

MICROBIAL ECOLOGY AND BIODEGRADATION POTENTIAL OF A  
SULFOLANE-CONTAMINATED, SUBARCTIC AQUIFER

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## Abstract

Contaminant biodegradation is one of many ecosystem services aquifer microbiota can provide to humans. Sulfolane is a water-soluble emerging contaminant that is associated with one of the largest contaminated groundwater plumes in the state of Alaska. Despite being widely used, the biodegradation pathways and environmental fate of sulfolane are poorly understood. In this study, we investigated the biodegradation of sulfolane by the microbial community indigenous to this contaminated subarctic aquifer in order to better understand the mechanisms and rates of loss, as well as the environmental factors controlling them. First, we conducted aerobic and anaerobic microcosm studies to assess the biodegradation potential of contaminated subarctic aquifer substrate and concluded that the aquifer microbial community can readily metabolize sulfolane, but only in the presence of oxygen, which is at low concentration *in situ*. We also investigated the impacts of nutrient limitations and hydrocarbon co-contamination on sulfolane biodegradation rates. To identify exactly which community members were actively degrading sulfolane, we combined DNA-based stable isotope probing (SIP) with genome-resolved metagenomics methods. We found a *Rhodoferrax* sp. to be the primary sulfolane degrading microorganism in this system and obtained a near-complete genomic sequence of this organism, which allowed us to propose a new metabolic model for sulfolane biodegradation. Finally, we assessed the distribution of sulfolane-degrading bacteria throughout the contaminated subarctic aquifer by sequencing 16S rRNA genes from 100 groundwater samples and two sulfolane treatment systems and screening for the sulfolane degrader previously identified using SIP. This assessment revealed that sulfolane biodegradation potential is widespread throughout the aquifer but is not likely occurring under normal conditions. However, the sulfolane-metabolizing *Rhodoferrax* sp. was the most dominant microbe in an effective experimental air-sparge system, suggesting that injecting air into the aquifer can stimulate sulfolane biodegradation *in situ*. These studies are the first to investigate sulfolane biodegradation potential in a subarctic aquifer. Through this work, we learn there are several important factors limiting

biodegradation rates, we expand the known taxonomic distribution of sulfolane biodegradation, and we shed insights into the mechanisms underlying an effective *in situ* sulfolane remediation system.

## Table of Contents

	Page
Title Page .....	i
Abstract .....	iii
List of Figures .....	vii
List of Tables .....	ix
Chapter 1: General Introduction .....	1
References .....	5
Chapter 2: Factors Limiting Sulfolane Biodegradation in Contaminated Subarctic Aquifer Substrate .....	7
Abstract .....	7
Introduction .....	8
Materials and methods .....	10
Aerobic microcosm studies .....	10
Anaerobic microcosms .....	13
Chemical analyses .....	15
Statistical analyses .....	15
Results .....	16
Aerobic microcosms .....	16
Anaerobic incubations .....	19
Discussion .....	19
Sulfolane concentrations reduced exclusively via aerobic biodegradation .....	19
Mineral nutrients stimulate biodegradation rates at high concentrations; no effect at low .....	20
Nutrient addition necessary for biodegradation in groundwater alone .....	22
Hydrocarbon co-contamination retards sulfolane biodegradation in alluvial substrate .....	23
Mineralization product of sulfolane biodegradation produced .....	23
Conclusion .....	25
Acknowledgements .....	26
References .....	26
Figures .....	30
Tables .....	32
Chapter 3: Identification and Characterization of a Dominant Sulfolane-Degrading <i>Rhodoferax sp.</i> via Stable Isotope Probing Combined with Metagenomics .....	35
Abstract .....	35
Introduction .....	36
Results .....	37

Isolation of <sup>13</sup> C-labeled DNA.....	37
Microbial Community Analysis.....	37
Metagenomic analyses .....	39
Discussion .....	40
Conclusion .....	43
Methods .....	43
Stable Isotope Probing .....	44
DNA extraction .....	45
Separation and detection of <sup>13</sup> C-DNA.....	45
16S rRNA gene sequencing .....	46
Metagenomic sequencing.....	47
Phylogeny .....	48
Acknowledgements.....	48
References.....	48
Figures .....	52
Chapter 4: Distribution of Sulfolane-metabolizing Microorganisms throughout a Contaminated Subarctic Aquifer and Groundwater Treatment Systems.....	59
Abstract.....	59
Introduction.....	60
Methods .....	63
Sampling .....	63
DNA extraction .....	66
Microbial community analyses .....	67
Statistical analyses .....	68
Results and Discussion .....	69
Distribution of sulfolane-assimilating species throughout plume and treatment systems .....	69
Community structure of contaminated aquifer and treatment systems .....	74
Comparison of sediment to groundwater community structure .....	77
Conclusion .....	79
References.....	80
Figures .....	85
General Conclusion.....	90

## List of Figures

Page

- Figure 2-1: Sulfolane concentration over time in aerobic microcosm incubations. (A) Sulfolane biodegradation is nutrient limited in high concentration sediment slurry microcosms. (B) Sulfolane biodegradation is not nutrient limited in low concentration sediment slurry microcosms. (C) Hydrocarbon co-contamination retards the rate of sulfolane biodegradation in sediment slurry microcosms. (D) Nutrient amendment is necessary to stimulate sulfolane biodegradation in groundwater only microcosms. Live slurries contained an active microbial community and sulfolane. Sterile controls were heat-killed. (N) indicates amendment with a dilute mineral nutrient solution. (H) indicates treatments amended with hydrocarbons. (O) indicates treatments amended with a complex organic nutrient solution. Error bars indicate standard deviation from the mean. .... 30
- Figure 2-2: Analysis of dissolved sulfur over time in high concentration, nutrient-amended sediment slurry microcosms. Solid lines indicate dissolved sulfur attributed to sulfolane. Dotted lines indicate dissolved sulfur attributed to sulfate. Sulfate values are normalized to starting concentrations. Error bars indicate standard deviation from the mean. .... 31
- Figure 3-1: A) Sulfolane loss over time in SIP microcosms. Dashed arrows indicate when triplicate microcosms were destructively harvested. Error bars represent standard deviation. B) Quantitative PCR results showing relative abundance of 16S rRNA gene copies in density gradient fractions after separation of the labeled and unlabeled DNA in representative microcosms amended with <sup>13</sup>C-labeled sulfolane. The <sup>13</sup>C-labeled fractions increase in relative abundance over time demonstrating the incorporation of <sup>13</sup>C into prokaryotic DNA as sulfolane is biodegraded. .... 52
- Figure 3-2: Abundance of OTU1 in all 16S rRNA gene amplicon community types. Since no differences were found between community structure over the course of incubation, all replicates from each timepoint (28, 32, and 36 days) were averaged together for the control (n=7), unlabeled fraction (n=9), and labeled fraction (n=9) communities. Time zero represents the subarctic aquifer substrate prior to sulfolane exposure (n=3). “Control” refers to the community incubated with unlabeled sulfolane. Error bars represent standard deviation from the mean. .... 53
- Figure 3-3: Phylogenetic tree of *Rhodoferax sp.* OTU1 compared to closest type strain bacteria. Tree is based on full-length 16S rRNA gene sequence similarity. SH-aLRT node confidence values  $\geq 0.70$  are shown. .... 54
- Figure 3-4: Phylogenetic tree of *Rhodoferax sp.* OTU1 and closest related non-type strain bacteria based on full-length 16S rRNA gene similarity. All non-type sequences represented here are  $>99\%$  similar to *Rhodoferax sp.* OTU1. SH-aLRT node confidence values  $\geq 0.70$  are shown. *Rhodoferax ferrireducens* is in bold for reference. .... 55

Figure 3-5: Order of alkanesulfonate metabolism genes in *Rhodoferax* sp. OTU1 and *Rhodoferax ferrireducens* strain T118. Open arrows indicate shared *ssu* genes. The checkered arrow is the monooxygenase *ssuD* which is present in this operon in strain T118 but not the sulfolane assimilating species. However, a homolog to *SsuD* is elsewhere in the genome of the sulfolane-assimilating *Rhodoferax* sp. The *dszA* homolog is present in both species at the end of the *ssu* operon..... 56

Figure 3-6: Schematic of proposed sulfolane biodegradation pathway. Protein identifications and Kegg Orthology values are as follows: Sulfonate transport system substrate-binding protein (*SsuA*; K15553), Sulfonate transport system ATP-binding protein (*SsuB*; K15555), Sulfonate transport system permease protein (*SsuC*; K15554), Dibenzothiophene sulfone monooxygenase (*DszA*; K22220). The flavin reductase refers to either *SsuE* (flavin reductase; K00299) or *DszD* (Genbank Accession AB051429.1; no KO or E.C. values available), which have coding regions in close proximity to *dszA* on the chromosome and plasmid respectively..... 57

Figure 4-1: NMDS of the aquifer monitoring well (MW), air sparge system (AS), and granular activated carbon (GAC) microbial communities. A: Marker size is proportional to the relative abundance of the sulfolane-assimilating *Rhodoferax* sp. in each sample. B: Identical NMS plot with equal marker sizes for each sample showing the microbial community group by sample type. A stable solution was reached after 58 iterations with an optimal dimensionality of 2 axes, a final stress of 18.6 and a final instability value of 0.00000. The proportion of variance explained by each axis was 36.7% for axis 1 and 16.9% for axis two. .... 85

Figure 4-2: Relative abundance of the sulfolane-assimilating *Rhodoferax* sp. in AS system after 10, 13, and 70 weeks of operation ..... 86

Figure 4-3: Plot of the linear regression between the relative abundance of the sulfolane degrading species and Mn during week 10 of AS system operation ( $p < 0.001$ ;  $R^2 = 0.88$ ). ..... 87

Figure 4-4: NMS plot of aquifer MW microbial communities showing correlations between explanatory variables and ordination axes. Axis 1 correlated with sample depth ( $R^2 = 0.329$ ) which was inversely correlated to Chao ( $R^2 = 0.616$ ) and Inverse Simpson ( $R^2 = 0.331$ ) diversity indices. A stable solution was reached after 45 iterations with an optimal dimensionality of 2 axes, a final stress of 16.2 and a final instability value of 0.00000. Axis 1 explains 49.1% of the community variability and axis 2 explains 16.3% of the community variability. .... 88

Figure 4-5: NMS plot of soil – groundwater comparison data showing differences in community composition based on sample type. A stable solution was reached after 45 iterations with an optimal dimensionality of 2 axes, a final stress of 11.61, and a final instability value of 0.00000. Axis 1 explains 56.8% of the community variability and axis 2 explains 25.8% of the community variability. .... 89

## List of Tables

	Page
Table 2-1: Experimental design for aerobic sulfolane biodegradation microcosm studies. ....	32
Table 2-2: Summary of the time required to achieve 95% sulfolane biodegradation for all microcosm studies in aquifer substrate from North Pole, Alaska.....	33



## Chapter 1: General Introduction

Humans are producing large quantities of synthetic chemicals every year for use in a variety of industrial and domestic applications. Through intentional or unintentional releases, these anthropogenic chemicals can enter surrounding ecosystems and associated food webs<sup>1</sup>. While some of these chemicals are unstable or easily degraded, others are chemically stable by design, which enables them to accumulate in the environment<sup>2</sup>. Remediation of environmental pollutants can be challenging, costly, and often is only employed when there is a risk to human health. Common contaminant clean-up methods involve chemical or physical treatments, which can be invasive, expensive, and not always feasible for large contaminated areas<sup>3</sup>. Fortunately, many xenobiotics are analogous in structure to naturally occurring organic compounds that can be metabolized or co-metabolized by environmental microorganisms<sup>4-6</sup>. For common environmental pollutants (e.g. hydrocarbons and tetrachloroethene), biodegradation has been well documented and successfully exploited to achieve effective, low-cost remediation alternatives<sup>3</sup>. However, there are many contaminants of emerging concern that are widely used but for which little or no information is available regarding their potential rates of natural attenuation. Understanding the biodegradation potential of emerging contaminants is valuable to determining their environmental fate and persistence, as well as to identifying effective remediation strategies. One such organic pollutant of emerging concern is sulfolane.

Sulfolane (tetrahydrothiophene 1,1-dioxide) is an organosulfur compound that was developed by the Shell Oil Corporation in the 1940s<sup>7</sup> and is now used in a variety of industrial applications with 18,000 – 36,000 tons produced worldwide annually<sup>8</sup>. Sulfolane has a chemically stable molecular structure, is non-volatile (0.0062 mm Hg at 27.6 °C), miscible in water, and has a low aquifer sorption coefficient ( $K_d = 0.008-0.14$ ) meaning sulfolane has the potential to be persistent and highly mobile in aqueous environments<sup>9-11</sup>. Despite widespread global use, sulfolane is not included in routine groundwater quality assessments, which can result in large areas of contamination by the time a problem is recognized. In

2009, sulfolane was first detected in a residential drinking well in North Pole, Alaska, and its source traced to a petroleum refinery where it was used as part of the refining process since 1985<sup>12</sup>. Further investigation revealed a groundwater plume that extended ~5 km downgradient from the source, was ~4 km wide, and affected hundreds of residential drinking supplies, which classified it as the largest contaminated groundwater plume in the state of Alaska to date (Alaska Department of Environmental Conservation, personal communication). At that time, there was limited information available regarding the ability of environmental microorganisms to degrade sulfolane<sup>13-15</sup> and all previous published research was conducted in temperate environments. It remained unclear if those studies would be relevant to a subarctic aquifer which is generally cold, oligotrophic, and situated within discontinuous permafrost.

This dissertation describes the microbial ecology and biodegradation potential of sulfolane in the contaminated portion of the North Pole aquifer and includes this general introduction, three research chapters, and overall conclusion of the results. The second chapter investigates the biodegradation potential of contaminated subarctic aquifer sediment and groundwater and identifies factors that simulate or inhibit degradation. Microcosms containing groundwater and sediment were established under aerobic and anaerobic ( $\text{NO}_3^-$ -, Fe (III) -, and  $\text{SO}_4^{2-}$ -reducing) conditions. We only observed sulfolane loss under aerobic conditions that contained the microbial community indigenous to groundwater and sediments and not in sterile controls, and we concluded that microbial biodegradation was the mechanism of action. We also assessed the impact of nutrient amendments on biodegradation rates and we found that at high sulfolane concentrations ( $100 \text{ mg L}^{-1}$ ) the addition of mineral nutrients increased sulfolane biodegradation rates. However, at sulfolane concentrations representative of those found in the North Pole aquifer, biodegradation was not nutrient limited. Since regions of the sulfolane-contaminated plume are co-contaminated with aliphatic hydrocarbons, we also investigated the impact of hydrocarbon co-contamination on sulfolane biodegradation rates. We found that an aliphatic hydrocarbon solution (kerosene) reduced biodegradation rates by ~30% suggesting that co-contaminated regions of the sulfolane plume will be more resistant to sulfolane biodegradation. These studies are the first to

investigate the sulfolane biodegradation potential of subarctic aquifer substrate and reinforces the importance for researchers modeling sulfolane half-lives to not only incorporate a biodegradation term in their models, but also to consider the variability of biodegradation rates associated with differing environmental conditions, including oxygen availability and co-contamination.

Microbiology has historically been hindered by the fact that only ~1% of microorganisms can be cultivated and evaluated in a laboratory setting<sup>16</sup>. In recent decades, DNA sequencing techniques have allowed insights into the unculturable microbial majority but linking microbial identity to specific functions in complex communities remains a challenge. Combining isotope-labeling techniques with advanced molecular methods allows identification and evaluation of the community members involved with the metabolism of a specific substrate<sup>17</sup>. In chapter 3, we demonstrate the power of integrating DNA-SIP with metagenomics to circumvent culture bias and enable characterization of the aquifer microbial community members responsible for the biodegradation of sulfolane. We found that one OTU comprised the majority (85%) of the sulfolane-assimilating community. After obtaining a 99.8% complete genome for this sulfolane metabolizing organism, we identified it as a *Rhodoferrax* sp. 99% identical to the type strain *Rhodoferrax ferrireducens* T118. We then searched the genome for genes associated with the 4S-dibenzothiophene desulfurization pathway, which was previously proposed to be the metabolic pathway for sulfolane biodegradation<sup>18</sup>. While we found an incomplete 4S pathway, we did find the gene that opens the dibenzothiophene ring structure (*dszA*) at the end of an alkane sulfonate transport operon and repeated five times on an associated plasmid. In addition to expanding the known taxonomic distribution of sulfolane biodegradation, we proposed a new metabolic model for sulfolane biodegradation based on these findings.

In chapter 4 we investigate the microbial ecology of the contaminated subarctic aquifer and two sulfolane remediation systems. One remediation system sorbs sulfolane from a homeowner's well water prior to consumption using granular activated carbon. The other was an experimental air sparge system that injected atmospheric air into the aquifer which locally lowered sulfolane concentrations to below

detection limits ( $6.88 \mu\text{g L}^{-1}$ ) in down-gradient test wells through unknown mechanisms. The microbial communities were characterized in 100 groundwater monitoring wells and the remediation systems using 16S rRNA gene sequencing. The community structure of the collection of samples was then correlated to environmental variables collected to at the time of sampling. We paid special attention to abundances of the sulfolane-assimilating *Rhodoferax* sp. identified in chapter 3 and found it to be widely distributed throughout the aquifer although generally in low abundance (average relative abundance  $0.59 \pm 0.77\%$ ). However, the *Rhodoferax* sp. was the most abundant species in the air sparge system and indicator species analysis identified it as a strong and significant indicator of the air sparge samples. Alternatively, the *Rhodoferax* sp. was only detected in half of the granular activated carbon samples at very low abundances (maximum relative abundance of 0.37%) indicating that sulfolane biodegradation is not likely occurring in the GAC systems. Although the entire microbial community decreased in richness and evenness with depth, the only environmental variable measured that correlated with the abundance of the sulfolane assimilator was dissolved manganese within the highly-oxygenated air sparge system where this species was most dominant. We also conducted a small-scale study to assess differences in soil and groundwater (i.e., attached and suspended) microbial communities and concluded that despite significant differences in composition, members of the *Rhodoferax* genus can be reliably detected in groundwater samples. These results demonstrate that sulfolane biodegradation potential is widespread throughout the aquifer and we conclude that air sparging appears to be a way to locally stimulate sulfolane biodegradation in-situ.

The information in this dissertation advances the fundamental understanding of sulfolane biodegradation and will benefit those trying to assess and remediate sulfolane-contaminated groundwater plumes or related efforts. The biodegradation rates generated under a variety of possible contamination scenarios may be useful in generating more accurate models of plume migration and longevity. Through genomic analysis of a sulfolane-metabolizing microorganism, we have proposed a new metabolic model for sulfolane biodegradation and highlight specific genes to target for future studies. In addition, we provide insights into the underlying mechanisms of an effective sulfolane remediation system and ways to

stimulate sulfolane biodegradation *in situ*. Overall, these studies advance both basic and applied science related to the environmental fate of the emerging contaminant, sulfolane.

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## Chapter 2: Factors Limiting Sulfolane Biodegradation in Contaminated Subarctic Aquifer Substrate<sup>1</sup>

### Abstract

Sulfolane, a water-soluble organosulfur compound, is used industrially worldwide and is associated with one of the largest contaminated groundwater plumes in the state of Alaska. Despite being widely used, little is understood about the degradation of sulfolane in the environment, especially in cold regions. We conducted aerobic and anaerobic microcosm studies to assess the biological and abiotic sulfolane degradation potential of contaminated subarctic aquifer groundwater and sediment from Interior Alaska. We also investigated the impacts of nutrient limitations and hydrocarbon co-contamination on sulfolane degradation. We found that sulfolane underwent biodegradation aerobically but not anaerobically under nitrate, sulfate, or iron-reducing conditions. No abiotic degradation activity was detectable under either oxic or anoxic conditions. Nutrient addition stimulated sulfolane biodegradation in sediment slurries at high sulfolane concentrations (100 mg L<sup>-1</sup>), but not at low sulfolane concentrations (500 µg L<sup>-1</sup>), and nutrient amendments were necessary to stimulate sulfolane biodegradation in incubations containing groundwater only. Hydrocarbon co-contamination retarded aerobic sulfolane biodegradation rates by ~30%. Our study is the first to investigate the sulfolane biodegradation potential of subarctic aquifer substrate and identifies several important factors limiting biodegradation rates. We concluded that oxygen is an important factor limiting natural attenuation of this sulfolane plume, and that nutrient amendments are unlikely to accelerate biodegradation within in the plume, although they may biostimulate degradation in *ex situ* groundwater treatment applications. Future work should be directed at elucidating the identity of indigenous sulfolane-degrading microorganisms and determining their distribution and potential activity in the environment.

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<sup>1</sup> Kasanke, C. P. & Leigh, M. B. Factors limiting sulfolane biodegradation in contaminated subarctic aquifer substrate. *PLoS One* **12**, 1–17 (2017).

## Introduction

Anthropogenic organic compounds are present as environmental contaminants throughout the world [1]. Many of these chemicals were engineered for industrial purposes, in which resistance to degradation is advantageous [2]. However, this desirable characteristic becomes problematic when compounds of this nature are released into the environment since recalcitrance correlates to persistence [3]. In addition, many synthetic organic compounds are designed for specific applications, which creates a diverse suite of potential environmental contaminants that are unique in their reactivity and persistence [4]. Often these compounds are not included in routine environmental monitoring protocols, as they are not regulated or well researched in terms of their toxicity or fate in the environment. This lack of understanding is a cause for concern when an unregulated industrial solvent enters a residential drinking water source. An example of this scenario occurred in Interior Alaska, where accidental industrial releases of sulfolane (tetrahydrothiophene 1,1 dioxide) from a petroleum refinery created one of the largest groundwater contamination plumes in the state (Alaska Department of Environmental Conservation, personal communication).

Sulfolane is an anthropogenic organosulfur compound used in various industrial processes, such as natural gas and petroleum refining, with 18,000 – 36,000 tons produced worldwide annually [5]. Sulfolane is miscible in water, has a low affinity for aquifer materials ( $K_d=0.008 - 0.14$ ), and is more stable than many common co-contaminants such as hydrocarbons and diisopropylamine [6,7]. These qualities make sulfolane a mobile and persistent groundwater contaminant once released into the environment [8]. Although the human health effects are unknown, toxicity studies, in which rats were exposed to sulfolane through their drinking water, found lowered white blood cell counts in females and neuropathy in males after 90 days [9]. No other studies have reported the effects of chronic, low-dose sulfolane exposures on humans or other animals [10].



There are no practical strategies to actively remediate such a large sulfolane plume in this region and remediation efforts have been recently replaced with groundwater monitoring [11]. However, previous research has demonstrated that sulfolane can be biodegraded by microorganisms found in sludge from wastewater treatment plants, biologically activated carbon, and in aquifer materials [12–14]. Exploiting the metabolic capabilities of microorganisms naturally occurring in areas of contamination, using techniques such as monitored natural attenuation or biostimulation, may be a way to remediate sulfolane-contaminated aquifers. Before employing bioremediation strategies, an understanding of the sulfolane biodegradation potential of microorganisms present in contaminated environments and the environmental factors controlling their activity must be achieved. Prior to this study no such information existed for subarctic aquifers.

The ability of indigenous microorganisms from a contaminated aquifer to perform sulfolane biodegradation has been reported previously in western Canada [13,15,16], and Australia [17]. Aerobic incubations using aquifer sediment from western Canada revealed that lower temperatures (i.e. 8 °C vs. 28 °C) limited sulfolane biodegradation and that the addition of nitrogen and phosphate stimulated biodegradation rates [13,15,18]. The biochemical pathway for sulfolane biodegradation has not yet been elucidated, but sulfate, one predicted end product of sulfolane biodegradation, was produced as sulfolane degraded [19]. Anaerobic sulfolane biodegradation studies that have been reported in the scientific literature have not generated consistent results. One study suggests sulfolane is readily degradable under unspecified anaerobic conditions [17], while another found inconsistent anaerobic biodegradation only under nitrate- and Mn(IV)-reducing conditions [15]. This discrepancy may be due to the difference in experimental methods and the biogeographic differences in microbial communities associated with the substrates tested (i.e. Australian and Canadian aquifer materials respectively) [20]. Subarctic aquifers are generally cold, a condition that is known to limit microbial activity. Therefore, it was necessary to assess the sulfolane biodegradation potential of the microbial community associated with subarctic aquifer substrate.

We conducted microcosm studies to assess the microbial (aerobic and anaerobic) and abiotic degradation potential in subarctic aquifer substrates from a contaminated groundwater plume in the interior Alaska city of North Pole. Our objectives were to identify degradative processes that contribute to the fate of sulfolane in the environment, and to identify environmental factors that may limit them *in situ*. Groundwater and sediment were combined as the inoculum in the majority of incubations since a greater portion of aquifer microbiota are thought to be associated with aquifer sediment [21]. Because the water table in North Pole, Alaska is shallow, and groundwater pumping followed by storage or treatment is often required for construction activities, we also conducted a comparative study to determine the biodegradation potential of the planktonic microbial community associated with the groundwater alone. We assessed the potential stimulatory effects of nutrients, including mineral nutrients and a complex organic amendment (beer fermentation settlings) on biodegradation rates in order to evaluate nutritional limitations and to possibly identify biostimulation strategies. Since hydrocarbon contamination (primarily jet fuel) co-exists with sulfolane in portions of the North Pole aquifer [22], we also examined the impact of aliphatic hydrocarbons on sulfolane biodegradation rates. Sterile microcosms were also run in parallel and in the dark to assess abiotic chemical degradation processes. We hypothesized that sulfolane degradation in subarctic aquifer substrate occurs primarily as the result of microbial processes, and that biodegradation is limited by oxygen and *in situ* nutrient availability.

## **Materials and methods**

### Aerobic microcosm studies

The North Pole, Alaska aquifer is part of the greater Tanana River aquifer, which is fed by the Alaska Range. Subsurface samples used as inoculum for aerobic microcosm studies were collected from Flint Hills Resources property located in North Pole, Alaska (64.7511° N, 147.3519° W) with permission of the property owners. Sulfolane use at this site began in 1985 and ended in 2014 when the plant stopped refining crude oil. The plume morphology and fate is impacted by the presence of discontinuous

permafrost in the aquifer and groundwater sulfolane levels range from 0 – 34.8 mg L<sup>-1</sup> [22]. All sediment used in this study was collected in March 2013, from one sampling event of augured material from the installation of a new monitoring well at depths between 3 and 9 m below ground surface. Sediment was stored at 4 °C (up to 13 months) and sieved through a 2 mm screen prior to use. Twenty liters of groundwater was collected in September 2012 using a peristaltic pump and stored at 4 °C until use (up to 18 months). Groundwater came from an existing monitoring well approximately 30 m from where sediment was collected. The well was screened 18.25 m below the ground surface and has stable historical sulfolane concentrations of approximately 125 µg L<sup>-1</sup> [22]. The top of the water table at time of sampling was 3 m below ground surface and the aquifer has an average temperature of 3.4 °C [22].

#### *Incubations of aerobic sediment-groundwater slurries ± mineral nutrients*

Aerobic sulfolane degradation rates were assessed at two different sulfolane concentrations. “High concentration” slurries contained 25 g of aquifer sediment, 100 ml of groundwater, and sulfolane to a target concentration of 100 mg L<sup>-1</sup>, and “low concentration” slurries contained 50 g of aquifer sediment, 250 ml of groundwater, and sulfolane to a target concentration of 500 µg L<sup>-1</sup> including background contamination. To observe the effects of nutrient amendment on biodegradation rates at high and low sulfolane concentrations, a Bushnell-Hass (BH) mineral nutrient solution was added to a subset of both slurry types (5 replicates). Each BH-amendment added 8 µg L<sup>-1</sup> magnesium sulfate, 1.8 µg L<sup>-1</sup> calcium chloride, 90 µg L<sup>-1</sup> monopotassium phosphate, 90 µg L<sup>-1</sup> dipotassium phosphate, 90 µg L<sup>-1</sup> ammonium nitrate, and 4.5 µg L<sup>-1</sup> ferric chloride. Two types of experimental controls were established: no-sulfolane controls and sterile controls (3 replicates) (Table 1). No-sulfolane controls were created exactly as described above, but without sulfolane addition. Sterile controls were autoclaved. Sterile aerobic conditions were maintained by loosely covering all incubation vessels with aluminum foil and shaking at 100 rpm at 4 °C, that temperature being the approximate year-round average of the North Pole aquifer [22]. Aliquots of liquid (1-2 ml) were routinely sampled every 5-7 days for sulfolane and sulfate analysis. High concentration incubations were monitored for 106 days, at which point monitoring ceased due to

logistical reasons. Low concentration incubations were monitored for 47 days; more time than was necessary to no longer detected sulfolane in the live slurries.

#### *Aerobic incubations using groundwater only*

It has been suggested that the majority of aquifer bacteria are attached to sediment particles rather than living as planktonic cells in the groundwater [23]. The biodegradation potential of sulfolane by planktonic microbes residing in groundwater alone was examined in microcosms similar to those described above, but with the omission of aquifer sediment. Groundwater-only microcosms were created by combining 150 ml of groundwater and sulfolane to a target concentration of 500  $\mu\text{g L}^{-1}$  including background contamination. The flasks were then divided into different treatment groups (Table 2-1). The effect of mineral nutrient and complex organic nutrient amendments was assessed separately. Microcosms amended with mineral nutrients contained BH mineral nutrient broth as described above and obtained by dilution. The complex organic nutrient solution used as an alternative amendment was created by autoclaving a four-fold dilution of fermentation settlings obtained from a local brewery. Microcosms amended with organic nutrients received 0.5 ml of the complex organic nutrient solution. Sterile controls were created by autoclaving a subset of each treatment group. Groundwater-only incubations were monitored for 80 days when sulfolane was no longer detectable in the nutrient amended treatment groups. All treatment groups were replicated in triplicate. Sampling conditions were the same as described above (Table 2-1).

#### *Microcosms co-contaminated with sulfolane and hydrocarbons*

Since petroleum hydrocarbons and sulfolane are both found in portions of the aquifer within the refinery property, co-contamination studies were conducted to assess the impacts of hydrocarbon co-contamination on sulfolane biodegradation. Sediment slurries were created using 25 g of aquifer sediment, 100 ml of groundwater, and sulfolane to a target concentration of 750  $\mu\text{g L}^{-1}$  including background contamination. Kerosene was selected as a surrogate for jet fuel and diesel fuel, which are the

primary forms of petroleum contamination onsite, since the mixtures are composed of a similar array of hydrocarbons (primarily aliphatic). Fifty  $\mu\text{L}$  of kerosene were added to a subset of the microcosms (5 replicates) after sterilization through a  $0.22\ \mu\text{m}$  filter. Although kerosene is not miscible with water, constant agitation on a shaker table ensured that it was uniformly mixed in the amended microcosms. Controls, incubation conditions, and sampling were the same as previously described (Table 2-1). Sulfolane concentration in co-contaminant incubations was monitored for 22 days, at which point sulfolane was no longer detectable in the live slurries.

### **Anaerobic microcosms**

Aquifer sediment used as inoculum in anaerobic microcosm studies was obtained from a capped soil core from a depth of 5.25 – 5.75 m below ground surface, collected from the refinery property described above. After collection, samples were placed in gas-tight containers equipped with septa, flushed with  $\text{N}_2$  to maintain an anoxic environment, and stored at  $4\ ^\circ\text{C}$  until use approximately 2 months after collection. Groundwater used in these incubations was collected from a pre-existing monitoring well on refinery property that had historical sulfolane concentrations of approximately  $500\ \mu\text{g L}^{-1}$ . Media bottles were filled to the top to eliminate oxygen in the headspace and stored at  $4\ ^\circ\text{C}$  overnight to allow biological consumption of dissolved oxygen. Resazurin was added to a final concentration of  $1\ \text{mg L}^{-1}$ . The groundwater was then degassed with  $\text{N}_2$  and reduced using sodium sulfide for the nitrate- and sulfate-reducing incubations. No reducing agent was added to the substrate used in iron-reducing microcosms.

### **Nitrate- and sulfate-reducing incubations**

To evaluate sulfolane biodegradation potential in anaerobic aquifers, sulfolane biodegradation test microcosms were established under anaerobic conditions. For each anaerobic microcosm, 50 g of sediment was combined with 75 ml of groundwater. Microcosms were divided into two groups for nitrate-reducing and sulfate-reducing incubations. In the nitrate-reducing microcosms,  $\text{KNO}_3$  was added to a final concentration of  $1.01\ \text{g L}^{-1}$ . In sulfate-reducing microcosms,  $\text{Na}_2\text{SO}_4$  was added to a final

concentration of 1.42 g L<sup>-1</sup>. There were 14 microcosm replicates of each reducing condition, which were divided into four treatment groups. Three microcosms were not amended and served as controls for background metabolic activity. To generate positive controls to verify the presence of an active microbial community, a relatively labile carbon source, benzoate was added to three microcosms to a final concentration of 50 mg L<sup>-1</sup> molecular carbon. The remaining eight microcosms were amended with sulfolane to a final concentration of 50 mg L<sup>-1</sup> molecular carbon, three of which were autoclaved as sterile biological controls and the other five were replicates to assess sulfolane biodegradation. All microcosms were incubated at 4 °C in the dark and were not disturbed until sampling, which occurred eight times in 1021 days. Aliquots from all microcosms were periodically taken for sulfolane and sulfate/nitrate analysis. All activity was conducted under strict anaerobic conditions. Nitrate- and sulfate-reducing incubations were monitored for 1021 days.

#### Iron-reducing incubations

Twelve iron-reducing microcosms were established by combining 50 g of aquifer sediment and 65 ml of groundwater. Amorphous iron oxide was made in house, checked by X-ray diffraction to confirm amorphous structure, and added to each microcosm resulting in a final concentration of 5.91 g L<sup>-1</sup> per microcosm [24]. Three of the twelve microcosms received no further amendment to monitor background metabolic activity. Three others were amended with benzoate (final concentration 50 mg L<sup>-1</sup> molecular carbon) and served as positive controls. The remaining six microcosms were amended with sulfolane to a final concentration of 50 mg L<sup>-1</sup> molecular carbon; three served as the treatment group and three were autoclaved and used as sterile controls. All microcosms were incubated at 4 °C in the dark and were not disturbed until sampling, which occurred four times in 391 days.

## **Chemical analyses**

### *Sulfolane extraction and quantification*

To quantify changes in sulfolane concentration over time and among treatment groups, three rinses of dichloromethane were used to perform an organic liquid – liquid extraction of aqueous aliquots from each microcosm. An aqueous solution of sulfolane-d8 was added to monitor extraction efficiency. Nitrobenzene - d8 was used as the internal standard. All sulfolane quantification was done on an Agilent 5975 gas chromatography mass spectrometer (GC-MS) (Santa Clara, California). A fluorinated 30-m RTX – 200 column (Restek) was used for these samples as it separated based on lone pair electrons, allowing for exclusion of potential hydrocarbon co-contaminants. Two GC-MS methods were developed to analyze sulfolane content in both high (100 mg L<sup>-1</sup>) and low (500 µg L<sup>-1</sup>) sulfolane concentration incubations. High concentration incubation samples were analyzed with a method using splitless injection. Low concentration samples were analyzed using a pulsed-splitless injection method where the injection pressure was increased to 40 psi and held for one minute. The lowest quantifiable amount of sulfolane detectable in water samples was 40 µg L<sup>-1</sup>.

### *Nitrate, sulfate, and Fe(II) quantification*

Nitrate and sulfate concentrations were analyzed using standard ion chromatography on a Dionex-200 liquid chromatograph coupled to a conductivity detector. A carbonate – bicarbonate buffer was used as the mobile phase. This instrument was demonstrated to be sensitive down to 1 mg L<sup>-1</sup> for each ion of interest. Dissolved iron (Fe(II)) was measured using the Ferrozine assay [25] and was demonstrated to be sensitive down to 10 µg L<sup>-1</sup>.

## **Statistical analyses**

To determine if sulfolane degradation had occurred, treatment groups within the same experiment (Table 1) were compared to each other at each time-point using analysis of variance (ANOVA); sulfolane

concentration being dependent on treatment type. A p-value of 0.05 or less was considered statistically significant. Due to the dramatic differences between treatment groups and low number of ANOVA tests performed, corrections for repeated measurements were not necessary. If significant differences in sulfolane concentrations were detected based on treatment (e.g. live slurry, sterile control) specific differences between treatments were identified using post-hoc Tukey tests. All values are reported  $\pm$  standard deviation from the mean. All statistical analyses were run using R statistical software [26].

## **Results**

### **Aerobic microcosms**

#### *Biological vs. abiotic degradation*

Biodegradation was the only mechanism of sulfolane removal observed in aerobic microcosms (Fig 2-1) with no statistically significant losses being detected in sterile controls. One nutrient-amended sterile control replicate from the high concentration microcosm study was excluded from analysis, as sulfolane loss due to microbial contamination was identified after 15 days of incubation. To rule out abiotic degradation, a 59-day follow-up incubation of six sterile control replicates under the exact same conditions (i.e. 100 mg L<sup>-1</sup> sulfolane amended with mineral nutrients) was performed. No sulfolane loss was observed in the sterile replicates, confirming biodegradation as the only mechanism of sulfolane loss in these incubations.

#### *Effect of nutrient amendments at high and low sulfolane concentrations*

The addition of a dilute mineral nutrient solution significantly increased the rate of sulfolane biodegradation in high concentration sediment-slurry microcosms (Fig 2-1A). For the first 10 days of incubation, there was no significant change in sulfolane concentration in any high concentration treatment groups. After 22 days of incubation, however, differences in sulfolane concentrations among treatments were detected (ANOVA,  $F_{3,10}=195$ ,  $p < 0.001$ ). By day 22, sulfolane in nutrient-amended live slurries had



dropped from the initial concentration of  $95.45 \pm 8.18 \text{ mg L}^{-1}$  to  $12.02 \pm 7.14 \text{ mg L}^{-1}$ , resulting in a significant concentration difference when compared to the sterile control ( $p < 0.001$ ). After 28 days of incubation, the mean sulfolane concentration in nutrient-amended slurries was below  $1 \text{ mg L}^{-1}$  ( $0.47 \pm 0.7$ ) with three of five replicates having no detectable sulfolane remaining. The unamended live slurries also contained less sulfolane than their sterile counterparts on day 28 ( $p=0.028$ ), and within 106 days of incubation, sulfolane concentrations had decreased from  $86.45 \pm 6.17 \text{ mg L}^{-1}$  to  $55.82 \pm 12.61 \text{ mg L}^{-1}$ . The highest biodegradation rate observed in high concentration, unamended incubations was  $2.93 \text{ mg L}^{-1} \text{ day}^{-1}$ , while that in the nutrient-amended slurries was  $6.19 \text{ mg L}^{-1} \text{ day}^{-1}$  (Fig 2-1A).

In contrast to the high-concentration sediment slurry microcosms incubated under aerobic conditions, the addition of a dilute mineral nutrient solution had no effect on the rate of sulfolane biodegradation at low sulfolane concentrations ( $p=0.97$ ) (Fig 2-1B). At 7 days of incubation, there were differences detected between the sterile controls and live slurries (ANOVA,  $F_{3,12}=275$ ,  $p < 0.001$ ) with the live treatment groups having significantly lower sulfolane levels than the sterile controls ( $p < 0.001$ ). By day 13, the sulfolane concentration dropped from  $462.07 \pm 54.41 \text{ } \mu\text{g L}^{-1}$  and  $506.53 \pm 19.75 \text{ } \mu\text{g L}^{-1}$  to below detection limits in all replicates of the nutrient amended and un-amended microcosms respectively. Sulfolane biodegradation occurred at an average rate of  $38.96 \text{ } \mu\text{g L}^{-1} \text{ day}^{-1}$ . There was no lag time detected in biodegradation activity in live slurries, and no loss of sulfolane was observed in the sterile controls over the course of the 47-day incubation.

#### *Effect of mineral and organic nutrients on sulfolane biodegradation in groundwater*

Sulfolane biodegradation did not occur in groundwater-only microcosms without nutrient amendment during the 80-day incubation period (Fig 2-1D). Sulfolane biodegradation occurred more quickly in the live microcosms amended with mineral nutrients than in those amended with complex organic nutrients. ANOVA testing revealed differences in sulfolane concentrations due to treatment after 39 days of incubation (ANOVA,  $F_{7,16}=10.83$ ,  $p < 0.001$ ) attributed to sulfolane loss in the mineral nutrient

treatment ( $p < 0.001$ ). Sulfolane loss was observed in microcosms amended with organic nutrients when compared to the sterile controls after 49 days of incubation (ANOVA,  $F_{7,15}=32.86$ ,  $p < 0.001$ ; post-hoc Tukey test,  $p < 0.001$ ). At that time (day 49) there was no detectable sulfolane remaining in the mineral nutrient treatment. After 80 days of incubation, sulfolane was no longer detected in microcosms amended with complex nutrients. Biodegradation rates were calculated to be  $33.3 \mu\text{g L}^{-1} \text{day}^{-1}$  and  $14 \mu\text{g L}^{-1} \text{day}^{-1}$  in the mineral and complex nutrient amended microcosms respectively. No sulfolane loss was observed in any of the sterile controls.

#### *Effect of hydrocarbon co-contamination on sulfolane biodegradation*

Sulfolane degraded more slowly in the presence of petroleum hydrocarbons when compared to the sulfolane-only microcosms (Fig 2-1C). Average initial sulfolane concentrations in all treatment groups were between  $730 \mu\text{g L}^{-1}$  and  $830 \mu\text{g L}^{-1}$ . After nine days of incubation, both live slurry treatments had significantly lower sulfolane concentrations than their sterile counterparts, indicating that biodegradation of sulfolane was occurring (ANOVA,  $F_{3,11}=59.88$ ,  $p < 0.001$ ; post-hoc Tukey,  $p < 0.001$ ). Furthermore, hydrocarbon-containing slurries had higher concentrations of sulfolane remaining than the sulfolane-only treatments ( $p=0.006$ ). Within 15 days of incubation, sulfolane concentrations in the treatment group without petroleum co-contamination dropped from  $730.96 \pm 32.85 \mu\text{g L}^{-1}$  to a non-detectable level in all replicates. In the treatment group containing hydrocarbon co-contamination, sulfolane levels declined to a lesser extent, from an initial sulfolane concentration of  $750.48 \pm 31.68 \mu\text{g L}^{-1}$  to  $205.25 \pm 150.17 \mu\text{g L}^{-1}$  and was no longer detected after 22 days of incubation. Sulfolane biodegradation rates were calculated to be  $48.7 \mu\text{g L}^{-1} \text{day}^{-1}$  in the non-hydrocarbon containing live slurries and  $34.09 \mu\text{g L}^{-1} \text{day}^{-1}$  in the live slurries containing hydrocarbons. No sulfolane loss was observed in the sterile controls.

#### *Dissolved sulfate increases as dissolved sulfolane biodegrades*

Dissolved sulfate, a predicted end-product of sulfolane biodegradation, increased in concentration as sulfolane biodegraded (Fig 2). However, much more sulfate was generated than could have originated

from sulfolane alone. The concentration of sulfur associated with dissolved sulfate increased from 28.8 mg L<sup>-1</sup> to 84.5 mg L<sup>-1</sup> after 28 days of incubation in the nutrient-amended live-treatment group. No significant change in sulfate concentration was observed in either the sterile control or the no-sulfolane control. The sulfur associated with sulfolane in the slurries decreased from a starting concentration of 25.5 mg L<sup>-1</sup> to 0.126 mg L<sup>-1</sup>. Therefore, no more than 25.4 of the 55.7 mg sulfur L<sup>-1</sup> that accumulated in the form of dissolved sulfate can be attributed to sulfolane degradation. Similar trends were observed in the non-nutrient amended incubations.

### **Anaerobic incubations**

Anaerobic sulfolane biodegradation by aquifer biota was not detected under nitrate, sulfate, or iron reducing conditions (Table 2-2). Dissolved sulfate and nitrate losses and Fe(II) generation were detected in the benzoate-amended samples indicating the presence of an active anaerobic microbial community. Yet, no sulfolane degradation was observed in any anaerobic microcosm throughout the course of these experiments (1021 days for nitrate and sulfate-reducing conditions and 391 days for iron-reducing conditions).

## **Discussion**

### **Sulfolane concentrations reduced exclusively via aerobic biodegradation**

These microcosm incubation studies demonstrated that aerobic sulfolane biodegradation potential exists in this subarctic aquifer and that biodegradation can occur at *in situ* temperature (4 °C) under aerobic conditions. This result is consistent with previous reports of aerobic sulfolane biodegradation in aquifer substrate from Western Canada [13,15,18]. However, in contrast to other studies, sulfolane did not biodegrade in the aquifer substrate under anaerobic (nitrate, sulfate, Fe(III)-reducing) conditions. This lack of degradation may be due to biogeographic and/or biogeochemical differences in the microbial communities in this Alaskan aquifer compared to Western Canada. Sulfolane was reported to readily biodegrade in Australian aquifer sediment bioreactors in the absence of oxygen at a temperature of 32 °C

[17]. Sulfolane also biodegraded in some anaerobic microcosm incubations conducted at 10 °C under nitrate- and Mn(IV)- reducing conditions in contaminated aquifer sediment from western Canada [15]. Although sulfolane biodegradation occurred in the Canadian studies, it was not observed in all treatment replicates or at a higher incubation temperature (28 °C). Differences in incubation temperatures do not fully account for the inconsistency observed between replicates in the Canadian microcosms. Rather, inconsistent results between treatment replicates suggest there may be an uneven distribution of anaerobic sulfolane degraders in the environment. We incubated samples at 4 °C, which is the approximate water temperature of the North Pole subarctic aquifer [22]. Repeating our experiments at higher temperatures might reveal anaerobic sulfolane biodegradation potential if it is present but being limited by temperature.

Although it was not examined in this study, previous research has found sulfolane to anaerobically biodegrade in the presence of Mn (IV) [15]. Since manganese is only sporadically dispersed throughout the aquifer we did not simulate Mn (IV) reducing conditions [22]. Future biodegradation experiments simulating Mn (IV) reducing conditions using subarctic aquifer substrate could reveal if such biodegradation potential exists in the aquifer, elucidate the importance of Mn (IV) on the persistence of sulfolane in this system, and help to reveal the geographic distribution of that trait.

#### **Mineral nutrients stimulate biodegradation rates at high concentrations; no effect at low**

Mineral nutrient amendment of sediment slurry microcosms stimulated aerobic biodegradation at high sulfolane concentrations (100 mg L<sup>-1</sup>), but not at low sulfolane concentrations (500 µg L<sup>-1</sup>) (Figs 2-1A and B). This difference is likely related to the difference in nutrient requirements necessary to process differing amounts of a substrate. Our studies are the first part-per-billion biodegradation assays on aquifer substrate attempting to mimic subarctic aquifer conditions. We report an average biodegradation rate of 38.96 µg L<sup>-1</sup> day<sup>-1</sup> in the low concentration sediment slurries regardless of nutrient amendments. This suggests that the North Pole aquifer has sufficient ambient nutrients to support microbial processing of small quantities of sulfolane. However, in an aquifer the movement of groundwater tends to replenish

contaminants at a given location. Therefore, it is unknown whether nutrient additions *in situ* would be necessary to maintain sulfolane biodegradation within the aquifer, given sufficient oxygen.

At high experimental sulfolane concentrations (100 mg L<sup>-1</sup>) we found that amendment with a mineral nutrient solution increased the aerobic biodegradation rate from 2.93 mg L<sup>-1</sup> day<sup>-1</sup> to 6.19 mg L<sup>-1</sup> day<sup>-1</sup> and promoted complete sulfolane removal (Fig 2-1 A). This is consistent with previous biodegradation findings in sediment and groundwater from a contaminated aquifer in Western Canada [13,18]. In aerobic shake flask microcosms containing 20 mg L<sup>-1</sup> sulfolane and incubated at 8 °C, biodegradation rates increased from 0.8-1 mg L<sup>-1</sup> day<sup>-1</sup> to 4 mg L<sup>-1</sup> day<sup>-1</sup> after the addition of N and P [13]. Another study from Western Canada using contaminated soil as the only inoculum found that N and P addition reduced the lag time in sulfolane biodegradation activity from 77 days to 2 days and increased biodegradation rates from 4.56 mg L<sup>-1</sup> day<sup>-1</sup> to 45.6 mg L<sup>-1</sup> day<sup>-1</sup> [18]. Observing similar results from different environmental samples suggests that nutrient limitation may be a universal constraint on aerobic sulfolane biodegradation at high sulfolane concentrations.

We also observed slightly higher degradation rates than found in aquifer substrate from Western Canada despite having a lower incubation temperature (4 °C vs. 8 °C) [13]. This was unexpected since temperature has been positively correlated with sulfolane biodegradation activity [13,15] and enzymatic activity in general [27,28]. The discrepancy may be due to differences in the amount of sediment used as the source of inoculum [29,30]. Fedorak and Coy (1996) used 50 g of aquifer sediment and 450 ml of groundwater (1:9 sediment water ratio) while we used 25 g of sediment and 100 ml of groundwater (1:4 ratio). Alternatively, differences between biodegradation rates might be accounted for by community composition differences between samples. Plate counts have shown that the abundance of sulfolane degraders is variable between samples [16,18]. Further investigations identified a *Variovorax sp.* as being capable of mineralizing sulfolane, although mixed cultures demonstrated greater mineralization than isolated degraders [19]. Although modern molecular techniques such as Next Gen sequencing have not yet been employed on this topic, determining the identity of sulfolane degraders and their *in situ*

distribution in respect to environmental variables may reveal other controls on sulfolane biodegradation; enabling more accurate estimates of plume longevity. Therefore, future work should be focused on examining the microbial community involved in active sulfolane biodegradation and determining the spatial distribution of specific sulfolane degraders.

#### **Nutrient addition necessary for biodegradation in groundwater alone**

The water table is close to the ground surface in North Pole, Alaska and often needs to be lowered during the construction season for building activities to occur. Water is pumped out of the ground and transported elsewhere through a process known as dewatering [31]. Typically, extracted groundwater is discharged to the ground surface in drainage ditches that connect to established stormwater flow systems (Alaska Department of Environmental Conservation, personal communication). If the groundwater contains sulfolane, this process could increase human exposure risk and contaminate previously uncontaminated areas. Therefore, examining the biodegradation potential of the planktonic community in groundwater has implications for dewatering waste management strategies. Since the majority of the microbial biomass in aquifers is thought to be associated with the aquifer sediment [21], we predicted that the biodegradation potential of sulfolane in groundwater alone would be lower than that of sediment slurries.

No sulfolane biodegradation occurred in groundwater without the addition of nutrients, indicating that sulfolane biodegradation is limited at *in situ* sulfolane concentrations (Fig 2-1C). The impact of nutrient amendment on groundwater-only microcosms was unexpected, as there was no difference in biodegradation rates between nutrient amended and non-nutrient amended microcosms containing sediment at similar sulfolane concentrations. This difference might be explained by the differences in biomass and/or microbial community composition between sediment and groundwater [21,32]. It is also possible that this difference is due to the presence of nutrients in aquifer sediment, which may be sufficient to support active microbial growth while those available in groundwater alone are too limited.

Mineral nutrients stimulated sulfolane biodegradation in groundwater more effectively than complex organic nutrients (beer fermentation settlings), but both nutrient additions were effective at increasing the biodegradation rate. This result may be related to the fact that mineral nutrients are more bioavailable than complex organics; ammonium being the preferred nitrogen source for bacteria [33]. Labile organic carbon might also have been preferentially utilized over the more recalcitrant sulfolane, slowing degradation rates [34].

#### **Hydrocarbon co-contamination retards sulfolane biodegradation in alluvial substrate**

Petroleum hydrocarbon contamination has also been found in the groundwater on the refinery and is mainly in the form of jet and diesel fuel [22]. We demonstrated that kerosene, which is similar in composition to onsite hydrocarbon contaminants, retards the rate of sulfolane biodegradation in aerobic sediment slurries by approximately 30% (Fig 1C). This finding agrees with previous research that found sulfolane biodegradation rates to be lower than those for common co-contaminants, such as diisopropylamine and hydrocarbons [6,7] although to our knowledge no competitive degradation experiments have previously been conducted. Our findings suggest co-contaminants are utilized preferentially over sulfolane and/or there is a toxic effect of co-contaminants on sulfolane-degrading microorganisms. Therefore, the suppressive effects of hydrocarbons on sulfolane biodegradation rates should be taken into consideration when modeling sulfolane biodegradation in co-contaminated aquifers.

#### **Mineralization product of sulfolane biodegradation produced**

The end products of sulfolane mineralization are proposed to be carbon dioxide and sulfate [19]. Previous biodegradation experiments found that up to 97% of the sulfur in sulfolane was converted into sulfate in mixed culture incubations, suggesting that complete mineralization of sulfolane was occurring. We also found that sulfate was produced while sulfolane biodegraded in our microcosm studies and similarly sulfate was the only biodegradation product we detected. However, much more sulfate was detected than could have originated from sulfolane alone (Fig 2-2). This discrepancy may be due to the

additional degradation of other organosulfur-compounds in the sediment, which contain functional groups such as sulfonates and sulfate esters [35–37]. It is also possible that microorganisms are liberating sulfur from sulfur containing minerals (e.g. pyrite) as a result of biological activity [38,39]. However, biological activity alone cannot account for this discrepancy, as there was no sulfate produced in the sulfolane-free live slurry, which also contained live microorganisms. It is well known that supplying a microbial community with an abundance of a specific substrate can stimulate the growth of organisms capable of utilizing the substrate and similar compounds while suppressing the growth of those that cannot [40]. We propose that a similar situation is occurring in our microcosm studies and that an amendment with the organosulfur molecule, sulfolane, stimulates the growth of microorganisms able to degrade many types of organosulfur molecules naturally occurring in aquifer sediment. In an aerobic aqueous solution, the end product of the sulfur atom removed from organosulfur compounds during biodegradation is often sulfate [36].

Our results suggest that an increase in sulfate concentrations observed in complex media is indicative of sulfolane biodegradation, yet represents a combination of sulfate liberated from sulfolane and other sulfur compounds found in the aquifer materials. Therefore, to conclusively determine if biodegradation has occurred, sulfolane concentrations should always be measured, rather than using sulfate production alone as a proxy. The degradation pathway for sulfolane has not yet been elucidated and it is not known what, if any, biodegradation intermediates accumulate. Isotopic analyses of sulfolane and its degradation products containing isotopically labeled sulfur could also be fruitful for identifying the pathways involved.

We found that sulfolane biodegraded only in the presence of oxygen. We also demonstrated that ambient nutrient concentrations were sufficient for sulfolane biodegradation to occur at the sulfolane concentrations being detected within the plume, but only when oxygen is available. The results of our research suggest that the sulfolane contamination associated with the Tanana aquifer in North Pole, Alaska is not likely to undergo biodegradation under ambient aquifer conditions, with possible exceptions



being locations where trace amounts of oxygen may be present, such as the leading edge of the contaminant plume, locations where groundwater and surface waters interact (e.g., edges of surface water bodies, such as ponds), or possibly in shallow portions of the aquifer susceptible to infiltration (e.g., oxygenated stormwater runoff). It has been shown that the leading edge of contaminant plumes tend to have dissolved oxygen that gets consumed as organic contaminants degrade; a phenomenon known as the plume “fringe” effect [41]. Groundwater monitoring wells along the North Pole, Alaska sulfolane plume fringe have dissolved oxygen concentrations up to 5 mg L<sup>-1</sup> [22]. Our studies were only conducted under fully aerated conditions, so it remains uncertain whether sulfolane biodegradation can occur under the low-oxygen conditions observed *in situ*, including at plume fringes. A similar effect may be observed at the groundwater-surface water interface of gravel ponds. Determining if sulfolane biodegradation occurs under suboxic conditions characteristic of those at the plume “fringe” or other oxygenated regions of the plume would allow for more accurate estimates of the contaminant’s fate and transport. Also techniques such as “air sparging”, where the aquifer material is flushed with atmospheric air to stimulate sulfolane biodegradation *in situ* should be further researched as a localized remediation strategy for sulfolane contaminated aquifer substrate [18,42].

## **Conclusion**

The subarctic aquifer that underlies North Pole, Alaska contains an active microbial community capable of performing aerobic sulfolane biodegradation, however oxygen is likely the primary limiting factor *in situ*. The presence of petroleum co-contamination retards aerobic sulfolane biodegradation and may contribute to low degradation rates in the subsurface. At the sulfolane concentrations prevalent in the plume, nutrient levels were sufficient to support biodegradation when sufficient oxygen was present, so nutrient addition would not be expected to accelerate biodegradation in the plume. Our study reinforces the importance for researchers modeling sulfolane half-lives under various aquifer conditions to not only incorporate a biodegradation term in their models, but also to consider the variability of biodegradation rates associated with differing environmental conditions, including oxygen availability and co-

contamination. The microbial community associated with groundwater alone has a lower biodegradation potential than that associated with groundwater-sediment mixtures, however nutrient amendments were successful in stimulating aerobic degradation in groundwater alone, which has implications for remediation of dewatering waste. Anaerobic conditions do not appear to support sulfolane biodegradation. Yet low oxygen conditions, such as those that often prevail at the leading edge of a plume, may have the potential to foster biodegradation activity as seen for some other organic contaminants [41], but warrants further investigation.

Future work should be directed at elucidating the identity of the microorganisms involved in sulfolane biodegradation. Doing so may reveal new taxa as well as provide taxonomic indicators of the potential for active sulfolane biodegradation *in situ* at a contaminated site. Determining the distribution and potential activity of sulfolane-degrading microorganisms under the range of redox and biogeochemical conditions present, including suboxic conditions, would also aid efforts to more accurately predict the fate of sulfolane in the environment and to perform monitored natural attenuation.

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## Figures

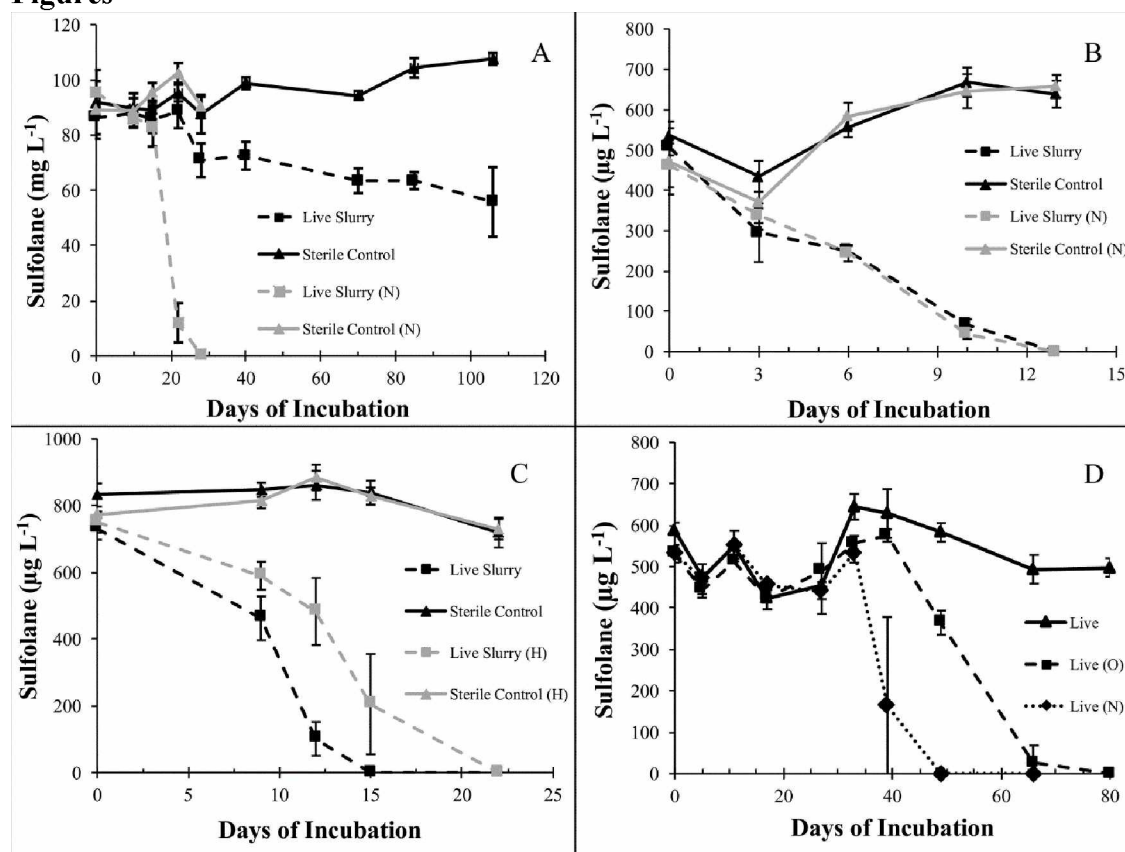


Figure 2-1: Sulfolane concentration over time in aerobic microcosm incubations. (A) Sulfolane biodegradation is nutrient limited in high concentration sediment slurry microcosms. (B) Sulfolane biodegradation is not nutrient limited in low concentration sediment slurry microcosms. (C) Hydrocarbon co-contamination retards the rate of sulfolane biodegradation in sediment slurry microcosms. (D) Nutrient amendment is necessary to stimulate sulfolane biodegradation in groundwater only microcosms. Live slurries contained an active microbial community and sulfolane. Sterile controls were heat-killed. (N) indicates amendment with a dilute mineral nutrient solution. (H) indicates treatments amended with hydrocarbons. (O) indicates treatments amended with a complex organic nutrient solution. Error bars indicate standard deviation from the mean.

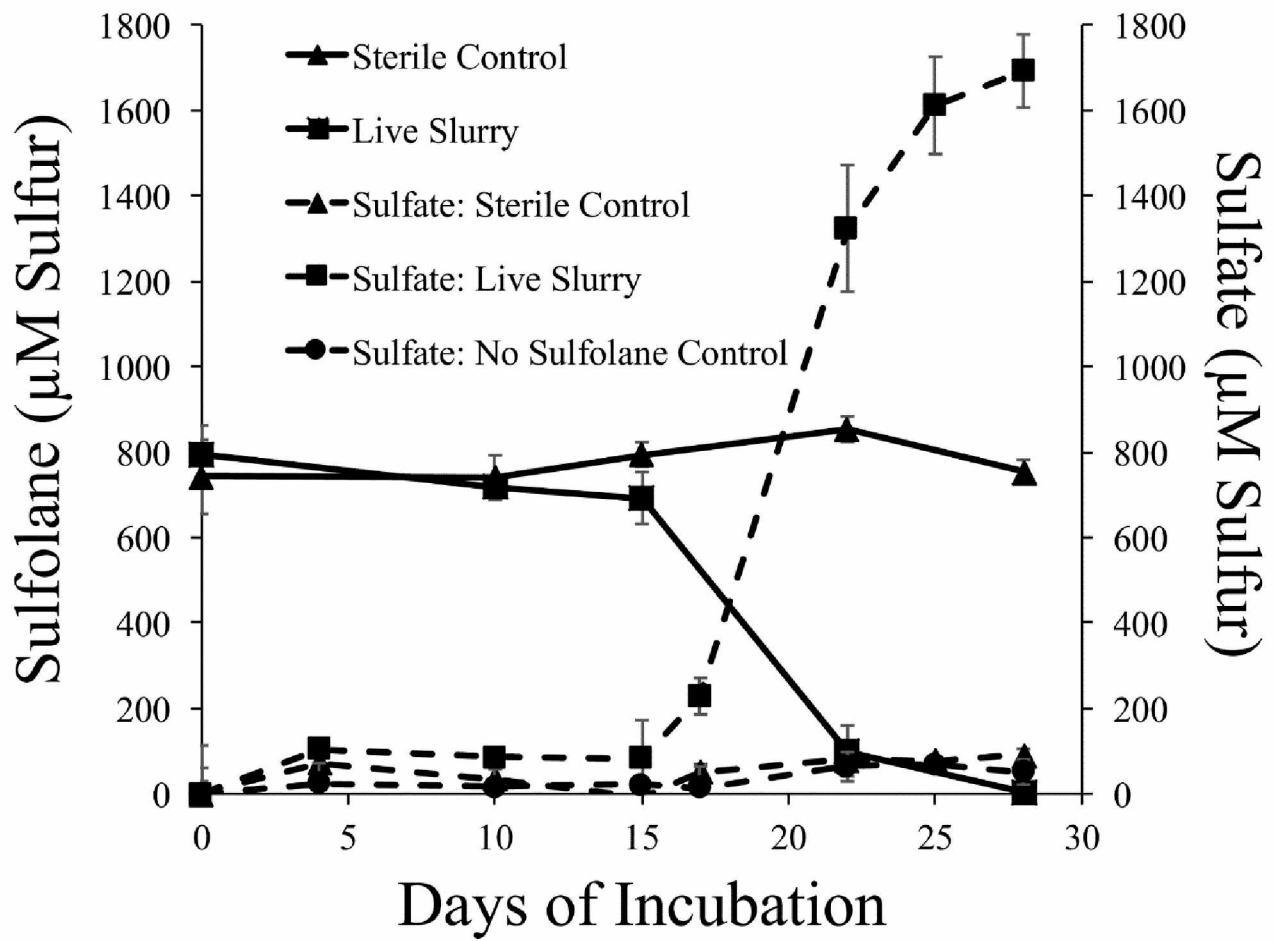


Figure 2-2: Analysis of dissolved sulfur over time in high concentration, nutrient-amended sediment slurry microcosms. Solid lines indicate dissolved sulfur attributed to sulfolane. Dotted lines indicate dissolved sulfur attributed to sulfate. Sulfate values are normalized to starting concentrations. Error bars indicate standard deviation from the mean.

## Tables

Table 2-1: Experimental design for aerobic sulfolane biodegradation microcosm studies.

	<b>Treatment groups</b>	<b>Replicates</b>	<b>Sulfolane</b>	<b>Microbes</b>	<b>Other amendment</b>
<b>High and Low Concentration Slurries</b>	Live slurry	5	+	+	None
	Live slurry (N)	5	+	+	Mineral Nutrients*
	Sterile control	3	+	-	None
	Sterile control (N)	3	+	-	Mineral Nutrients*
	No sulfolane control	3	-	+	None
	No sulfolane control (N)	3	-	+	Mineral Nutrients*
<b>Hydrocarbon Co-contaminant Slurries</b>	Live slurry	5	+	+	None
	Live slurry (K)	5	+	+	Kerosene
	Sterile control	3	+	-	None
	Sterile control (K)	3	+	-	Kerosene
	No sulfolane control	5	-	+	None
<b>Groundwater Only</b>	Live slurry	3	+	+	None
	Live slurry (N)	3	+	+	Mineral Nutrients*
	Live slurry (O)	3	+	+	Organic Nutrient**
	Sterile control	3	+	-	None
	Sterile control (N)	3	+	-	Mineral Nutrients*
	Sterile control (O)	3	+	-	Organic Nutrient**

Conditions tested were high ( $100 \text{ mg L}^{-1}$ ) and low sulfolane concentrations ( $500 \text{ } \mu\text{g L}^{-1}$ ) in sediment slurries, hydrocarbon and sulfolane co-contamination in sediment slurries, and biodegradation in groundwater only. (N) indicates treatments amended with mineral nutrients. (K) indicates kerosene amendment. (O) indicates amendment with organic nutrients. \* Amended with an 11-fold dilution of a 1X Bushnell-Haas mineral nutrient broth. \*\* Amended with a complex organic nutrient solution. In the high concentration incubations there were only two replicates of the nutrient amended sterile control while there were three replicates in the low concentration incubations (refer to the results section for a detailed explanation).



Table 2-2: Summary of the time required to achieve 95% sulfolane biodegradation for all microcosm studies in aquifer substrate from North Pole, Alaska.

<b>Incubation type</b>	<b>95% Sulfolane degraded (days)</b>
High concentration Slurry	Not achieved: ~ 40% in 106 days
	28*
Low Concentration Slurry	13
	13*
Groundwater Only	No activity
	49*
	80**
Hydrocarbon Co-Contaminated Slurry	22
Anaerobic Sulfate Reducing	No activity
Anaerobic Nitrate Reducing	No activity
Anaerobic Iron Reducing	No activity

\* Indicates amendment with a dilute mineral nutrient solution. \*\* Indicates amendment with an organic nutrient source



## Chapter 3: Identification and Characterization of a Dominant Sulfolane-Degrading *Rhodoferax* sp. via Stable Isotope Probing Combined with Metagenomics <sup>1</sup>

### Abstract

Sulfolane is an industrial solvent and emerging organic contaminant affecting groundwater around the world, but little is known about microbes capable of biodegrading sulfolane or the pathways involved. We combined DNA-based stable isotope probing (SIP) with genome-resolved metagenomics to identify microorganisms associated with sulfolane biodegradation in a contaminated subarctic aquifer. In addition to 16S rRNA gene amplicon sequencing, we performed shotgun metagenomics on the <sup>13</sup>C-labeled DNA to obtain functional and taxonomic information about the active sulfolane-degrading community. We identified the primary sulfolane degrader, comprising ~85% of the labeled community in the amplicon sequencing dataset, as closely related to *Rhodoferax ferrireducens* strain T118. We obtained a 99.8%-complete metagenome-assembled genome for this strain, allowing us to identify putative pathways of sulfolane biodegradation. Although the 4S dibenzothiophene desulfurization pathway has been proposed as an analog for sulfolane biodegradation, we found only a subset of the required genes, suggesting a novel pathway specific to sulfolane. DszA, the enzyme likely responsible for opening the sulfolane ring structure, was encoded on both the chromosome and a plasmid. This study demonstrates the power of integrating DNA-SIP with metagenomics to characterize emerging organic contaminant degraders without culture bias and expands the known taxonomic distribution of sulfolane biodegradation.

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<sup>1</sup> Kasanke, C.P., Collins, R.E., and Leigh, M.B. (2019). Identification and Characterization of a Dominant Sulfolane-Degrading *Rhodoferax* sp. via Stable Isotope Probing Combined with Metagenomics. *Scientific Reports* 9, 3121 (2019).

## Introduction

Sulfolane is an anthropogenic organo-sulfur molecule used in some oil and natural gas refineries, resulting in contamination of groundwater at industrial sites around the world<sup>1-3</sup>, including in North Pole, Alaska, where it has contaminated hundreds of private drinking water wells. Despite its emerging importance as a groundwater contaminant, little is known about the environmental fate of sulfolane. Sulfolane biodegradation potential exists in activated sludge, contaminated aquifer substrate, and pristine soil, but the identity of the microorganism(s) responsible remains largely unknown<sup>4-6</sup>. Three sulfolane degraders have been previously identified through pure-culture-based studies, with mixed enrichment cultures reportedly degrading sulfolane more quickly than pure cultures<sup>7-9</sup>. It remains unknown how diverse, widespread, or abundant sulfolane degraders are in the environment, particularly in contaminated aquifers, where this information is valuable in assessing plume longevity and identifying remediation strategies, including natural attenuation and accelerated bioremediation.

Molecular techniques like stable isotope probing (SIP) are powerful tools for examining the active members from environmental microbial communities involved in the biodegradation of emerging organic contaminants such as sulfolane. <sup>13</sup>C-based SIP is the process of exposing a microbial community to a chemical compound highly enriched in <sup>13</sup>C, which otherwise accounts for roughly 1% of all carbon. The microorganisms that metabolize the <sup>13</sup>C-labeled substrate will incorporate the heavy isotope into their biomolecules<sup>10</sup>. Analyzing the <sup>13</sup>C-enriched DNA enables the identification of functionally relevant community members through DNA sequencing approaches including 16S rRNA gene amplicon and shotgun-metagenomic sequencing. Shotgun metagenomic sequencing can also shed light on the functional capabilities of the active organisms and identify metabolic pathways potentially being utilized<sup>11,12</sup>.

The environmental microorganisms known to be capable of degrading sulfolane to date have all been isolated from temperate regions. They include a *Shinella sp.* from Okinawa Japan, a *Variovorax sp.* from Alberta Canada, and a *Pseudomonas sp.* from Illinois USA<sup>7-9</sup>. To our knowledge, the identity of

sulfolane degrading microbes in a subarctic aquifer, like that found in North Pole, Alaska, have not previously been reported. We performed DNA-SIP with  $^{13}\text{C}$ -labeled sulfolane in subarctic aquifer substrate to elucidate the identity of subarctic sulfolane degraders while circumventing culture bias. We combined DNA-SIP with shotgun metagenomics to taxonomically identify microbes involved in sulfolane degradation as well as to gain insights into their genetic potential and possible degradation pathways that may be being used to process sulfolane. To our knowledge, this study is the first to combine these techniques to identify and examine active sulfolane-degrading microorganisms from environmental samples.

## **Results**

### **Isolation of $^{13}\text{C}$ -labeled DNA**

Quantitative PCR results showed a clear separation between the heavy and light DNA in the density gradient (Figure 3-1). In addition, there was an increase in the relative abundance of labeled DNA over the course of the incubation in the microcosms amended with labeled sulfolane. That indicated the labeled carbon was being assimilated by members of the microbial community. As expected, there was no quantifiable heavy ( $^{13}\text{C}$ -labeled) DNA in any of the control microcosms amended with  $^{12}\text{C}$ -sulfolane.

### **Microbial Community Analysis**

The microbial community associated with the  $^{13}\text{C}$ -labeled DNA fractions was very low in richness (Chao1  $65.6 \pm 27.8$ ) and diversity (Inverse Simpson index  $1.4 \pm 0.3$ ) with only one OTU (OTU1) comprising  $85.7 \pm 8.7\%$  of the total labeled microbial community (Figure 3-2). A BLAST comparison of the 253-bp partial 16S rRNA gene sequence for OTU1 showed this gene fragment was 99% identical to five different species from three genera of the *Comamonadaceae* family (*Rhodoferax ferrireducens*, *Rhodoferax saidenbachensis*, *Limnohabitans parvus*, *Acidovorax facilis* and *Acidovorax radicans*). Analysis of the full 16S rRNA gene uniquely identified OTU1 as a *Rhodoferax* sp., as detailed below.

The next two most abundant community members were *Lysobacter sp.* and *Bacteriovorax sp.*, which, when combined, comprised less than 3% of the total labeled community ( $1.3 \pm 2.0\%$  and  $1.1 \pm 0.9\%$  respectively). The dominant phylotype in the unlabeled or “light” fractions of the  $^{13}\text{C}$ -sulfolane incubation was OTU15 from the *Sphingomonadaceae* family, which represented 7.4% of the unlabeled community. OTU1 comprised 4.9% of the light fraction’s total community.

Although OTU1 (presumably *Rhodoferax sp.*) was uniquely dominant in the  $^{13}\text{C}$ -labeled microbial community, it was not the only dominant organism detected in the total community analysis of the control microcosms that were exposed to  $^{12}\text{C}$ -sulfolane. In the  $^{12}\text{C}$ -control cultures, OTU1 was co-dominant with OTU3 (identified as *Arthrobacter sp.*), which represented  $23.2 \pm 3.4\%$  of the total microbial community, while OTU1 constituted  $21.3 \pm 6.0\%$ . OTU3 was also significantly more abundant than OTU1 in the starting community (paired t-test,  $df = 2$ ,  $t = -8.28$ ,  $p = 0.014$ ). Even though OTU3 was a dominant member of the total community in  $^{12}\text{C}$ -sulfolane control cultures, it was ruled out as a sulfolane degrader due to its lack of incorporation of  $^{13}\text{C}$  from sulfolane into DNA.

Statistical analyses showed that the microbial communities in the SIP “heavy” fractions, SIP “light” fractions, and  $^{12}\text{C}$ -sulfolane controls were all significantly different from each other (MRPP,  $p < 0.05$ ). These groups were also significantly different from the time-zero total community samples with the largest difference between T0 and the  $^{13}\text{C}$ -labeled communities ( $A=0.66$ ,  $p \ll 0.05$ ). However, once a community shift occurred and sulfolane degradation was initiated by day 28, there was no significant change in the microbial community profile over the course of the incubation within the SIP “heavy” fractions, SIP “light” fractions, and  $^{12}\text{C}$ -sulfolane controls. As expected, the microbial community showed more OTU richness and diversity prior to sulfolane exposure (Chao1  $2209.0 \pm 89.1$ , Inverse Simpson  $140.2 \pm 12.7$ ). The dominant  $^{13}\text{C}$ -enriched OTU1 was the 18<sup>th</sup> most abundant phylotype in the T0 total community and comprised less than 0.1% of the microbial community prior to sulfolane exposure and incubation. The two most abundant T0 total community phylotypes were identified as *Geobacter spp.* and represented 4.2 % and 3 % of the total community, respectively. These OTUs were not found in the SIP

“heavy” community. Although there was a small subset of archaea in the T0 microbial community, no labeled archaeal DNA was detected in the sequence data.

### **Metagenomic analyses**

By shotgun sequencing the low-diversity heavy SIP fractions we were able to obtain a high-quality draft metagenome assembled genome (MAG) of the putative sulfolane-degrading microorganism. A total of 2.9 M paired-end 2×250 bp reads (1.5 Gbp) remained after quality control and trimming, which produced an assembly containing 12,437 nodes and a total length of 14 Mbp. A single large connected scaffold made up 49% of the total size (6.9 Mbp) with a mean sequencing depth of 53x (Supplementary Figure 3-1). This component was composed of 395 contigs with an N50 of 146 kbp and a longest contig of 614 kbp. An additional 262 kbp were contained in 41 contigs on 5 additional connected components, of which two were closed circular plasmids and one was complete but not circular (Supplementary Figure 3-2). The average length of the 11,998 unconnected contigs was 572 bp; the mean k-mer depth of these contigs was 0.7x. The 395 contigs from the large connected component were defined as a MAG and annotated in JGI-IMG/ER. A total of 6537 protein coding genes were identified; the GC content was 60.84%. CheckM analysis found the genome to be 99.8 % complete with only 0.50 % contamination (defined as redundancy of putative single-copy genes).

The 16S rRNA gene for the MAG was aligned to the representative sequence of OTU1, the sulfolane-degrading *Comamonadaceae* sp. identified in the community dataset, using BLAST<sup>13</sup>. The 16S rRNA gene assembled from the metagenome was 100% identical to the amplicon, matching 253 of 253 bases and identifying this draft genome as the genome of the labeled sulfolane degrader. BLAST comparisons of the full 16S rRNA gene sequence from the metagenome-assembled genome identified the sulfolane degrader as a *Rhodoferrax* sp., being 99% identical to *Rhodoferrax ferrireducens* strain T118. A phylogenetic tree based on the 16S rRNA gene shows this sulfolane degrader as a member of the *Rhodoferrax* clade (Figure 3-3).

We searched the MAG for genes that may be involved with sulfolane degradation. It has been suggested that sulfolane biodegradation may follow the 4S-pathway described for diobenzothiophene desulfurization<sup>8</sup>. This pathway employs the genes *dszA*, *dszB*, and *dszC* to oxidize the sulfur to sulfite, which abiotically oxidizes to sulfate in aerobic conditions. A protein BLAST search found a gene that is homologous to *dszA* (42% identical, 59% positive, e-value of  $10^{-109}$ ) in the genome and a second, distantly related homolog (Supplementary Figure 3-3), repeated 5 times on an associated plasmid (Supplementary Figure 3-4). The MAG does not contain *dszB*, which cleaves the sulfite moiety to complete the remineralization of sulfur in the 4S-desulfurization pathway<sup>14,15</sup>. The MAG also lacks the *thdF* gene, which is associated with the degradation of sulfolane-related compounds in *Escherichia coli* mutants<sup>16,17</sup>. However, the genome of the *Rhodoferrax sp.* does have 84 genes involved with sulfur metabolism, including complete sulfur oxidation (*sox*) and alkanesulfonate utilization (*ssu*) pathways (Supplementary Figure 3-5).

## Discussion

Using DNA-SIP combined with metagenomics, we have identified a single OTU as the primary sulfolane-degrading organism in subarctic aquifer substrate and provided DNA evidence that strongly suggests it is a member of the *Rhodoferrax* genus (Figure 3-3). Of the 178 OTUs detected in the <sup>13</sup>C-labeled SIP fractions, OTU1 was identified as the dominant microorganism incorporating carbon from sulfolane in this subarctic aquifer substrate. Although initial analysis of the 253-bp amplicon data revealed OTU1 as a member of the *Comamonadaceae* family, the full 16S rRNA gene provided a more rigorous genus-level taxonomic resolution. Phylogenetic analysis showed that OTU1 was 99% similar to type strain *Rhodoferrax ferrireducens* T118 (Figure 3-3), which was isolated from subsurface sediments collected in Oyster Bay, VA, USA. This relationship was also supported by whole genome database comparisons. When non-type strains were included in the phylogenetic analysis, most of the closest relatives were found in contaminated soil or groundwater with the top 20 originating from freshwater or



terrestrial environments (Figure 3-4). Although this is the first report of a *Rhodoferax sp.* degrading sulfolane, it is not surprising since members of the *Rhodoferax* genus are commonly found in contaminated freshwater environments and have been implicated in the degradation of other contaminants including herbicides, naphthalene, and benzene<sup>18–20</sup>.

Prior to identifying *Rhodoferax sp.* OTU1 as a sulfolane degrader, the only environmental microorganisms known to degrade sulfolane originated from subtropical, humid subtropical, or humid continental climates. In Western Canada a sulfolane degrader was isolated from a contaminated aquifer and identified as a *Variovorax sp.* which is in the same family (*Comamonadaceae*) as *Rhodoferax sp.* OTU1<sup>8</sup>. Two other environmental isolates have been reported to degrade sulfolane. *Pseudomonas maltophilia* was isolated from the soil of an abandoned strip mine near Cambria, Illinois; USA and grew on sulfolane as the sole carbon source<sup>7</sup>. A novel *Shinella sp.* was isolated from soil in the Yambaru area of Okinawa Main Island; Japan and grew on sulfolane as the sole sulfur source<sup>9</sup>. To our knowledge, the only other report of a sulfolane-degrading bacterial species was a mutated strain of *Escherichia coli*, which was not isolated from the environment but did yield insights into sulfone degradation pathways<sup>16</sup>. A mutation in the *thdA* gene allowed this *E. coli* strain to degrade sulfolane via a novel sulfone oxidase enzyme. The authors proposed *thdA* to be a regulator gene for several genes involved in the metabolism of organo-sulfur compounds, including *thdF* thiophene oxidase<sup>16</sup>. The novel sulfone oxidase was never identified and *thdA* has not been sequenced, but the genetic sequence for *thdF* has been published<sup>17</sup>. We did not find *thdF* homologs in the MAG of the degrader, but that does not rule out the unidentified sulfone oxidase as being involved in sulfolane metabolism.

It has been proposed that the biodegradation of sulfolane followed the 4S-desulfurization pathway for dibenzothiophene due to structural relatedness of the compounds and the production of sulfate as a mineralization product<sup>8</sup>. This pathway involves the use of the *dsz* operon involving genes *dszA*, *dszB*, and *dszC*<sup>21</sup>. The gene product of *dszA* opens the ring structure after dibenzothiophene is converted to dibenzothiophene-5,5-dioxide by *dszC*<sup>15</sup>. We found no evidence of *dszB* being present in the MAG for

the degrader, suggesting that sulfolane biodegradation in this strain does not utilize the 4S-desulfurization pathway. However, the MAG does encode a homolog to *dszA* at the end of an alkanesulfonate utilization pathway (Figure 3-5). In addition, 5 copies of another *dszA* homolog are present on an IncP-family plasmid in the metagenomic co-assembly (Supplementary Figures 3-2, 3-3, and 3-4). This plasmid has a copy number of about 3 relative to the chromosome, suggesting that the plasmid-borne *dszA* homolog is present at 15 times the copy number of the genomic *dszA* homolog. This result is perhaps not surprising considering that the dibenzothiophene pathway is also generally plasmid-borne<sup>22,23</sup>. If either of these DszA homologs can act on sulfolane as DszA does to dibenzothiophene, the remaining compound would be 4-hydroxy-butane sulfinic acid. Under aerobic conditions sulfinic acids can oxidize to sulfonic acids, with aliphatic sulfinic acids being more reactive than aromatic ones<sup>24</sup>. Although speculative, it is feasible that the resulting product (4-hydroxy-butane-sulfonic acid) is then degraded in a similar fashion to other alkanesulfonates and/or taurine, which this species is genetically equipped to process (Figure 3-6, Supplementary Figure 3-5).

Greene et al. previously found that mixed cultures are more efficient at degrading sulfolane than isolates, but it was unclear if this was due to the exchange of specific nutrients between organisms, the removal of growth-inhibiting products, the combined metabolic attack on the substrate, or some combination<sup>8</sup>. We were surprised to find no compelling evidence of sulfolane assimilation in any species other than *Rhodoferrax sp.* OTU1 throughout the course of this labeling study. Although there were other labeled microbes in this community, they were in extremely low abundance and were likely labeled due to the scavenging of labeled biomolecules<sup>25</sup>. In addition, the <sup>13</sup>C-labeled community structure did not change substantially over the entire SIP incubation period, which might be expected if sulfolane metabolites were being degraded in series. Although *Rhodoferrax sp.* OTU1 represented ~25% of the community in the control (unlabeled sulfolane) microcosms, it was co-dominant with an *Arthrobacter sp.* Despite the abundance of the *Arthrobacter sp.* in the controls, we found no labeled *Arthrobacter* DNA, which strongly suggests it is not involved in sulfolane biodegradation. However, it is possible that other

members of the community were able to degrade sulfolane yet were not assimilating the carbon and therefore were not identified with the methods we used. We are currently working to isolate *Rhodoferax* sp. OTU1 into pure culture in order to enable definitive characterization of the sulfolane degradation pathway.

## Conclusion

We have identified a *Rhodoferax* sp. as being the dominant and likely the exclusive sulfolane-degrading microorganism in enrichment cultures from contaminated subarctic aquifer substrate. By combining  $^{13}\text{C}$ -DNA-SIP with 16S rRNA gene amplicon sequencing and shotgun metagenomics, we were able not only to resolve the taxonomy of this degrader, but also gain insights into how it may be metabolizing sulfolane. We also suggest that sulfolane degradation does not proceed following the previously proposed model<sup>8</sup>. Although an *Arthrobacter* sp. comprised ~25% of the total community in the  $^{12}\text{C}$ -sulfolane controls during active sulfolane biodegradation, it was not assimilating sulfolane carbon into its DNA and therefore likely not involved in the biodegradation process. We caution that simply analyzing changes in the microbial community profile during sulfolane biodegradation studies is not sufficient to determine species function and may be misleading. Obtaining a pure culture of *Rhodoferax* sp. OTU1 would allow of the confirmation of its degradation abilities and investigations into the pathways utilized. The genomic data obtained in this study could further assist in that pursuit. Although this study cannot confirm that *Rhodoferax* sp. OTU1 actively degrades sulfolane within the contaminated aquifer, it enables further studies of biodegradation potential *in situ*, such as through characterizing the environmental distribution of this organism and identifying environmental factors driving its abundance.

## Methods

Subsurface samples used as inoculum for SIP studies were collected from Flint Hills Resources property located in North Pole, Alaska (64.7511° N, 147.3519° W) where groundwater sulfolane concentrations ranged from 0 to 34.8 mg L<sup>-1</sup><sup>26</sup>. The sediment and groundwater samples used in this study

came from a sulfolane-contaminated subarctic aquifer and a detailed site characterization and history of sulfolane use can be found in Kasanke and Leigh<sup>3</sup>. The aquifer sediment used in this study consisted of augured material from the installation of a new monitoring well at depths between 3 and 9 m below ground surface. All sediment was stored at 4 °C (the approximate average aquifer temperature) and was sieved through a 2-mm screen prior to use. Groundwater used in this study came from an existing monitoring well approximately 30 m from where sediment was collected. The well was screened 18.25 m below the ground surface and has stable historical sulfolane concentrations of approximately 125 µg L<sup>-1</sup><sup>26</sup>. Groundwater was collected in September 2012 and sediment in March 2013. Both were stored at 4 °C until use in February 2016. The top of the water table at time of sampling was 3 m below ground surface and the aquifer has an average temperature of 3.4 °C<sup>26</sup>.

### **Stable Isotope Probing**

SIP microcosms each contained 12.5 g of soil and 40 ml of groundwater combined in a 160-ml serum bottle. Since previous sulfolane biodegradation studies showed more predictable degradation curves when nutrients were added, 5 ml of a 1X Bushnell-Haas mineral nutrient solution was added to each microcosm (final concentration: magnesium sulfate 0.022 g L<sup>-1</sup>, calcium chloride 0.0022 g L<sup>-1</sup>, monopotassium phosphate 0.11 g L<sup>-1</sup>, diammonium hydrogen phosphate 0.11 g L<sup>-1</sup>, potassium nitrate 0.011 g L<sup>-1</sup>, ferric chloride 0.006 g L<sup>-1</sup>). To examine the composition of the initial microbial community, three of these microcosms were immediately harvested and stored at -80 °C for DNA extraction and 16S rRNA gene sequencing. The remaining microcosms were assigned to two treatment groups containing nine microcosms each. One group was amended with a sterile aqueous solution of custom-synthesized <sup>13</sup>C-labeled sulfolane (Microbial Insights, TN) and the other was amended with commercially available (predominantly <sup>12</sup>C) sulfolane (Acros Organics, Belgium) as a control. The target starting sulfolane concentration in all microcosms was 100 mg L<sup>-1</sup>. All microcosms were incubated under aerobic conditions at 4 °C. Aliquots (1 ml) of the liquid phase were periodically sampled from each microcosm for sulfolane quantification using gas chromatography-mass spectrometry<sup>3</sup>. Once significant sulfolane

loss was detected (39.15 % removed at 28 days of incubation), three microcosms from each treatment group were destructively harvested for microbial community characterization (Figure 3-1a). The remaining microcosms were harvested in the same manner for two additional timepoints (days 32 and 36 of incubation) when additional sulfolane loss was observed (89.64 % and 100 % respectively) (Figure 3-1a).

### **DNA extraction**

To obtain enough bacterial DNA from these low-biomass samples for density gradient separation ( $>1 \mu\text{g}$ ), DNA was extracted using a modified version of the MO BIO PowerSoil DNA isolation kit (MO BIO Laboratories #1288-100). For each DNA extraction, 0.5 g of microcosm sediment and 100  $\mu\text{l}$  of microcosm supernatant was combined into a single PowerSoil Bead Tube, with five DNA extractions performed for every harvested microcosm. Extractions were performed following the MO BIO PowerSoil protocol except that DNA was concentrated by combining all five extracts from a microcosm onto a single PowerSoil Spin Filter and eluted into a single collection tube using 100  $\mu\text{l}$  ( $2 \times 50 \mu\text{l}$ ) of C6 elution buffer. Double-stranded DNA was quantified using a Qubit fluorometer. All DNA extracts contained between 1.84 and 4.8  $\mu\text{g}$  total DNA.

### **Separation and detection of $^{13}\text{C}$ -DNA**

$^{13}\text{C}$ -labeled DNA was separated from the unlabeled DNA via isopycnic centrifugation in cesium trifluoroacetate (CsTFA) following a previously described protocol <sup>27</sup>. Briefly, between 1.8 and 3 ng of total DNA was added to 5 ml of a CsTFA solution (Amersham 17-0847-02) diluted to a buoyant density of  $\sim 1.62 \text{ g ml}^{-1}$ . Density gradients were created by ultracentrifugation in a Beckman Coulter Optima L-100 XP ultracentrifuge using the fixed-angle Beckman NVT 100 rotor at 45,600 r.p.m. and  $25^\circ \text{C}$  for 72 hours. The gradients were divided into 20 fractions (buoyant density 1.28 – 1.82) and qPCR targeting the bacterial and archaeal 16S rRNA gene was performed on each fraction <sup>28</sup>. The normalized abundance values for targeted genes were calculated by dividing the abundance of each fraction by that of the most

abundant fraction within the same gradient. Fractions containing heavy DNA (labeled) were pooled together and those containing light DNA (unlabeled) were pooled together (Figure 3-1) within each individual gradient. The primarily  $^{12}\text{C}$ -sulfolane controls were also fractionated and pooled similarly to control for any DNA contamination in heavy fractions. Pooled fractions from all samples were then subjected to 16S rRNA gene sequencing as described below.

### **16S rRNA gene sequencing**

The V4 region of the bacterial and archaeal 16S rRNA gene was amplified using Illumina fusion primers as described by Caporaso et al.<sup>29</sup>. PCR output for all samples was normalized using a Life Technologies SequelPrep Normalization plate. The normalized products were pooled. After Ampure clean up, QC and quantitation the pool was loaded on a 500-cycle reagent cartridge (v2) Illumina MiSeq flow cell and sequenced in paired end 2x250 bp format using custom V4 sequencing and index primers<sup>29</sup>. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA demultiplexed and converted to FastQ with Illumina Bcl2fastq v1.8.4.

FASTQ files were analyzed using mothur software (1.35.1) following a modified version of the standard MiSeq SOP (accessed March 2016)<sup>30,31</sup> as described by Martinez et al.<sup>28</sup>. Briefly, all sequences had a quality score of 25 or greater and the maximum contig length was set to 275. All unique sequences were aligned against the SILVA SEED v119 database and chimera checking was performed using the mothur implementation of UCHIME<sup>31,32</sup>. Unique operational taxonomic units (OTUs) were defined at a level of 97% sequence similarity and taxonomy was assigned using SILVA SEED v119 taxonomy database<sup>32</sup>. To account for differences in sequence coverage, the number of sequences was subsampled to the number of sequences in the least covered sample (8142). Differences between microbial communities were assessed using nonparametric Multi-Response Permutation Procedures (MRPP) from a Bray-Curtis distance matrix<sup>33</sup>. All statistical analyses were performed with R statistical software using the vegan package<sup>34</sup>.

## **Metagenomic sequencing**

Shotgun metagenomic sequencing was performed on one <sup>13</sup>C-labeled DNA extract from each of the sampling timepoints. The DNA was prepared for sequencing using a Nextera XT DNA Library Prep Kit and sequencing was performed on an Illumina MiSeq using a standard v3 flow cell and paired end 2x300 bp sequencing format with an average insert size of 275 bp. Raw reads were trimmed and quality filtered using bbdduk in the bbmap package (<https://jgi.doe.gov/data-and-tools/bbtools/bb-tools-user-guide/>). After trimming and filtering, raw sequences were error corrected and assembled using SPAdes version 3.10.1<sup>35</sup>. The de Bruijn graph assembly was visualized with Bandage<sup>36</sup>, which indicated the presence of a large connected component containing a single metagenome assembled genome (MAG) and several complete plasmids (Supplementary Figure 3-2). As a check, contigs were also binned by tetranucleotide frequency using VizBin<sup>37</sup> into a single MAG. For further processing we used the large connected component MAG. MAG bin quality was assessed for contamination and completeness using CheckM and the MAG and plasmids were annotated using RAST, PATRIC, and JGI/M ER pipelines<sup>38-41</sup>. Phage and plasmid genes were also identified with PHASTER<sup>42</sup>. The full-length 16S rRNA gene (1541 bp) was extracted from the MAG and queried against Genbank and RDP databases using BLASTN to determine the relationship of this genome to other known microorganisms<sup>43-45</sup>. Using BLASTN, the full-length 16S rRNA gene from the MAG was queried against the representative OTU sequences in the 16S rRNA amplicon dataset to identify the OTU of the genome we obtained<sup>46</sup>. Raw reads from the 16S rRNA amplicon sequencing and shotgun metagenomic sequencing are available in the sequence read archive (SRA) under accession #SRP136637. This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under the accession QEII0000000. The version described in this paper is version QEII01000000. The MAG assembly and annotation is publicly available in the JGI IMG/ER database under accession # 181102. Sequence assemblies for the plasmids extracted from the MAG are available in the supplementary materials.

## **Phylogeny**

We compared the full-length 16S rRNA gene (1541 bp) of the dominant <sup>13</sup>C-labeled organism to that of both the type strain and non-type strains of the closest relatives. The 16S rRNA sequences of close relatives were obtained from the RDP and GenBank databases <sup>43,44</sup>. Prior to tree construction the sequences were aligned using the RDP tree builder program and manually checked using Seaview version 4 <sup>43,47</sup>. The maximum likelihood phylogenetic tree was constructed using PhyML <sup>48</sup> in SeaView under the GTR model <sup>47</sup> after alignment in MUSCLE <sup>49</sup> and edited online using iTOL <sup>50</sup>. Shimodaira-Hasegawa approximate likelihood ratio test (SH-aLRT) node confidence values were calculated during tree construction <sup>51</sup>.

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## Figures

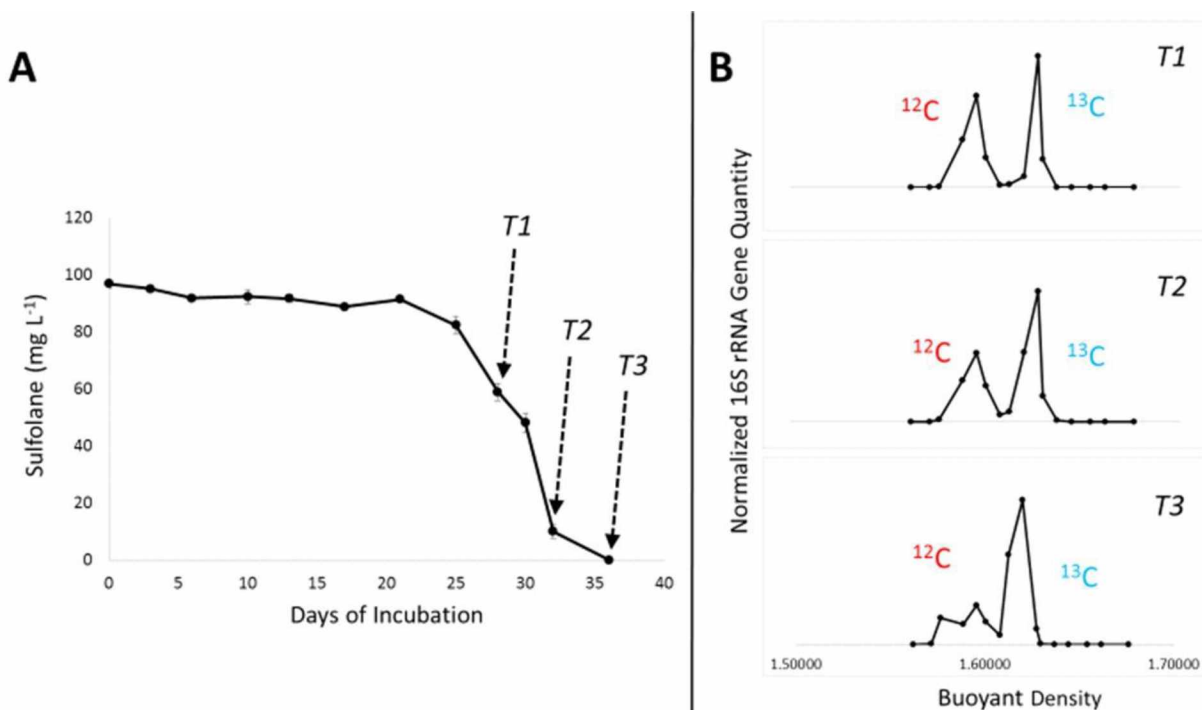


Figure 3-1: A) Sulfolane loss over time in SIP microcosms. Dashed arrows indicate when triplicate microcosms were destructively harvested. Error bars represent standard deviation. B) Quantitative PCR results showing relative abundance of 16S rRNA gene copies in density gradient fractions after separation of the labeled and unlabeled DNA in representative microcosms amended with <sup>13</sup>C-labeled sulfolane. The <sup>13</sup>C-labeled fractions increase in relative abundance over time demonstrating the incorporation of <sup>13</sup>C into prokaryotic DNA as sulfolane is biodegraded.

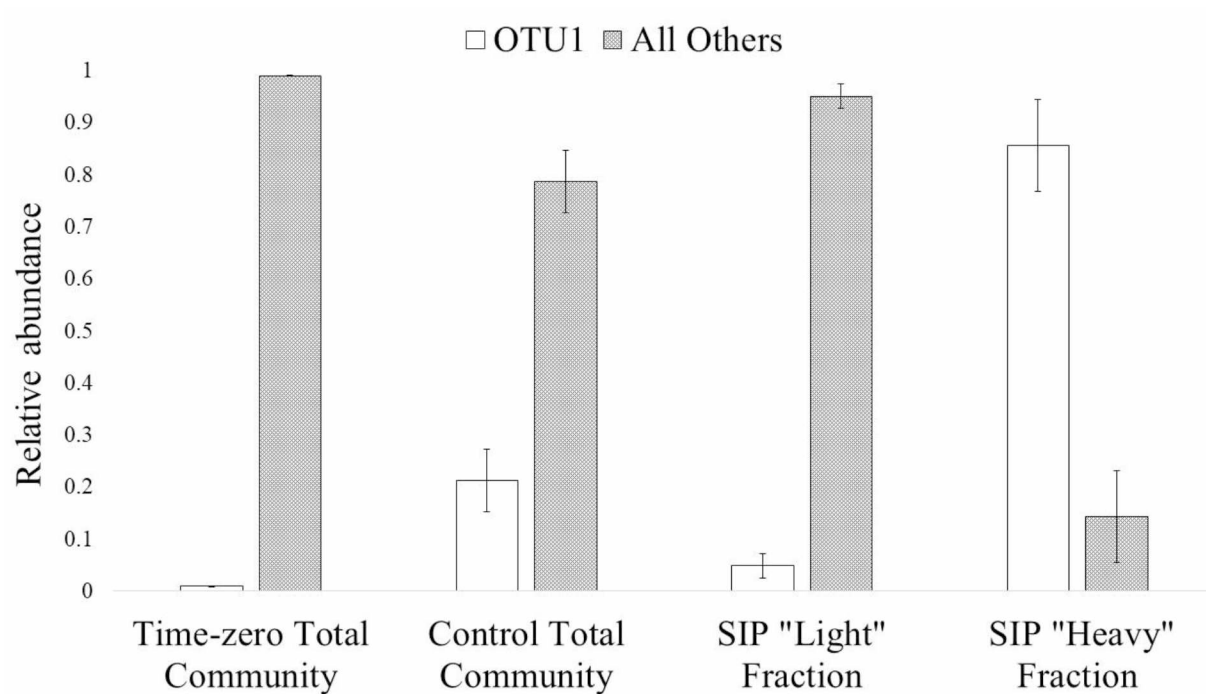


Figure 3-2: Abundance of OTU1 in all 16S rRNA gene amplicon community types. Since no differences were found between community structure over the course of incubation, all replicates from each timepoint (28, 32, and 36 days) were averaged together for the control (n=7), unlabeled fraction (n=9), and labeled fraction (n=9) communities. Time zero represents the subarctic aquifer substrate prior to sulfolane exposure (n=3). "Control" refers to the community incubated with unlabeled sulfolane. Error bars represent standard deviation from the mean.

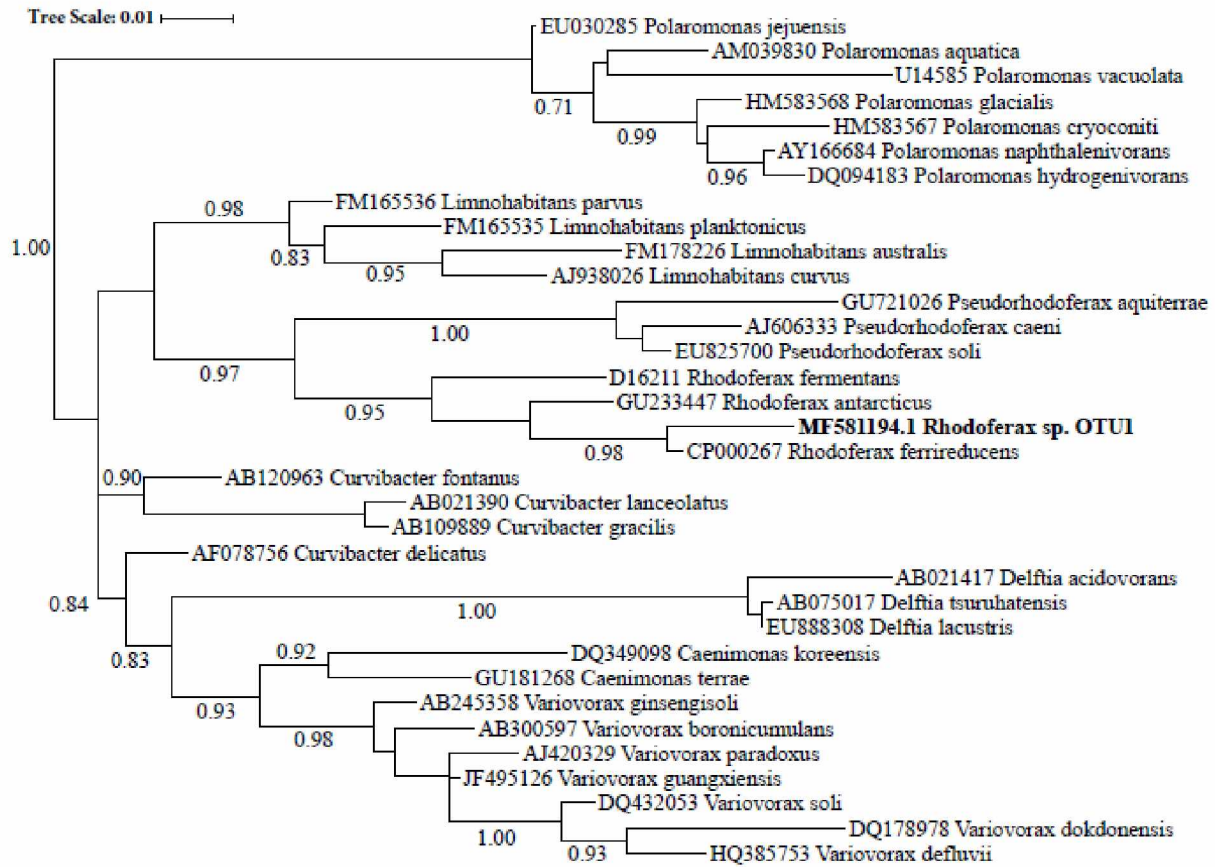


Figure 3-3: Phylogenetic tree of *Rhodofex sp. OTU1* compared to closest type strain bacteria. Tree is based on full-length 16S rRNA gene sequence similarity. SH-aLRT node confidence values  $\geq 0.70$  are shown.

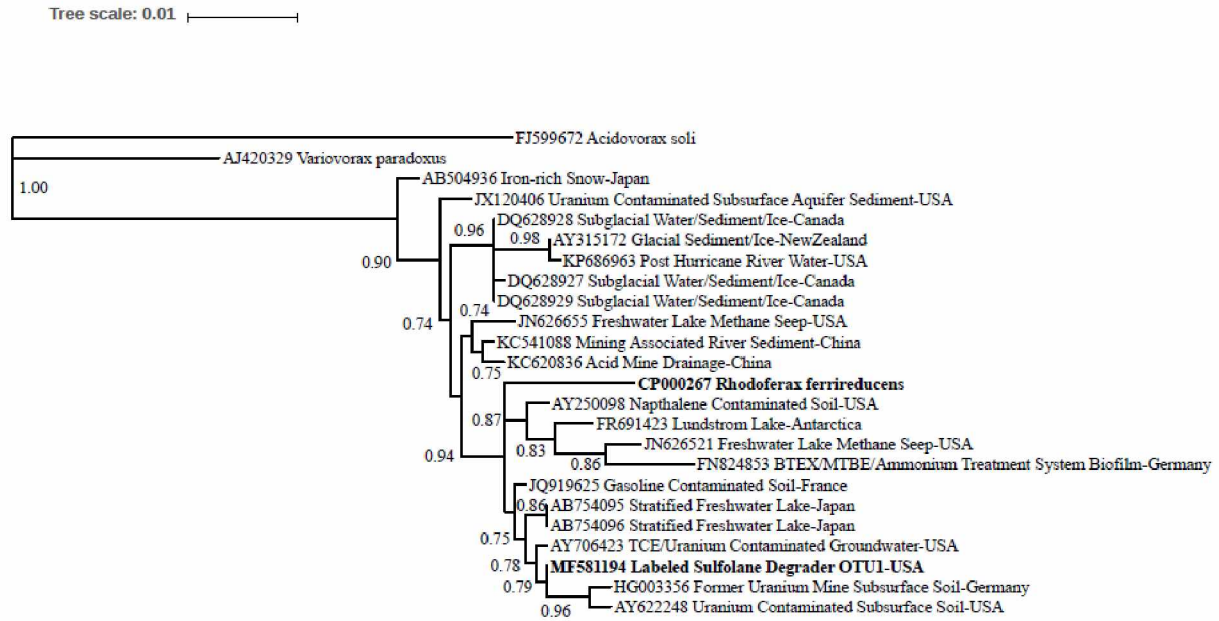


Figure 3-4: Phylogenetic tree of *Rhodoferrax* sp. OTU1 and closest related non-type strain bacteria based on full-length 16S rRNA gene similarity. All non-type sequences represented here are >99% similar to *Rhodoferrax* sp. OTU1. SH-aLRT node confidence values  $\geq 0.70$  are shown. *Rhodoferrax ferrireducens* is in bold for reference.

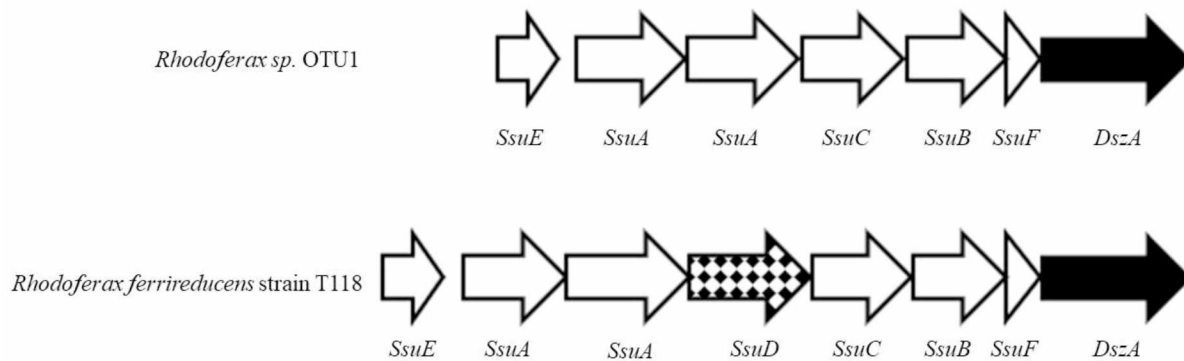


Figure 3-5: Order of alkanesulfonate metabolism genes in *Rhodoferrax* sp. OTU1 and *Rhodoferrax ferrireducens* strain T118. Open arrows indicate shared *ssu* genes. The checkered arrow is the monooxygenase *ssuD* which is present in this operon in strain T118 but not the sulfolane assimilating species. However, a homolog to *SsuD* is elsewhere in the genome of the sulfolane-assimilating *Rhodoferrax* sp. The *dszA* homolog is present in both species at the end of the *ssu* operon.



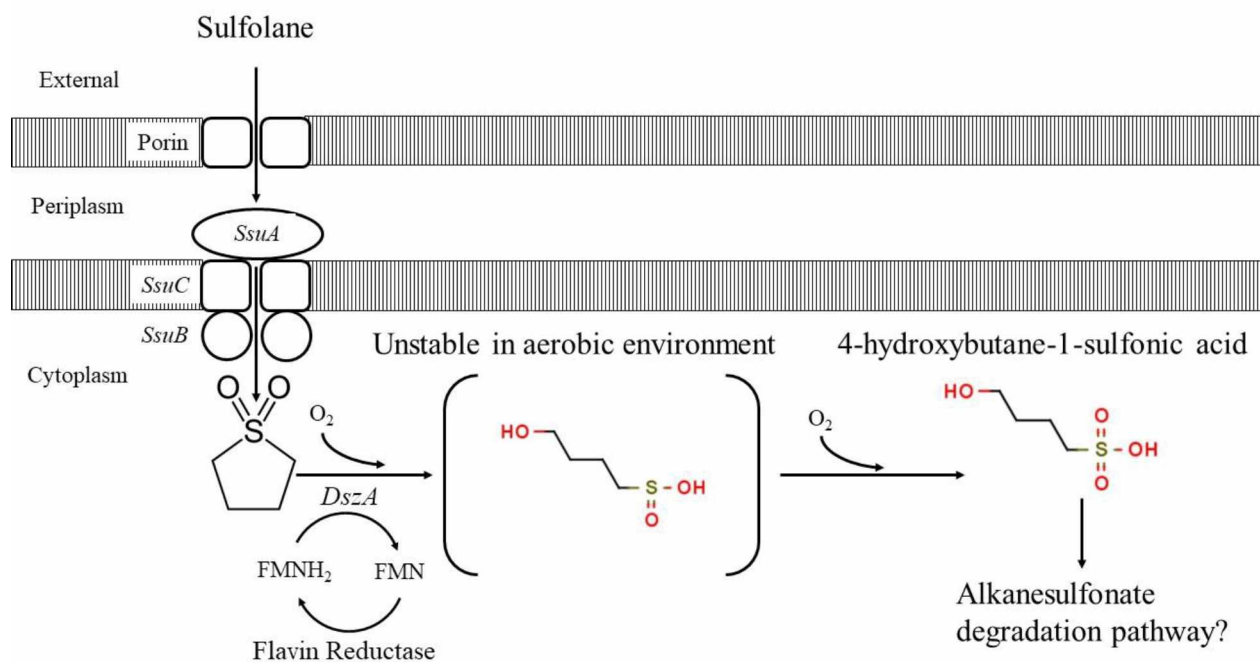


Figure 3-6: Schematic of proposed sulfolane biodegradation pathway. Protein identifications and Kegg Orthology values are as follows: Sulfonate transport system substrate-binding protein (*SsuA*; K15553), Sulfonate transport system ATP-binding protein (*SsuB*; K15555), Sulfonate transport system permease protein (*SsuC*; K15554), Dibenzothiophene sulfone monooxygenase (*DszA*; K22220). The flavin reductase refers to either *SsuE* (flavin reductase; K00299) or *DszD* (Genbank Accession AB051429.1; no KO or E.C. values available), which have coding regions in close proximity to *dszA* on the chromosome and plasmid respectively.

(Supplementary information available online)



## Chapter 4: Distribution of Sulfolane-metabolizing Microorganisms throughout a Contaminated Subarctic Aquifer and Groundwater Treatment Systems<sup>1</sup>

### Abstract

Sulfolane, an emerging contaminant, has been found in the groundwater surrounding a refinery in Interior Alaska, where it was used for decades and has migrated over three kilometers from the source. Recently, a microorganism from this aquifer was identified with the capability to metabolize sulfolane under aerobic conditions. We assessed the distribution of this sulfolane-assimilating *Rhodoferax* sp. throughout the contaminated subarctic aquifer using 16S-rRNA-amplicon-sequence-based microbial community analyses of ~100 samples collected from groundwater monitoring wells and two sulfolane treatment systems. One treatment system was an air sparging system where air was injected directly into the aquifer and the other was a granular activated carbon filtration system for treatment of water from private wells. We found that this organism was present in low abundances throughout the aquifer but was significantly more abundant in the groundwater associated with the experimental air sparge system, especially 13 weeks after system start-up. We found no correlation between relative abundance of the sulfolane-assimilating *Rhodoferax* sp. and the environmental variables collected (D.O., sulfolane, ORP, pH, conductivity, depth, location) in the monitoring wells, however, the abundance of the sulfolane assimilator correlated strongly with dissolved manganese after 10 weeks of air sparging. The sulfolane-assimilating *Rhodoferax* sp. was not a major component of the granular activated carbon filtration systems. Community analysis of the aquifer monitoring well samples revealed that depth was inversely related to community richness and evenness. We also investigated the community differences between subarctic groundwater and aquifer sediment. Although there were significant differences between

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groundwater and sediment microbial communities, it appears that the sulfolane-assimilating *Rhodoferax* sp. is associated with groundwater as no members of the genus were detected in sediment samples. This research is the first to characterize the environmental distribution of a subarctic sulfolane-degrading microbe and sheds light on the potential species detection biases when characterizing the microbial community in a subarctic aquifer using groundwater samples exclusively. We conclude that air sparging appears to be a way to enhance the abundance of aerobic sulfolane-degraders and potentially to locally stimulate sulfolane biodegradation *in situ*.

## **Introduction**

Sulfolane is an anthropogenic organosulfur compound used in industrial applications worldwide (Tindal et al., 2006). Developed in the 1950s, sulfolane can now be classified as a contaminant of emerging concern due to its persistence, mobility, continued widespread use, and lack of inclusion in routine contaminant assessments (Lapworth et al., 2012; Sauve and Desrosiers, 2014). In 2009, an environmentally and economically notable case of sulfolane contamination occurred in North Pole, Alaska when sulfolane was detected in a residential well near a refinery that used the solvent. Further investigation revealed a groundwater plume that was 5.6 km long, 3.2 km wide, over 90 m deep and that affected hundreds of residential drinking wells (Magdziuk and Andresen, 2018). The effects of sulfolane exposure on humans is unknown, but a study that exposed rats to sulfolane via their drinking water resulted in lowered white blood cell counts in females and neuropathy in males (Petersen et al., 2012). The refinery currently offers drinking water solutions to the affected residents including providing them with alternative drinking water or granular activated carbon (GAC) filters to remove the sulfolane from contaminated well water (Magdziuk et al., 2016).

Although sulfolane generally does not readily biodegrade under anaerobic conditions often found in subsurface environments, aerobic sulfolane biodegradation has been reported in laboratory incubation studies (Fedorak and Coy, 1996; Greene et al., 2000; Kasanke and Leigh, 2017). In North

Pole, Alaska, aerobic and anaerobic microcosm studies using groundwater and sediment from the contaminated aquifer as inoculum found aerobic biodegradation to be the only observable mechanism of sulfolane loss (Kasanke and Leigh, 2017). As part of remediation efforts, an *in-situ* experimental air sparge (AS) system was employed that injected atmospheric air into a small section of the North Pole aquifer to limit sulfolane from migrating off of refinery property (Angermann and DeJournett, 2013). Although effective at lowering sulfolane concentrations, the mechanisms behind the reduction in contaminant concentrations were never conclusively determined (Kurapati et al., 2014).

Recently, a DNA-based stable isotope probing (SIP) experiment using  $^{13}\text{C}$ -labeled sulfolane and North Pole aquifer substrate identified a *Rhodoferrax* sp. as the dominant sulfolane-metabolizing species and showed that this organism comprised  $85.7 \pm 8.7\%$  of the  $^{13}\text{C}$ -labeled microbial community (Kasanke et al. 2019). Other sulfolane-degrading bacteria have been identified through aerobic culturing studies, including a *Variovorax* sp. isolated from Alberta; Canada (Greene et al., 2000), *Pseudomonas maltophilia* isolated from Illinois; USA (Lee and Clark, 1993), a novel *Shinella* sp. isolated from Okinawa Main Island; Japan (Matsui et al., 2009), and most recently a strain of *Cupriavidus plantarum* isolated from a petrochemical wastewater treatment plant (Yang et al., 2019). Although researchers have identified environmental microorganisms that can biodegrade sulfolane, the abundance and distribution of these microbes throughout a contaminated aquifer and the environmental parameters that may control their abundance has not been assessed. Characterizing the distribution of sulfolane-degrading microbes throughout a contaminated aquifer and remediation systems informs plume longevity estimates, identifies potential areas of active biodegradation, and provides insight into the fundamental mechanisms of effective remediation systems.

GAC has been used for the physical removal of sulfolane in home water treatment systems in North Pole, Alaska (Magdziuk et al., 2016). Prior to home installation, the North Pole GAC point-of-entry water treatment systems installed to treat private wells were tested for effectiveness in sulfolane removal (BARR, 2011) and a separate study of the same GAC substrate found evidence of rapid sulfolane

sorption (Diaz, 2015). However, another research group found that GAC required inoculation with an aerobic, sulfolane-degrading enrichment culture to effectively remediate contaminated groundwater in California (Ying et al., 1994). They concluded that biodegradation, not sorption, was responsible for the effectiveness of their system. However, that work was done in the presence of the co-contaminants dibromochloropropane and ethylene dibromide, which were thought to preferentially bind to the GAC over sulfolane. Although microcosm studies using GAC from a North Pole point-of-entry remediation system as inoculum found no biodegradation after 10 weeks of incubation under aerobic conditions (Janda, 2016), the GAC is replaced in six-month intervals and it is unknown if sulfolane biodegradation occurs after a prolonged lag-time (BARR, 2011). Understanding the mechanisms underlying treatment system function lends insight into the potential generation of byproducts, such as degradation intermediates, that could be relevant to human health.

It is commonly found that environmental parameters are important determinants of microbial community composition. For example, pH is often reported as the most significant parameter controlling community structure across environments (Barberán et al., 2015; Fierer et al., 2007; Fierer and Jackson, 2006). Other variables that have been shown to drive the structure of aquifer microbial communities include oxygen (Franklin et al., 1999), depth (Probst et al., 2018), and nutrient availability (Hubalek et al., 2016). In addition, groundwater (suspended) and aquifer sediment (attached) microbial communities have been reported to differ (Alfreider et al., 1997; Anantharaman et al., 2016; Flynn et al., 2013). Therefore, when conducting aquifer microbial community assessments another important environmental variable to consider is aquifer substrate specificity.

In this study, we examined the microbial community in 100 groundwater monitoring wells (MW) distributed throughout the North Pole aquifer for the distribution of a recently identified sulfolane-metabolizing *Rhodoferrax* sp. as well as other known sulfolane degraders. We also investigated two effective sulfolane remediation systems, an experimental AS system and a fully-operational GAC filtration system, for the presence and abundance of these degraders. A more general microbial

community assessment was conducted to describe the dynamics within and between the MW, AS, and GAC communities. To determine the environmental controls on the subarctic aquifer microbiome, we measured DO, pH, and several other environmental parameters and compared them to sulfolane-degrading species abundances as well as overall community composition. To determine if sulfolane-degrading species were attached to aquifer substrate or planktonic, we conducted a supplemental sampling campaign to identify differences between subarctic groundwater and aquifer sediment microbial communities. Through this effort we were also able to identify and describe potential biases involved with sampling groundwater for microbial community characterizations.

## **Methods**

### **Sampling**

#### **Site description**

This study focuses on a sulfolane-contaminated groundwater plume located in North Pole, Alaska, USA (64.7511° N, 147.3519° W), which is part of the greater Tanana River aquifer supplied by the Alaska Range. The sulfolane plume originated at a refinery where sulfolane use began at this site in 1985 and ceased in 2014 when refinery operations ended. The size of the plume is currently estimated to be 5.6 km long, 3.2 km wide, and 91.4 m deep and it continues to migrate to the north-northwest (Magdziuk and Andresen, 2018). Sulfolane concentrations throughout the plume ranged from 0-34.8 mg L<sup>-1</sup> at the time of sampling (Kurapati et al., 2014).

#### **Plume-wide sampling**

Groundwater samples were collected from MWs installed throughout the contaminated groundwater plume. One hundred groundwater samples were collected from two routine sampling campaigns by environmental consultants Shannon and Wilson, Inc. under contract from refinery owners Flint Hills Resources. Eighty-two samples were collected between October 2<sup>nd</sup> and December 20<sup>th</sup>, 2013,

and 18 samples were collected between January 7<sup>th</sup> and March 27<sup>th</sup>, 2014. Wells were constructed of 5-cm diameter pipe with 0.05-cm screens, which were between 1.2 and 1.5 m in length with half of the screen above and half below the targeted depth. Well depths ranged from 4 to 46 m below ground surface. Prior to sample collection, groundwater was purged until geochemical parameters stabilized or three well volumes of groundwater were pumped from the well. A YSI ProPlus multiprobe or equivalent was used to monitor geochemical parameters, including temperature, conductivity, dissolved oxygen (DO), pH, and oxidation-reduction potential (ORP). One liter of groundwater was collected from each well and stored in a sterile 1-L bottle at 4 °C until being filtered through a sterile 0.22- $\mu$ m filter within 24 hours for microbial analysis. A separate liter of groundwater was submitted to SGS Laboratories of Anchorage, Alaska for sulfolane analysis using isotope dilution gas chromatography/mass spectrometry following a modification of USEPA Method 1625B.

#### *Air sparge system*

The air sparge (AS) system pumped atmospheric air into the aquifer through eight injection wells with a 0.6-m well screen positioned 6.1 m below ground surface in brown/gray gravelly sand to sandy gravel soil with trace amounts of silt (Angermann and DeJournett, 2013). The air flow rate at each air sparge point was slightly variable and ranged from 42.5 to 76.5 m<sup>3</sup> h<sup>-1</sup>. Eight monitoring wells like those described above but surrounding the experimental AS system were also sampled for microbial community analysis. One well was placed ~12 m upgradient of general aquifer flow, three wells were down the center line of the system, two wells were ~4.5 m outside the system to the west, and two wells were ~4.5 m outside the system to the east. When samples were collected from the AS wells sulfolane, temperature, conductivity, DO, pH, and ORP were analyzed as described above for MW samples. In addition, dissolved iron, dissolved manganese, total organic carbon, and total phosphorus were measured in AS samples (Angermann and DeJournett, 2013). Although the upgradient well was intended to be out of the influence of the system, it had D.O. concentrations between 0.11 to 1.45 mg L<sup>-1</sup> indicating it was also impacted by the AS system and fluctuated between being oxic and suboxic (Langmuir, 1997). The D.O.



concentrations in the other AS wells ranged between 0.49 and 17.56 mg L<sup>-1</sup> with an average of 9.77 ± 5.51 mg L<sup>-1</sup>. Initial sulfolane concentrations in the AS system prior to activation ranged from 71.7 to 278 µg L<sup>-1</sup> and were lowered to below detection limits (6.88 ug L<sup>-1</sup>) in down-gradient test wells after 4 -15 weeks of operation (Angermann and DeJournett, 2013). The air sparge system was started on March 7<sup>th</sup>, 2012 and was shut down on July 10, 2013 after 70 weeks of operation (Kurapati et al., 2014). We obtained samples from 10, 13, and 70 weeks after startup but were unable to acquire samples prior to system initiation.

#### Granular activated carbon from point-of-entry treatment systems

We also examined the microbial community in a point of entry (POE) treatment systems where granular activated carbon (GAC) was used to sorb sulfolane from private wells (Supplementary Figure 2) (BARR, 2011). On May 15<sup>th</sup>, 2014, a GAC canister from a POE system was received for microbial analysis. The canister had treated 78,160 L of sulfolane-contaminated water prior to replacement. The canister was divided into thirds (top, middle, and bottom) and two 500-g samples were taken from each section for microbial community analysis (Janda, 2016). The GAC samples were stored at -80°C until the DNA was extracted.

#### Groundwater-sediment comparison

A small-scale assessment was conducted to compare the microbial community associated with groundwater to that associated with aquifer sediment. On October 12, 2014, soil cores 1.5 meters in length with an inside diameter of 5 cm were collected from a small area within the sulfolane-contaminated aquifer (Latitude: 64.74663400; Longitude: -147.36561100) using a Geoprobe push core sampler. Once a desired depth was achieved (between 4.25 and 10 meters), the sampler was equipped with a sterile collection tube and pushed down for sample collection. Seven soil cores were collected from between 4.25 and 6 m below ground surface and one soil core was collected from 7.6-9.1 m below ground surface. These eight soil cores were subsampled every 0.3 meters resulting in a total of 21 sediment

samples suitable for DNA analysis. Once subsampled, the sediment was stored at -80 °C until DNA was extracted for community analysis as described below. Soil particle size was determined using the hydrometer method as described in Gee and Bauder (1986). The depth to the water table was 2.75 m below ground surface at the time of sampling.

The same borehole from sediment core collection was then used for groundwater sampling. Groundwater samples were collected using a Geoprobe ® sampler equipped with a SP16 water sample system utilizing a 0.9-meter screen with an expendable point. The system was pushed 0.45 meters past the desired depth then the screen was exposed so that 0.45 meters was below the interval and 0.45 meters was above. The drill rod was pulled up a total of 0.9 meters exposing the screen to the appropriate sample interval. Once the screen was exposed, a tube (inside diameter = 1.5 cm) was inserted through the sampler and a peristaltic pump was used to retrieve the sample. Groundwater chemistry was measured using a YSI 556 Multiprobe system with a 5083-flow cell attachment as described above. After the chemical values stabilized, 1 L of groundwater was collected for microbial community analysis. Groundwater samples were filtered and stored as described above for MW samples.

### **DNA extraction**

DNA was extracted from the groundwater filters using a phenol-chloroform extraction described in Miller *et al.* (1999) as modified by Hazen *et al.* (2010). Soil DNA was extracted from sediment solids after sieving through a 2 mm screen using a MoBio Power Soil DNA isolation kit (MO BIO, Carlsbad, CA) in accordance with manufacturer's instructions. To obtain a more representative sample, three randomly selected 0.25-g soil subsamples were independently extracted from each 0.3-meter core section and pooled prior to sequencing. DNA was isolated from GAC samples using a MoBio PowerMax Soil DNA Isolation Kit following the manufacturer's instructions. GAC samples were extracted in duplicate and pooled for sequencing. All DNA extracts were stored at -20 °C prior to sequencing.

## **Microbial community analyses**

### *Monitoring well, air sparge, and GAC*

The bacterial and archaeal community structure was assessed in MW, GAC, and AS samples by sequencing a ~250-bp segment of the V4 region of the 16S rRNA gene using an Illumina MiSeq as described in (Kasanke et al. 2019). MW, GAC, and AS samples were sequenced at Michigan State University. FastQ files were analyzed using mothur software (1.35.1) following a modified version of the standard MiSeq SOP (accessed March 2016) (Kozich et al., 2013; Schloss et al., 2009) as described by Martinez et al. 2017 (Martinez-Cruz et al., 2017). All sequences had a quality score of 25 or greater and the maximum contig length of 275. All unique sequences were aligned against the SILVA SEED v132 database and chimera checking was performed using the mothur implementation of Uchime (Kozich et al., 2013; Quast et al., 2013). Unique operational taxonomic units (OTUs) were defined at a level of 99% sequence similarity and taxonomy was assigned using SILVA SEED v132 taxonomy database (Quast et al., 2013). BLASTN was used to obtain higher-resolution taxonomic assignment for dominant community members (Mount, 2007). To account for differences in sequence coverage, the number of sequences was subsampled to the number of sequences in the least covered sample after quality control steps (8142). After subsampling, Chao and Inverse Simpson diversity indices were calculated. MW, GAC, and AS samples were analyzed along with a larger dataset including a previously reported DNA-SIP experiment where <sup>13</sup>C-labeled sulfolane was added to North Pole aquifer substrate identifying a *Rhodoferrax* sp. as the dominant sulfolane assimilating microorganism (Kasanke et al. 2019). That dataset was generated using the same methods and sequencing center as the MW, GAC, and AS samples allowing for direct OTU comparison of the labeled sulfolane-assimilating species throughout these samples. Sequence files used in this analysis are publicly available on the sequence read archive (SRA) under accession #PRJNA504308.

### Groundwater-sediment comparison

The DNA extracts from the sediment and groundwater comparison were sequenced at the Institute of Arctic Biology Genomics Core Laboratory at the University of Alaska Fairbanks using an Illumina Miseq and the Illumina v3 reagent kit. The standard Illumina iTRU two-step 16S rRNA gene sequencing protocol was used (Illumina Inc., 2013). A 460-bp segment from the V3-V4 region of the 16S rRNA gene was targeted using the primers recommended for Illumina sequencing by Klindworth *et al.* (2013). Fastq files were analyzed using mothur software as described above with the maximum contig length set to 480 bp. To account for differences in sequence coverage, the number of sequences was subsampled to the number of sequences in the least covered sample after quality control steps (3390). After subsampling, Chao and Inverse Simpson diversity indices were calculated. Although direct OTU comparison was not possible between this dataset and the DNA-SIP dataset described above, the genome of the sulfolane-assimilating *Rhodoferox* sp. has been sequenced, including the full 16S rRNA gene. To determine if the sulfolane-assimilating *Rhodoferox* sp. was present in the groundwater-sediment comparison dataset, the representative sequence of OTUs assigned to the genus *Rhodoferox* were aligned against the full 16S rRNA gene of the degrader using BLASTN (Altschul et al., 1990; Mount, 2007). Sequence files used in this analysis are publicly available on the sequence read archive (SRA) under accession #PRJNA504308.

### Statistical analyses

All multivariate statistical analyses were conducted using PCORD Version 6 statistical analysis software (McCune and Mefford, 2011). Differences between microbial communities were assessed using nonparametric Multi-Response Permutation Procedures (MRPP) from a rank-transformed Bray-Curtis distance matrix (McCune and Grace, 2002). To help identify species differences between the MW, AS, and GAC microbial communities, indicator species analysis was performed. Indicator values were calculated using the method of Dufrêne and Legendre (1997) and significance of the indicator value was

determined using 4,999 randomized Monte Carlo simulations. Community data was visualized using nonmetric multidimensional scaling (NMS) based on a Bray-Curtis distance matrix and random starting configurations with dimensionality of the data determined by comparison of 250 runs with real data and 250 randomized Monte Carlo simulations (Kruskal, 1964; Mather, 1976). Pearson and Kendall correlations of environmental variables with ordination axes was performed and variables with  $R^2$  values of 0.2 or greater were reported. R statistical software was used to conduct Welch's two-sample t-tests, one-way analysis of variance (ANOVA) tests, post-hoc Tukey tests, and simple linear regressions (The R Foundation for Statistical Computing, 2015). For all analyses, a p-value or equivalent of 0.05 or less was considered significant. All reported values are the mean  $\pm$  standard deviation.

## **Results and Discussion**

### **Distribution of sulfolane-assimilating species throughout plume and treatment systems**

One of the primary goals of this study was to assess the abundance of known sulfolane-metabolizing bacteria throughout a contaminated aquifer and two groundwater treatment systems and to investigate environmental factors controlling their relative abundance. One treatment system we examined sorbs sulfolane from private wells prior to consumption using GAC (BARR, 2011). The other was an experimental AS treatment system which successfully lowered sulfolane concentrations to below detection limits ( $6.88 \text{ ug L}^{-1}$ ) in down-gradient test wells after 4 -15 weeks of operation (Angermann and DeJournett, 2013). Although we found significant differences in overall microbial community composition between the AS, GAC, and MW samples as discussed later (MRPP, significance of delta  $\ll$  0.001, A= 0.20), the initial focus was on the distribution of a *Rhodoferax* sp. identified as the dominant, if not exclusive, sulfolane-assimilating species in a DNA-SIP study that used substrate from this aquifer as inoculum (Kasanke et al. 2019). We also examined the AS, GAC, and MW samples for the distribution of other known sulfolane-degrading microorganisms reported in the literature.

Plumewide abundance and environmental drivers of *Rhodoferax* sp. distribution

In our plume-wide survey of aquifer monitoring wells (MW), we detected a total of 253 OTUs that were classified as *Rhodoferax* spp., including the third most abundant groundwater bacterium detected. By re-analyzing a sulfolane SIP dataset along with the MW, AS, and GAC samples we were able to identify one of these OTUs as a previously described sulfolane-assimilating *Rhodoferax* sp. (Kasanke et al. 2019). Based on our detection of this known subarctic sulfolane-assimilating *Rhodoferax* sp. in 70% of the MW samples, we concluded that sulfolane biodegradation potential was widely distributed throughout the aquifer. Despite being widely distributed throughout the plume, the sulfolane-assimilating *Rhodoferax* sp. was generally present in relatively low abundance (maximum relative abundance of 4.1%; average  $0.59 \pm 0.77\%$ ). Because this aquifer is generally anoxic (Kurapati et al., 2014), it is unlikely that this organism is degrading sulfolane under normal aquifer conditions. Our prior incubation studies demonstrated that sulfolane biodegradation potential exists in aquifer sediment and water under aerobic conditions. Although there are reports of anaerobic sulfolane biodegradation at other sites (Greene et al., 1998; Kim et al., 1999), our anaerobic sulfolane biodegradation microcosm studies using substrate from this aquifer as the inoculum failed to result in sulfolane loss despite incubating for over 1000 days (Kasanke & Leigh, 2017).

We found that the AS system fostered significantly higher abundances of the sulfolane-assimilating *Rhodoferax* sp. than the surrounding aquifer (Welches two-sample t-test,  $df=23.6$ ,  $t=4.66$ ,  $p=0.0001$ ) (Figure 4-1A) with this bacterium being the most abundant OTU in the AS samples (maximum relative abundance 10.5%; average  $3.6 \pm 3.2\%$ ). Indicator species analysis found the sulfolane-assimilating *Rhodoferax* sp. to be a strong indicator of the AS samples when compared to MW and GAC samples with an indicator value of 80.9 ( $p = 0.0002$ ) (Supplementary Table 4-1). Previous microcosm studies involving air sparging of sulfolane-contaminated soil attributed sulfolane loss exclusively to aerobic biodegradation (Greene and Fedorak, 2001) and sulfolane losses have never been reported in sterile controls among all sulfolane biodegradation studies published to date (Greene et al., 2000, 1998;

Kasanke and Leigh, 2017). In fact, all reports of abiotic sulfolane degradation involved the use of strong oxidizers, radiation, or both (Agatonovic and Vaisman, 2005; Izadifard et al., 2017); none of which are present in the North Pole aquifer. Although difficult to definitively conclude, the dominance of the known sulfolane degrader in this system combined with the reduction in sulfolane concentration (Angermann and DeJournett, 2013) suggest that the AS system stimulated sulfolane biodegradation *in situ*.

A one-way ANOVA found significant differences in the relative abundance of the *Rhodofexax* sp. in the AS communities with respect to the amount of time the AS system was active (ANOVA,  $F_{2,21}=14.96$ ,  $p < 0.001$ ). As the sulfolane concentration decreased from week 10 to week 13, the sulfolane-assimilating *Rhodofexax* sp. increased in abundance ( $p < 0.009$ ) suggesting biodegradation as a mechanism of sulfolane loss in this system (Figure 4-2). After 70 weeks of operation, the abundance of the *Rhodofexax* sp. was significantly reduced ( $p < 0.001$ ) (Figure 4-2). This decrease may be a result of community resilience after contaminant exposure ceases (Boivin et al., 2006) since sulfolane was not detected down-gradient of the AS system after 15 weeks of operation (Angermann and DeJournett, 2013). The decrease in abundance could also result from a depletion of specific nutrients necessary to support the growth of this species (Bren et al., 2013), which would be expected if prolonged biodegradation was occurring (Kasanke and Leigh, 2017).

The environmental variables we measured (i.e., sulfolane, temperature, conductivity, DO, pH, and ORP) did not explain variations in the relative abundance of this species throughout the aquifer. Although statistically significant correlations of the *Rhodofexax* sp. with temperature and sulfolane concentration were detected, the goodness of fit value was extremely low ( $R^2 = 0.065$  and  $0.061$  respectively) indicating these variables are not reliable predictors of the distribution of this species. Although there were generally higher abundances of the *Rhodofexax* in the AS system than elsewhere in the plume, we also found no correlations with the environmental variables collected for the AS system when combining the data from weeks 10, 13, and 70 of system operation. However, during week 10 of AS system operation there was a significant ( $p < 0.001$ ) and strong ( $R^2 = 0.88$ ) positive correlation

between dissolved Mn concentration and the relative abundance of the *Rhodoferrax* sp. (Figure 4-3). This correlation did not exist for weeks 13 and 70 of AS system operation. We were unable to measure dissolved Mn concentrations in the MW samples, but availability of this element may play a role in either stimulating or limiting the growth of this sulfolane-assimilating bacterium. Future attempts to determine environmental controls on the distribution of sulfolane degrading microorganisms should include a more thorough elemental analysis, including measuring dissolved Mn concentrations.

In the separate sample set in which parallel groundwater and sediment samples were compared, we found no OTUs that were exact matches to the sulfolane-assimilating *Rhodoferrax* sp., although we did identify one OTU that was 98% identical (E-value 0.0) to the sulfolane assimilator. Despite this OTU being the only *Rhodoferrax* detected in the groundwater-sediment comparison dataset, it was present in low abundances exclusively in the groundwater samples (maximum relative abundance 0.15%) and was not detected in any of the aquifer sediment samples. In combination, these results suggest that despite significant differences between attached and suspended microbial communities (described below), groundwater is an appropriate aquifer substrate to detect and quantify members of the *Rhodoferrax* genus. This finding is consistent with reports that freshwater environments are the described natural habitat for members of the *Rhodoferrax* genus (Hiraishi et al., 2015).

#### Role of sulfolane degraders in granular activated carbon water treatment systems

In the GAC treatment system, the sulfolane-assimilating *Rhodoferrax* sp. was only detected in low abundance in three of six GAC samples (relative abundance of 0.37%, 0.11%, and 0.025%). The obligate anaerobe *Ferribacterium limneticum* (99% identical E value =  $2e-128$ ) was the most dominant bacterium representing  $38.6 \pm 19.2\%$  of the GAC community suggesting that the GAC system was primarily anoxic. (Cummings et al., 1999). Although thermodynamically feasible, anaerobic sulfolane biodegradation has only been observed in 4 of 60 anaerobic microcosms inoculated with sediment from Western Canada (Greene et al., 1998). Anaerobic incubations conducted using aquifer substrate from North Pole, Alaska



resulted in no sulfolane loss after 1021 days of incubation (Kasanke and Leigh, 2017). Column studies conducted prior to home installation in North Pole, Alaska have shown predictable sulfolane breakthrough curves consistent with sorption models (BARR, 2011). All these findings support the conclusion that sorption, not biodegradation, is the mechanism of sulfolane removal in the North Pole GAC systems. This conclusion is in contradiction with previous research that found it necessary to inoculate GAC with microorganisms obtained from sewage plant effluent to remediate sulfolane (Ying et al., 1994). However, that research was done in the presence of the co-contaminants dibromochloropropane and ethylene dibromide, which preferentially sorb to GAC. In the absence of co-contaminants or other organics that compete for sorption sites on GAC, it does not appear that biodegradation is a necessary component of GAC treatment in order to achieve the removal of sulfolane from contaminated water.

#### *Distribution of other known degraders in the plume and treatment systems*

In addition to screening for the sulfolane-metabolizing *Rhodofera* strain found in our earlier study of this aquifer, we also queried our dataset for other previously reported sulfolane degraders from other geographic regions. To our knowledge, only four other sulfolane degrading microorganisms have been isolated from the environment. *Pseudomonas maltophilia* was isolated from the soil of an abandoned strip mine near Cambria, Illinois; (Lee and Clark, 1993) a novel *Shinella* sp. was isolated from soil in the Yambaru area of Okinawa Main Island; Japan (Matsui et al., 2009), and a strain of *Cupriavidus plantarum* was isolated from a petrochemical wastewater treatment plant in Taiwan (Yang et al., 2019). One OTU in our dataset was classified as a *Cupriavidus* sp. that was 99% identical (E-value = 4e-131) to the known degrader. That OTU was only detected in one MW sample although it did represent 1.9% of the community relative abundance. We did not detect any *Shinella* spp. in the dataset and among the 36 *Pseudomonas* spp. detected, none were matches to *Pseudomonas maltophilia*. In western Canada, a sulfolane-degrading *Variovorax* sp. described as being closely related to *Variovorax paridoxus* was isolated from sulfolane contaminated aquifer substrate (Greene et al., 2000). The sulfolane-assimilating

*Rhodoferax* sp. was detected in all AS wells except one from week 10 where a *Variovorax* sp. 99% identical to *Variovorax paradoxus* (E-value =  $2e-128$ ) represented 86.1% of the community. The elevated abundance of this species in a portion of the AS system relative to the MW samples (max relative abundance 0.04 %) and close relation to a known sulfolane degrader suggests this bacterium also may have been degrading sulfolane. By week 13 a community shift occurred, and this AS well also became dominated by the sulfolane-assimilating *Rhodoferax* sp.

### **Community structure of contaminated aquifer and treatment systems**

To better understand the overall microbial ecology of subarctic aquifers, we investigated community structure and environmental factors controlling it across the plume and treatment systems. The total prokaryotic microbial community in all samples was primarily dominated by bacteria, with archaea only comprising  $1.57 \pm 1.88\%$  of the total community relative abundance. The microbial communities differed significantly between MW, AS, and GAC samples, (MRPP, significance of delta  $\ll 0.001$ , A= 0.20) (Figure 4-1B). Those associated with the AS samples and the GAC samples were the least similar (MRPP, significance of delta  $\ll 0.001$ , A=0.37), which may be explained by the substantial differences in environmental conditions between air sparged subsurface groundwater and the water-treatment-system-associated GAC. Indicator species analysis identified several strong (indicator value  $> 70$ ) and significant ( $p < 0.05$ ) indicator species for the AS and GAC treatment systems, however none were identified for the aquifer MW communities (Supplementary Table 4-1).

The AS, GAC, and MW communities also varied in species richness with differences between AS (Chao  $1872.89 \pm 1025.32$ ), GAC (Chao  $137.3865 \pm 42.45261$ ), and MW (Chao  $1341.17 \pm 1288.75$ ) samples (ANOVA,  $F_{2,127} = 5.13$ ,  $p = 0.007$ ) exclusively attributed to a lower species richness in the GAC communities ( $p < 0.05$ ). There were no statistically significant differences in the community evenness as measured by Inverse Simpson (InvSim) diversity index between AS (InvSim  $96.31 \pm 67.80$ ), GAC

(InvSim  $3.47 \pm 1.37$ ), and MW (InvSim  $112.79 \pm 125.32$ ) microbial communities (ANOVA,  $F_{2, 127} = 2.66$ ,  $p = 0.07$ ), which we attribute to the high variability of species distributions in the MW samples.

### Plume-wide Community Structure

The aquifer MW dataset was dominated by a *Ralstonia* sp. that was 99% identical to the plant pathogen *Ralstonia syzygii* (E value =  $5e-128$ ) with a relative abundance of  $5.9\% \pm 12.4\%$  throughout the aquifer. *Ralstonia syzygii* is a plant parasite that is reported to live exclusively in the xylem of plants and is only transmitted by sucking insects (Purcell and Hopkins, 1996). However, *R. syzygii* is a member of the *Ralstonia solanacearum* species complex consisting of plant parasites that are genetically similar yet diverse in host specificity, pathogenicity, and transmission routes including soilborne pathogens that infect open wounds in plant roots (Remenant et al., 2011). Other relatively dominant members of the aquifer microbial community include *Sulfuricurvum kuijense* (97% identical; E value  $2e-118$ ), *Rhodoferrax antarcticus* (99% identical; E value  $1e-128$ ), *Burkhoderia singularis* (100% identical; E value  $7e-132$ ), and *Pelobacter propionicus* (98% identical; E value  $3e-120$ ) which respectively represented  $1.5\% \pm 3.6\%$ ,  $1.5\% \pm 5.0\%$ ,  $1.2\% \pm 2.5\%$ , and  $1\% \pm 2.4\%$  of the MW community relative abundance.

The MW microbial community decreased in species richness and evenness with depth (Figure 4-4A). NMS analysis of groundwater MW data found an optimal dimensionality of 2 axes with a final stress of 16.2 and a final instability value of 0.00000 with the proportion of variance explained by each axis in the MW NMS being 49.1% for NMS1 and 16.3% for NMS2. Pearson and Kendall correlations with ordination axes and MW explanatory variables showed MW community structure correlated with depth (NMS1  $R^2 = 0.329$ ). Depth was inversely correlated with Chao and Inverse Simpson diversity indices (NMS1  $R^2 = 0.616$  and  $0.331$  respectively). This correlation is consistent with studies of terrestrial aquifer microbial communities in other locations and is generally attributed to increasingly oligotrophic conditions at depth (Lee et al., 2018; Lin et al., 2012a). We did observe a higher alpha diversity in the near-surface samples in this subarctic aquifer (Chao1 max = 4176) than is reported for a South Korean

aquifer (Chao1 max = 2113) (Lee et al, 2018) or an aquifer in Eastern Washington, USA (Chao1 max = 772) (Lin et al., 2012a). This is somewhat unexpected since those aquifers are much warmer (both >15 °C) than this subarctic aquifer (4 °C).

#### Air Sparging System Community Structure

As noted previously, the AS community was dominated by the sulfolane-assimilating *Rhodoferrax* sp. Other dominant AS community members include two *Sideroxydans* species, which BLAST comparisons found to be 98% (e-value 2e-123) and 97% (e-value 3e-120) identical to the chemolithoautotrophic bacterium *Sideroxydans lithotrophicus* first isolated from groundwater in Lansing, MI, USA (Emerson and Moyer, 1997). These two *Sideroxydans* spp. represented  $2.5 \pm 3.3\%$  and  $1.4 \pm 1.3\%$  of the AS microbial community respectively. Sampling date was associated with microbial community shifts in the AS samples (MRPP significance of delta 0.0001; A = 0.25), which were not observed in the broader MW survey. These differences are likely in response to abrupt changes in the environmental parameters over time in relation to the length of oxygen exposure within the AS system.

Initial NMS analysis suggested a 1-dimensional solution was most appropriate to visualize the differences in the AS community data (final stress = 0.00003, final instability = 0.00000) with one sample as an extreme outlier from the other samples. This sample was from week 10 and, as described above, had a *Variovorax* sp. represent 86.1% of the sample community. After removing this sample from the AS dataset, NMS analysis found an optimal dimensionality of two axes with a final stress of 10.9 and a final instability value of 0.00000 (Supplementary Figure 4-1). The proportion of variance explained by each axis was 62.1% for axis 1 (NMS1) and 18.9% for axis two (NMS2) with the differentiation in weeks occurring primarily along NMS1. Pearson and Kendall correlations with ordination axes and air sparge environmental variables showed the AS community structure was correlated with water table elevation (NMS1  $R^2 = 0.388$ ), manganese (NMS1  $R^2 = 0.227$ ), and temperature (NMS1  $R^2 = 0.213$ ).

### Granular activated carbon water treatment system community structure

The GAC community was primarily dominated by two species, the obligate anaerobe *Ferribacterium limneticum* (99% identical E value =  $2e-128$ ) and facultative anaerobe *Noviherbaspirillum denitrificans* (99% identical; E value =  $2e-126$ ) (Cummings et al., 1999; Ishii et al., 2017). These species constituted a relative abundance of  $38.6 \pm 19.2\%$ , and  $30.6 \pm 6.9\%$ , respectively, and were strong and significant indicators of the GAC samples (indicator value = 99.7 and 99.1 respectively;  $p = 0.0002$  both). The dominance of these bacteria in the GAC community suggests the GAC environment is primarily anaerobic and unfavorable for sulfolane biodegradation (Kasanke and Leigh, 2017). We were unable to obtain environmental data for the GAC samples and were therefore unable to assess environmental correlations with GAC community composition.

### Comparison of sediment to groundwater community structure

To assess the reliability of performing subarctic aquifer microbial community assessments using groundwater samples, we examined how similar groundwater communities were to the surrounding aquifer sediment. We found that the groundwater and sediment communities are significantly different in composition (MRPP significance of  $\delta < 0.0001$ ;  $A = 0.37$ ) (Figure 4-5), sharing only 21.7% of the total 3752 unique OTUs. Despite having differing species abundance distributions between sample types, these shared OTUs represented  $88.7 \pm 3.9\%$  and  $74.7 \pm 15.9\%$  of the groundwater and sediment community relative abundances respectively. This is a larger shared proportion than was previously reported for a temperate aquifer (Flynn et al., 2013), but more comparative studies are needed to determine if this is a consistent trend. The groundwater community in North Pole, Alaska, was found to be more species rich (two-sample  $T_{15,2} = 6.29$ ,  $p < 0.001$ ) (Chao  $885.31 \pm 159.70$ ) and have a more even species distribution (two-sample  $T_{7,9} = 3.79$ ,  $p = 0.006$ ) (InvSim  $76.96 \pm 32.73$ ) than sediment (Chao  $388.87 \pm 232.71$ ; InvSim  $26.78 \pm 21.82$ ). Archaea represented  $0.15\% \pm 0.26\%$  of the total community across all samples.

The most abundant groundwater community members in this comparison dataset appear to be relatively novel bacteria with poor matches to the BLAST database. The three most abundant groundwater OTUs included *Pelolinea submarina* (90% identical E-value  $2e-164$ ) that represented  $4.8 \pm 2.0\%$  of the total community and two OTUs similar to *Bellilinea caldifistulae* that represented  $4.4 \pm 2.0\%$  (91% identical E-value  $9e-168$ ) and  $3.5 \pm 3.2\%$  (90% identical E-value  $2e-164$ ) of the total community. Phylogenetic analysis showed that these three OTUs form a single clade with *Pelolinea submarina* and *Bellilinea caldifistulae*, both anaerobic, non-motile bacteria isolated from marine benthic sediment, forming a separate sister clade (Supplementary Figure 4-2) (Imachi et al., 2014; Yamada et al., 2007). Other dominant groundwater bacteria include *Gaiella occulta* (95% identical E-value 0.0) that represented  $3.3 \pm 2.0\%$  of the total community. *Gemmatimonas phototrophica* (90% identical E-value  $6e-170$ ) that represented  $2.5 \pm 1.4\%$  of the total community. Apart from the bacteria most similar to *Gaiella occulta*, all these species were strong and significant indicators of the groundwater community (Supplementary Table 4-2). The differences in dominant groundwater community members between this small aquifer subset and MW samples could be due spatial heterogeneity of the community (Lin et al., 2012b) (Supplementary Figure 4-3). However, one factor that complicates a direct comparison of the two groundwater community datasets was our use of different sequencing primers, which introduces the potential for differential amplification biases (Thijs et al., 2017).

In contrast to groundwater, the dominant members of the attached microbial community were generally well represented in the BLAST database enabling more accurate taxonomic identification. The dominant sediment community members include *Burkholderia tropica* (99% identical, E-value 0.0) with a relative abundance of  $14.2 \pm 20.3\%$  (maximum 61.1%); *Stenotrophomonas rhizophila* (99% identical E-value 0.0) with a relative abundance of  $6.8 \pm 4.8\%$ ; *Sphingomonas adhaesiva* (99% identical E-value 0.0) with a relative abundance of  $4.4 \pm 3.0\%$ ; *Acidovorax delafieldii* (99% identical E-value 0.0) with a relative abundance of  $4.2 \pm 3.8\%$ ; and *Hydrogenispora ethanolica* (86% identical E-value  $8e-134$ ) with a

relative abundance of  $3.1 \pm 2.3\%$  of the community. Indicator analysis found that all these bacteria are strong and significant indicators of sediment communities in this sample set (Supplementary Table 4-2).

To investigate drivers of sediment community composition, NMS analysis was performed on sediment microbial community. NMS analysis of the sediment communities stabilized on a two-dimensional solution after 52 iterations with a final instability of 0.00000 and a final stress of 12.68 (Figure 4-4B). NMS1 explained 50.2% of the community variability and NMS2 explained 35.7% of the community variability. Pearson and Kendall correlations revealed NMS1 correlated with soil moisture ( $R^2 = .265$ ), percent gravel ( $R^2 = 0.254$ ), and percent sand ( $R^2 = 0.251$ ) and axis 2 correlated with depth ( $R^2 = 0.376$ ). The importance of these variables on community dynamics is logical since they are influential in dictating aquifer hydrological movement in addition to the types and availability of nutrients (Heath, 1983; Maamar et al., 2015).

## **Conclusion**

This study characterizes the microbial community in a sulfolane-contaminated aquifer via 16S rRNA gene sequencing and is the first microbial community analysis of a sulfolane-contaminated aquifer not relying on culture-based methods. Our approach enabled us to characterize changes in the overall microbial community composition while paying special attention to the distribution of known sulfolane-degrading microorganisms. Sulfolane biodegradation potential is widely distributed throughout this contaminated aquifer although not likely occurring under the normal, suboxic conditions based on community abundance values of a known sulfolane degrading organisms and the persistence of the sulfolane plume. However, air sparging effectively reduced sulfolane levels concomitantly with an increase in the relative abundance of a *Rhodoferrax* sp. known to metabolize sulfolane under aerobic conditions, suggesting that aerobic biodegradation was the mechanism for sulfolane removal. Extensive community analyses throughout the aquifer revealed that depth is a main factor driving microbial community structure in both subarctic aquifer sediment and ground water. Our comparative analysis of

microbial communities associated with subarctic groundwater to sediment revealed substantial yet incomplete overlap in community composition and significantly different community structure between the two sample types. Although we confirmed that members of the *Rhodoferrax* genus can be reliably detected in groundwater samples, this finding has implications for the reliability of using groundwater analyses for characterizing overall aquifer microbial communities. This research provides novel insights into the basic microbial ecology of a subarctic aquifer and the fundamental mechanisms behind effective sulfolane remediation systems, which will be useful in assessing and remediating sulfolane contaminated sites worldwide.

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## Figures

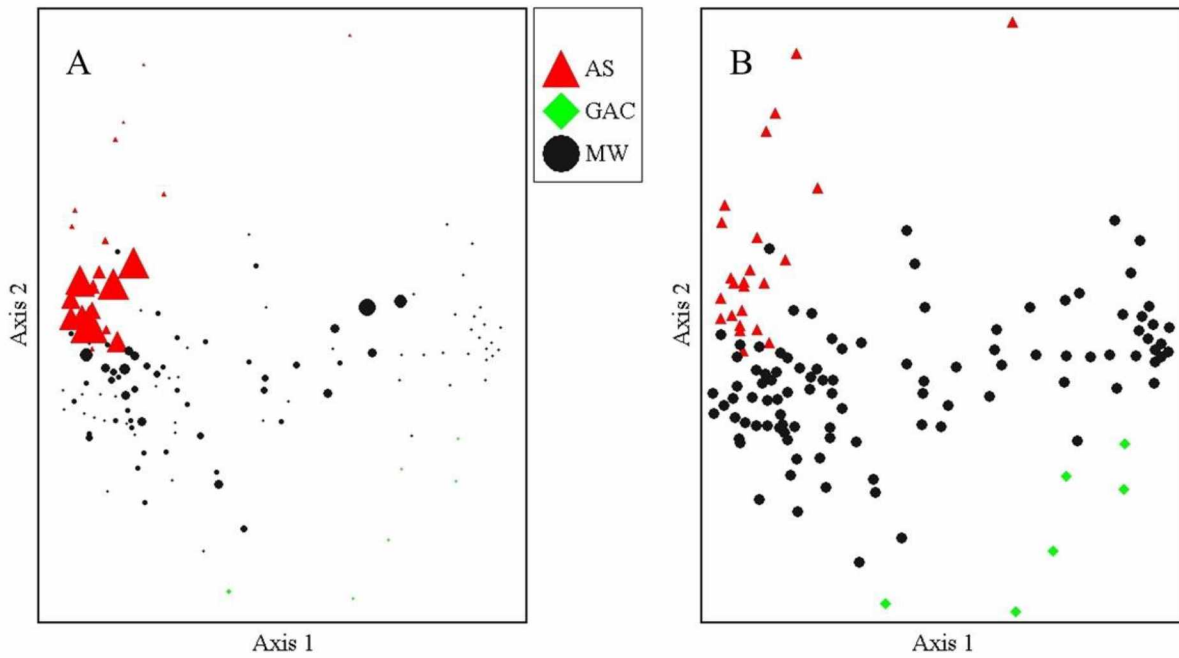


Figure 4-1: NMDS of the aquifer monitoring well (MW), air sparge system (AS), and granular activated carbon (GAC) microbial communities. A: Marker size is proportional to the relative abundance of the sulfolane-assimilating *Rhodoferrax* sp. in each sample. B: Identical NMS plot with equal marker sizes for each sample showing the microbial community group by sample type. A stable solution was reached after 58 iterations with an optimal dimensionality of 2 axes, a final stress of 18.6 and a final instability value of 0.00000. The proportion of variance explained by each axis was 36.7% for axis 1 and 16.9% for axis two.

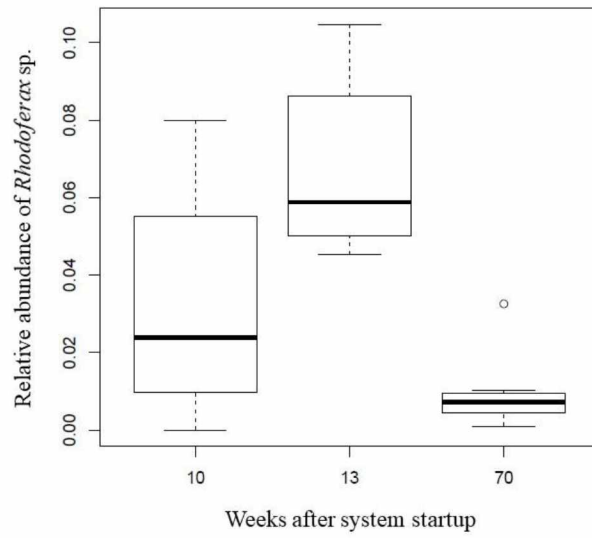


Figure 4-2: Relative abundance of the sulfolane-assimilating *Rhodoferrax* sp. in AS system after 10, 13, and 70 weeks of operation

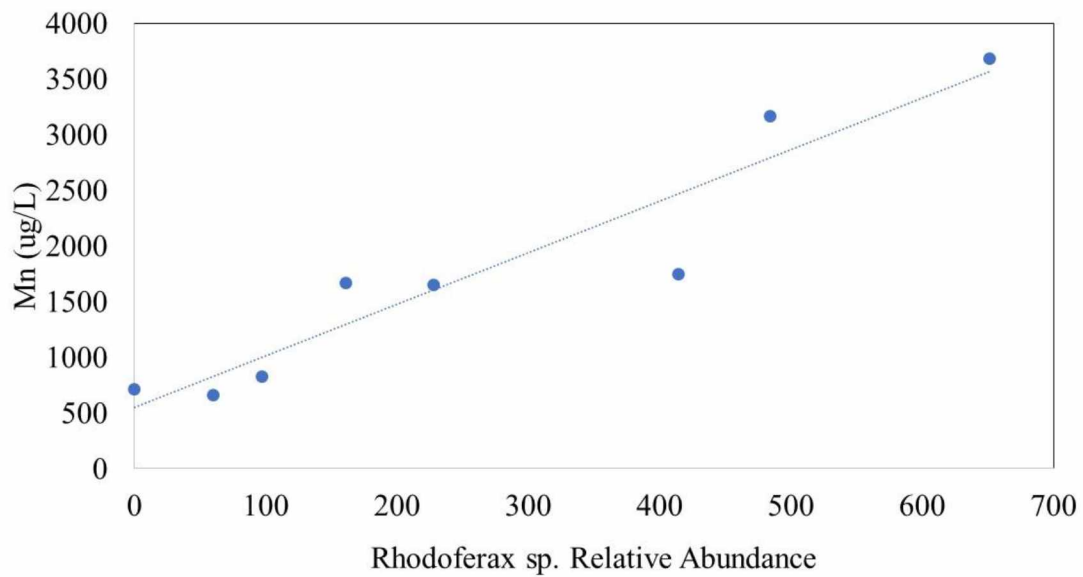


Figure 4-3: Plot of the linear regression between the relative abundance of the sulfolane degrading species and Mn during week 10 of AS system operation ( $p < 0.001$ ;  $R^2 = 0.88$ ).

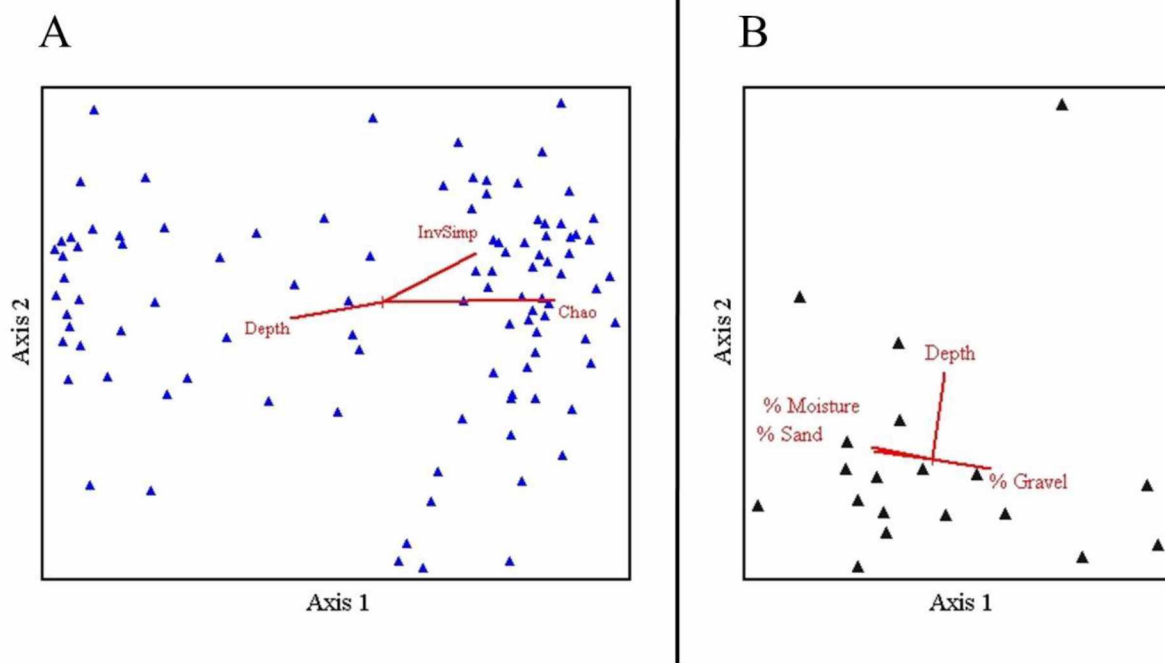


Figure 4-4: A) NMS plot of the suspended groundwater microbial communities from the MW samples showing correlations between explanatory variables and ordination axes. Axis 1 explains 49.1% of the community variability and axis 2 explains 16.3% of the community variability. B) NMS plot of the attached aquifer sediment microbial community data showing correlations between explanatory variables and ordination axes. Axis 1 explained 50.2% of the community variability and axis 2 explained 35.7% of the community variability.



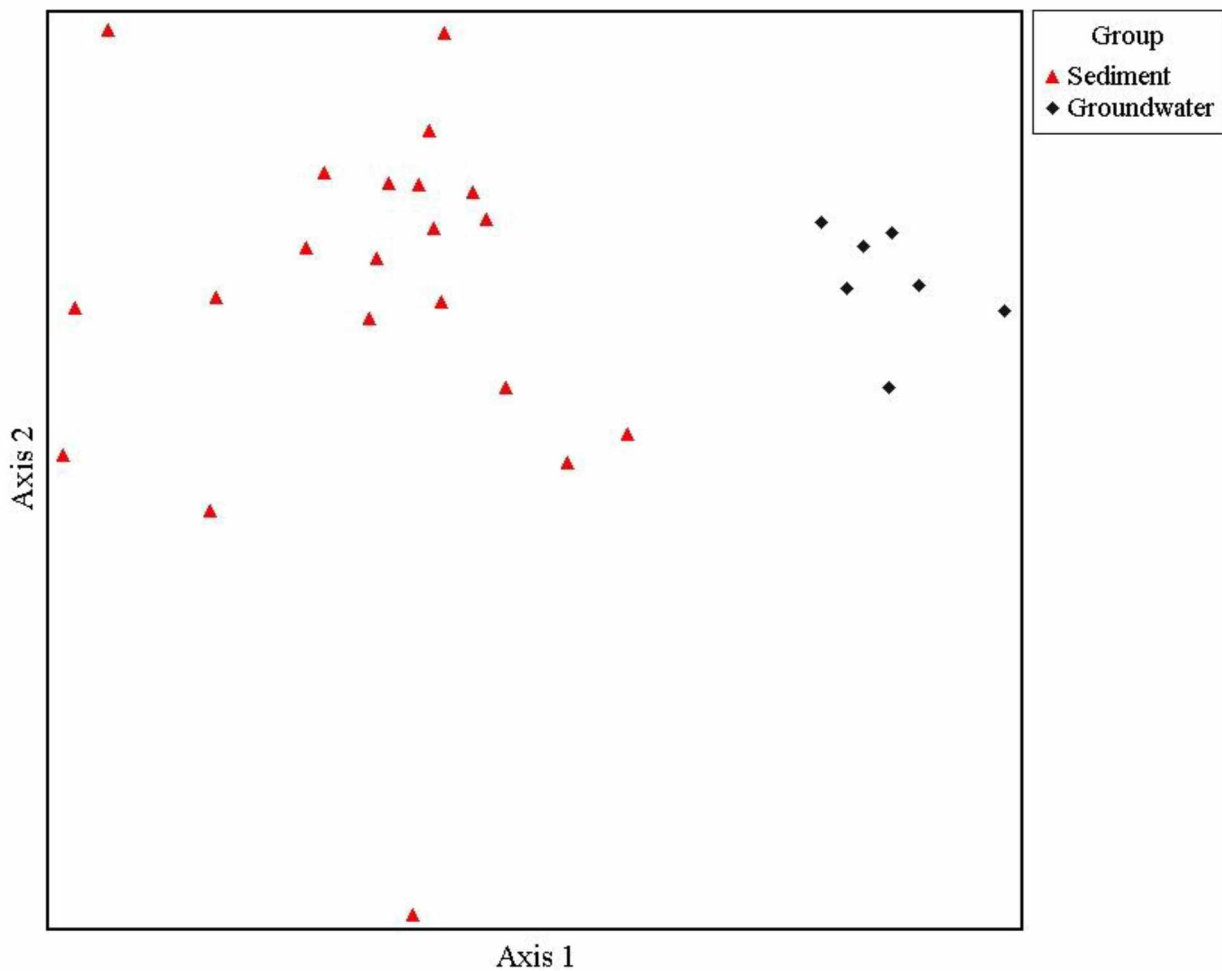


Figure 4-5: NMS plot of soil – groundwater comparison data showing differences in community composition based on sample type. A stable solution was reached after 45 iterations with an optimal dimensionality of 2 axes, a final stress of 11.61, and a final instability value of 0.00000. Axis 1 explains 56.8% of the community variability and axis 2 explains 25.8% of the community variability.

(Supplementary information available online)

## General Conclusion

This dissertation has described the microbial ecology of a subarctic aquifer polluted with the emerging contaminant, sulfolane, including a characterization of the sulfolane biodegradation potential of the aquifer and two remediation systems. We also revealed environmental controls on sulfolane biodegradation rates and the overall aquifer microbial community composition. In addition, we identified a dominant and novel sulfolane-assimilating bacterium and potential genes involved with sulfolane metabolism. Our studies provide new basic research into sulfolane biodegradation that can be valuable to the applied sciences of predicting contaminant fate and accelerating biodegradation. In addition, this series of studies demonstrates the use of a combination of traditional and advanced molecular methods that can be applied to other emerging organic contaminants for which limited biodegradation information exists.

In our series of studies, we first revealed that sulfolane biodegradation potential exists within this aquifer and identified aerobic biodegradation as the only observable mechanism of sulfolane degradation, which suggests that oxygen limitation may explain the persistence of sulfolane in this aquifer. We also quantified and reported the rates of sulfolane biodegradation in subarctic substrate under a variety of conditions that can often be found at contaminated sites (e.g. high/low contaminant concentrations, co-contamination), which may be useful in projecting the fate of sulfolane and in assessing potential remediation options in future scenarios. After identifying the importance of aerobic biodegradation in sulfolane attenuation, we conducted labeling studies to identify the members of the aquifer microbial community actively involved with sulfolane biodegradation. By doing so, we identified one *Rhodoferax* sp. as the primary sulfolane-assimilating organism in this system and expanded the known taxonomic distribution of sulfolane-degrading microorganisms. We then generated a near-complete genome for this *Rhodoferax* sp. which led us to propose a new metabolic model for sulfolane biodegradation. Finally, we conducted a microbial community analysis of the sulfolane-contaminated aquifer and two remediation

systems, allowing insights into the biodegradation potential of the aquifer, mechanisms underlying effective sulfolane treatment systems, and drivers of microbial community structure in a subarctic aquifer.

Understanding the microbial ecology of a contaminant plume is important for predicting plume longevity and ecosystem resilience to contaminant exposure, and, through these efforts, we have gained novel insights into the fate of sulfolane in the environment. We also demonstrated the power of combining modern molecular techniques to identify and characterize the microorganisms involved with the biodegradation of an emerging contaminant. Future work should be directed at elucidating conclusively the biochemical pathway(s) for and genetic basis of sulfolane metabolism. The isolation of the sulfolane-assimilating *Rhodoferrax* sp. we describe into pure culture may help toward this goal and the genomic information generated from our research could benefit such efforts. The use of controlled laboratory experiments would aid in conclusively determining if sulfolane is completely mineralized by this strain and would enable more focused genetic and biochemical analyses into the pathways involved. Inserting the genes we propose to be involved with sulfolane metabolism into model laboratory microorganisms could also yield insights into the mechanisms of sulfolane biodegradation. Overall, a better understanding of the microorganisms and genes involved with sulfolane metabolism would advance efforts to fully understand the environmental fate of sulfolane and identify alternative remediation options.