies in which label incorporation is incomplete.

In conclusion, ^{15}N-DNA SIP combined with large-scale sequencing determined the relative incorporation of nitrogen between bacterial groups in a contaminated Arctic soil. *Alphaproteobacteria* showed the highest degree of nitrogen incorporation, were dominant members of the fertilized contaminated soil, and were therefore the greatest beneficiaries of this biostimulation treatment. It is important that research into new bioremediation treatments proceed with a better understanding of how these treatments are specifically affecting microbial ecology and interactions, a sentiment that has been expressed in recent reviews of advances in environmental biotechnology (Madsen 2006, Lovley 2011). Further studies on the nitrogen-utilizing and hydrocarbon-mineralizing capacity of specific groups of Arctic bacteria will help in determining whether biostimulation is in fact producing the most efficient bioremediating community.

4.6. Acknowledgements

This study was financially supported by the NRCan PERD Program. We thank the staff at CFS-Alert, Drew Craig, Don Kovanen, Diane Labbé, Sylvie Sanschagrin, Claude Masson, Josée Sirois, and Julie Champagne for their technical advice and support for this project.

Connecting Text

The study outlined in Chapter 4 demonstrated that the bacteria in hydrocarbon-contaminated Arctic soils do not assimilate added nitrogen equally. Several known hydrocarbon-degrading groups effectively incorporated heavy nitrogen into their DNA, but it was the *Alphaproteobacteria* that were most enriched in the heavier fractions of the CsCl gradient, as determined by both 16S rRNA and *alkB* gene abundances. It was also observed that four major groups, the *Actinobacteria*, and the *Alpha-*, *Beta-*, and *Gammaproteobacteria,* shared resources within the contaminated soils. But what determined the relative abundance of these groups? Does a wide array of hydrocarbon compounds necessitate activity from all of these groups, or do other environmental factors drive species patterns in contaminated soils? In Chapter 5, soils from across the Arctic were contaminated with diesel and amended with monoammonium phosphate, in an attempt to identify consistent patterns in bacterial species abundance and bioremediation potential.

5

Predictable patterns in bacterial community composition and hydrocarbon degradation following disturbance of Arctic soils with diesel and nutrients

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In revisions for *The ISME Journal*

This chapter was written by the candidate, with helpful comments provided by all co-authors. The candidate also performed most of the experimental work, designed the study, and analyzed the data. Christine Maynard conducted the majority of the sequencing for this study. Dr. Etienne Yergeau provided valuable input to the experimental design, direction, and data analysis.

5.1. Abstract

Increased exploration and exploitation of resources in the Arctic is leading to a higher risk of petroleum contamination. A number of Arctic microorganisms can use petroleum for growth-supporting carbon and energy, but traditional approaches for stimulating these microorganisms (e.g. nutrient addition) have varied in effectiveness between sites. Consistent environmental controls on microbial community response to

disturbance from petroleum contaminants and nutrient amendments across Arctic soils have not been identified, nor is it known whether specific taxa are universally associated with efficient bioremediation. In this study, we contaminated 18 varied Arctic soils with diesel and treated subsamples of each with monoammonium phosphate (MAP). Bacterial community composition of uncontaminated, diesel-contaminated, and diesel + MAP soils was assessed through multiplexed 16S rRNA gene sequencing on an Ion Torrent Personal Genome Machine, while hydrocarbon degradation was measured by GC analysis. Diversity of 16S rRNA gene sequences was reduced by diesel, and more so by the combination of diesel and MAP. *Actinobacteria* dominated soils with less than 10% organic matter, while *Proteobacteria* dominated higher organic matter soils, and this pattern was exaggerated in contaminated soils treated with MAP. Degradation with and without MAP was predictable by initial bacterial diversity and the abundance of specific assemblages of *Betaproteobacteria*, respectively. High *Betaproteobacteria* abundance was positively correlated with high diesel degradation in MAP-treated soils, suggesting this may be an important group to stimulate. The predictability with which bacterial communities respond to these disturbances suggests that costly and time-consuming contaminated site assessments may not be necessary in the future.

5.2. Introduction

The thawing of Arctic sea ice is facilitating the exploration and exploitation of Arctic resources by northern countries. Petroleum is a targeted resource, and is also used to power many aspects of Arctic work, so there is an increasing risk of contamination in a biome that has had limited exposure to human industrial activity. In many environments,

a variety of native microorganisms are capable of petroleum degradation (Greer et al 2010), but petroleum-degrading activity is primarily limited to the few months in which soil temperatures exceed 0°C (Walworth et al 2001). In the interest of decreasing the lifespan of these pollutants in the environment, nutrients are often added to contaminated Arctic soils to stimulate microbial growth and activity, although studies have shown that treatment effectiveness can vary substantially between geographically similar soil environments (Greer 2009, Yergeau et al 2009).

Several recent studies have determined that pH and nitrogen concentration are major determinants of microbial diversity across a variety of soils in uncontaminated ecosystems (Lauber et al 2009, Chu et al 2010, Ramirez et al 2012), but the factors governing diversity in hydrocarbon-contaminated soils have not been firmly established. Comparisons of microbial community composition across sites have mostly been conducted in soils with existing petroleum contamination (Greer et al 2010, Powell et al 2010), but the discovery of consistent trends has often been confounded by variations in pollutant composition and age. In addition, despite the identification of many hydrocarbon-degrading taxa across ecosystems, it is generally not known which indigenous microorganisms should be promoted to optimize the rate and extent of bioremediation in contaminated environments. Nutrient amendment use by bacterial taxa in hydrocarbon-contaminated soils has been shown to be unequal (Chapter 4; Bell et al 2011), and due to the fact that many taxa are able to compete within contaminated soils, it is not clear that the most effective hydrocarbon-degrading taxa are favoured by bioremediation treatments or the natural environment.

Ecological disturbance is generally defined as a distinct event that alters the taxonomic composition, resource availability, or physical properties of an environment (Horner-Devine et al 2004), which classifies both the petroleum contamination of soils and the subsequent application of nutrients as disturbance events. Although petroleum contaminants can provide growth-supporting energy and carbon to many bacteria, they are toxic in high concentrations, primarily due to negative interactions with cell walls (Ramos et al 2002, Kang and Park 2010), and thus require adaptations and resource tradeoffs. Microbial communities can also be disrupted by nitrogen inputs, and reductions in respiration, biomass, and diversity have been observed following nitrogen addition (Allison et al 2007, Ramirez et al 2012). Experiments with protists have shown that competition can intensify at high levels of disturbance, when multiple surviving organisms are at the limit of their tolerance (Violle et al 2010), so a competitive advantage in a specific environment may play more of a role in taxonomic dominance than the ability to quickly metabolize the petroleum carbon source.

In this study, we collected soil samples from across the Arctic and sub-Arctic, and treated them with equal amounts of diesel, both with and without a nutrient amendment. The amendment that we selected is comparable in both type and concentration to those that have been used previously at hydrocarbon-contaminated Arctic sites (Thomassin-Lacroix et al 2002, McCarthy et al 2004, Greer 2009, Chapter 4; Bell et al 2011). We expected that the success of hydrocarbon-degrading bacteria in disturbed soils would be governed by adaptations to the soil environment, and that we would observe clear differences in the relative abundance of hydrocarbon-degrading groups across soil gradients. In addition, we expected to see a shift in the extent of hydrocarbon degradation

depending on which taxa dominated the bacterial community. A clear understanding of the factors that drive microbial community shifts following disturbance with petroleum and nutrient amendments will help in predicting optimal bioremediation treatments without site-specific assessments that are time-consuming and expensive (Dorn and Salanitro 2000).

5.3. Methods

5.3.1. Soil collection and analyses

The top 15 cm of soil was collected from 18 Arctic locations between June and August 2011. We aimed to find patterns in microbial community response to petroleum contamination and nutrient amendment regardless of where a contamination event occurred, so we collected soils that varied widely in pH and organic matter content. Soil water content was determined by drying overnight at 100°C, total soil organic matter content was measured as mass loss on ignition for two hours at 600°C, and pH was measured using a 10:1 ratio of water to soil. Both total soil nitrogen and nitrates/nitrites were determined at Agridirect Inc. (Longueuil, Quebec, Canada). Total soil nitrogen was determined via combustion using a Leco CNS-2000 analyzer (Leco, St. Joseph, Michigan, USA) at 1350 °C in the presence of oxygen. Combustion gases were collected in a ballast, and passed to a thermal conductivity detector that measures the difference in conductivity between the produced gas and helium. Using a colourimetric autoanalyzer, nitrates were converted to nitrites via reduction in a copper-cadmium coil, and were then reacted with sulfanilamide and N-1 naphthylethylenediamine dihydrochloride. Total

nitrate/nitrite concentrations were determined at 520 nm. Soil sampling locations and properties are presented in Table 5.1. and Appendix 1.

Table 5.1. Soil characteristics and percent diesel degradation.

		Soil characteristics					Diesel degradation (%)	
Sample code	*Coordinates*	*pH*	*Water content (%)*	*Organic matter (%)*	*Nitrate+nitrite (mg/kg)*	*Total nitrogen (mg/kg)*	*DSL*	*DSL-MAP*
AH1	79°N, 90°W	7.20	70.70	27.15	0.64	1230.78	28.32	52.97
AH2	79°N, 90°W	7.30	12.33	5.20	<0.05	876.73	22.67	41.54
AK1	69°N, 150°W	5.95	80.97	96.25	0.20	1560.34	51.84	55.75
AK2	69°N, 150°W	6.74	66.19	50.07	1.18	4868.21	38.67	41.66
AK3	69°N, 150°W	6.97	88.53	79.45	2.80	1994.91	27.76	34.86
AL1	82°N, 62°W	8.10	21.44	9.35	5.99	2356.67	4.85	41.01
AL2	82°N, 62°W	8.58	16.21	4.13	1.82	586.52	12.91	20.72
AL3	82°N, 62°W	8.49	15.88	3.44	1.88	672.94	8.57	28.04
AVK	61°N, 78°W	6.63	76.66	80.52	1.87	3734.74	0.00	50.22
BDE	61°N, 73°W	6.25	23.30	11.07	<0.05	1840.87	15.95	30.09
BY1	73°N, 78°W	6.61	9.35	6.73	<0.05	1722.31	22.11	26.68
BY2	73°N, 78°W	6.72	10.93	0.67	0.75	178.13	24.33	4.29
EBA	64°N, 82°W	7.67	1.88	3.73	44.16	11587.60	17.39	44.06
IQA	63°N, 68°W	6.29	76.27	76.42	0.35	5765.36	8.75	53.67
NOR	70°N, 19°E	6.00	65.61	79.81	0.33	1684.98	17.91	43.48
RAN	62°N, 92°W	6.93	45.51	22.47	<0.05	7682.68	33.22	50.22
RUS	69°N, 70°E	7.18	22.40	4.20	1.45	1552.06	23.97	48.25
THU	76°N, 68°W	7.83	24.28	8.14	0.90	984.30	0.77	8.73

Abbreviations: DSL, diesel-treated soils; DSL-MAP, diesel + monoammonium phosphate-treated soils.

5.3.2. Hydrocarbon analysis

Hydrocarbon analyses were adapted from the Canada-Wide Standard for Petroleum Hydrocarbons in Soil, as used previously for the determination of hydrocarbon concentrations in sub-Arctic soils (Chang et al 2011). Two grams of wet soil was extracted from three replicates of each sample using a 20 ml mixture of acetone:hexane (1:1 v/v) in glass bottles, with 20 ppm of octacosane (C28) added to determine extraction efficiency. Bottles were placed in a sonicator bath for 30 min, after which soil particles

were allowed to settle overnight. In a clean glass bottle, 5 ml of clear supernatant was mixed with 1 g of anhydrous Na_2SO_4 to remove any remaining water, and was then removed and shaken with 0.1 g of grade 62 activated silica gel to purify the sample. Prior to GC analysis, 20 ppm of triacontane (C30) was added to each sample as an internal standard. Analysis of F2 (C_{10}-C_{16}) and F3 (C_{16}-C_{34}) hydrocarbon fractions was performed on a Hewlett Packard 6890 gas chromatograph connected to a flame ionization detector. Using an automatic sampler, 1 µl of sample was injected on a DB-1 capillary column (15 m x 530 um x 0.15 um) from Agilent technologies (Santa Clara, CA, USA). Oven temperature was maintained at 35°C for 2 min, and was then raised by 30°C min^{-1} to 300°C, which was held for 5 min, using helium as the carrier gas. The injector was maintained at 35°C for 0.1 min, and was increased to 350°C at a rate of 500°C min^{-1}. The detector was then maintained at 350°C during quantification. Quantification of F2 and F3 fractions was performed using a calibration curve made of decane (C10), hexadecane (C16), and tetratriacontane (C34). Percent F2+F3 degradation was calculated separately for each replicate as: 100-[(final F2+F3)/(baseline average F2+F3)*100].

5.3.3. Microcosm setup and incubation

For each sample, 250 g of soil was contaminated to a final concentration of 5500 mg/kg of ultra low sulphur diesel fuel. We selected diesel fuel since it is a common contaminant of polar soils, as is jet fuel (Aislabie et al 2006, Chapter 4; Bell et al 2011, Yergeau et al 2012c), which is similar in composition to diesel. Soils were incubated for one week at 1°C to allow abiotic loss of the volatile components of diesel, as most short-term evaporative weathering can occur within one week of contamination (Neff et al

2000). Following weathering, a 50 g sample was removed to determine baseline hydrocarbon concentrations. Each soil was then split into 2 x 100 g portions, and one portion was treated with 250 mg monoammonium phosphate (MAP) per kg wet soil, a nutrient amendment that has previously been shown to effectively stimulate bioremediation in hydrocarbon-contaminated Arctic soils (Greer 2009). This led to three separate treatments per soil: 1) initial soil, 2) DSL (diesel added), and 3) DSL-MAP (both diesel and MAP added). Both DSL and DSL-MAP treatments were split into 5 replicates of 20 g, and were incubated at 10°C under ambient moisture conditions in loose-top 50 ml Falcon tubes for 4 weeks to simulate the high point of the Arctic summer. At the end of the incubation, soil was used for both hydrocarbon analysis and total DNA extraction.

5.3.4. Soil DNA extraction, 16S rRNA gene amplification and Ion Torrent sequencing

Total soil DNA was extracted from three replicates of each soil under each treatment condition using the protocol described in Yergeau et al. (2007). To 250 mg of 0.1 and 0.5 mm (1:1) zirconia:silica beads, 500 mg of soil was added, and extracted with 500 mL of phenol:chloroform:isoamyl alcohol (25:24:1; TRIS saturated, pH 8.0) and 500 mL of extraction buffer (12.2 mM KH_2PO_4, 112.8 mM K_2HPO_4, 5% w/v CTAB, 0.35 M NaCl; pH 8.0), and were bead-beaten for 30 s at 50 m s^{-1}, followed by 5 min of centrifugation at 10 000 g and 4°C. The supernatant was mixed with 500 mL of chloroform:isoamyl alcohol (24:1) and centrifuged as previously. The supernatant was precipitated for 2 h at room temperature using two volumes of a 30% w/v PEG 6000 and

1.6M NaCl solution. The resulting pellet was ethanol-washed and resuspended in 100 µl of deionized water, and frozen prior to downstream analyses.

Partial 16S rRNA gene amplicons were produced using the universal primers E786 (5'-GATTAGATACCCTGGTAG-3') and U926 (5'-CCGTCAATTCCTTTRAGTTT-3') which target the V5 variable region (Baker et al 2003). These primers were initially compared with other 16S rRNA gene sets that produced amplicons below the Ion Torrent size threshold (at the time, 220 bp including adaptors). The primers were compared for inclusivity in the RDP database, and matched between 95.5% and 98.3% of all bacteria with 1 mismatch or less, with no clear bias against any specific groups. We conducted a sequencing run with the candidate primers, and selected the set that produced the highest bacterial diversity.

Primers for each sample contained unique multiplex identifier codes (MID) and adaptor sequences for Ion Torrent sequencing. Reactions were carried out using 12 µl of KAPA2G Robust DNA Polymerase mix (KapaBiosystems Inc., Boston, MA, USA), 12 µl of nuclease-free deionized water, and 40 pmol of each of the appropriate forward and reverse primers. PCR cycling conditions involved an initial 5 min denaturing step at 95°C, followed by 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and a final elongation step of 7 min at 72°C. Amplicons were gel purified using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Piscataway, NJ, USA), and then quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Burlington, ON, Canada), pooled in equimolar ratios, and diluted to a concentration of 5×10^7 molecules for sequencing. Sequencing was conducted on an Ion Torrent Personal Genome Machine™ using the Ion Xpress™ Template Kit and the Ion 314™ chip

following manufacturer's protocols. Ion Torrent sequencing has been shown to produce microbial community profiles that are highly comparable to those produced by 454 sequencing (Yergeau et al 2012b).

5.3.5. Sequence classification and OTU analysis

Using an in-house Perl pipeline, sequences were binned by MID, after which MIDs and Ion Torrent adaptor codes were trimmed from each sequence. Sequences were then filtered using a moving average Q15 cutoff, to ensure that many high quality bases did not conceal very low quality bases, as can occur with an average overall quality score. If the average of 5 consecutive bases along a sequence fell below Q15, the sequence was trimmed at that point. Reads of less than 75 bp were removed from later analysis. Since it has been shown that 100 sequences are sufficient to detect ecological patterns (Kuczynski et al 2010), we removed 2 sample replicates that contained less than 100 usable sequences after filtering (1 replicate each of AH1-DSL and AK2). This left us with 160 sample replicates, representing 54 samples (3 treatments per soil) for downstream analysis.

Taxonomic identities were assigned to sequences using the RDP Pipeline Classifier, Release 9 (Wang et al 2007). A bootstrap of 50% was used as is recommended for sequences of less than 250 bp (Claesson et al 2009). At each taxonomic level, the proportion of sequence identities was calculated as a percent of all sequences classified to that level. OTU analysis was performed in mothur (Schloss et al 2009), and was used to determine Shannon diversity values and UniFrac distance between samples. To standardize between samples, the number of sequences representing each sample was

reduced to the lowest number among all sample replicates (156 sequences) using random numbers as in Fortunato et al. (2012). Analysis was performed following mainly the workflows outlined on the mothur website with the Sogin et al. (2006) and Costello et al. (2009) datasets.

Samples were assembled into a single group file, and unique sequences were identified using the 'unique.seqs' algorithm. Unique sequences were then aligned against the Green Genes core set (Lauber et al 2009) with 'align.seqs', using the following parameters: ksize=9, align=needleman, gapopen=-1. Sequences were filtered with 'filter.seqs', putatively noisy sequences were removed with the 'pre.cluster' algorithm that is based on the procedure by Huse et al. (2010), and possible chimeras were removed with 'chimera.uchime' and 'remove.seqs'. A phylip-formatted distance matrix was created using 'dist.seqs', and average-neighbour clustering was performed using 'cluster.split' and setting method=average. A shared file was created using 'make.shared', and Shannon diversity was obtained using 'summary.single'. To calculate UniFrac distances, a tree was created using the phylip-formatted distance matrix in the 'clearcut' algorithm that is based on the method of Evans et al. (2006). OTUs from replicates for each sample were merged with 'merge.groups', and weighted UniFrac values between samples were calculated using 'unifrac.weighted' with default parameters. A principle coordinate analysis (PCoA) matrix was created with 'pcoa', and was exported to R for graphing.

5.3.6. Statistical analysis

Average Shannon diversity was calculated using a 3% dissimilarity cutoff. A global analysis of this data was performed by ANOVA in JMP 8.0 (SAS Institute, Cary, NC), and paired Student's t-tests were used to compare differences between treatments, as each was comprised of the same initial soil samples. This same analysis was performed for average UniFrac distance between samples within each treatment. Classified sequence data were transformed using the Bray-Curtis distance prior to creation of a PCoA matrix as recommended by Legendre and Gallagher (2001). A PCoA matrix of the transformed data, and ordination plots for both the taxonomic and UniFrac data were produced using the 'vegan' package in R (v.2.15.0, The R Foundation for Statistical Computing, Vienna, Austria). To confirm organic matter as the most explanatory environmental variable, forward selection in canonical redundancy analysis was performed as described by Blanchet et al. (2008) in R using the 'vegan' package. To compare shifts in species abundances following disturbance in low and high organic matter soils, the relative abundance of each taxa in each initial soil was subtracted from the abundance in the corresponding treated soils (i.e. DSL - initial and DSL-MAP - initial). Similarly, the abundance within each DSL soil was subtracted from the abundance in the corresponding DSL-MAP soil, to demonstrate shifts as a result of nutrient additions alone. Low and high organic matter groups were compared for each phylum using unpaired t-tests, as these groups contained separate sample sets. Families from the *Actinobacteria* and *Proteobacteria* were also compared in this way if they were among the ten most abundant families within these phyla in one of the three treatments. Correlations between environmental variables, diversity, and taxonomic abundances were also performed in R.

Since the *Actinobacteria* and *Betaproteobacteria* responded most strongly to diesel and MAP disturbance, we also looked for correlations between these groups and degradation, using a Bonferroni-adjusted p-value for each pairwise comparison.

5.4. Results

5.4.1. Soil characteristics

Soil parameters are presented in Table 5.1. Organic matter content co-varied with both water content ($R=0.921$, $p<0.001$) and pH ($R=-0.640$, $p=0.004$), and pH was also correlated with water content ($R=-0.549$, $p=0.018$). Total nitrogen was correlated with nitrates/nitrites ($R=0.721$, $p<0.001$). Diesel degradation in DSL-MAP soils correlated with water content ($R=0.565$, $p=0.015$), log total nitrogen ($R=0.670$, $p=0.002$), and log organic matter content ($R=0.657$, $p=0.003$). Degradation in DSL soils was not linearly related to any of the environmental variables measured, but peaked at ~ pH 7.

5.4.2. Diversity and community composition across soils

After sequence filtering, we obtained a total of 154 418 usable reads, with an average of 903 reads per sample replicate. Shannon Diversity declined with increasing disturbance ($p<0.001$) using a 3% dissimilarity cutoff to define operational taxonomic units (Figure 5.1.), and all treatments were significantly different from each other according to paired Student's t-tests ($p<0.05$). PCoA ordination showed that microbial community composition spread farther across the first axis in the DSL-MAP soils than in the initial or DSL soils (Figure 5.2.ab). Average weighted UniFrac distances between soils within the same treatment were higher in the DSL-MAP soils than in the initial or

DSL soils (Figure 5.2.c; $p<0.05$). Bacterial community composition grouped almost exclusively based on whether the soil of origin contained more or less than 10% organic matter (Figure 5.2.d).

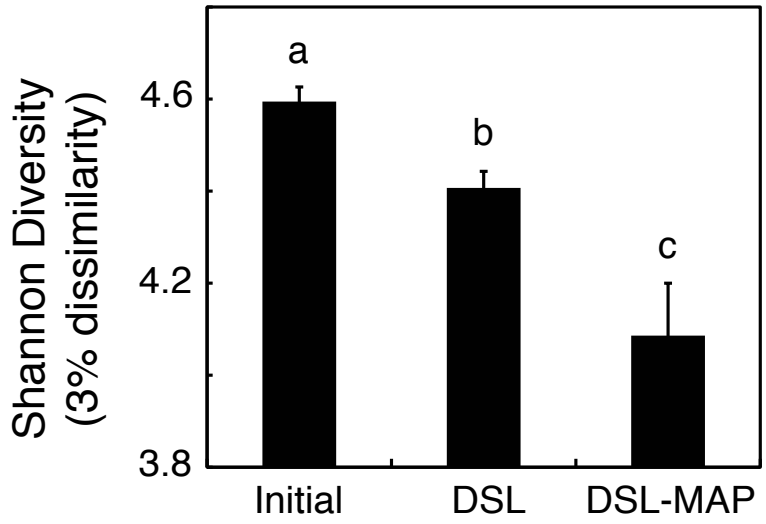

Figure 5.1. Decline in Shannon diversity following soil disturbance with diesel (DSL) and diesel + monoammonium phosphate (DSL-MAP), as measured by differences in 16S rRNA gene composition using a 3% dissimilarity cutoff. Error bars represent standard error. Different letters above columns indicate significant differences between means ($p<0.05$), as determined by paired Student's t-tests.

The abundance of *Actinobacteria* and *Proteobacteria* correlated strongly and significantly with organic matter (Figure 5.3.). Despite the strong covariance between organic matter and water content, organic matter correlated better than water content with the relative abundance of the two major phyla, *Actinobacteria* and *Proteobacteria*, with the exception of the *Proteobacteria* in the DSL soils (Appendix 2). This was further confirmed using forward selection on all environmental variables in canonical redundancy analysis ($R_{adj}=0.471$, $p=0.003$ in reduced model). Before treatment, *Actinobacteria* dominated soils with low organic matter, but abundance dropped off quickly with increasing organic matter content ($R=-0.790$, $p<0.001$), giving way to the *Proteobacteria* ($R = 0.792$, $p<0.001$)(Figure 5.4.a).

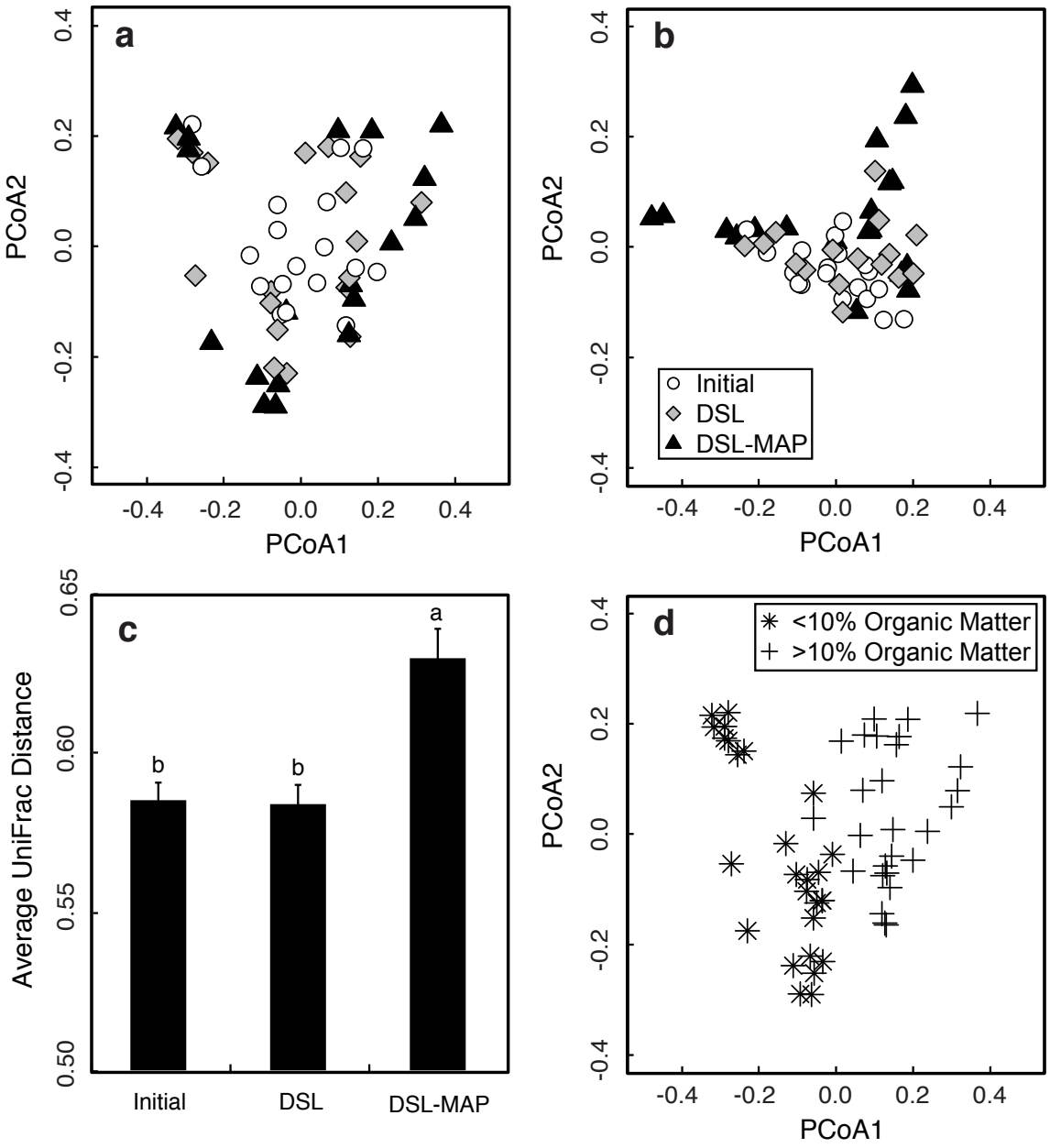

Figure 5.2. Representation of differences between samples using distance matrices. Principle coordinate analysis (PCoA) ordination plots show relatedness of samples separated by treatment using a) weighted UniFrac distances, and b) Bray-Curtis distances of classified 16S rRNA gene sequences at the phylum level (including classes of *Proteobacteria*). The average weighted UniFrac distance between samples is displayed in c) as a column graph. Error bars represent standard error while different letters above columns indicate significant differences between means ($p<0.05$), as determined by paired Student's t-tests. Weighted UniFrac distances were also used to create an ordination plot of d) samples separated by % organic matter.

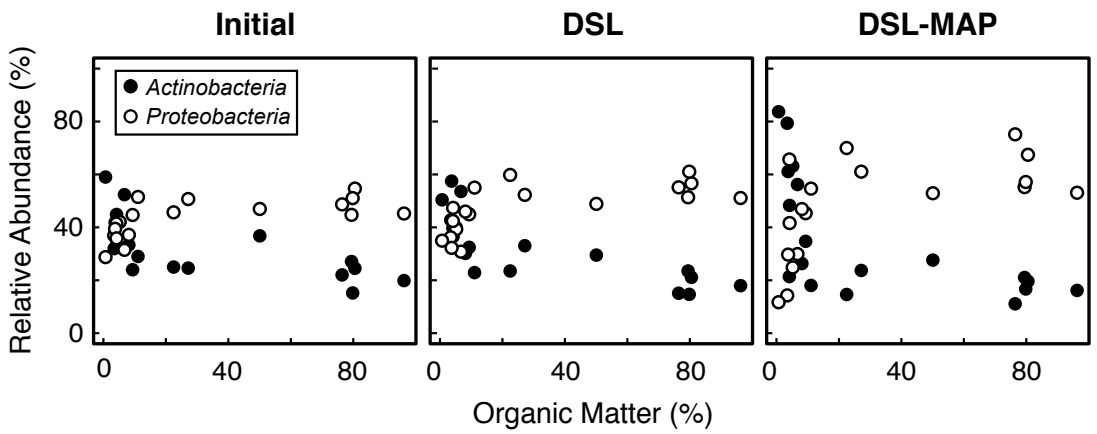

Figure 5.3. Correlations between *Actinobacteria*, *Proteobacteria* and organic matter content in initial, diesel-treated (DSL), and diesel + MAP-treated (DSL-MAP) soils.

Following disturbance, *Actinobacteria* represented an even larger proportion of the microbial communities in low organic matter soils ($R_{DSL} = -0.849$, $p_{DSL} < 0.001$; $R_{DSL\text{-}MAP} = -0.807$, $p_{DSL\text{-}MAP} < 0.001$), while *Proteobacteria* represented a greater proportion of the communities in high organic matter soils ($R_{DSL} = 0.763$, $p_{DSL} < 0.001$; $R_{DSL\text{-}MAP} = 0.759$, $p_{DSL\text{-}MAP} < 0.001$). This was especially true in DSL-MAP soils, with *Actinobacteria* representing 11.10% - 83.71% and *Proteobacteria* representing 11.70% - 75.16% of classified sequences, as compared with 15.20% - 59.02% and 28.76% - 54.66%, respectively, in the initial soils.

No major taxonomic group shifted in the same direction following disturbance in all samples (Table 5.2.). When samples were grouped by percent organic matter, The *Betaproteobacteria* and *Alphaproteobacteria* were both significantly more successful in soils with over 10% organic matter in response to DSL and DSL-MAP treatments ($p<0.05$), although on average, the *Alphaproteobacteria* still declined in abundance in both soil types (Figure 5.4.a).

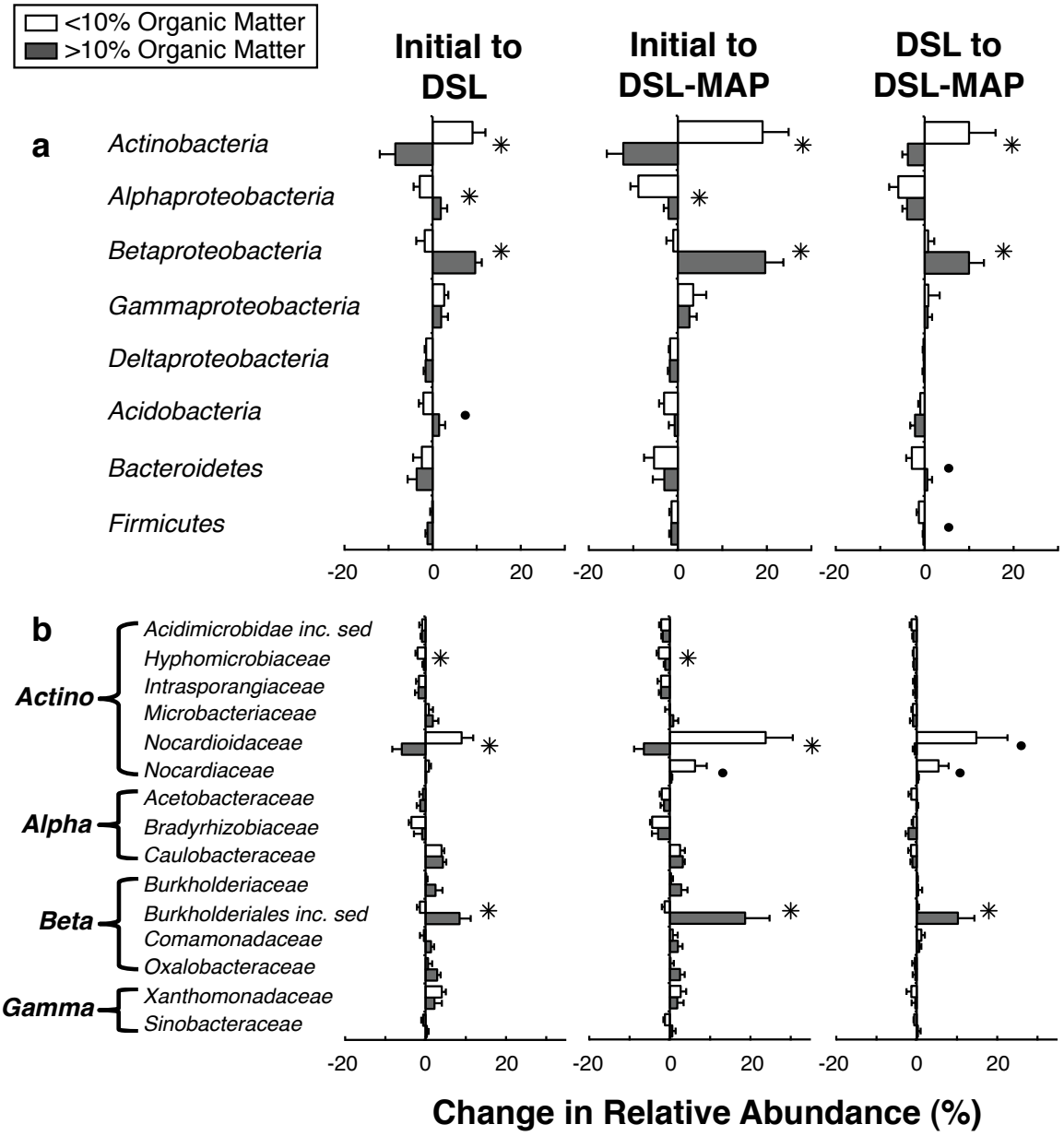

Figure 5.4. Percent shifts in the diesel-treated (DSL) and diesel + MAP-treated (DSL-MAP) soils in the relative abundance of classified sequences of a) the major phyla (and classes of *Proteobacteria*), and b) families of *Actinobacteria* and *Proteobacteria* that were among the ten most abundant in at least one of the treatments. Significant differences in sequence abundance shifts between soils with low and high organic matter content are denoted by stars ($p<0.05$) or circles ($p<0.10$) as determined by the Student's t-test.

Table 5.2. Number of samples in which major taxonomic groups increased or decreased between treatments.

Taxonomic Group	*Δ Initial to DSL*		*Δ Initial to DSL-MAP*		*Δ DSL to DSL-MAP*	
	+	-	+	-	+	-
Alphaproteobacteria	10	8	2	16	1	17
Betaproteobacteria	12	6	11	7	13	5
Gammaproteobacteria	12	6	11	7	7	11
Deltaproteobacteria	2	16	1	17	7	11
Actinobacteria	9	9	8	10	8	10
Acidobacteria	6	12	4	14	3	15
Firmicutes	7	11	2	16	4	14
Bacteroidetes	6	12	5	13	7	11

Abbreviations: DSL, diesel-treated soils; DSL-MAP, diesel + monoammonium phosphate-treated soils
.

There were also significant differences in the relative abundance of *Actinobacteria* between low and high organic matter soils ($p<0.05$), as they increased dramatically in abundance in low organic matter soils, and declined in high organic matter soils in response to disturbance, while the *Gammaproteobacteria* increased marginally in both low and high organic matter soils. Most other phyla tended to decline following disturbance (Figure 5.4.a). At the family level, shifts in the *Nocardioidaceae* (*Actinobacteria*) varied significantly between low and high organic matter soils ($p<0.05$), as they increased the most of any group in low organic matter soils in the presence of disturbance, but declined in high organic matter soils, while the reverse was true for *Burkholderiales inc. sed.* (*Betaproteobacteria*) ($p<0.05$; Figure 5.4.b). *Hyphomicrobiaceae* (*Actinobacteria*) declined in both low and high organic matter soils, but the decline was significantly greater in low organic matter soils ($p<0.05$; Figure 5.4.b).

5.4.3. Predictability of diesel degradation

Since the *Actinobacteria* and *Betaproteobacteria* responded most strongly to disturbance from diesel and nutrients, we decided to further investigate these groups for correlations with degradation across soils. For correlations with these two phyla and degradation, we used a Bonferroni-corrected p-value cutoff of 0.025 (α=0.05/2 pairwise comparisons). There was a negative correlation between the abundance of *Actinobacteria* in the initial soils and degradation in DSL-MAP soils (R=-0.564, p=0.0149), whereas the abundance of *Betaproteobacteria* in the initial soils was not predictive of degradation in either DSL or DSL-MAP soils.

We also investigated correlations between degradation and each bacterial family that made up ≥ 5% of sequences identified from either the *Actinobacteria* or *Betaproteobacteria* in at least one sample. Since different families are likely promoted depending on environmental factors, we also examined assemblages of all families meeting the above criteria (≥ 5% of sequences in at least one sample) from each phylum, grouping by those that correlated either positively or negatively with degradation. For these comparisons, we used a Bonferroni-corrected p-value cutoff of 0.0026 (α=0.05/19 pairwise comparisons). No single family in the initial soils was significantly predictive of degradation in either DSL or DSL-MAP soils, but when the six most commonly identified *Betaproteobacteria* families were normalized to the total abundance of *Betaproteobacteria* in each sample, the relative abundance of *Burkholderiaceae* + *Oxalobacteraceae* + *Nitrosomonadaceae* in the initial soils was significantly and positively correlated with degradation in DSL soils (R=0.775, p<0.001), while the relative abundance of *Burkholderiales inc. sed.* + *Comamonadaceae* + *Rhodocyclaceae* in the

initial soils was significantly and negatively correlated with degradation in DSL soils ($R=-0.771$, $p<0.001$). The resulting abundance of these assemblages following disturbance in DSL soils was not significantly correlated with degradation (Figure 5.5.a), nor were these assemblages predictive of degradation in DSL-MAP soils (data not shown). Degradation in DSL-MAP soils could be predicted by bacterial diversity in the initial soils ($R=0.660$, $p<0.003$), but diversity in DSL-MAP soils did not correlate with degradation (Figure 5.5.b).

Although the relative abundance of *Betaproteobacteria* in the initial soils was not predictive of degradation in DSL-MAP soils (Figure 5.5.b), the abundance of *Betaproteobacteria* in DSL-MAP soils was significantly correlated with DSL-MAP degradation ($R=0.597$, $p=0.009$). The relative abundance of *Rhodocyclaceae* (*Betaproteobacteria*) in DSL-MAP soils was negatively correlated with DSL-MAP degradation ($R=-0.740$, $p<0.001$).

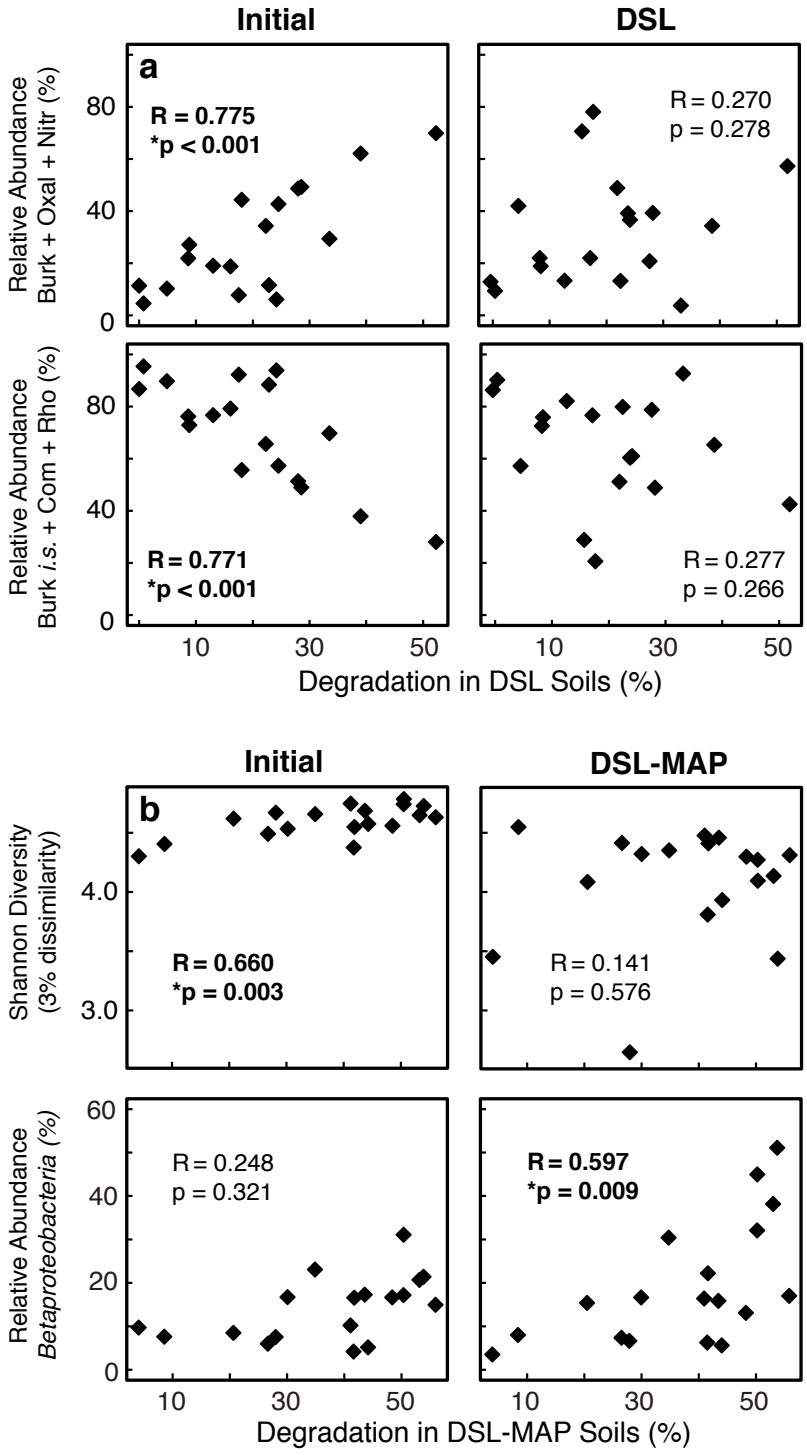

Figure 5.5. Correlations between a) degradation in diesel-treated (DSL) soils and the relative abundance of major families of *Betaproteobacteria* (Burk = *Burkholderiaceae*, Oxal = *Oxalobacteraceae*, Nitr = *Nitrosomonadaceae*, Burk i.s. = *Burkholderiales incertae sedis*, Com = *Comamonadaceae*, Rho = *Rhodocyclaceae*) as a proportion of all *Betaproteobacteria*, b) degradation in diesel + MAP-treated (DSL-MAP) soils and Shannon Diversity (3% dissimilarity), and degradation in DSL-MAP soils and the relative abundance of *Betaproteobacteria*.

5.5. Discussion

5.5.1. Environmental controls on disturbed soil communities

From both an applied and ecological standpoint, it was interesting to observe that disturbance and environment combined to reliably shape bacterial communities across soils collected from geographically disparate Arctic sites. Other recent studies have also shown that disturbance from shifts in climate (Yergeau et al 2012c), differences in local environmental factors (Pommier et al 2012), and even soil compartment type (Schulz et al 2012) have a greater effect than geography in determining microbial community composition. Metagenomic studies have shown that discrete taxonomic units exist within bacteria (Caro-Quintero and Konstantinidis 2012), and despite the fact that some key genes involved in hydrocarbon degradation are located on mobile elements (Whyte et al 1997, Ma et al 2006) and may potentially be exchanged between taxa, our data show that taxonomic affiliation does affect which organisms will dominate in particular contaminated soils. Hydrocarbons are found almost ubiquitously across natural environments, so the disturbance of soil communities by petroleum contaminants may in fact be an exaggerated analogy to natural fluxes in the Arctic environment, such as biomass decay, plant inputs, and the disruption of organic matter by freeze-thaw. Similarly, the addition of nutrients may resemble fluxes such as nutrient pulses at snowmelt and the periodic input of animal waste products. In many biological systems, community composition shifts temporally in response to changing environmental conditions, helping to support a greater range of taxa in a single environment through time (Fuhrman et al 2006, Magurran and Henderson 2010). Thus, the bacteria that responded positively to diesel and nutrient inputs may have a history of increasing in

abundance with such fluxes. Bacteria that live in variable-resource environments have been shown to quickly process pulsed carbon sources (Lennon and Cottingham 2008), so resource history could be important in predicting bioremediation capacity.

While some bacteria may be naturally adapted to high carbon and nutrient sources, diesel contamination and subsequent nutrient amendments were shown to be significant disturbances to bacterial communities in Arctic soils. Shannon diversity of OTUs declined in both DSL and DSL-MAP soils, with the greatest declines observed in DSL-MAP soils. The addition of both diesel and MAP appeared to have a disruptive effect on soil microbial communities, as the average weighted UniFrac distance between samples increased relative to both initial and DSL soils, and community composition was polarized depending on the soil environment. Only *Actinobacteria* and the major classes of *Proteobacteria* represented large proportions of the microbial community in DSL and DSL-MAP soils. Although pH has previously been shown to shape phylum-level microbial community structure in uncontaminated Arctic soils (Fierer and Jackson 2006, Chu et al 2010), the effect was less prominent in our study. This may be due to the fact that most pH effects on community composition have been observed at pH values of less than 6 (Fierer and Jackson 2006, Lauber et al 2009, Chu et al 2010), while the lowest pH in our soils was 5.95.

Bioaugmentation, the inoculation of contaminated soils with additional hydrocarbon-degrading microorganisms, has done little to increase rates of bioremediation (e.g. Thomassin-Lacroix et al 2002, van Herwijnen et al 2006). This lack of success has been blamed on a failure to consider other environmental controls on hydrocarbon degraders (Thompson et al 2005), and is not surprising in light of our results.

While hydrocarbon contamination can reduce the number of bacterial taxa in Arctic soils, there are still many that remain active (Chapter 4; Bell et al 2011), and competitive interactions between these remaining groups are likely to be governed by other environmental parameters. Our experimental setup controlled for contaminant composition, age, and concentration, and found that differences in phylum-level community composition was best explained by soil organic matter content, especially in DSL-MAP soils. Total carbon was previously identified as one of several factors that linked with community composition in hydrocarbon-contaminated Antarctic soils, but this effect was confounded by variability in soil contaminants (Powell et al 2010). In our study, organic matter primarily affected the relative abundance of *Actinobacteria* and *Proteobacteria*, with *Actinobacteria* dominating in soils with less than 10% organic matter, and *Proteobacteria* dominating higher organic matter soils. In an alpine tundra soil, *Actinobacteria* dominated at snowmelt, while Gram-negative bacteria dominated during the growing season when plant carbon inputs were higher (Björk et al 2008). *Actinobacteria* have also been linked with low carbon concentrations by PLFA in temperate soils, and are reduced when higher concentrations of carbon are present or added (Griffiths et al 1999, Fierer et al 2003).

5.4.2. Soil resource history

Ramirez et al. (2012) recently showed that *Actinobacteria* reliably increase in abundance across nitrogen gradients and environments in response to nitrogen inputs, and suggest that the *Actinobacteria* may represent a copiotrophic group. However, our observation of dominant *Actinobacteria* populations in low organic matter soils suggests

that this may not be the case. It has been suggested that fast-growing copiotrophic microorganisms that primarily use labile carbon sources are likely to most rapidly and positively respond to nutrient inputs (Ramirez et al 2012), and that intrinsic growth rate is the main factor leading to success following disturbance (Haddad et al 2008). However, we show that positive response to nutrient addition in the presence of hydrocarbon contaminants is not limited to specific groups, but that different bacterial taxa are favoured depending on environmental factors; in this case, organic matter content. Copiotrophs are expected to outcompete oligotrophs in the presence of abundant resources (Fierer et al 2007), so the fact that *Proteobacteria* and *Actinobacteria* respond positively to carbon and nutrient inputs in different soil environments suggests that they have either separately adopted the copiotrophic life strategy, or that each has capitalized on reduced competition following soil disturbance. Fierer et al (2007) suggest that copiotrophism may be a fixed characteristic of bacterial phyla, in which case the latter hypothesis appears more probable.

Degradation in DSL-MAP soils correlated linearly and positively with organic matter, water content, and total nitrogen, while degradation in DSL soils was not linearly related to any of the environmental variables that we examined. Despite the fact that low water content has previously been correlated with ineffective nutrient stimulation of degradation (Walworth et al 1997), we observed successful stimulation in soils with water contents as low as 1.88%, although our fertilizer concentration was below what is generally considered to be inhibitory. It was interesting to note that higher total nitrogen content in soils indicated higher degradation in the presence of added nutrients, which suggests that microorganisms that are adapted to using nitrogen naturally may more

effectively use nitrogen additives. Historical resource regimes in soils have been shown to affect future microbial function, suggesting that evolved community adaptations are important even in the face of a changing soil environment (Keiser et al 2011). Similarly, microbial communities that have evolved in high organic matter environments may be better adapted to quickly process added diesel.

When microbial communities were inoculated into new litter environments, the resource regimes under which they had historically evolved affected decomposition rates, but these communities became more effective at decomposing particular substrates following successive 100-day exposures (Keiser et al 2011). Since hydrocarbon contaminants can persist in Arctic soils (Atlas 1986), exposure to the contaminant may be so prolonged that the resource history of the microbial communities prior to contamination may no longer be relevant. Metagenomic analysis of a site that had experienced chronic heavy-metal contamination over several decades showed that the microbial community had simplified significantly, both in terms of functional potential and taxonomic diversity (Hemme et al 2010). Further studies of extended hydrocarbon contamination will help in determining how important it is to consider microbial resource history when designing bioremediation treatments.

5.4.3. Predictability of hydrocarbon degradation in Arctic soils

We found bacterial diversity in the initial soils to be predictive of degradation in DSL-MAP soils, but not in DSL soils. Traditionally, the debate of how diversity influences productivity centres around the theories of sampling effect and niche complementarity; essentially whether the selection of a single efficient organism or a

group of complementary organisms are responsible for a given function (Bell et al 2005, Fargione et al 2007). Interestingly, diversity in DSL-MAP soils was not related to degradation, suggesting that diversity may be important in the initial selection of organisms, but less important for actual hydrocarbon degradation. Other recent studies have also shown that the diversity-function relationship depends on the function being considered, and that in some cases, the presence of specific taxa is more important than actual diversity (Salles et al 2009, Peter et al 2011). Most of the major taxonomic groups were present in all soils, so it may be that evenness increased the number of groups that could potentially respond to nutrient addition, as the presence of dominant bacterial taxa can sometimes limit the establishment of other bacteria (Heczko et al 2000).

We were also able to link specific taxonomic assemblages with diesel degradation. Although a number of taxa correlated significantly with degradation in both DSL and DSL-MAP soils, groupings of *Betaproteobacteria* were the best predictor of degradation across all soils. When the proportion of *Betaproteobacteria* consisting of *Burkholderiaceae*, *Oxalobacteraceae* and *Nitrosomonadaceae* in the initial undisturbed Arctic soils was high, hydrocarbon degradation could be expected to proceed well without treatment, while the opposite was true when mostly *Burkholderiales inc. sed.*, *Comamonadaceae* and *Rhodocyclaceae* were present. Members of most of these families are known to be capable of degrading hydrocarbons (Pepi et al 2003, Castorena et al 2006, Owsianiak et al 2009, Mbadinga et al 2011), so it was interesting that the relative abundance of these assemblages in contaminated soils did not correlate with degradation. Instead, they likely identify an initial soil parameter that is important for hydrocarbon degradation, such as moderate pH.

In contrast to this was the relationship between *Betaproteobacteria* and hydrocarbon degradation in DSL-MAP soils. Whereas specific *Betaproteobacteria* families were predictive of degradation in DSL soils, *Betaproteobacteria* abundance in the initial soils could not predict the extent of degradation in the presence of added nutrients. However, the relative abundance of *Betaproteobacteria* in DSL-MAP soils was strongly and positively related to degradation, suggesting a potentially important active role for this group. In a study that aimed to determine optimal bioremediation cultures for diesel degradation using microautoradiography, *Betaproteobacteria* represented over half of radioactively labeled cells (Hesselsoe et al 2008), and *Betaproteobacteria* have previously been shown to be positively correlated with carbon mineralization across a range of soils (Fierer et al 2007), suggesting that this correlation could represent a functional relationship. If *Betaproteobacteria* can be confirmed as the most efficient petroleum degraders in nutrient-amended soils, future approaches to bioremediation should attempt to favour *Betaproteobacteria*, or limit the growth of competing taxa that may hinder the success of this group.

5.4.4. Conclusions

Because petroleum contamination is increasingly likely throughout the Arctic, it is important to understand the factors that will shape petroleum hydrocarbon-degrading microbial communities, as well as rates of contaminant degradation in soils with different physicochemical characteristics. Despite the apparent geographic isolation between the soils used in our study, we observed consistent microbial community shifts when both diesel and nutrients were added, towards *Actinobacteria* dominance in low organic matter

soils and *Proteobacteria* dominance in high organic matter soils. Bioremediation potential was also predictable across soils, based on the initial 16S rRNA gene diversity or *Betaproteobacteria* composition of uncontaminated soils. In addition, total *Betaproteobacteria* abundance was correlated with effective degradation in the presence of nutrient amendments, suggesting that this may be an important taxonomic group to stimulate. Site-specific assessments of bioremediation potential are expensive and time-consuming (Dorn and Salanitro 2000), so predictability will be invaluable in responding to future contamination events. Since the concentration, age, and composition of petroleum contaminants at affected sites is highly variable, future work should aim to identify how consistently these patterns apply, especially in soils with aged contaminants in which the original resource history of soils may no longer be relevant or available for study.

5.6. Acknowledgements

We thank Matt Wallenstein, Claudia Boot, Nicolas Lecomte, Emilie Champagne, Jen Allan, Isabelle Laurion, Chantal Lemieux, Karita Neghandi, Philippe Galipeau, Marie-Jeanne Rioux, Katryne Larrivée, JF Lamarre, Elise Bolduc, and the Centre d'Études Nordiques for soil collection, Julie Champagne for sequencing assistance, and Chantale Beaulieu and Stéphane Deschamps for assistance with GC analysis. This work was supported by the NRCan PERD Program, and an NSERC postgraduate scholarship to TH Bell.

Connecting Text

In Chapter 5, it was shown that there are consistent environmental controls on bacterial abundance in hydrocarbon-contaminated and nutrient-amended soils. *Actinobacteria* tended to dominate soils with low organic matter content (<10%), while *Proteobacteria* dominated soils that were high in organic matter. Bacterial diversity and specific taxonomic assemblages in uncontaminated soils could predict eventual bioremediation efficiency, while *Betaproteobacteria* were found to be associated with effective bioremediation in contaminated soils that had been amended with monoammonium phosphate. Since the environment has such a strong effect on promoting hydrocarbon-degrading microorganisms, the question remains as to whether more efficient taxa could be promoted. Chapter 6 describes a study in which targeted bacterial inhibitors were used to determine whether a change in relative taxonomic abundance within a soil from CFS-Alert could alter rates of diesel degradation.

6

The use of bacterial inhibitors alters microbial composition and increases diesel degradation in an Arctic soil

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In revisions with *FEMS Microbiology Ecology*.

This chapter was written by the candidate, with helpful comments provided by all co-authors. The candidate also performed most of the experimental work, designed the study, and analyzed the data. Dr. Etienne Yergeau provided valuable contributions to the experimental design, direction, and data analysis.

6.1. Abstract

A wide array of microbial taxa are active in hydrocarbon-contaminated Arctic soils, and many are capable of hydrocarbon metabolism. However, the most effective hydrocarbon degraders may not be dominant, meaning shifts in microbial abundance could improve contaminant reduction. In this study, we contaminated an Arctic soil with diesel, and used gentamicin and vancomycin to selectively inhibit portions of the microbial community. We measured diesel degradation using gas chromatography, bacterial and fungal abundance using qPCR, and assessed bacterial diversity and

community composition through Ion Torrent sequencing of 16S rRNA gene amplicons. Combined addition of both antibiotics increased diesel degradation significantly relative to the no antibiotic treatment, despite reduced bacterial and fungal abundance, while degradation was comparable to the control when nutrients were also added. All treatments produced unique bacterial communities, and addition of both antibiotics led to dominance by *Xanthomonadaceae* and *Micrococcineae*. Although the bacterial communities resulting from gentamicin and vancomycin addition were similar with and without nutrients, nutrient addition produced a much larger fungal population, possibly reducing hydrocarbon-degrading activity through competition. These results suggest that the most efficient hydrocarbon-degrading community is not promoted naturally, and approaches that target specific microbial taxa may be required to promote *in situ* bioremediation performance.

6.2. Introduction

Hydrocarbon-degrading microorganisms inhabit many ecosystems (Greer et al 2010) and can metabolize and degrade petroleum contaminants for use as energy and carbon sources in a process known as bioremediation. Numerous *in situ* treatments are currently used to increase rates of hydrocarbon breakdown in contaminated soils, including supplementation with nutrients and/or oxygen to stimulate the growth of the existing microbial community (Powell et al 2006a, Delille and Coulon 2008, Yergeau et al 2009), or the addition of cultured hydrocarbon-degrading microorganisms (Thomassin-Lacroix et al 2002, Thompson et al 2005). Effective treatments are especially important in hydrocarbon-contaminated Arctic soils, as significant hydrocarbon degradation may

only occur when temperatures exceed 0°C (Walworth et al 2001), which translates to roughly two months each year in the high Arctic (Chapter 4; Bell et al 2011). However, the effectiveness of such treatments has varied across contaminated polar soils (Powell et al 2006a, Delille and Coulon 2008, Yergeau et al 2009, Chapter 5; Bell et al submitted), suggesting that more targeted approaches may be required.

The role of cooperative interactions between microbial species in hydrocarbon-contaminated soils has been discussed by several authors (Brennerova et al 2009, Ciric et al 2010, Vilchez-Vargas et al 2010), but little attention has been paid to the potentially negative effects of interspecies competition. The growth and activity of some organisms can be reduced in the presence of competing species (Case and Gilpin 1974, Mille-Lindblom et al 2006, Meidute et al 2008, Violle et al 2011), so the inhibition of taxa that are less efficient at hydrocarbon degradation may in fact increase the bioremediation capacity of a mixed microbial community. It is expected that most of the energy flux in hydrocarbon-contaminated soils comes from a small number of highly active taxa (Dejonghe et al 2001), and while specialized microorganisms are required to transform substances such as uranium (Gihring et al 2011) and chlorinated solvents (Tas et al 2010), the ubiquity of natural hydrocarbons means that many co-existing taxa have evolved pathways that can be used to metabolize petroleum contaminants. In addition, species that are not directly involved in hydrocarbon degradation may obtain carbon and energy through cross-feeding (DeRito et al 2005), and will still occupy critical space and resources. Despite extreme climatic conditions, microbial diversity in Arctic soils is similar to what is found at lower latitudes (Neufeld and Mohn 2005, Chu et al 2010), and

hydrocarbon-contaminated Arctic soils have also been shown to maintain a variety of active taxa (Greer 2009, Chapter 4; Bell et al 2011).

A positive relationship between microbial richness/diversity and functional productivity has sometimes been observed, supporting the biodiversity and ecosystem productivity hypothesis (Bell et al 2005, Gravel et al 2011), however, this relationship is function-specific (Griffiths et al 2000, Peter et al 2011), and can depend more on the presence of specific taxa than diversity *per se* (Salles et al 2009, Peter et al 2011). Reduced microbial diversity has even been shown to increase productivity under some circumstances. Following the reduction of soil microbial diversity through chloroform fumigation, decomposition of added straw and ryegrass has increased, as has thymidine and leucine incorporation (Degens 1998, Griffiths et al 2000). This reverse relationship has even been observed in the context of bioremediation, as chloroform fumigation of soil containing the pesticide 2,4-dichlorophenoxyacetic acid prior to inoculation with *Alcaligenes xylosoxidans* led to more than a 10-fold increase in contaminant reduction (Gunalan and Fournier 1993). Dominant taxa that do not significantly contribute to specific metabolic processes still use important resources, potentially limiting the effectiveness of more functional microorganisms (Jiang 2007). This means that the reduction of diversity could actually reduce limitations on productivity. This mirrors well-studied relationships among macroorganisms, whereby the loss of key species that maintain ecological equilibrium can lead to the rapid depletion of resources (Beschta and Ripple 2009), which is precisely the goal in bioremediation.

In this study, we used antibiotics targeting different portions of the microbial community to determine whether shifts in the relative abundance of taxa would affect the

degradation of added diesel in a high Arctic soil. Although these antibiotics are not 100% effective in the inhibition of entire phylogenetic groups, they can predictably shift the bacterial composition of complex communities (Antonopoulos et al 2009, Robinson and Young 2010), as opposed to methods such as dilution and chloroform fumigation which are less targeted. In this study, we used gentamicin and vancomycin to target mainly Gram-negative and Gram-positive microorganisms, respectively, as most of the identified hydrocarbon degraders in contaminated Arctic soils are members of the phyla *Proteobacteria* (Gram-negative) and *Actinobacteria* (Gram-positive) (Whyte et al 2002a, Chapter 4; Bell et al 2011, Chapter 5; Bell et al submitted). Since the soil environment has been shown to select the composition of hydrocarbon-degrading communities (Chapter 5; Bell et al submitted), this study aimed to determine whether changing the microbial community within a single soil could affect rates of hydrocarbon breakdown.

6.3. Methods

6.3.1. Soil collection and microcosm setup

The top 15 cm of an uncontaminated soil was collected from CFS-Alert, Nunavut. Details of the CFS-Alert site are provided in Bell et al. (Chapter 4; 2011). The soil had an average pH of 7.88, an organic matter content of 4.90 ± 0.16%, and a starting water content of 12.20 ± 0.35%. Bulk soil was contaminated to a final concentration of 5500 mg/kg of ultra low sulphur diesel. This soil was then incubated for one week at 1°C to allow the weathering of volatile components as in Bell et al. (Chapter 5; submitted). Homogenized soil was split in half, with one half receiving 250 mg/kg of monoammonium phosphate (MAP), which has been used successfully as a biostimulation

treatment in hydrocarbon-contaminated soils at CFS-Alert (Greer 2009). Four antibiotic treatments were then applied to each half of the treated soil, using gentamicin and vancomycin (Table 6.1.).

Table 6.1. Treatments and corresponding abbreviations.

Antibiotic	*Concentration (mg/g soil)*	*MAP added*	*Abbreviation*
Baseline (untreated soil)	n/a	n/a	BAS
None	n/a	-	NAN
	n/a	+	NAM
Gentamicin	2.0	-	GEN
	2.0	+	GMA
Vancomycin	0.8	-	VAN
	0.8	+	VMA
Gentamicin + vancomycin	2.0 / 0.8	-	GVA
	2.0 / 0.8	+	GVM

Vancomycin and gentamicin are bactericidal antibiotics (Shelburne et al 2004). Vancomycin is a glycopeptide antibiotic that inhibits the synthesis of peptidoglycan for bacterial cell walls, and primarily targets Gram-positive bacteria (Reynolds 1989), while gentamicin is an aminoglycoside that acts mostly through protein synthesis inhibition, and is particularly effective against many Gram-negative bacteria (Jana and Deb 2006). Three 30 g replicates of each treatment were incubated at 10°C for 6 weeks in loose-top Falcon tubes. To ensure that soil water content did not limit microbial activity, 2.5 ml of sterile water was added equally to treatments after the first 3 weeks of incubation. The final average water content was 18.7 ± 0.25%. At the end of the incubation, soil was used for DNA extraction and hydrocarbon analysis. Quantification of hydrocarbons was performed as described in Bell et al. (Chapter 5; submitted).

6.3.2. Soil DNA extraction, 16S rRNA gene amplification, and Ion Torrent sequencing

Total soil DNA was extracted from 10 g of soil from each of three replicates per treatment using the MoBio UltraClean® Mega Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). Partial 16S rRNA gene amplicons were produced from each extract with the universal bacterial primers E786 (5'-GATTAGATACCCTGGTAG-3') and U926 (5'-CCGTCAATTCCTTTRAGTTT-3') (Baker et al 2003), and were each labeled with adaptor sequences and unique multiplex identifier codes (MID) for Ion Torrent sequencing. PCR reactions were performed using 12 µl of KAPA2G Robust DNA Polymerase mix (KapaBiosystems Inc., Boston, MA, USA), 12 µl of nuclease-free deionized water, and 40 pmol of each of the appropriate forward and reverse primers. PCR cycling consisted of an initial 5 min denaturing step at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C, and a final elongation step of 7 min at 72°C. Gel purification of amplicons was performed using the illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Piscataway, NJ, USA), and DNA in the purified eluate was quantified using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, Burlington, ON, Canada), pooled in an equimolar ratio, and diluted to a concentration of 5×10^7 molecules for sequencing. Sequencing was performed on an Ion Torrent Personal Genome Machine™ using the Ion Xpress™ Template Kit and the Ion 314™ chip following manufacturer's protocols. Ion Torrent sequencing produces microbial community profiles that are very similar to those produced by Roche 454 sequencing (Yergeau et al 2012b).

6.3.3. qPCR

Quantitative real-time PCR (qPCR) was performed to determine relative 16S rRNA and fungal ITS/5.8s gene copy numbers. All qPCR reactions were performed in 20-µl volumes using the SYBR green QuantiTect PCR mix (Qiagen, Mississauga, Ontario, Canada) in a Rotor-Gene 3000 apparatus (Corbett Life Science, Sydney, New South Wales, Australia). We used the primer sets described in Fierer *et al*. (2005) for quantifying total bacteria (Eub338/Eub518) and fungal (5.8s/ITS1f) populations. Conditions were as described in Yergeau *et al*. (2010a). Standards were made from 10-fold dilutions of linearized plasmids containing the gene fragment of interest that was cloned from amplified pure culture DNA (16S rRNA gene, $R^2 = 0.999$; fungal ITS/5.8s, $R^2 = 0.990$). Tests with Lambda phage DNA as in Yergeau *et al*. (2009) showed little to no qPCR inhibition, so values were not adjusted (data not shown).

6.3.4. Sequence and statistical analysis

Sequences were binned by multiplex identifier (MID) code, and MID codes were trimmed from each sequence. Filtering of sequences was performed using a moving average Q15 cutoff; if the average of 5 bases along the sequence fell below Q15, the sequence was trimmed at that point. Reads of less than 100 bp were removed from analysis. Taxonomic identities were assigned to sequences using the RDP Pipeline Classifier (http://pyro.cme.msu.edu/). The maximum relative abundance within treatments was determined for taxonomic assignments at the family level. The thirty most abundant taxa were selected, each was normalized to the maximum value within

samples for that taxonomic assignment, and these were visualized using a heat map created in R with the 'gclus' and 'vegan' packages.

The relationship between samples was determined by mapping sequences against the Green Genes core dataset using Fast UniFrac (Hamady et al 2010), and the resulting distance matrix was visualized using PCoA analysis in R. Shannon Diversity values were calculated directly from unclassified sequences, after the number of sequences was standardized between samples by eliminating sequences with random numbers as in Fortunato et al. (2012). Sequence processing was performed mostly in MOTHUR (Schloss et al 2009). Sequences were aligned to the Green Genes core set, putatively noisy sequences were removed using the 'pre.cluster' algorithm that is based on the procedure by Huse et al. (2010), and potential chimeras were removed with Uchime. Sequences were then converted to a distance matrix, and clustered using an average-clustering algorithm. Average operational taxonomic unit (OTU) diversity at the 5% dissimilarity cutoff was visualized for each treatment using rarefaction curves.

ANOVA was used to statistically test for differences between treatment means in hydrocarbon degradation, qPCR results, and the relative abundance of major taxonomic groups. Differences between specific means were assessed using Tukey's post-hoc test. Bacterial 16S rRNA and fungal ITS/5.8s gene copy numbers, as well as bacterial:fungal gene quantification ratios were log transformed prior to ANOVA, since values spanned several orders of magnitude. All ANOVAs were performed in JMP 8.0 (SAS Institute, Cary, NC).

6.4. Results

6.4.1. Diesel degradation

The amount of diesel degraded during the 6-week incubation varied between treatments (ANOVA; p=0.0083), and was significantly higher in the GVA treatment than NAN and VAN treatments (Figure 6.1.). Excluding GVA and GVM, MAP addition led to increased diesel degradation under each antibiotic scenario, although these differences were not found to be significant by Tukey's post-hoc test.

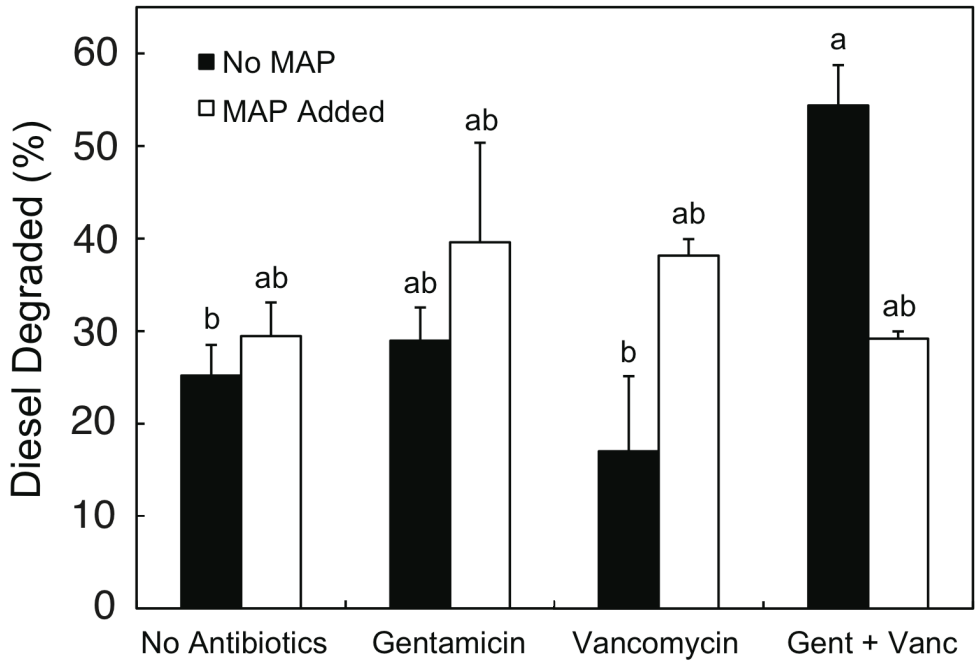

Figure 6.1. Diesel degraded (%) in soils over the 6-week incubation period following treatment with various antibiotic treatments, and with or without monoammonium phosphate (MAP) added. Bars represent standard error. Different letters above columns indicate significant differences between means ($p<0.05$), as determined by Tukey's post-hoc test.

6.4.2. qPCR

Bacterial 16S rRNA gene copy number varied significantly between treatments ($p < 0.0001$). The addition of gentamicin led to copy numbers that were approximately two orders of magnitude lower than were quantified in other treatments, and there were no obvious differences between soils with and without MAP added (Figure 6.2.a). Fungal ITS/5.8s gene copy number also varied between treatments ($p<0.0001$). Similar to 16S rRNA gene quantification, gene copies were lowest in the GEN and GVA treatments, although MAP addition did increase fungal populations in the GMA and GVM treatments. MAP addition reliably increased fungal copy numbers regardless of antibiotic treatment, and gene copies were highest in VMA (Figure 6.2.b). The ratio of 16S rRNA gene to fungal ITS/5.8s copies differed between treatments ($p<0.0001$), and was lowest in the presence of both antibiotics and MAP, irrespective of antibiotic treatment (Figure 6.2.c).

6.4.3. 16S rRNA gene diversity and composition

After filtering, we obtained a total of 138 968 usable reads, with an average of 5147 reads per replicate. Rarefaction curves showed a general decline in species diversity in all treatments relative to the initial soil, with the exception of VAN (Figure 6.3.). The lowest plateaus were observed when gentamicin was present, and in the NAM treatment. The rarefaction curves for NAN and VAM were comparable, and indicated that OTU diversity was intermediate to that of the other groups. Mean OTU diversity was not correlated to total hydrocarbon degradation. Fast UniFrac analysis showed that OTU composition separated primarily by whether or not gentamicin was present (Figure 6.4.).

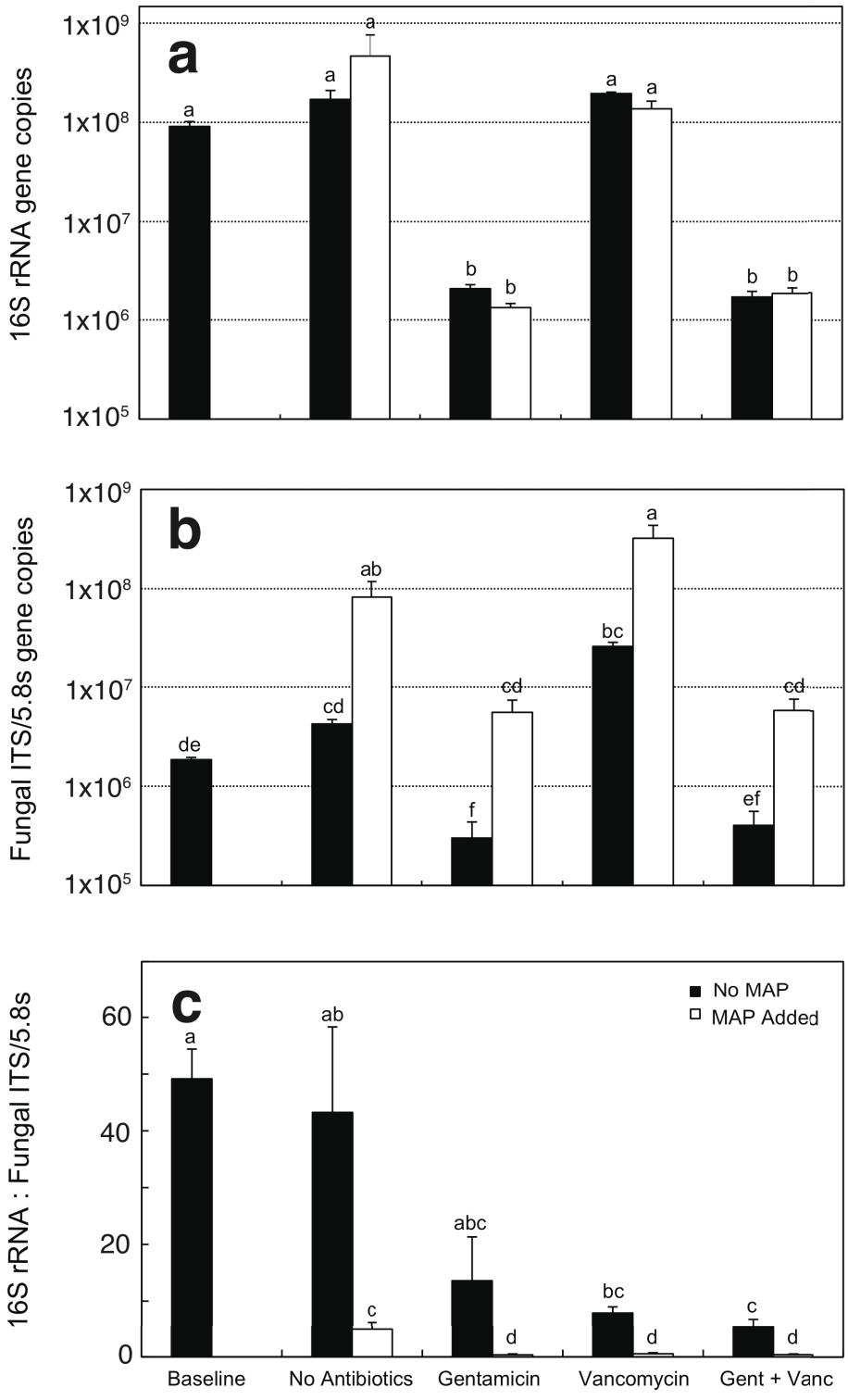

Figure 6.2. Gene quantification from qPCR assays of a) bacterial 16S rRNA, and b) fungal ITS/5.8s genes. Also shown is c) the ratio of 16S rRNA and fungal ITS/5.8s genes. Bars represent standard error. Different letters above columns indicate significant differences between means ($p<0.05$), as determined by Tukey's post-hoc test.

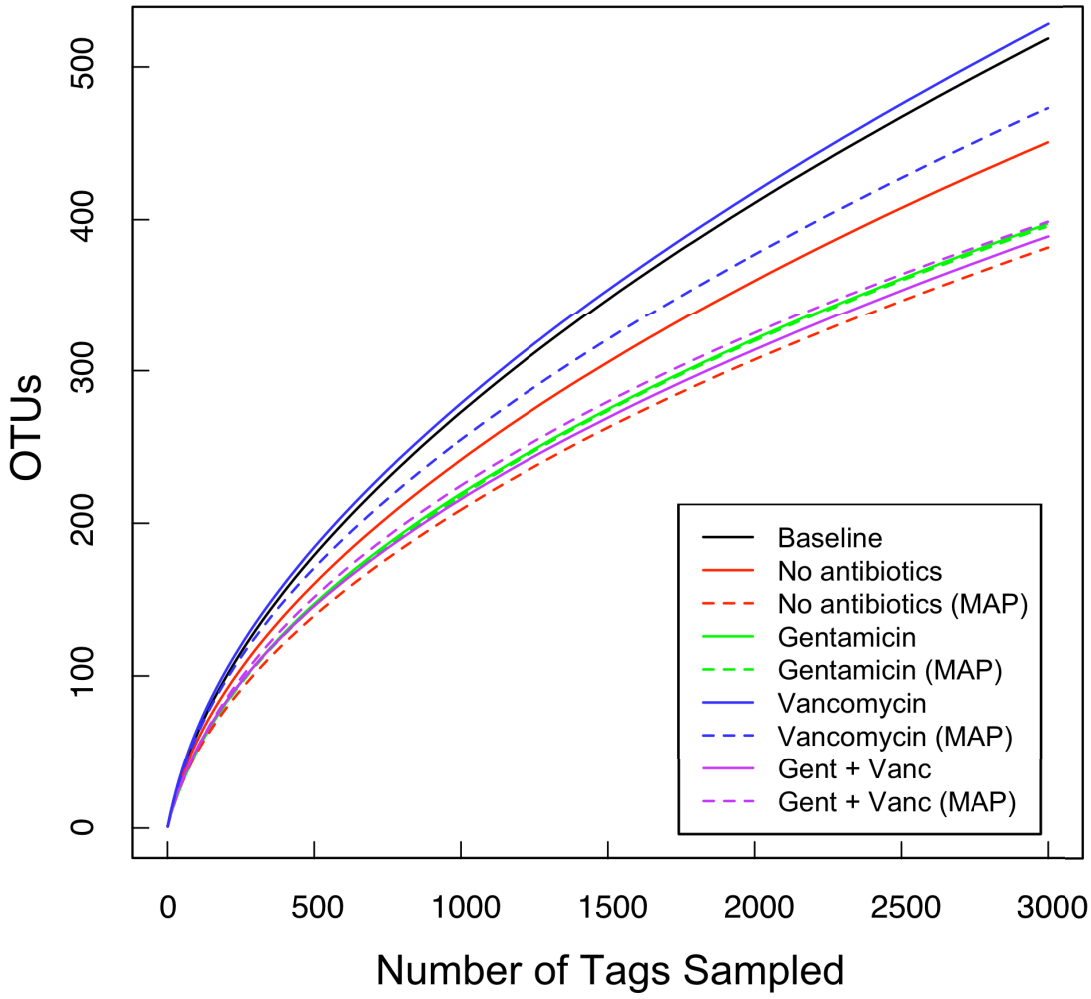

Figure 6.3. Rarefaction curves showing differences in bacterial 16S rRNA gene OTU diversity between treatments using a 5% dissimilarity cutoff.

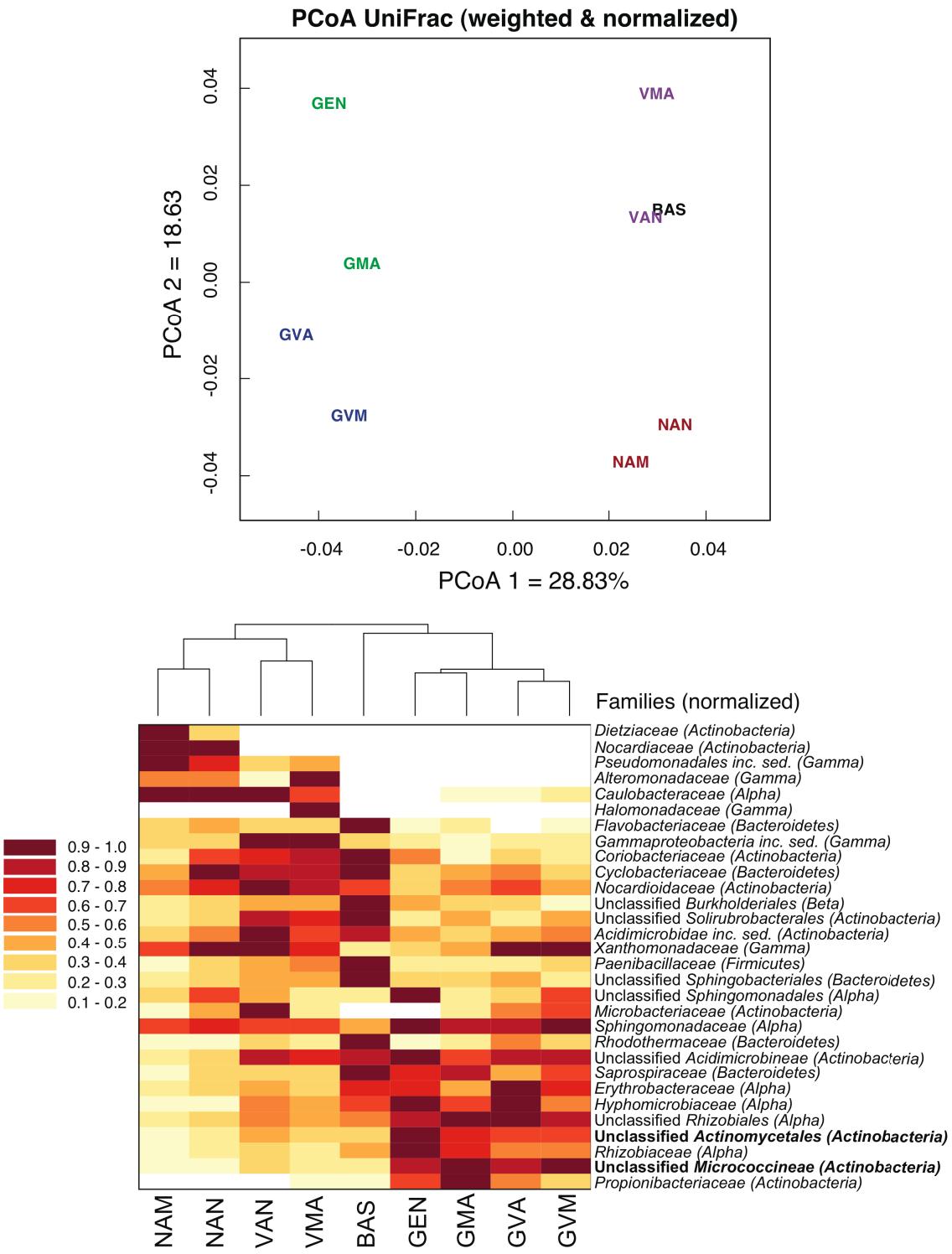

Figure 6.4. Bacterial community composition as assessed by Ion Torrent sequencing of 16S rRNA gene amplicons. Samples were separated through principal component analysis using a distance matrix created in Fast UniFrac (upper panel). A heat map demonstrates variation in the relative abundance of the thirty most commonly identified taxa classified to the family level, with each taxa normalized to the maximum abundance for all treatments (lower panel). The majority of unclassified *Actinomycetales* had a best match of *Micrococcineae*, so both of these groups are represented in bold.

Samples separated on the secondary axis based on antibiotic treatment, and then by the presence or absence of MAP.

Comparison of the thirty most abundant groups at the family level showed differences between all treatments (Figure 6.4.). Following diesel contamination, many groups within the *Bacteroidetes* and *Firmicutes* declined. The most striking shift within the contaminated soils was the complete absence of the *Dietziaceae* and *Nocardiaceae* when antibiotics were added, as they were some of the most abundant groups in the no antibiotic treatments. Sequences related to the *Micrococcineae* suborder of *Actinobacteria* were highly represented in all soils treated with gentamicin. Unclassified *Actinomycetales* were also prevalent in these soils, and over 75% of these sequences had a best match of *Micrococcineae* that fell below the 0.50 classification cutoff. All soils receiving gentamicin were also characterized by a higher relative abundance of most of the major families of *Alphaproteobacteria*, with the exception of the *Caulobacteraceae* which were more abundant in the vancomycin-treated and no antibiotic soils. *Xanthomonadaceae* were more abundant in VAN, VMA, GVA and GVM than in the other treatments.

The ratio of Gram-negative to Gram-positive sequences varied by treatment ($p=0.0067$). There was a significant decline in the proportion of Gram-negative sequences in the GEN treatment relative to NAN, while the proportion of Gram-positive sequences in the VMA treatment was comparable to NAN (Figure 6.5.a). The Gram-negative to Gram-positive ratio was about 50% higher in the VMA treatment than the VAN treatment, although this difference was not significant.

The most abundant *Gammaproteobacteria* and *Actinobacteria* classifications were compared at the family level, and on average constituted over 50% of the sequences in all soils treated with gentamicin, as well as in the NAM treatment (Figure 6.5.b). As mentioned earlier, the *Nocardiaceae* and *Dietziaceae* were two of the most abundant families following contamination in the absence of antibiotics, particularly following the addition of MAP, but were absent when antibiotics were present. ANOVA comparisons of the contaminated samples showed a significant difference between treatments in the abundance of *Xanthomonadaceae* ($p=0.0366$), but treatments could not be separated by a Tukey's post-hoc test. There were also differences in the abundance of all unclassified *Micrococcineae* and *Actinomycetales* sequences ($p<0.001$), with a significant difference between soils receiving gentamicin and all other treatments.

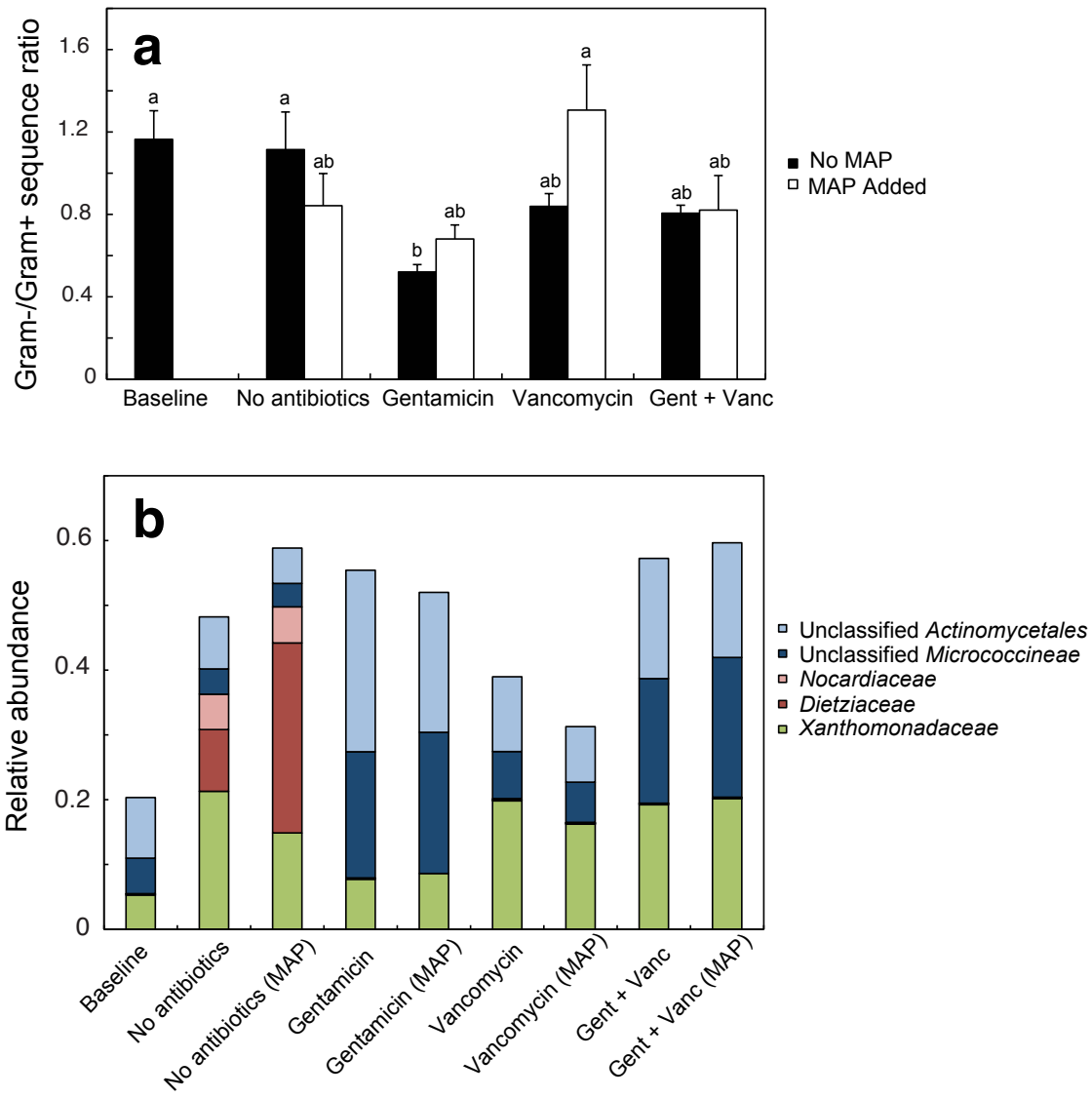

Figure 6.5. The main effects of antibiotics on microbial community composition are shown by a) changes in the Gram-negative/Gram-positive sequence ratio, and b) the unnormalized abundance of the main groups of *Actinobacteria* and *Gammaproteobacteria* identified across soils. Bars represent standard error. Different letters above columns indicate significant differences between means ($p<0.05$), as determined by Tukey's post-hoc test.

6.5. Discussion

In this study, we used diesel to contaminate a low organic matter soil from the Canadian high Arctic, and observed that the addition of targeted antibiotics altered soil microbial composition, and in some cases, increased the amount of diesel degraded during a 6-week incubation. Interestingly, total degradation was not tied to estimates of fungal and bacterial biomass, suggesting that many microorganisms may not contribute substantially to hydrocarbon metabolism under standard bioremediation conditions. Environmental factors appear to control the relative abundance of microorganisms in hydrocarbon-contaminated Arctic soils (Chapter 5; Bell et al submitted), so adaptations to specific soil environments, such as the ability to rapidly acquire nutrients and colonize substrate surfaces, may play an important role in determining the relative abundance of microbial taxa. This may result in lower abundances of highly efficient hydrocarbon degraders.

6.5.1. Microbial population size and degradation

We observed significantly higher diesel degradation in the GVA treatment when compared with NAN, even though the number of 16S rRNA gene copies in GVA was roughly two orders of magnitude lower. This result was surprising, but could indicate that a large percentage of the microbial population is not actively involved in degradation, as has been observed in a diesel-degrading mixed culture (Hesselsoe et al 2008). Microbial respiration has been linked to carbon availability as opposed to microbial biomass (Wang et al 2003), and the reduction of biomass in the GVA treatment may have increased the hydrocarbon surface area that was bioavailable to certain taxa. A number of

other studies have also shown microbial activity and biomass to be decoupled in soils. Following chloroform fumigation of an agricultural soil, microbial respiration increased significantly over the first 40 days of incubation, despite a halving of microbial biomass carbon (Griffiths et al 2000). Similarly, heterotrophic colony-forming units varied by more than an order of magnitude between biopiles from CFS-Alert, with no observed difference in ^{14}C-hexadecane mineralization (Greer 2009), and 16S rRNA gene copy number in frozen high Arctic permafrost can actually exceed that observed in the corresponding active layer (Wilhelm et al 2011). An increased carbon mineralization to biomass ratio in metal contaminated soils has been assumed to be related to increased energy expenditure associated with heavy metal tolerance (Nakatsu et al 2005), which may also be the case in our study as a result of resistance adaptations to antibiotics. Even cultured isolates that are grown without added stressors vary substantially in terms of the amount of carbon that they divert to CO2, biomass, and metabolites during hydrocarbon degradation (Bouchez et al 1996).

6.5.2. Fungal response to MAP

Biostimulation treatments in polar environments have generally been designed to target bacterial hydrocarbon degraders, so less is known about how fungi respond to nutrient additions in these soils (Hughes and Bridge 2009). However, in temperate soils, addition of mixed nutrient compost and NPK fertilizer to hydrocarbon-contaminated soils led to increases in fungal CFU counts and PLFA quantification, respectively (Margesin et al 2007, Li et al 2012). We observed that MAP addition significantly increased fungal abundance under all antibiotic scenarios, despite only minimal effects of MAP on

bacterial abundance. High bacterial growth rates following amendment in uncontaminated soils can limit fungal abundance (Meidute et al 2008), and a number of studies have demonstrated strong competitive interactions between fungi and bacteria in natural soil environments (Mille-Lindblom et al 2006, Meidute et al 2008, Siciliano et al 2009), suggesting that the limited bacterial growth response may have been to the benefit of fungal populations. Although not significant, we observed increases in hydrocarbon degradation in MAP-treated soils that were concurrent with fungal population increases (excluding GVA and GVM), but this does not necessarily indicate that fungi are efficient hydrocarbon degraders in these soils. Selective inhibition of soil bacteria and fungi using streptomycin and cycloheximide showed that bacteria were responsible for 82% of hexadecane mineralization, while fungi were responsible for only 13%, while both groups contributed equally to consumption of added glucose (Song et al 1986). Substantial and direct involvement of Arctic fungi in the bioremediation of hydrocarbon-contaminated soils remains to be demonstrated, as carbon from soil organic matter and bacterial biomass and metabolites may also supplement fungal metabolism. However, fungi are known to indirectly assist hydrocarbon degradation in some cases, as bacterial dispersal in unsaturated soil matrices can be greatly facilitated by the presence of fungal mycelia (Furuno et al 2010).

6.5.3. Shifts in bacterial diversity

The effect of antibiotics on bacterial diversity was mixed. We saw that in the absence of antibiotics, diesel addition led to a lower rarefaction curve than that obtained from the initial soil, while the NAM curve was lower still, a trend that is consistent with

that observed in other low organic matter Arctic soils (Chapter 5; Bell et al submitted). As would be expected, gentamicin addition reduced diversity, while the addition of vancomycin alone appeared to limit the drop in diversity caused by diesel and MAP addition. A mixed culture study showed that high disturbance intensity increased *Pseudomonas* morphotype diversity, as it prevented dominance by a single well-adapted group (Hall et al 2012), and vancomycin may have played a similar role in our study by limiting the success of species that respond rapidly to diesel and nutrient input. However, the same study showed low diversity at the highest disturbance frequency and intensity (Hall et al 2012), possibly explaining the opposing effect of gentamicin addition on diversity, since it simultaneously reduced bacterial populations. Many other studies have also suggested that moderate levels of disturbance can help in maintaining the diversity of organisms (Petraitis et al 1989, Biswas and Mallik 2010, Miller et al 2011). The fact that no relationship was observed between diversity and hydrocarbon degradation suggests that altering the abundance and relationships of specific microbial taxa is more important than non-specifically modifying diversity.

6.5.4. Distinct bacterial community composition across treatments

Bacterial community composition varied between all treatments, although antibiotic treatment was a more important factor than MAP treatment. Other studies of contaminated soils at CFS-Alert have identified *Pseudomonas* and *Rhodococcus* as dominant, and potentially important, hydrocarbon-degrading genera (Whyte et al 2002a, Yergeau et al 2012c). In this study, *Nocardiaceae*, the family to which *Rhodococcus* belongs, and the closely related *Dietziaceae,* dominated NAN and NAM soils, as did

Xanthomonadaceae, which is closely related to the *Pseudomonadaceae*. The disappearance of the two *Actinobacteria* families under all antibiotic treatments is not surprising, since *Dietzia* are highly sensitive to a variety of antimicrobials (Pilares et al 2010), as are *Rhodococcus* and other closely related genera, particularly to gentamicin and vancomycin (Soriano et al 1995). In the absence of these groups, hydrocarbon degradation was equal if not greater, demonstrating that a variety of effective hydrocarbon degraders are present in CFS-Alert soils. Although *Pseudomonas* and *Xanthomonas* have been shown to possess some gentamicin resistance (Heuer et al 2002), the *Xanthomonadaceae* declined in the GEN and GMA treatments. However, they remained dominant in the GVA and GVM treatments, possibly due to inhibition by vancomycin of competing taxa that were highly resistant to gentamicin alone.

The bacterial communities in the soils treated with both gentamicin and vancomycin were surprisingly similar to a mixed enrichment culture that degraded more than 50% of 10 000 mg l^{-1} crude oil in 7 days (Zhao et al 2011). The enrichment consisted of 7 genera including *Pseudomonas*, *Microbacterium* (*Micrococcineae*), and 3 genera of *Alphaproteobacteria*, two of which classify as *Rhizobiales*, all of which are closely related to groups that increased in relative abundance in the gentamicin + vancomycin treatments, especially GVA, while many other taxa declined in abundance. Nevertheless, nearly twice as much diesel was degraded in the GVA treatment when compared with the GVM treatment, suggesting that there were important differences between these two communities. Aside from a higher abundance of certain groups of *Alphaproteobacteria* in GVA, there was a much larger fungal population in GVM, which may have led to negative competitive interactions. When fungi are able to establish

themselves in an environment ahead of bacteria, they can be far more effective in direct competition for substrates (Mille-Lindblom et al 2006). Several polar bacterial isolates have been shown to exhibit substantial antifungal activity (Shekh et al 2011). Mesophilic species of *Xanthomonas*, *Pseudomonas*, and *Microbacterium* are known to engage in active competition with fungi, and to produce a variety of fungistatic compounds (Radtke et al 1994, Cavaglieri et al 2005, Weise et al 2012), and it is thought that the signalling activity of fungi and *Xanthomonas* may have deleterious effects on the communication abilities of both groups (Wang et al 2004). Active competition may force a reduced investment in hydrocarbon-degrading activity by bacteria that are already exposed to multiple stressors, namely diesel and antibiotics. *Xanthomonadaceae* are also known to obtain carbon from co-occurring microorganisms (Lueders et al 2006), so a reduction in other taxa may promote metabolism that is more focused on hydrocarbon contaminants.

6.5.5. Other contributing factors

Several other factors could also have influenced the observed shifts in diesel degradation. Firstly, increased microbial turnover could have led to faster substrate use. Predation from protists is known to increase microbial growth rates in contaminated environments (Kinner et al 2002, Head et al 2006), and cell-specific alkylbenzene and toluene degradation was between three and four times more effective when predators were present in culture (Mattison and Harayama 2001, Mattison et al 2005). A similar role may be played by phage, which are known to control microbial biomass in Arctic soils (Allen et al 2010a). Secondly, the addition of antibiotics can directly promote success in some microbial species. It is now well known that low concentrations of

antibiotics can act as signalling compounds, and have been specifically shown to stimulate motility and surface colonization (Linares et al 2006). *Pseudomonas* and *Burkholderia* have even been shown to use some antibiotics as sole carbon and nitrogen sources (Allen et al 2010b). The addition of vancomycin and gentamicin may also have differentially modified microbial transcription and translation in our soils, as sub-inhibitory concentrations of antibiotics have been shown to up and down-regulate a wide variety of bacterial genes and promoters (Goh et al 2002, Yergeau et al 2010b). The taxonomic composition of bacteria that were active in biofilm communities was also shown to differ widely based on the presence of various antibiotics, despite minimal differences in DNA abundance (Yergeau et al 2012d).

6.5.6. Conclusions

Most current approaches to the bioremediation of hydrocarbon-contaminated soils involve blanket treatments that aim to increase the biomass and activity of whole microbial communities. However, we have demonstrated that direct manipulation of microbial community composition, even following declines in microbial abundance, can lead to increased diesel degradation in an Arctic soil. Such increased degradation may result from a higher abundance of efficient hydrocarbon degraders, paired with a decline in the abundance of specific competitors. This suggests that future research should focus on identifying the most efficient hydrocarbon-degrading consortia in soils, and on finding safe and effective ways of modifying the structure and activity of bacterial populations, *in situ*.

6.6. Acknowledgements

We thank Christine Maynard and Julie Champagne for sequencing assistance and Chantale Beaulieu and Stéphane Deschamps for assistance with GC analysis. We also thank Dr. David Conrad for advice regarding antibiotic selection. This work was supported by the NRCan PERD Program, and an NSERC postgraduate scholarship to TH Bell.

7

General discussion and conclusions

Bioremediation, the harnessing of natural microbial metabolism to degrade or otherwise detoxify pollutants, is the method of choice for the remediation of hydrocarbon-contaminated polar soils. In general, this involves blanket treatments to soil microbial communities, especially biostimulation with major nutrients, in an attempt to increase overall microbial biomass and activity, but the effectiveness of such treatments has varied between sites, and the influence of blanket treatments on individual microbial taxa is mostly unknown. This thesis explored how microbial communities respond to biostimulation, specifically MAP, in hydrocarbon-contaminated Arctic soils. In Chapter 3, some of the factors potentially affecting the relative success of hydrocarbon-degrading taxa were discussed, as well as how this may affect rates of bioremediation. Some of these ideas were then tested and presented in the experimental chapters (Chapter 4-6), and general conclusions for these are provided in the following sections in the context of the thesis objectives. The main findings are also summarized graphically in Figure 7.1.

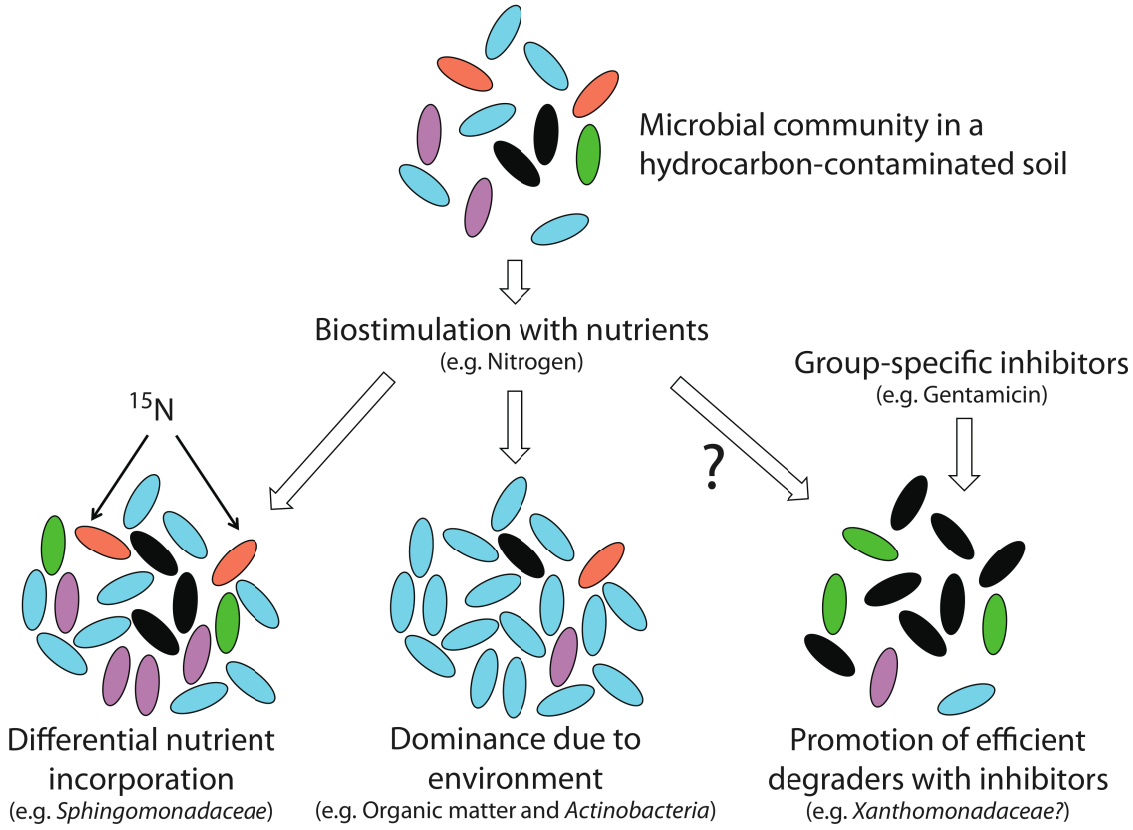

Figure 7.1. Although nutrients such as nitrogen are commonly added to stimulate hydrocarbon-degrading microbial communities in contaminated Arctic soils, a number of factors affect the relative success of competing taxa, including the ability to assimilate nutrients, and adaptation to the specific soil environment. The promotion of more efficient hydrocarbon degraders may be possible through the use of targeted microbial inhibitors, which may or may not require additional biostimulation.

7.1. Bacterial incorporation of added nitrogen

Although the addition of key nutrients such as nitrogen to hydrocarbon-contaminated soils can promote the growth and activity of microbial communities when these nutrients are limiting, individual taxa are likely to vary in the extent to which they can benefit from such treatments. If the beneficiaries are not efficient hydrocarbon degraders, they may slow rates of pollutant breakdown by directly competing with other microbial taxa, or by modifying the soil environment.

By using a novel ^{15}N DNA-SIP approach in which ^{15}N-MAP was added to hydrocarbon-contaminated soil from CFS-Alert as a biostimulation treatment, it was shown that many hydrocarbon-degrading taxa were active, and did incorporate the labeled nitrogen. Incorporation did vary between groups, however, and the *Alphaproteobacteria* were shown to be by far the most effective nitrogen-incorporating phylum, as determined by the extent of 16S rRNA and *alkB* gene sequence enrichment in heavy CsCl gradient fractions. It was also demonstrated that families within phyla incorporated added nitrogen differentially. Within the *Gammaproteobacteria* for example, the known hydrocarbon-degrading families *Pseudomonadaceae* and *Xanthomonadaceae* incorporated substantial amounts of nitrogen, while the *Sinobacteraceae*, a group that has not been identified as being directly involved in hydrocarbon degradation, did not.

The fact that *Alphaproteobacteria* are efficient at incorporating nitrogen is perhaps not surprising, as this group has previously been shown to respond well to high concentration inputs of nitrogen. However, the identified hydrocarbon-degrading members of this group, such as the *Sphingomonadaceae*, are typically known to be involved in the degradation of more recalcitrant compounds. Other work on hydrocarbon-contaminated CFS-Alert soils has shown that untreated soils contained a high ratio of alkane to PAH-degrading genes, and that groups such as *Pseudomonas* are associated with efficient degradation (Yergeau et al 2009, Yergeau et al 2012c). This suggests that less effective degraders may be efficiently siphoning resources, and that reducing this activity could potentially lead to more rapid bioremediation.

7.2. Factors affecting bacterial community structure in Arctic soils following hydrocarbon contamination

Extensive profiles of microbial populations using next-generation sequencing have started to reveal some of the controls on the relative abundance of microbial taxa in the environment. Nitrogen and pH are known to be especially important in the shaping of uncontaminated soil bacterial communities. What is not known is whether these controls continue to affect bacterial community structure following disturbance from hydrocarbon contaminants, and subsequent bioremediation treatments, especially nitrogen. Do the most efficient hydrocarbon degraders succeed, or is taxonomic abundance still determined primarily by the environment?

Disturbance from added diesel was shown to reduce bacterial 16S rRNA gene diversity in soils collected from across the Arctic, but diversity was reduced even more by the addition of MAP. The treatment of diesel-contaminated soils with MAP was shown to essentially polarize the relative abundance of bacterial taxa according to pre-existing physico-chemical controls. The relative abundance of *Actinobacteria* tended to increase in soils with less than 10% organic matter, while the abundance of *Proteobacteria* increased in higher organic matter soils. Bacterial diversity was also better preserved in higher organic matter soils following MAP addition, while a substantial drop was observed in soils with less than 10% organic matter. The fact that the environment plays such a powerful role in shaping microbial community structure in contaminated soils shows that the most efficient hydrocarbon degraders will not necessarily be promoted if they are not as competitive in a given soil environment. On the other hand, hydrocarbon-

degrading ability may not be confined to specific taxa across all environments, and could vary depending on resource and evolutionary history.

7.3. Correlation of *in situ* taxa with bioremediation efficiency

Since biostimulation effectiveness varies between soils, site-specific assessments of hydrocarbon-degrading capacity are generally conducted in order to determine an appropriate course of action, and these can be time-consuming and expensive. As a result, the ability to use sequence data to predict remediation capacity via bioattenuation and following specific treatments, would be invaluable.

The efficiency of bioattenuation in diesel-contaminated Arctic soils was predictable by the presence of specific family assemblages of *Betaproteobacteria* in uncontaminated soils, although the abundance of these assemblages following four weeks of diesel contamination did not correlate with the amount of hydrocarbon degradation. Bacterial diversity in uncontaminated soils predicted how effectively soils would degrade diesel in the presence of MAP, but diversity in contaminated MAP-treated soils was not correlated with degradation. These results imply that specific taxonomic assemblages and bacterial diversity play a role early in the degradation process, or that they are indicative of other soil parameters that are key to effective bioremediation. On the other hand, the relative abundance of *Betaproteobacteria* in contaminated soils treated with MAP did correlate with degradation efficiency, suggesting that this group may be actively involved in efficient hydrocarbon degradation, and could be an important group to promote at future contaminated sites.

7.4. The effect of altering microbial community structure on rates of bioremediation

Since the environment, especially organic matter, has been shown to affect the relative abundance of microbial species in hydrocarbon-contaminated Arctic soils, it is not clear that the most efficient hydrocarbon-degrading species are dominant following contamination and subsequent bioremediation treatments. Even if effective species do dominate a community, their activity may be inhibited by competition from other active co-occurring species. It would be expected that altering the relative abundance of microorganisms within a given soil community should affect rates of hydrocarbon degradation, unless competition did not play a role in limiting rates of bioremediation, or many species were functionally redundant with respect to hydrocarbon degradation.

Using targeted antibiotics, relative taxonomic abundance within contaminated soil from CFS-Alert was altered, and this led to subsequent shifts in total diesel degradation over the course of 6 weeks. The addition of MAP was shown to increase fungal abundance under all antibiotic treatments, while bacterial abundance was mostly unaffected by MAP addition, and declined in the presence of gentamicin. Quite surprisingly, the simultaneous addition of both gentamicin and vancomycin led to the highest amount of hydrocarbon degradation after the 6-week incubation. The bacterial community in this soil was characterized by a high abundance of *Xanthomonadaceae*, *Micrococcaceae*, and several groups of *Alphaproteobacteria*, including groups related to the *Rhizobiales*. Diesel degradation was lower when MAP was also added with gentamicin and vancomycin, and this may have been related to competition with a larger fungal population. Overall, these results suggest that the direct manipulation of microbial

communities can impact function in soils, including the bioremediation of hydrocarbon contaminants.

7.5. Future directions

In the near future, it will undoubtedly be possible to conduct *in situ*, high-throughput functional assessments of individual microbial taxa, and even individual microorganisms. These techniques will finally enable quantification of the contributions of specific taxa under natural conditions, but in an applied field such as bioremediation, it will be important to understand the full potential contribution of different strains to metabolic processes. For this to be possible, advances in culturing methods and in altering the relative abundance and/or activity of specific groups in soil will be essential.

As is the case in systems of higher organisms, it appears that the reduction of interspecies competition can lead to a more rapid reduction in resources; in the case of hydrocarbon-contaminated Arctic soils, this refers to the contaminants themselves. Obviously the widespread application of antibiotics to hydrocarbon-contaminated Arctic soils is neither a safe nor realistic approach for altering microbial community structure, so less toxic alternatives should be explored. Sterilization of soil through irradiation has been applied to forest soil, leading to increased bacterial re-growth following fungal inhibition, as well as increased carbon substrate use (McNamara et al 2007). Soil fumigation is widely applied in agriculture (Ruzo 2006), and has been shown to sometimes result in higher plant infection, possibly because competitive relationships between pathogenic and other species are reduced (Shiomi et al 1999, van Elsas et al

2002), however, this approach is currently being phased out due to fumigant toxicity (Yates et al 2003).

Many aspects of microbial ecology in hydrocarbon-contaminated soils remain to be resolved, including whether the bioremediation efficiency of certain microbial groups changes depending on the chemical composition and concentration of contaminants, and what circumstances require specialist microorganisms. Nevertheless, a framework that considers the impact of all active microorganisms, and one that aims to identify efficient and targeted assessments and treatments of hydrocarbon contaminants, should be a priority. This will be especially important in extreme and remote terrestrial environments such as the Arctic, in which treatments can be expensive, and the annual window for bioremediation is limited by environmental factors. Ultimately, efficient bioremediation strategies will allow quick and effective responses to future contamination events in the Arctic, and will help to protect this fragile ecosystem from a growing human footprint.

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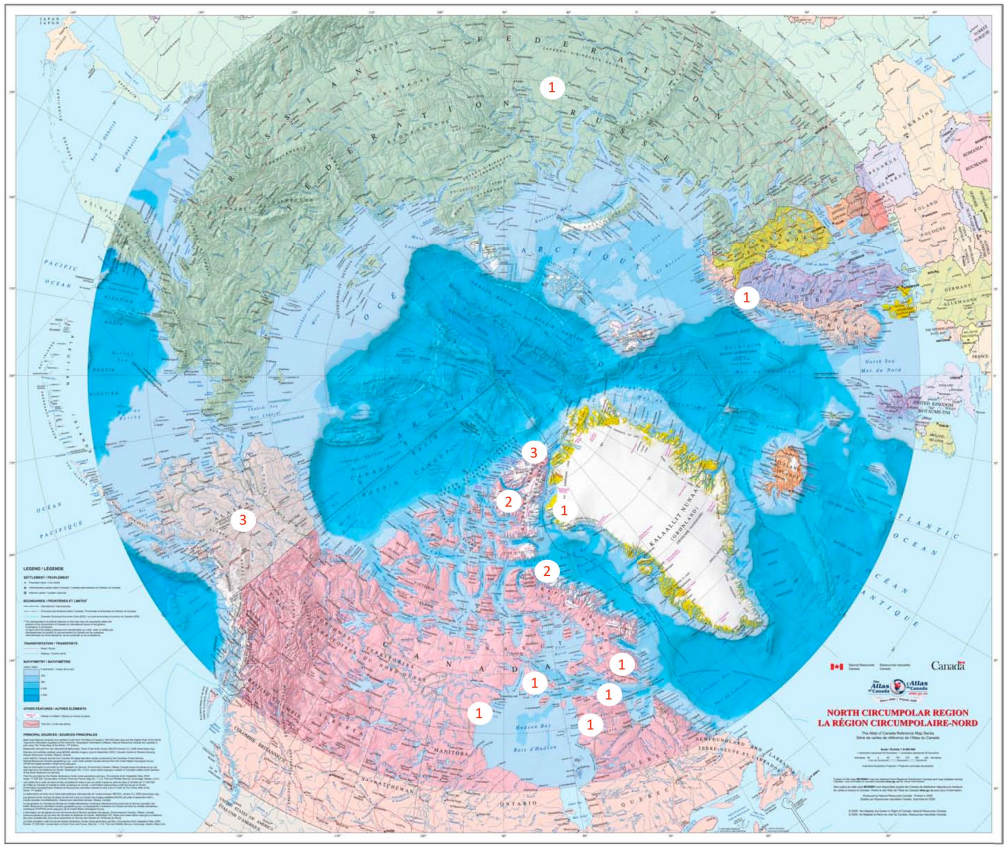
Appendices

Appendix 1. A map showing the distribution of soil samples used in this study. Numbers inside circles indicate the number of soil samples taken from a particular region. Adapted from the Atlas of Canada North Circumpolar Region Map of 2008 (http://atlas.nrcan.gc.ca/site/english/maps/archives/reference/circumpolar/MCR0001_circumpolar_2008).



Appendix 2. Correlations between the abundance of *Actinobacteria* and *Proteobacteria*, and three co-related environmental variables: organic matter, water content, and pH.