Feasibility of Biosurfactant Enhanced Bioremediation of Residual Petroleum Hydrocarbon Fractions in Contaminated Soils from Lac Mégantic

Aleksandra Kasprzyk
Degree of Master of Engineering

Department of Civil Engineering and Applied Mechanics
McGill University, Montreal

A Thesis submitted to the Graduate and Postdoctoral Studies Office in partial fulfilment of the requirements of the degree of Master of Engineering.

July 2018

Abstract

Soils contaminated with petroleum hydrocarbons, like those that resulted from the Lac Mégantic disaster, are often treated using cost-effective bioremediation technologies such as biopiles. As the soil undergoes aging, the contaminants become more resistant to mass transfer, remediation slows, and residual hydrocarbon fractions remain in the soil. Biosurfactants are sometimes added to increase bioavailability to soil microbes for enhanced bioremediation performance. These surface active compounds produced by microorganisms are able to improve the solubilisation, mobilization, and emulsification of hydrophobic or insoluble organic contaminants. However, although positive biosurfactant influence is often reported, there have been numerous cases where no or negative effects were observed. Limited information is currently available on the biosurfactant inhibition of biodegradation – the phenomenon is not well understood. It is likely linked to interaction of the biosurfactant in question with the microbial community and the hydrocarbon degraders. Studies investigating microbial interactions with biosurfactants typically use an uncontaminated soil sample spiked with fresh hydrocarbons rather than aged and weathered hydrocarbon-contaminated soils. This study investigated the effects of selected biosurfactants on microbial community dynamics as well as hydrocarbon biodegradation in aged hydrocarbon contaminated biopile soils from Lac Mégantic.

One biopile soil demonstrated a significant reduction in total petroleum hydrocarbons (TPH) for biosurfactant treated systems after 34 days, but no significant difference between nutrient amended and unamended systems. Another biopile soil over a longer period of 80 days meanwhile found that the amended system demonstrated high TPH reduction, with one of the best overall reduction trends. The low to medium biosurfactant (rhamnolipid) doses had comparable performances to the amended system. A high rhamnolipid dose reduced the biodegradation extent to the same low level as unamended controls. The different results between the soils, as well as between the different biosurfactant doses, were most likely due to the biosurfactant influence on the microbial community

during the process of biodegradation. Analysis of the microbial community indicated changes in community composition with biosurfactant dose. The *Proteobacteria* phyla dominated overall throughout the 80 days, though the *Gamma* subclass tended to increase early and then decrease as biodegradation plateaued. Generally, with a few exceptions, higher microbial diversity was found in the better performing TPH reducing systems. The *Alpha* subclass seemed to become enriched and increased in relative abundance with higher rhamnolipid doses. Experiments on pure cultures isolated from the same soil provided evidence of hydrocarbon degraders using the rhamnolipids as a carbon source, suggesting the possibility of potential preferential degradation of biosurfactants over residual hydrocarbons in the weathered soils.

Resumé

Les terrains contaminés avec des hydrocarbures pétroliers, comme ceux qui ont résulté de la catastrophe du Lac Mégantic, sont souvent traités à l'aide des technologies rentables de biorestauration comme les biopiles. Quand le sol vieillit et est exposé aux intempéries, les contaminants deviennent plus résistants au transfert de masse, la remédiation ralentit et les fractions d'hydrocarbures résiduelles demeurent dans le sol. Afin d'améliorer la performance de la biorestauration, les agents bio-surfactants sont parfois ajoutés pour augmenter la biodisponibilité des contaminants aux microbes du sol. Ces tensioactifs biologiques sont capables d'améliorer la solubilisation, la mobilisation et l'émulsification de contaminants organiques hydrophobes ou insolubles. Cependant, bien que l'influence positive du biosurfactant soit souvent signalée, il y a eu de nombreux cas où aucun effet ou un effet négatif étaient observés. Les informations concernant l'activité d'inhibition de la biodégradation exercée par les biosurfactants sont insuffisantes et incomplètes - le phénomène n'est pas bien compris. Il est probablement lié à l'interaction du biosurfactant avec la communauté microbienne et les bio dégradeurs d'hydrocarbures. Les études portant sur les interactions microbiennes avec les biosurfactants utilisent généralement des échantillons de sol non-contaminés dans lesquels ils ajoutent des hydrocarbures frais,

plutôt que des sols contaminés par des hydrocarbures exposés aux intempéries et altérés. Cette étude a effectué des recherches sur les effets des biosurfactants sélectionnés sur la dynamique des communautés microbiennes ainsi que sur la biodégradation des hydrocarbures pétroliers résiduels dans les sols provenant des biopiles contaminés et âgés du Lac Mégantic.

Un sol de biopile a démontré une diminution considérable des hydrocarbures pétroliers totaux (HPT) pour les systèmes traités par biosurfactant après 34 jours, mais aucune différence marquée entre les systèmes amendés et non amendés. Un autre sol de biopile examiné pendant une période de 80 jours a révélé que le système amendé présentait une baisse très substantielle de HPT, avec l'une des meilleures tendances générales. Les concentrations faibles et moyennes de biosurfactant (rhamnolipide) ont eu des performances comparables à celle du système amendé. Une forte dose de rhamnolipide a réduit l'étendue de la biodégradation au même niveau que le système non amendé. Les différents résultats entre les sols, ainsi qu'entre les différentes doses de biosurfactants, étaient très probablement dus à l'influence des biosurfactants sur la communauté microbienne au cours du processus de biodégradation. L'analyse de la communauté microbienne a indiqué des changements dans la composition de la communauté parmi les différentes doses de biosurfactant. Les phyla de protéobactéries ont globalement dominé tout au long des 80 jours, bien que la sous-classe *Gamma* ait eu tendance à augmenter tôt puis à diminuer à mesure que la biodégradation atteignait un plateau. En général, à quelques exceptions, une plus grande diversité microbienne a été observée dans les systèmes les plus performants de réduction HPT. La sous-classe *Alpha* semblait s'enrichir et augmenter en abondance relative avec des doses de rhamnolipides plus élevées. Des expériences sur des cultures bactériennes pures isolées à partir du même sol ont mis en évidence des bio-dégradeurs d'hydrocarbures utilisant les rhamnolipides comme source de carbone, suggérant la possibilité potentielle de dégradation préférentielle des biosurfactants par rapport aux hydrocarbures résiduels dans les sols exposés aux intempéries.

Acknowledgments

I would like to thank my supervisor, Dr. Subhasis Ghoshal, for providing me with this amazing opportunity, and for his continued support and guidance throughout my graduate program.

I would like to acknowledge the financial support from the McGill Masters Top-Up Award (Hydro-Quebec Masters in Engineering Award), the Fonds de recherche Nature et technologies (bourse de maitrise en recherche), and financial contributions from Maxxam Analytique to the research project.

I would like to acknowledge Dr. Rosa Galvez and her research team at Université Laval for financial assistance, research insights, and environmental samples.

I greatly appreciate the assistance provided by Dr. Ali Akbari, a postdoctoral scholar under the direction of Dr. Ghoshal. His mentorship and support have been invaluable in helping with data analysis methods, designing the experiments, and in developing laboratory protocols and techniques.

I would like to thank Mariana Umpiérrez, Arshath Abdul Rahim, and Yanyan Zhang for their patience, support, and help with experimental methods, instruments, and procedures. I would also like to acknowledge my other colleagues and friends at Benedek Laboratories, Sarayu Rao, Bing Guo, Sarah Outayek, Mehrnoosh Azodi, Roberta Maffettone, Elena Penkarski-Rodon, Lamia Nouroudine Hassanaly, Claire Gibson, Vinicius Bueno, and Sourjya Bhattacharjee, as well as Sanaz Alizadeh at École Polytechnique Montréal.

Thanks to Prof. Jinxia Liu for allowing me to use her laboratory instruments at McGill's Civil Engineering department. A special thanks to Daina Brumelis, Jorge Sayat, Sun Chee Wong and the administrative staff of the Civil Engineering department for their support. I would also like to thank Prof. Maria Elektorowicz at Concordia University for her support and advice.

Finally, I would like to thank my family, friends, and boyfriend, for their love, encouragement, inspiration, and support over the last two years.

Table of Contents

Preface

This thesis is presented in a manuscript-based format in accordance with the "Guidelines for Thesis Preparation" of the Graduate and Postdoctoral Studies, McGill University. Chapter 1 presents an introduction and literature overview. Chapter 2 presents the research methods, results, discussions of results, and conclusion. Chapter 3 presents the contributions to new knowledge. Chapter 2 is prepared as a manuscript for submission to a peer-reviewed journal. The author of the thesis is the primary author of the publication to be submitted. Below is a detailed description of the efforts of all contributing authors:

Kasprzyk, A., Akbari A., Ghoshal, S. "Feasibility of Biosurfactant Enhanced Bioremediation of Residual Petroleum Hydrocarbon Fractions in Contaminated Soils from Lac Mégantic". *In preparation for submission to* **Chemosphere**.

A. Kasprzyk: Execution of the experiments, analysis of data, and wrote the manuscript draft.
A. Akbari: Provided input on experimental methods, procedure, and design; analyzed microbial community sequencing data.
S. Ghoshal: Supervision of the research and revision of the manuscript.

Chapter 1 Literature Review

1.1 Introduction

Petroleum contamination represents a major risk to the health of humans and ecosystems. The Lac Mégantic rail disaster of the summer of 2013 resulted in extensive contamination of the surrounding area with crude oil and other contaminants. The train was transporting approximately 7.68 million liters of crude oil when it derailed in the town of Lac Mégantic (southeastern Quebec, Canada) (MDDEFP, 2014). A devastating explosion and major oil fire ensued (Mejia-Avendano et al., 2017). It is estimated that 5.98 million liters of the oil was released into the environment or burned, of which at least 100 000 liters went directly into the Chaudière river (MDDEFP, 2014).

The oil in question was Bakken Formation shale light crude oil from North Dakota. The transportation company, Montreal, Maine and Atlantic Railways (MMA), did not release more detailed information about the exact oil composition. However, it is known that the Bakken crude was extracted through hydraulic fracturing, which most likely introduced hundreds of possible compounds into the oil through impurities, natural gas, and fracking additives (Galvez-Cloutier et al., 2014). Crude oil is mainly composed of saturated hydrocarbon compounds with straight or branched chains (alkanes and cycloalkanes). It also contains polycyclic aromatic hydrocarbons (PAH); monocyclic aromatic hydrocarbons (MAH); benzene, toluene, ethylbenzene and xylene (BTEX) volatile organic compounds; nitrogen-sulfur-oxygen compounds (NSO); and polar compounds (asphaltenes and resins) (Galvez-Cloutier et al., 2014; Kuhad et al., 2009). Light crude oils typically contain high levels of saturated and aromatic hydrocarbons, and much smaller proportions of asphaltenes and resins (Kuhad et al., 2009). Crude oil specifically extracted from shale, known as “tight oil”, also tends to be more volatile (Wybenga, 2014). Bakken crude from North Dakota is light with an average API gravity of 40 - 43° (810 - 824 kg/m^3), “sweet” (negligible amounts of corrosive H_2S gas) with an average of 0.1 wt.% sulfur, and volatile with an average vapor pressure of 11.5 - 11.8 psi (Auers et al., 2014).

During the fire, combustion by-products were formed, and firefighting foams (fluoroalkyl surfactants) were added to the site (Mejia-Avendano et al., 2017). The combustion, in combination with the processes of environmental weathering, sorption, volatilization, leaching and photo-oxidation, resulted in more changes in the contamination's composition (Galvez-Cloutier et al., 2014; Kuhad et al., 2009).

The total petroleum hydrocarbon concentrations varied throughout the site, ranging from 4000 to 77 000 mg/kg in the impact zone (Galvez-Cloutier et al., 2014). As expected, the surface soils had the highest levels of contamination. The highly contaminated soils (hydrocarbon levels exceeding Quebec soil quality criteria) were excavated. The most severely contaminated soils were disposed of at a disposal site approved by the governmental Ministère du Développement Durable, de l'Environnement et de la Lutte contre les Changements Climatiques (MDDELCC) (Millette et al., 2014). The rest of the soils were transported by environmental engineering firms for bioremediation treatment in off-site biopile facilities**.** The treatment focused particularly on the bioremediation of the hydrocarbon contamination (Mejia-Avendano et al., 2017).

1.2 Bioremediation of Petroleum Hydrocarbons

Biodegradation is the process of organic compounds being broken down into smaller compounds by microorganisms. Bioremediation involves harnessing this process for the treatment of environmental contamination. There are a number of treatment methods, but several factors are common among them. For effective biodegradation rates, the microorganisms must have: a carbon source (compost, molasses, hydrocarbons, etc.); electron acceptor (oxygen for aerobic, or nitrate, sulfate, carbon dioxide, iron, etc. for anaerobic); pH 6-8; sufficient nutrients (nitrogen and phosphorus); and appropriate ambient temperature (Kuhad et al., 2009; Surridge et al., 2009). It is also beneficial for the soil to have

adequate moisture (>40%), and minimal toxic compounds (e.g., high heavy metal concentrations) (Surridge et al., 2009). Biopiles are a particularly cost-effective engineered composting system that can be successfully used for the remediation of petroleum hydrocarbon contaminated soils.

For biopiles, such as the ones in the Lac Mégantic case, the contaminated soils are excavated, moved, and then treated off-site. The soil is sifted, mixed with bulking agents, and heaped into piles on top of protective impermeable liners. Bulking agents can consist of straw, wood chips, sawdust, compost or sewage sludge; they improve soil aeration. Along the bottom of the pile there is a network of perforated piping for collecting leachates, for either injecting or extracting air for aeration or to remove evaporating volatile compounds. Extracted air is filtered prior to discharge. Moisture and nutrients are provided via irrigation. Biopiles are sometimes also augmented with surfactants, additional microorganisms, and/or heating. The pH can be adjusted to near neutral using either lime (if pH < 6) or elemental sulfur/ ammonium sulfate (if pH > 8) (Kuhad et al., 2009; Singh et al., 2009). The biopile is usually covered with protective membranes, which help regulate temperature, limit water evaporation, and contain volatile constituents. The soil is periodically turned over or tilled for continued biodegradation. The biopile soils are analyzed to demonstrate contaminant concentrations have reached regulatory goals (Kuhad et al., 2009; Singh et al., 2009; Surridge et al., 2009).

The rate of bioremediation is usually limited by the available nutrients, i.e., the amount of nitrogen or phosphorus (Singh et al., 2009; Surridge et al., 2009). Sometimes it can also be impeded by an insufficiency among the micronutrient balance (Surridge et al., 2009).

1.3 Endpoints & residual fractions

Even if ideal bioremediation conditions are provided, after a period of time the contaminant concentration will stop decreasing and reach a plateau referred to as the 'endpoint'. The time at which this happens and the endpoint concentration itself varies from case to case, but is strongly influenced by the bioavailability of the compounds (soil structure and composition, contaminant entrapment in micro and nanopores, strong binding to organic matter with aging, aqueous solubility of compounds, etc.) (Pignatello, 2009).

At the beginning of treatment, lighter compounds volatilize and degrade. Contaminants desorb and dissolve into the aqueous phase present within the soil matrix, and continue to biodegrade. During the final phase of soil bioremediation treatments for hydrocarbon contamination the rate of biodegradation becomes especially reduced. This last stage is the most difficult to bioremediate, and as a result the heaviest molecular weight hydrocarbons are more likely to remain in the soil (Alexander, 2000; Kuhad et al., 2009).

1.3.1 Bioavailability

Mostly, biodegradation of organic compounds occurs when they are in the aqueous phase. For biodegradation to occur, the contaminants in question need to be available in the aqueous phase to the microorganisms. The mechanisms of desorption, diffusion and dissolution of the contaminant within the soil matrix can therefore control the rate of biodegradation. Over the long term, the contaminants become more resistant to mass transfer and less bioavailable as they undergo chemical oxidation reactions, slow chemical diffusion into small pores, and sorption into organic matter (Pignatello, 2009; Van Hamme, 2004). Some compounds may bind irreversibly to soil minerals and/or clays (Akbari and Ghoshal, 2015). Contaminants such as PAHs have high hydrophobicity and low solubility in aqueous

media. These contaminants are even more likely to associate with hydrophobic components in soil and undergo strong soil sorption due to their own hydrophobicity/low water solubility and thus have limited bioavailability to bacteria in the aqueous phase (Bezza & Chirwa 2016; Van Hamme, 2004; Zhu and Aitken, 2010).

The bacteria themselves can also become attached to soil particles, and so become limited in their movement and access to the contaminants (Pignatello, 2009). The heterogeneous distribution of the bacteria within the soil might not correspond with the contamination gradients. Furthermore, the bacteria may experience electrostatic interactions with soil minerals (silica, clays, alumina, and mineral carbonates) which can change the bacterial viability/physiology (Akbari and Ghoshal, 2015). All of these factors limit the access of the microorganisms to the contaminants in the aqueous phase.

One important exception to bioavailability being driven by the aqueous concentration of contaminants, are non-aqueous phase liquids (NAPLs) of long chain alkanes such as hexadecane and other petroleum hydrocarbons with very low aqueous solubility. For example, the aqueous solubility of hexadecane is 2.1×10^{-5} mg/L (Coates et al. 1985; Mackay et al., 2006), and the dissolved fraction alone is insufficient to support growth of bacteria. These compounds are biodegraded through direct contact of bacterial cells at the organic liquid-water interface, rather than uptake in the aqueous phase only. Such biodegradation activity is directly associated with bacterial adhesion at oil–water interfaces (Akbari and Ghoshal, 2015; Zoueki et al., 2010).

1.3.2 Pore Size

Petroleum NAPLs are generally biodegraded through direct bacterial contact. In these cases especially, the pore size distribution of the soil matrix greatly influences the bioavailability of hydrocarbons.

Essentially, if NAPL/oil droplets are trapped in non-bioaccessible pores (too small for bacteria to enter) then there can be no direct contact, and therefore no biodegradation (Akbari and Ghoshal, 2015). Based on experimental data, Akbari and Ghoshal (2015) found *Dietzia maris* bacteria were able to access and mineralize hexadecane NAPL when separated with 5, 8, or 12 µm pore diameter membranes, but not when the pore diameters were 0.4 and 3 µm. As such, 4 µm was assumed as the minimum required pore diameter for bioaccessibility. The same study (Akbari and Ghoshal, 2015) then compared two soils: sandy soil from Resolution Island, Nunavut, and aggregated clayey soil from the Northwest Territories. Both soils had low background organic matter contents (2.4 and 2.3%, respectively). X-ray micro-CT scanning was used to find the spatial distribution, connectivity, and diameter of pores in both soils. The ratio of non-bioaccessible to bioaccessible pore volume was found to be 0.04 and 0.32 for the Nunavut sandy soils and NWT clayey, respectively. This reflected the results of bioremediation performance, which demonstrated much better bioremediation of non-volatile petroleum hydrocarbons (>C16−C34) in the Nunavut sandy soil with low endpoints (102.5 ± 20.5 mg/kg) while the NWT clayey soil had relatively high endpoints (525.8 ± 77.1 mg/kg). The difference in biodegradation extent was attributed primarily to pore size structure (Akbari and Ghoshal, 2015).

1.4 Microbial Community

Different microorganisms are more capable than others of uptaking different contaminants. As the contaminant composition changes over time and the abundance of certain compounds become available, the microorganisms capable of utilizing those compounds become more predominant (Van Hamme, 2004). Since the biodegradation of petroleum hydrocarbons in contaminated soils entails a changing TPH composition, a lack of biodegradation may be associated with a lack of change in the microbial community composition (Akbari and Ghoshal, 2014). It is unlikely that microbial community

compositions would remain unchanged if biodegradation were taking place (Akbari and Ghoshal, 2014). As such, it is important to analyze and track the microbial community structure over time.

In unpolluted environments soil microbial communities are relatively evenly distributed (Surridge et al., 2009). Zhou et al. (2002) found for low-carbon soils the surface soil tends to have even diversity distribution, while subsurface soil has more distinct patches and patterns. The same study found that high-carbon soils instead tend to have uniform diversity throughout the soil layers. Microbial diversity in an environment is based on both the total number of species present (species richness/ abundance) and species distribution (species equitability/ dominance) (Dejonghe et al., 2001; Surridge et al., 2009).

Generally, higher microbial diversity is proportional to increased catabolic potential, and subsequently also indicative of more effective contaminant removal (Dejonghe et al. 2001). Low contamination levels (TPH < 1000 mg/kg) and aged soil with weathered contamination generally have more diverse TPH compositions than fresh petroleum contamination. These types of soils with more diverse TPH composition require a more diverse microbial community for effective TPH biodegradation (Akbari and Ghoshal, 2014). Fresh oil contamination, on the other hand, tends to have a large pool of normal alkanes and a less diverse TPH composition. As such, selective species are augmented in cases of fresh oil contamination, and microbial biodiversity becomes reduced (Akbari and Ghoshal, 2014; Dell'Anno et al., 2012). Diversity and bioremediation capacity can be improved through bioaugmentation – the addition of specific microbial strains or consortia (Dejonghe et al., 2001; Singh et al., 2009). Soil and environmental conditions can also be optimized for specific degrading microorganisms, although that might not necessarily lead to better degradation rates, as noted for cases requiring more biodiverse consortia.

1.4.1 Hydrocarbon Degraders

There are a large number and variety of microorganisms capable of degrading hydrocarbons. They have been found around the world, in all types of natural environments (Kuhad et al., 2009; Van Hamme et al., 2003). These microorganisms are able to utilize hydrocarbons as their sole source of carbon, and they include bacteria, fungi, yeast, algae, cyanobacteria and some protozoan organisms (Paul et al., 2005). Most of the hydrocarbon-degrading bacteria are Gram-negative, but there have also been Gram-positive bacteria identified as hydrocarbon degraders (Kuhad et al., 2009).

The most common and efficient hydrocarbon-degrading bacteria reported in the literature include species of *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Corynebacterium*, *Enterobacter, Flavobacterium, Mycrococcus, Nocardia, Pseudomonas, Sphingomonas,* and *Rhodococcus* (Kuhad et al., 2009; Singh et al., 2012). Among the fungi are species of *Aspergillus, Mortiecerella, Penicillium, Phanerochaete*, and *Trichoderma* (Leahy and Colwell, 1990). Yeasts include *Aureobasidium, Candida, Rhodotorula* and *Sporobolomgers* (Kuhad et al., 2009).

In recent years, many interesting studies have isolated and characterized new hydrocarbon degraders. According to Kuhad et al. (2009), "some of these include species of *Alcanivorax, Cycloclasticus, Oleiphilus, Oleispira, Thalassolituus* and some members of the genus *Planomicrobium* (previously known as *Planococcus*). *Alcanivorax spp., Oleiphilus spp., Oleispira spp., Thalassolituus spp.* and *Planomicrobium spp.* use a variety of branched- and/or straight-chain saturated hydrocarbons, whereas *Cycloclasticus spp.* can use a range of polycyclic aromatic hydrocarbons."

Additionally, there are certain genes in microorganisms that are functional biomarkers for hydrocarbon degradation. Some of the more common genes include: alkane monooxygenases *alkB* (C5 to C12 alkane

degradation); *alkM* (C10 to C20 alkane degradation); *alkB1* and *alkB2* (C12 to C16 alkane degradation); catechol-2,3-dioxygenase *xylE* (xylene and toluene degradation); naphthalene dioxygenase *ndoB* (naphthalene degradation); and pyrene dioxygenase *nidA* (pyrene degradation) (Ali and Ghoshal, 2014; Margesin et al., 2003).

1.4.2 Microbial Uptake Mechanisms

As mentioned, most biodegradation of organic compounds occurs when they are in the aqueous phase, while poorly soluble compounds are biodegraded through direct bacterial contact. There are a number of key steps before microorganisms metabolize any contaminants. A microorganism needs to sense, move towards, bind, and use active or selective systems to transport the contaminant compound molecules into the cell (Van Hamme, 2004; Van Hamme & Urban, 2009). Some microorganisms also release extracellular enzymes to oxidize contaminants prior to uptake, initialize catabolism using membrane-bound oxidases and/or emit biosurfactants (Van Hamme & Urban, 2009). For cases of direct uptake, adhesion of bacteria to the oil phase is essential. Biosurfactants produced by microorganisms are particularly important for the biodegradation of hydrocarbons, and they have a number of functions that can improve mineralization capacity.

1.5 Biosurfactants

Surfactants are soap-like surface-active compounds – the ones produced by biological organisms such as bacteria, cyanobacteria, fungi and yeast are called biosurfactants. Surfactants are amphiphilic molecules, meaning they have hydrophobic tails and hydrophilic heads. Surfactants can be classified by the polarity of their head groups: non-ionic, anionic, cationic, or zwitterionic. Regardless of charge, the hydrophilic heads tend to remain in contact with the water phase while the hydrophobic tails prefer any present hydrophobic compounds (Souza et al., 2014). Through this mechanism, surfactants reduce the

surface and interfacial tension between two different compounds (two liquids; gas and a liquid; or a liquid and a solid). At low concentrations, surfactants are soluble in water. When the concentration of surfactant is high enough, the molecules begin to form micelles. The concentration at which this occurs is called the critical micelle concentration (CMC) (Bordoloi and Konwar, 2009). A hydrophobic contaminant molecule would then partition into the center of the hydrophobic core, while the micelle remains distributed in the aqueous phase where a microbe would be able to more easily access the contaminant (Van Hamme & Urban, 2009). In this way, biosurfactants increase the solubilization of hydrophobic compounds (Liu, 2005). Some biosurfactants have also been known to increase the aqueous solubility of specific hydrocarbons below CMC concentrations (Bordoloi and Konwar, 2009). At concentrations below CMC, surfactants can mobilize residual NAPLs entrapped in pores through the reduction of surface and interfacial tension, which alters capillary forces, wettability, and contact angles (Bezza and Nkhalambayausi-Chirwa, 2015). Once mobilized from pores, the NAPL-water interface may be accessible to bacteria for attachment and biodegradation through direct uptake. Generally, low–molecular weight biosurfactants perform better at increasing solubilisation and mobilization, while high–molecular weight biosurfactants are more effective at promoting emulsification (Dhanarajan and Sen, 2014).

Thus, when microbes produce biosurfactants during the biodegradation process the bioavailability of hydrophobic or insoluble organic compounds is often improved (Singh et al., 2007; Van Hamme et al., 2004). These biosurfactants can also change the surface properties of the bacterial cell; dissolve and emulsify the compounds; release entrapped hydrocarbons from porous medium; and generally improve the solubility and mobility of compounds (Singh et al., 2007; Hazra et al., 2012; Xia et al., 2014).

Biosurfactants are classified primarily based on their major structural features and molecular weight, and to a lesser extent their microbial origin (Van Hamme & Urban, 2009). There are five major classes of biosurfactants: glycolipids (rhamnolipids, sophorolipids, trehalolipids, etc.); lipopeptides and lipoproteins (surfactin, lichenysin, iturins, fengycins, etc.); phospholipids, fatty acids and neutral lipids (corynomycolic acid, spiculisporic acid, phosphatidylethanolamine, etc.); polymeric biosurfactants (emulsan, liposan, serrawettin, etc.); and particulate biosurfactants (Desai and Banat, 1997; Dhanarajan and Sen, 2014; Pacwa-Plociniczak et al., 2011; Van Hamme & Urban, 2009). Biosurfactants are predominantly produced by hydrocarbon degrading microorganisms and have several advantages over synthetic surfactants; they have better biodegradability, lower toxicity, higher foaming, can be made from renewable sources or even industrial wastes, and show greater stability and performance at extreme temperatures, pH, and salinity (Bordoloi and Konwar, 2009; Mulligan, et al. 2001; Souza et al., 2014).

1.5.1 Biosurfactant Enhanced Bioremediation

In bioremediation applications, (bio)surfactants are either added externally (influent, spraying, injection) or produced by microorganisms within the soil itself (augmentation and supporting growth of biosurfactant producers) (Ławniczak et al., 2013). Studies regarding the use of surfactants and biosurfactants in bioremediation generally begin by assessing the (bio)surfactant's ability to solubilize hydrocarbons (solubilization, above CMC), its emulsification activity (higher molecular weight biosurfactants), and/or its surface tension lowering activity (mobilization, below CMC) (Pacwa-Płociniczak et al., 2011). It is accepted that (bio)surfactants disperse hydrocarbons mainly through these three mechanisms, and are especially useful for aged contaminated sites. Despite these assumptions, there have been a number of studies with conflicting results.

Although positive influence of biosurfactants is often reported, there have been numerous cases where no effects or negative effects were observed (Das and Mukherjee 2007; Ławniczak et al., 2013). Additionally, there is not always the expected correlation between pollutant desorption/solubilization and biodegradation (Ławniczak et al., 2013). For example, Adrion et al. (2016) found relatively low levels of PAH desorption but significant increases to biodegradation rates. Meanwhile Vipulanandan and Ren (2000) compared four surfactants in freshly spiked samples and found that although the rhamnolipid biosurfactant increased naphthalene's solubility by 30 times (highest rate of the four), this case took 40 days to biodegrade compared to only 100 hours using the surfactant Triton X-100. The authors assumed, based on turbidity measurements, that this was due to the biosurfactant being biodegraded along with the naphthalene as a competing substrate, while the Triton X-100 was not. Therefore, desorption/solubility is not always reliable as a predictor of biodegradation effectiveness.

1.7.2 Biosurfactant Impact on Microorganisms

There are in fact a number of other mechanisms and factors that are not usually accounted for in biosurfactant enhanced bioremediation studies. Most biosurfactant-enhanced bioremediation studies focus on a biosurfactant's physico-chemical properties, such as its ability to solubilize hydrocarbons and its emulsification activity. While important, these studies tend to neglect the effect on the microorganisms themselves, whether individual cells or community population dynamics. As pointed out by Cappello et al. (2012), the efficacy of a biosurfactant in enhancing biodegradation does not depend exclusively on its physicochemical characteristic but also its effect (interaction, stimulation, etc.) on the microbial community.

It should be noted that biosurfactants are being investigated in the medical field for antibacterial and antifungal properties. Although seemingly contradictory, in natural habitats biosurfactants are used to

gain a competitive advantage in interactions with other microorganisms and against nematodes and protozoan predators. For example, when tested *in vitro*, biosurfactants produced by *Pseudomonas* and *Bacillus* species demonstrated growth-inhibition and lysis effects against a range of competitive microorganisms, including viruses, mycoplasmas, bacteria, fungi and oomycetes (Raaijmakers et al., 2010; Van Hamme, 2004).

Biosurfactants are also used for motility and cell differentiation. In a number of studies, it was found that surface motility was lost in *B. subtilis* mutants deficient in surfactin production (*sfp* gene), and then swarming was restored by re-introduction of the *sfp* gene. In fact, for several *Pseudomonas* and *Bacillus* mutants deficient in lipopeptide biosurfactant production, the addition of purified lipopeptide was able to restore their reduced surface motility. Similarly, the swarming motility of deficient *S. marcescens* mutants was restored not only by the addition of their own biosurfactant serrawettin, but also by the addition of either surfactin or rhamnolipids. As such, the addition of structurally related and unrelated biosurfactants have been found to restore motility in other species where synthetic surfactants NP40 and Triton X-100 failed. This suggests that surface tension reduction alone is not enough for motility but also the physical– chemical nature of the surfactant itself (Raaijmakers et al., 2010).

The use of surfactants in motility is vital in allowing bacteria to travel from depleted environments towards nitrogen and phosphorus rich environments. This has been especially observed in the case of rhamnolipids, and one of the reasons why rhamnolipid production is boosted in nitrogen-limited environments. Rhamnolipids can also increase nutrient uptake for specific groups of bacteria. Additionally, while some microorganisms evolved to survive using rhamnolipids, other non-biosurfactant producing microorganisms employed coexistence with the biosurfactant-producing bacteria to survive (Christova and Stoineva, 2014; Raaijmakers et al., 2010).

Generally, biosurfactants are all synthesized from the same basic structural components: amino acids, sugars, fatty acids and lipids. As a result, biosurfactants can be used by microorganisms as a form of 'nutrient storage' – the microbes metabolizing the sugars, lipids and amino acids as needed. This is especially supported by the fact that biosurfactant production can be induced by limiting access to nutrients (nitrogen, phosphorous or iron) (Amézcua-Vega et al. 2007; Soberón- Chávez et al. 2005; Teichmann et al. 2007). During bioremediation, there is a possibility that the biosurfactants might then be used as a preferred carbon source instead of the contaminants (Chrzanowski et al., 2012; Ławniczak et al., 2013; Van Hamme & Urban, 2009). In some other cases, however, using the biosurfactants as a carbon source could be beneficial to bioremediation by supporting the growth of hydrocarbon degraders (Hickey et al. 2007).

A few studies have mentioned that efficiency in enhancement of biodegradation by biosurfactants could be linked to the biosurfactants decreasing bacterial surface adhesion and dislodging biofilms of certain strains. Adherent bacteria are more efficient degraders than suspended bacteria (Congiu et al., 2015). Excessive levels of biosurfactant have the potential to inhibit bacterial adhesion altogether (Zoueki et al., 2010). On the other hand, biosurfactants also play an important role in surface attachment and biofilm formation for other bacteria, such as *Bacillus* and *Pseudomonas*. For instance, *B. subtilis* requires surfactin to form a biofilm, while *P. aeruginosa* produces and employs rhamnolipids to initiate biofilm formation and for migration within the biofilm itself (Raaijmakers et al., 2010). Biosurfactants are also used to maintain the liquid-filled channels in the maturing biofilm that in turn helps facilitate the distribution of nutrients and oxygen (Pamp and Tolker-Nielsen, 2007; Raaijmakers et al., 2010; Van Hamme & Urban, 2009). Meanwhile, the surfactin produced (and required) by *B. subtilis*, inhibited

biofilm formation of *Salmonella enterica sv. Typhimurium* (Raaijmakers et al., 2010). Thus, this may be another case of an evolutionary competitive edge over other microorganisms.

Essentially, microorganisms can use their biosurfactants to regulate their surface properties in order to attach or detach from surfaces according to needs. It has been found that during early growth, hydrophobic bacterial cells directly attached to hydrocarbon drops. During late exponential growth, adhesion decreased, the cells became more hydrophilic and excreted bioemulsifier, which allowed the now hydrophilic cells to attach to the hydrophilic outer layer of emulsified oil droplets (Christova and Stoineva, 2014; Van Hamme, 2004). This is possible because biosurfactants can be oriented with the hydrophilic part facing the bacterial cell surface, (facilitating attachment to hydrophobic surfaces) but can also be oriented with the hydrophobic part facing the bacterial cell surface, (facilitating cell attachment to hydrophilic surfaces) (Neu, 1996; Raaijmakers et al., 2010). Microbes using biosurfactants to control their cell surface properties also allow them to protect themselves and avoid toxic levels of organic or metal pollutants (Van Hamme, 2004; Van Hamme & Urban, 2009).

Biosurfactants are also more likely to form different micellar and emulsion structures compared to synthetic surfactants. Martienssen and Schirmer (2007) compared the emulsion formation and biodegradation of gasoline in water with the addition of either a synthetic surfactant Triton X-100 or a biosurfactant Bioversal FV. Within one hour, both formed stable emulsions between 10 µm and 100 µm in diameter. After 24 hours, the synthetic surfactant samples comprised mostly of very small emulsions less than 10 µm in diameter, while the biosurfactant sample contained a variety of large emulsions being progressively settled by microorganisms. After 3 days, the biosurfactant case's initial emulsion structure had become a complex multimolecular structure, occupied by a variety of large bacterial clusters. The synthetic surfactant emulsions displayed no bacterial growth after 3 days and not even after 7 days.

Thus, the roles biosurfactants play in biological processes include: bacterial toxicity/pathogenesis, motility, biofilm formation, cellular differentiation, bacterial cell signaling, possible carbon source, protection against toxic compounds, and accessing substrate/nutrients (Christova and Stoineva, 2014; Cameotra and Makkar 2004; Kitamoto et al. 2002; Lang 2002; Van Hamme et al. 2006). For *Bacillus*, in fact, lipopeptides have been shown to function as signal molecules for coordinated growth and differentiation (Raaijmakers et al., 2010).

Overall, while biosurfactant improvement of biodegradation is generally observed, in the cases where negative results occurred, the respective studies admitted that biosurfactant inhibition of biodegradation is not well understood. The data from an investigation adding different concentrations of biosurfactants, and tracking the effect to microbial community dynamics as well as hydrocarbon biodegradation, could be used to further understanding on the mechanisms of biosurfactant enhanced biodegradation. Regardless of whether the results are positive or negative, the corresponding microbial community can be compared. It could be that the microbial community, for instance, is primarily made up of surfactin-producers and the addition of rhamnolipids does not provide the biofilm stimulation required.

1.8 Literature Cited

Adrion, A., Nakamura, J., Shea, D., & Aitken, M.D. (2016). "Screening Nonionic Surfactants for Enhanced Biodegradation of Polycyclic Aromatic Hydrocarbons Remaining in Soil After Conventional Biological Treatment." *Environmental Science & Technology*, **50**(7), 3838−3845.

Alexander, M. (2000). "Aging, bioavailability, and overestimation of risk from environmental pollutants." *Environmental Science & Technology* **34**(20): 4259-4265.

Akbari, A. and S. Ghoshal (2014). "Pilot-scale bioremediation of a petroleum hydrocarbon-contaminated clayey soil from a sub-Arctic site." *Journal of Hazardous Materials* **280**: 595-602.

Akbari, A. and S. Ghoshal (2015). "Bioaccessible Porosity in Soil Aggregates and Implications for Biodegradation of High Molecular Weight Petroleum Compounds." *Environmental Science & Technology* **49**(24): 14368-14375.

Amézcua-Vega, C., Poggi-Varaldo, H.M., Esparza-Garcia, F., Rios-Leal, E., & Rodriguez-Vazquez, R. (2007). "Effect of culture conditions on fatty acids composition of a biosurfactant produced by *Candida ingen*s and changes of surface tension of culture media." *Bioresource Technology* **98**(1):237–240.

Auers, J. R., Couture, R. M., and Sutton, D. L. (2014). *The North Dakota Petroleum Council Study on Bakken Crude Properties.* Bakken Crude Characterization Task Force. Bismarck, ND: North Dakota Petroleum Council.

Ayed, H.B., Jemil, N., Maalej, H., Bayoudh, A., Hmidet, N., Nasri, M. (2015). "Enhancement of solubilization and biodegradation of diesel oil by biosurfactant from *Bacillus amyloliquefaciens* An6." *International Biodeterioration & Biodegradation*, **99**: 8-14.

Bezza, F.A., and Nkhalambayausi-Chirwa, E.M. (2015). "Desorption kinetics of polycyclic aromatic hydrocarbons (PAHs) from contaminated soil and the effect of biosurfactant supplementation on the rapidly desorbing fractions." *Biotechnology & Biotechnological Equipment*, **29**(4): 680-688.

Bezza, F. A. and Chirwa, E.M.N. (2016). "Biosurfactant-enhanced bioremediation of aged polycyclic aromatic hydrocarbons (PAHs) in creosote contaminated soil." *Chemosphere* **144**: 635-644.

Bordoloi, N. K. and Konwar, B. K. (2009). "Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons." *Journal of Hazardous Materials* **170**(1): 495-505.

Cameotra, S.S. and Makkar, R.S. (2004). "Recent applications of biosurfactants as biological and immunological molecules." *Current Opinion of Microbiology* **7**:262–266.

Cappello, S., Crisari, A., Hassanshahian, M., Genovese, M., Santisi, S. & Yakimov, M. (2012). "Effect of a Bioemulsificant Exopolysaccharide (EPS_{2003}) on Abundance and Vitality of Marine Bacteria." *Water, Air and Soil Pollution*, **223**(7): 3903-3909.

Chang, W., Akbari, A., Snelgrove, J., Frigon, D., & Ghoshal, S. (2013). "Biodegradation of petroleum hydrocarbons in contaminated clayey soils from a sub-arctic site: The role of aggregate size and microstructure." *Chemosphere* **91**(11): 1620-1626.

Chikere, C. B., Surridge, K., Okpokwasili, G. C., Cloete, T. E., (2012). "Dynamics of indigenous bacterial communities associated with crude oil degradation in soil microcosms during nutrient-enhanced bioremediation." *Waste Management & Research* **30**(3): 225-236.

Christova N., and Stoineva I. (2014). "Trehalose Biosurfactants" in *Biosurfactants: research trends and applications.* Ed. Mulligan, Catherine N., Ackmez Mudhoo, and Sanjay K. Sharma. Boca Raton, FL: CRC Press.

Chrzanowski, Ł., Ławniczak, Ł., & Czaczyk, K. (2012). "Why do microorganisms produce rhamnolipids?" *World Journal of Microbiology and Biotechnology*, **28**(2): 401-419.

Coates, M., Connell, D.W., and Barron, D.M. (1985). "Aqueous solubility and octan-l-ol to water partition coefficients of aliphatic hydrocarbons." *Environmental Science and Technology*, **19**: 628-632.

Congiu, E., Parsons, J.R., & Ortega-Calvo, J.J. (2015). "Dual partitioning and attachment effects of rhamnolipid on pyrene biodegradation under bioavailability restrictions." *Environmental Pollution*, **205**: 378-384.

Das, K., and Mukherjee, A.K. (2007). "Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: role of biosurfactants in enhancing bioavailability." *Journal of Applied Microbiology,* **102**(1):195–203.

de la Cueva, S. C., Rodriguez, C. H., Cruz, N. O. S., Contreras, J. A. R., & Miranda, J. L. (2016). "Changes in Bacterial Populations During Bioremediation of Soil Contaminated with Petroleum Hydrocarbons." *Water, Air, and Soil Pollution* **227**(3): 12.

Dell'Anno, A., Beolchini, F., Rocchetti, L., Luna, G.M. and Danovaro, R. (2012) "High bacterial biodiversity increases degradation performance of hydrocarbons during bioremediation of contaminated harbor marine sediments." Environmental Pollution **167**: 85–92.

Dejonghe, W., Boon, N., Seghers, D., Top, E.M., Verstraete, W. (2001). "Bioaugmentation of soils by increasing microbial richness, missing links". *Environmental Microbiology* **3**(10):649–657.

Desai, J.D., and Banat, I.M. (1997). "Microbial production of surfactants and their commercial potential". *Microbiology and Molecular Biology Reviews* **61**(1):47–64.

Dhamodharan, D. & Jayapriya, J. (2015). "Integrated Approach for Polycyclic Aromatic Hydrocarbon Solubilization from the Soil Matrix to Enhance Bioremediation" *Bioremediation Journal* **19** (4): 287-295.

Dhanarajan G. and Sen R. (2014). "Amphiphilic Molecules of Microbial Origin" in *Biosurfactants: research trends and applications.* Ed. Mulligan, Catherine N., Ackmez Mudhoo, and Sanjay K. Sharma. Boca Raton, FL: CRC Press.

Galvez-Cloutier, R., Guesdon, G., & Fonchain, A. (2014). "Lac-Mégantic: Analysis of environmental emergency, trials and evaluation of impacts." *Canadian Journal of Civil Engineering* **41**(6): 531-539.

Guibert, L., Loviso, C., Marcos, M. Commendatore, M., Dionisi, H., & Lozada, M. (2012). "Alkane biodegradation genes from chronically polluted sub-Antarctic coastal sediments and their shifts in response to oil exposure". *Microbial Ecology* **64**(3)**:** 605–616.

Hazra, C., Kundu, D., & Chaudhari, A., (2012). "Biosurfactant-assisted bioaugmentation in bioremediation". *Microorganisms in Environmental Management*. Springer, Netherlands, pp.631–664.

Hickey, A.M., Gordon, L., Dobson, A.D.W., Kelly, C.T., & Doyle, E.M. (2007). "Effect of surfactants on fluoranthene degradation by *Pseudomonas alcaligenes* PA-10". *Applied Microbiology & Biotechnology* **74**(4):851–856.

Kavitha, V., Mandal, A.B., & Gnanamani, A. (2014). "Microbial biosurfactant mediated removal and/or solubilization of crude oil contamination from soil and aqueous phase: An approach with *Bacillus licheniformis* MTCC 5514." *International Biodeterioration & Biodegradation*, **94**: 24-30.

Kitamoto, D., Isoda, H., and Nakahara, T. (2002). "Functions and potential applications of glycolipid biosurfactants—From energy saving materials to gene delivery carriers." *Journal of Bioscience Bioengineering* **94**(3):187–201.

Kuhad, R.C., and Gupta, R., "Biological Remediation of Petroleum Contaminants" in Singh, A., Kuhad, R. C., & Ward, O. P. (2009). *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg, pp.173-183

Lang, S. (2002). "Biological amphiphiles (microbial biosurfactants)." *Current Opinion in Colloid and Interface Science* **7**(1-2):12–20.

Ławniczak, Ł., Marecik, R., and Chrzanowski, Ł. (2013). "Contributions of biosurfactants to natural or induced bioremediation." *Applied Microbiology and Biotechnology*, **97**(6): 2327-2339.

Leahy, J.G., and Colwell, R.R. (1990). "Microbial degradation of hydrocarbons in the environment". *Microbiological Reviews* **54**(3):305–315.

Li, F., Zhu, L., and Zhang, D. (2014). "Effect of surfactant on phenanthrene metabolic kinetics by *Citrobacter* sp. SA01." *Journal of Environmental Sciences*, **26**(11): 2298-2306.

Mackay, D., Shiu, W., Ma, K., and Lee, S. (2006). *Handbook of Physical-Chemical Properties and Environmental Fate for Organic Chemicals, Second Edition*. Boca Raton, FL: CRC Press.

Malavenda, R., Rizzo, C., Michaud, L., Gerce, B., Bruni, V., Syldatk, C., Hausmann, R., & Lo Giudice, A. (2015). "Biosurfactant production by Arctic and Antarctic bacteria growing on hydrocarbons." *Polar Biology* **38**(10): 1565-1574.

Margesin, R., Labbe, D., Schinner, F., Greer, C.W., & Whyte, L.G. (2003). “Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils.” *Applied and Environmental Microbiology* **69**(6): 3085–3092.

Martienssen, M. & Schirmer, M.. (2007). “Use of Surfactants to Improve the Biological Degradation of Petroleum Hydrocarbons in a Field Site Study.” *Environmental Technology*, **28**(5): 573-582.

Mejia-Avendano, S., Munoz, G., Duy, S. V., Desrosiers, M., Benoit, P., Sauve, S., & Liu, J. (2017). "Novel Fluoroalkylated Surfactants in Soils Following Firefighting Foam Deployment During the Lac-Megantic Railway Accident." *Environmental Science & Technology* **51**(15): 8313-8323.

Millette, D., Gosselin, C., & Beliveau, E. (2014). “Clean-Up at Lac-Megantic.” Canadian Consulting Engineer. https://www.canadianconsultingengineer.com/features/clean-up-at-lac-megantic/

Ministère du Développement durable, Environnement, Faune et Parcs (MDDEFP) (2014). « Tableau-Synthèse : Estimation au 22 juin 2014 des quantités de pétrole brut léger impliquées dans l’accident à Lac-Mégantic » http://www.mddefp.gouv.qc.ca/lac-megantic/tableau_synthese/2014078-tableau-synthese-petrole.pdf

Mulligan, Catherine N., Ackmez Mudhoo, and Sanjay K. Sharma. (2014). *Biosurfactants: research trends and applications.* Boca Raton, FL: CRC Press.

Mulligan, C.N., Yong, R.N., and Gibbs, B.F. (2001). “Surfactant-enhanced remediation of contaminated soil: A review.” *Engineering Geology* **60**(1-4): 371-380.

Neu T.R. (1996). “Significance of bacterial surface-active compounds in interaction of bacteria with interfaces.” *Microbiological Reviews* **60**(1):151-166.

Nikolopoulou, M. and Kalogerakis, N. (2008). "Enhanced bioremediation of crude oil utilizing lipophilic fertilizers combined with biosurfactants and molasses." *Marine Pollution Bulletin* **56**(11): 1855-1861.

Pacwa-Plociniczak, M., Plaza, G. A., Piotrowska-Seget, Z., and Cameotra, S. S. (2011). "Environmental Applications of Biosurfactants: Recent Advances." *International Journal of Molecular Sciences* **12**(1): 633-654.

Pamp, S.J., and Tolker-Nielsen, T. (2007). “Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*.” *Journal of Bacteriology* **189**(6):2531–2539.

Paul, D., Pandey, G., Pandey, J., Jain, R.K. (2005). “Accessing microbial diversity for bioremediation and environmental restoration.” *Trends in Biotechnology* **23**(3):135–142.

Pignatello, J.J., “Bioavailability of Contaminants in Soil” in Singh, A., Kuhad, R. C., & Ward, O. P. (2009). *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 35-64

Raaijmakers, J.M., De Bruijn, I., Nybroe, O., and Ongena, M. (2010). “Natural functions of lipopeptides from Bacillus and Pseudomonas more than surfactants and antibiotics.” *FEMS Microbiology Reviews*, **34**(6): 1037-1062.

Singh, A., Kuhad, R.C., and Ward, O.P. (2009). “Biological Remediation of Soil: An Overview of Global Market and Available Technologies” in Singh, A., Kuhad, R. C., & Ward, O. P. (2009). *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 1-17

Singh, A., & Ward, O. P. (2004).”Biotechnology and Bioremediation - An Overview” in Singh, A., & Ward, O. P. *Biodegradation and Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 1-17

Singh, A., Van Hamme, J.D., Ward, O.P., (2007). “Surfactants in microbiology and biotechnology : part 2. Application aspects.” *Biotechnology Advances* **25**(1): 99–121.

Soberón-Chávez, G., Lepine, F., and Deziel, E. (2005). “Production of rhamnolipids by *Pseudomonas aeruginosa*.” *Applied Microbiology and Biotechnology* **68**(6):718–725.

Souza, E.C., Vessoni-Penna, T.C., and Oliveira, R.P.D. (2014). "Biosurfactant-enhanced hydrocarbon bioremediation: An overview." *International Biodeterioration & Biodegradation* **89**: 88-94.

Surridge, A.K.J., Wehner, F.C., and Cloete, T.E. (2009). “Bioremediation of Polluted Soil” in Singh, A., Kuhad, R. C., & Ward, O. P. (2009). *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp.103-117

Teichmann, B., Linne, U., Hewald, S., Marahiel, M.A., and Bolker, M. (2007). “A biosynthetic gene cluster for a secreted cellobiose lipid with antifungal activity from *Ustilago maydis*”. Molecular Microbiology **66**(2):525–533.

Van Hamme, J.D. (2004). “Bioavailability and Biodegradation of Organic Pollutants - A Microbial Perspective” in Singh, A., & Ward, O. P. *Biodegradation and Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 37-56

Van Hamme, J., Singh, A., and Ward, O.P. (2003). “Recent advances in petroleum microbiology.” *Microbiology and Molecular Biology Reviews* **67**(4):503–549.

Van Hamme, J.D., Singh, A., and Ward, O.P. (2006). “Physiological aspects. Part I in a series of papers devoted to surfactants in microbiology and biotechnology.” *Biotechnology Advances* **24**(6):604–620.

Van Hamme, J.D., and Urban, J., (2009). “Biosurfactants in Bioremediation” in Singh, A., Kuhad, R. C., & Ward, O. P. *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp.73-86

Vipulanandan, C., & Ren, X.P. (2000). “Enhanced solubility and biodegradation of naphthalene with biosurfactant.” *Journal of Environmental Engineering – ASCE*, **126**(7): 629-634.

Wybenga, F. (2014). “A Survey of Bakken Crude Oil Characteristics Assembled for the U.S. Department of Transportation.” Dangerous Goods Transport Consulting Inc., American Fuel & Petrochemical Manufacturers.

Xia, W., Du, Z., Cui, Q., Dong, H., Wang, F., He, P., and Tang, Y. (2014). “Biosurfactant produced by novel *Pseudomonas sp.* WJ6 with biodegradation of n-alkanes and polycyclic aromatic hydrocarbons.” *Journal of Hazardous Materials* **276**: 489–498.

Zhou, J., Xia, B., Treves, D.S., Wu, L.Y., Marsh, T.L., O’Neill, R.V., Palumbo, A.V., and Tiedje, J.M. (2002). “Spatial and resource factors influencing high microbial diversity in soil.” *Applied and Environmental Microbiology* **68**(1):326–334.

Zhu, H., & Aitken, M.D., (2010). “Surfactant-enhanced desorption and biodegradation of polycyclic aromatic hydrocarbons in contaminated soil”. *Environmental Science & Technology* **44**(19): 7260–7265.

Zoueki, C. W., Ghoshal, S., and Tufenkji, N. (2010). "Bacterial adhesion to hydrocarbons: Role of asphaltenes and resins." *Colloids and Surfaces B: Biointerfaces* **79**(1): 219-226.

Chapter 2 – Feasibility of Biosurfactant Enhanced Bioremediation of Residual Petroleum Hydrocarbon Fractions in Contaminated Soils from Lac Mégantic

2.1 Introduction

Petroleum contamination represents a major risk to the health of humans and ecosystems. One such example can be seen in the case of the Lac Mégantic rail disaster, which occurred in the summer of 2013 and resulted in the release of an estimated 5.98 million liters of Bakken Formation light crude oil into the environment (MDDEFP, 2014). The surrounding area was severely contaminated; at least 100 000 liters of crude oil went directly into the Chaudière river, and the total petroleum hydrocarbon concentrations in the soil ranged from 4000 to 77 000 mg/kg in the impact zone (Galvez-Cloutier et al., 2014; MDDEFP, 2014). These soils, like many soils contaminated with petroleum hydrocarbons, were treated using cost-effective engineered bioremediation systems. As the soil undergoes aging the rate of biodegradation slows over time and becomes especially reduced during the final phase of bioremediation. Eventually the contaminant concentration stops decreasing and a plateau referred to as the 'endpoint' is reached (Singh et al., 2009; Surridge et al., 2009). Over the long term, the contaminants become more resistant to mass transfer and less bioavailable as they slowly diffuse and get entrapped within micro and nanopores, sorb into organic matter, and bind irreversibly to soil minerals and/or clays (Akbari and Ghoshal, 2015; Pignatello, 2009; Van Hamme, 2004). These processes within the soil matrix can thus limit the microorganisms' access to the pollutants and therefore control the rate of biodegradation.

In some bioremediation scenarios, surfactants or biosurfactants are used to improve performance, either by being added externally (influent, spraying, injection) or produced by microorganisms within the soil itself (augmentation and supporting microbial growth) (Ławniczak, 2013). Biosurfactants are predominantly produced by hydrocarbon-degrading microorganisms and have several advantages over

synthetic surfactants; they have better biodegradability, lower toxicity, can be made from renewable sources or even industrial wastes, and show greater stability and performance at extreme temperatures, pH, and salinity (Bordoloi and Konwar, 2009; Mulligan, et al. 2001; Souza et al., 2014). Biosurfactants can improve the bioavailability of hydrophobic or insoluble organic compounds by: changing the surface properties of the bacterial cell; dissolving and emulsifying the compounds; releasing entrapped hydrocarbons from small pores; and generally improving the solubilization and mobilization of compounds (Singh et al., 2007; Hazra et al., 2012; Van Hamme et al., 2004; Xia et al., 2014).

There are many available biosurfactants; rhamnolipids, belonging to the glycolipids category, are one of the most characterized biosurfactants. Rhamnolipids are mainly produced by *Pseudomonas aeruginosa* and are reasonably cost competitive compared to synthetic surfactants (Maier and Soberón-Chávez, 2000). They are generally a mix of one (monorhamnolipid) or two (dirhamnolipid) rhamnose sugars linked to one or two 3-hydroxydecanoic acid moieties. Sophorolipids (also glycolipids) are becoming more popular in commercial applications as their production costs drop (Pekin et al., 2005). Sophorolipids are produced by yeasts, mainly by the *Candida sp.*, and they are either in acidic form (free fatty acid tail) or in lactonic form (fatty acid carboxylic end connected to sophorose head). Finally, the most studied lipopeptide biosurfactant is surfactin. Surfactin is mainly produced by strains of *Bacillus subtilis*, and is an extremely powerful surface-active compound (Arima et al., 1968). It is gaining commercial application in the biomedical field (Dhanarajan and Sen, 2014).

The hypothesis to be evaluated is whether the addition of biosurfactants to aged biopile soil contaminated with hydrocarbons results in higher degradation, and to determine the corresponding shift of the microbial community.

Studies examining bioremediation enhanced with biosurfactants generally begin by assessing the biosurfactant's ability to solubilize hydrocarbons, its emulsification activity, and/or its surface tension lowering activity (Pacwa-Płociniczak et al., 2011). It is assumed that biosurfactants improve hydrocarbon bioavailability mainly through these three mechanisms, and are especially useful for aged contaminated sites. Despite these assumptions, there have been a number of studies with conflicting results. Although positive influence of biosurfactants is often reported, there have been numerous cases where no or negative effects were observed (Das and Mukherjee 2007; Ławniczak, 2013). Additionally, there is not always the expected correlation between pollutant desorption/solubilization and biodegradation (Ławniczak et al., 2013). For example, Adrion et al. (2016) found relatively low levels of PAH desorption but significant increases to biodegradation rates. Meanwhile Vipulanandan and Ren (2000) compared four surfactants in freshly spiked samples and found that although the rhamnolipid biosurfactant increased naphthalene's solubility by 30 times (highest rate of the four), that case took 40 days to biodegrade compared to only 100 hours using the surfactant Triton X-100. The authors assumed, based on turbidity measurements, that this was due to the biosurfactant being biodegraded along with the naphthalene as a competing substrate, while the Triton X-100 was not. Therefore, a biosurfactant's desorption/solubility ability is not always reliable as a predictor of its biodegradation effectiveness.

Most biosurfactant-enhanced biodegradation studies focus on a biosurfactant's physico-chemical properties. While important, these studies tend to neglect the effect on the microorganisms themselves, whether individual cells or community population dynamics. Cappello et al. (2012) suggested that the efficacy of a biosurfactant in enhancing biodegradation does not depend exclusively on its physicochemical characteristic but also its effect (interaction, stimulation, etc.) on the microbial community. For example, some biosurfactants have the potential to inhibit bacterial adhesion, which in

turn can reduce biodegradation since adherent bacteria are more efficient degraders than suspended bacteria (Congiu et al., 2015; Zoueki et al., 2010).

Limited information is currently available on the biosurfactant inhibition of biodegradation. The data from an investigation applying different concentrations of biosurfactants, and tracking the effect to microbial community dynamics as well as hydrocarbon biodegradation, could be used to further understanding on the mechanisms of biosurfactant enhanced biodegradation. Furthermore, many laboratory studies that have assessed the effects of biosurfactants on hydrocarbon biodegradation, have used uncontaminated soil samples spiked with hydrocarbons, rather than use soils where the hydrocarbon contamination has been environmentally aged and weathered .

The specific objectives of this study were (i) to determine if biosurfactants influenced biodegradation of aged petroleum hydrocarbon contamination in a concentration dependant manner, (ii) and to determine the influence of biosurfactants on the soil microbial community during biodegradation.

A comparison of three different biosurfactants applied at identical concentrations was performed with environmentally aged, contaminated Lac Mégantic biopile soil samples. Based on the results of these microcosm experiments, a biosurfactant that influenced biodegradation positively was chosen for a longer experiment where it was applied at different concentrations to similar aged Lac Mégantic biopile soil samples. These microcosms tracked changes over time (80 days) of the total petroleum hydrocarbon contamination and the microbial community. A secondary line of research resulted in the isolation of several indigenous hydrocarbon-degrading bacteria of the Lac Mégantic contaminated soils.

2.2 Methods and Materials

2.2.1 Materials

The Lac Mégantic hydrocarbon contaminated soil for the Biopile A and for the Biopile B experiments was obtained from Université Laval and Englobe, respectively. Both biopile soils were collected in 2015, two years after the derailment. Excavation and bioremediation treatments were ongoing at the time. Biopile A soil was sieved with 4 mm sieves; Biopile B soil was likewise sieved, mixed, and homogenized. Soils were kept in frozen storage at −20°C before sample preparation. The pH was 8.29 ± 0.01 and 7.89 ± 0.01 for Biopile soils A and B, respectively. The iron concentration was 15.44 ± 0.50 and 21.54 ± 4.11 mg/ g wet soil, calcium was 12.26 ± 2.76 and 5.72 ± 1.21 mg/ g wet soil, and magnesium was 7.60 ± 0.53 and 6.49 ± 0.13 mg/ g wet soil for Biopile soils A and B, respectively. The total phosphorous concentration was 410 and 400 mg/ kg soil, inorganic phosphorous was 350 and 330 mg/ kg soil, for Biopile soils A and B, respectively. The total Kjeldahl nitrogen was 390 and 760 mg/kg soil, nitrogen in ammonia form (NH_3-N) was below detectable limits, while nitrate and nitrite combined were <1.0 and 5.9 mg/kg soil, for Biopile soils A and B, respectively. The total organic carbon was 0.59 and 1.7% g/g soil, for Biopile soils A and B, respectively.

The Bushnell-Haas media (B5051) was purchased from Fluka Analytical. The rhamnolipids (R90) were manufactured by AGAE Technologies, and purchased from Sigma-Aldrich. The sophorolipids (diacetylated lactonic sophorolipids from yeast) were manufactured by Cayman Chemical, and purchased from Cedarlane Labs. The surfactin (from *Bacillus subtilis*) was obtained from Sigma-Aldrich. Agar plates used R-2A agar purchased from Fluka Analytical. All prepared media, broth, and agar was autoclaved prior to use.

2.2.2 Biosurfactant comparison with Biopile Soil A

Microcosms were prepared in triplicate with different types of biosurfactants added at identical concentrations. The microcosms each contained 5 g soil and 40 mL added liquid. One control was 5 g soil

and 40 mL of distilled water (unamended control); another control was 5 g soil and 40 mL Bushnell-Haas (BH) media (amended control). The 4 treated systems were dosed with 80 mg/L rhamnolipids in distilled water (RL w/o nutrient), 80 mg/L rhamnolipids in BH media (RL w/ nutrient), 80 mg/L sophorolipids in BH media (SOPH w/ nutrient), and 80 mg/L surfactin in BH media (SURF w/ nutrient), respectively. Bushnell-Haas media was used because it is an effective nutrient amendment for hydrocarbon degraders (Bushnell and Haas, 1941; Alfred et al., 1963). The microcosms were incubated in a shaker set at 175 rpm and 17°C. The incubation temperature was based on the average summer temperature at the Lac Mégantic biopile site. After 34 days the microcosms were analyzed using total petroleum hydrocarbon (TPH) analysis at Maxxam Analytique.

2.2.3 TPH Bioremediation in Biopile Soil B

Microcosms were prepared in triplicate with the same rhamnolipid biosurfactant added at different concentrations. The 3 control systems consisted of: 5 g soil in 35 mL of distilled water (unamended control); 5 g soil in 35 mL BH media (amended control); and a killed control of 5 g soil in 35 mL BH media dosed to 86.87 mg/L rhamnolipids (killed control). The 4 treated systems were dosed to 20 mg/L (1 CMC), 80 mg/L (4 CMC), 600 mg/L (30 CMC), and 2000 mg/L (100 CMC) rhamnolipids, respectively. All treated systems contain 5 g soil in 35 mL BH media. The microcosms were incubated with mixing at 175 rpm, and maintained at 17°C. Each sacrificial microcosm was analyzed for TPH at Maxxam Analytique at pre-determined time points.

Slurry microcosms were chosen for two main reasons. It was advantageous to check first if biosurfactants were able to enhance biodegradation under favourable conditions of sufficient mixing before scaling up or using unsaturated reactors. Secondly, a previous bioremediation study performed by Akbari and Ghoshal (2014) found that although mixing in a slurry reactor led to faster biodegradation, the overall biodegradation endpoint for the slurries (F3: 578.6 mg/kg, TPH: 696.2 mg/kg) was very

comparable to their pilot-scale biopile reactor (F3:525.8 mg/kg, TPH: 620.9 mg/kg) which was operated without mixing and under unsaturated conditions.

2.2.4 Chemical Composition Analysis

The Quebec governmental 'Centre d'expertise en analyse environnementale du Québec' MA. 400 – HYD. 1.1 method of petroleum hydrocarbon (C10-C50) analysis was followed, using hexane extraction (CEAEQ, 2016). The sample extracts were analyzed by gas chromatography–flame ionization detectors (Agilent GC-FID 6890 RACER, Agilent GC-FID 6890 NO RACER, and Agilent GC-FID 7890 RACER), all using an Agilent DB-1 Column. The resulting GC-FID response files and chromatograms were forwarded to McGill University for further data analysis using ChemStation software.

2.2.5 Microbial Community Analysis

For the Biopile Soil B experiments, prior to being taken to Maxxam Analytique laboratory, 1.33 mL of the mixed slurry was removed from each microcosm in a sterile environment. This slurry was kept frozen at -80°C, and later used for DNA analysis. DNA extraction was performed using the Qiagen PowerSoil DNA isolation kit, using 1.0 mL slurry in each 0.1 mm glass bead beating tube. Soil samples from Biopile Soil A were also analyzed for microbial community DNA. Once extracted, DNA samples were sent to Genome Quebec for Next Generation Sequencing (Illumina MiSeq). The PCR amplification used New England Biolabs (NEB) Q5® High-Fidelity DNA Polymerase, as well as bacterial primers 779 forward (5'- AACMGGATTAGATACCCKG -3') and 1115 reverse (5'- AGGGTTGCGCTCGTTG -3').

2.2.6 Pure Culture Isolation

To isolate the indigenous hydrocarbon-degrading bacteria of the Lac Mégantic contaminated soils, triplicate flasks were each prepared with 7 g soil, 75 mL BH media, and 750 µL sterile Bakken Crude oil to serve as the sole carbon source. The Bakken crude oil was provided by Université Laval, who obtained it from the Valero Energy Inc. Jean Gaulin refinery in Lévis, QC. The crude oil was sterilized using a glass

syringe equipped with an EMD Millipore Swinny 13mm Filter Holder and a 0.22 µm membrane (TefSep Teflon laminated membrane from GE Water and Process Technologies).

The flasks were maintained in a shaker operated at 175 and at 25°C. After 4 days, flask dilutions were pipetted, spread onto R-2A agar plates, incubated at 25°C, and monitored for growth. Distinct colonies were isolated and re-streaked onto clean plates until pure culture isolations were obtained.

Prior to extraction, liquid cultures were grown in small amounts of Luria Bertani (LB) Lennox broth, purchased from Sigma-Aldrich. The liquid cultures were centrifuged, the supernatant discarded, and the resulting pellet of each culture then used for DNA extraction.

DNA extraction was performed using the Zymo Research ZR Fungal/Bacterial DNA MiniPrep isolation kit. The PCR amplification used Thermo Scientific DreamTaq DNA polymerase, as well as the 16S universal bacterial primers 338 forward (5'-ACTCCTACGGGAGGCAGC-3') and 1390 reverse (5'-GACGGGCGGTGTGTACAA-3'). Clear single bands in gel electrophoresis confirmed that PCR amplification was successful. The unpurified PCR samples were then sent to Genome Quebec for Sanger Sequencing. Gene sequences obtained were compared to the online databases of NCBI with BLAST.

Subsequent experiments with the isolated cultures involved attempting to grow individual liquid cultures using rhamnolipids as the sole carbon source. Sterile Bushnell-Haas media dosed to 600 mg/L rhamnolipids (30 CMC) was used. Capped tubes with this broth were inoculated, incubated at 25°C with mixing at 175 rpm, and monitored for growth. The rhamnolipid solution was filter syringe sterilized using a BD syringe with a Fisherbrand 0.22 µm membrane filter (all sterile).

2.3 Results and Discussion

2.3.1 Biosurfactant comparison with Biopile Soil A

Three different biosurfactants were added to Biopile soil A microcosms to evaluate whether TPH reduction would be improved, and to compare the performance between the three. Rhamnolipids, sophorolipids and surfactin were chosen as the three biosurfactants, these being well studied, effective, and available.

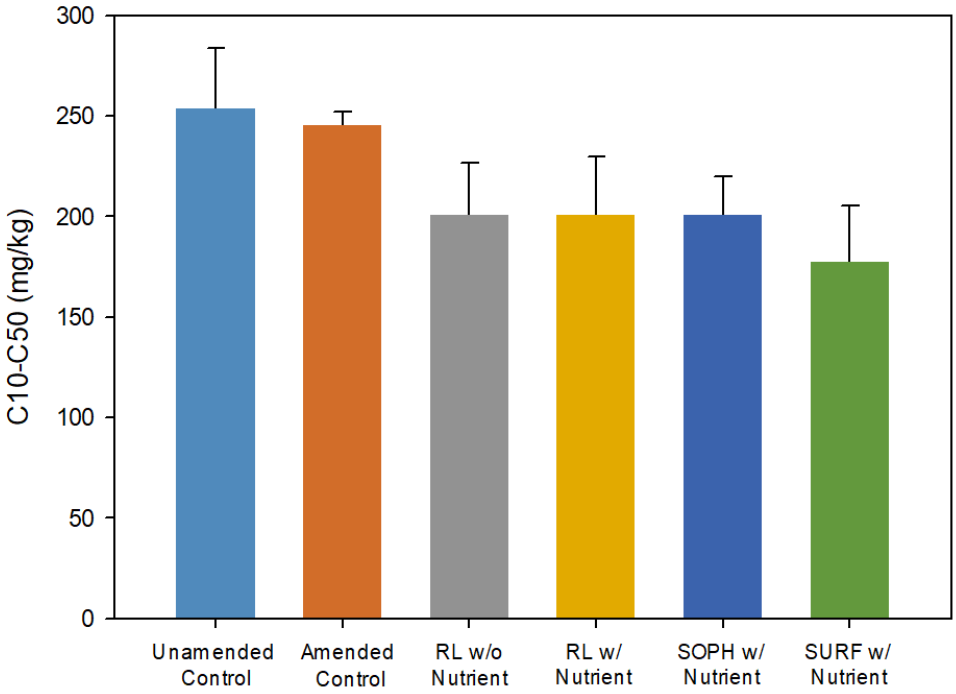


Figure 1 : Biopile A soil microcosm TPH concentrations after 34 days

Note that the TPH concentrations at day 0 and day 34 in the unamended control were almost identical. As shown in Figure 1, after a run time of 34 days, a statistically significant higher TPH reduction (based on t-tests performed in SigmaPlot; results are in the Appendix Table A1) was found among all the biosurfactant systems as compared to the controls. While surfactin resulted in the greatest reduction, the difference between the biosurfactants themselves was not large. Although surfactin was the most powerful surface-active compound amongst the three, it did not demonstrate a proportionally large

increase in TPH reduction. As a result, the more economical rhamnolipids were chosen for the subsequent experiments.

Interestingly, it can be seen that the two controls, unamended vs amended, were not significantly different. Likewise, the two treated systems of rhamnolipids without vs with nutrient were also not significantly different. Based on these observations, the addition of BH media for these systems did not appear to affect TPH degradation.

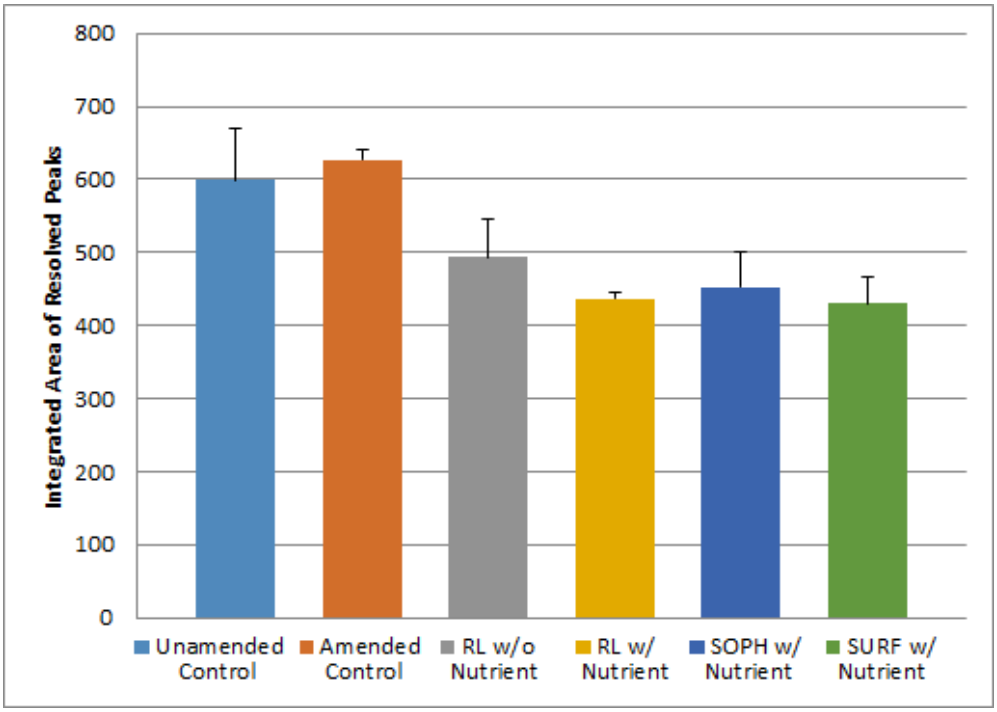


Figure 2 : GC response for the integrated area of resolved peaks of Biopile A samples

Above is Figure 2, which shows the GC response for the integrated area of resolved peaks for the respective TPH chromatograms. These were analysed using ChemStation software. Chromatograms with unaltered baselines and with resolved peaks (Figures A1-A7) have been included in the Appendix. Overall the GC response for the resolved peaks in Figure 2 shows similar trends to the TPH values of Figure 1. The main two differences between the above figures are that in Figure 2, the surfactin treated

system is not evidently the strongest performing, and the rhamnolipid without nutrient system has less TPH reduction than the rhamnolipid with nutrient system.

Thus the Biopile A experiments demonstrated a clear beneficial effect of biosurfactant addition on TPH reduction. Based on the data in Figures 1 and 2, RL w/ nutrient was chosen when moving forward with Biopile B soil experiments.

2.3.2 TPH Bioremediation in Biopile Soil B

Microcosms with Biopile soil B were prepared with BH media and rhamnolipids at different concentrations. These were tracked over a longer period of time, and compared against each other as well as against three controls.

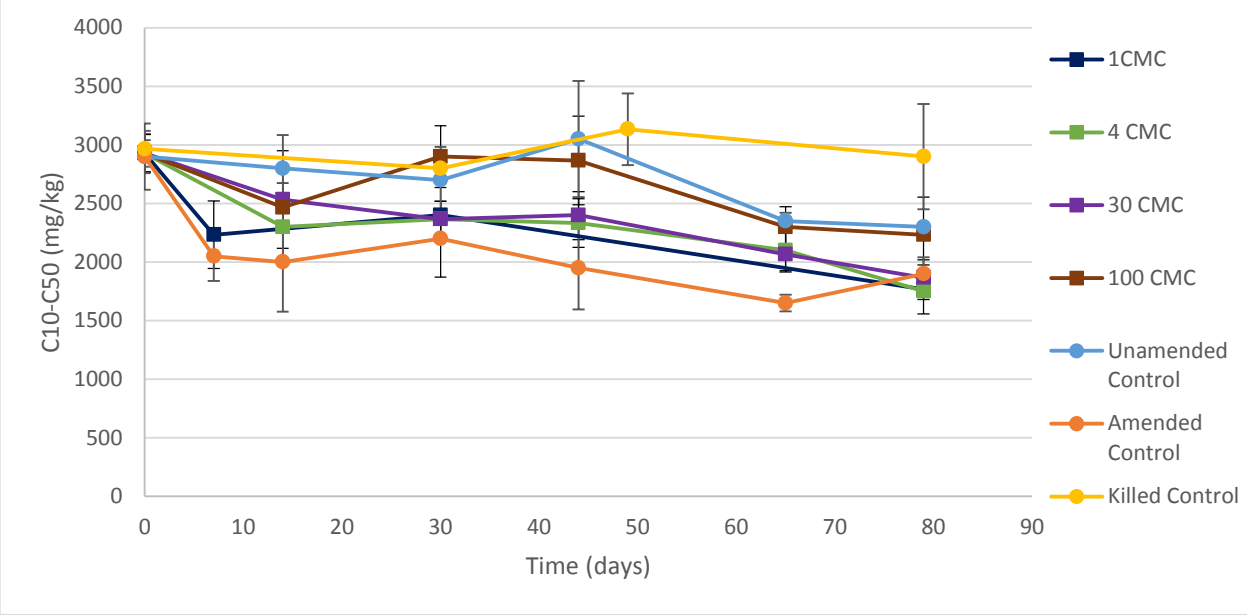


Figure 3 : Biopile B soil microcosm TPH concentrations over 80 days

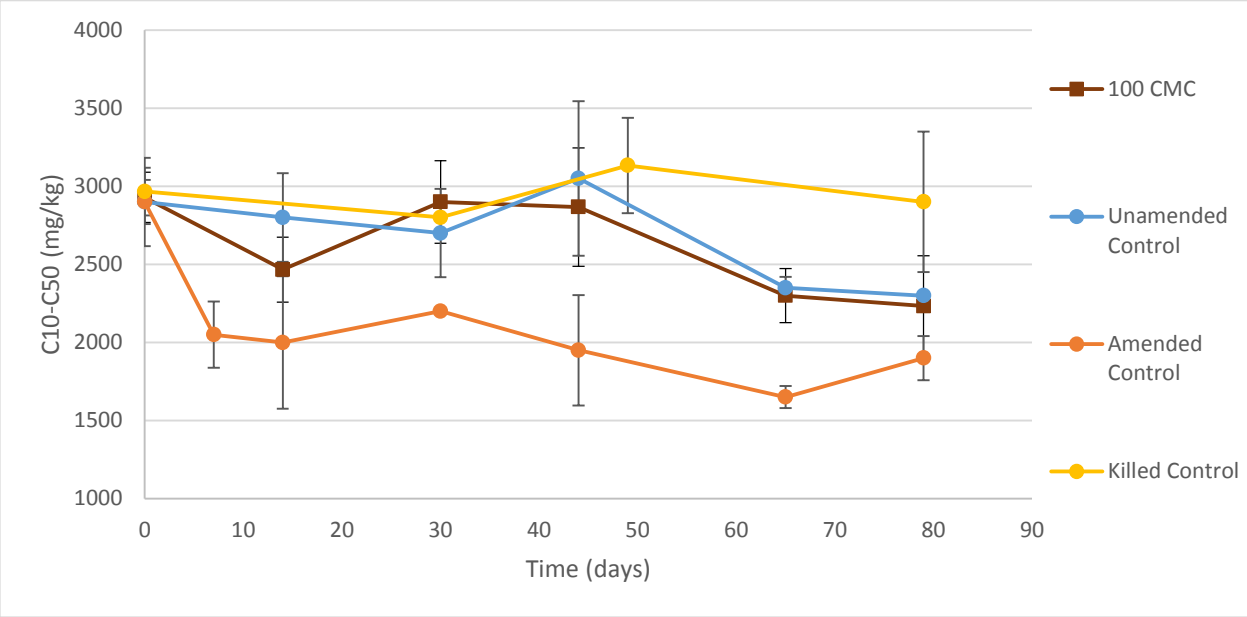


Figure 4 : Biopile B soil microcosm TPH concentrations for Control Systems and 100 CMC

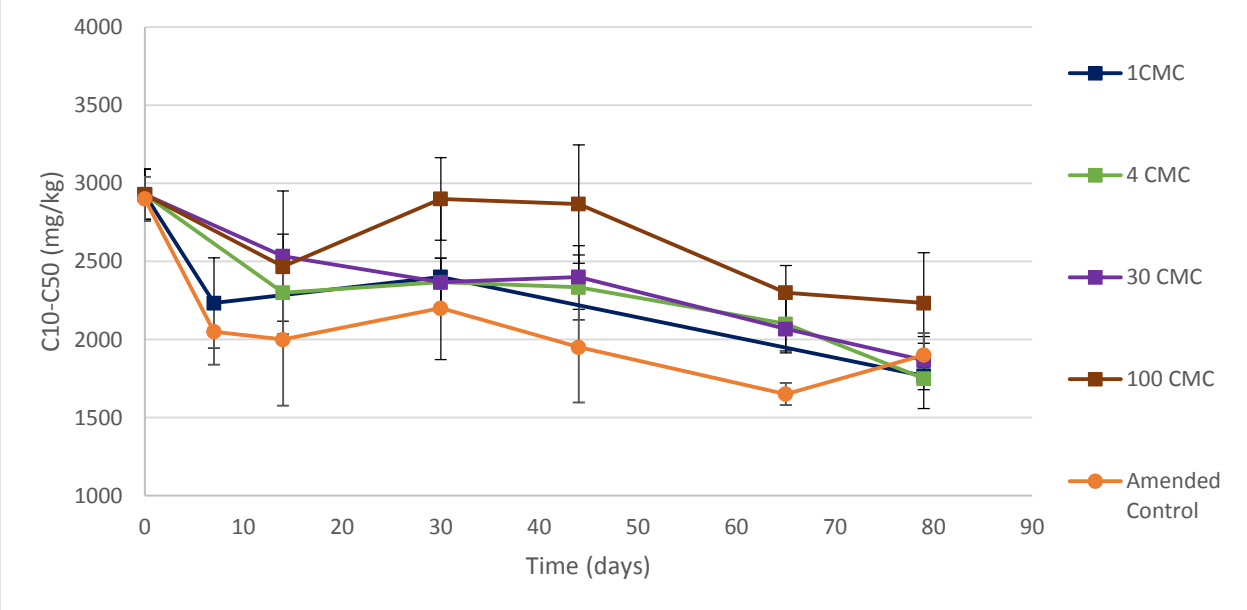


Figure 5 : Biopile B soil microcosm TPH concentrations for Treated Systems and Amended Control

As shown in Figures 3 and 5, the amended control demonstrated the overall best performance of all the systems, including the biosurfactant treated microcosms. Its final endpoint was also around the same level as 1 CMC, 4 CMC and 30 CMC microcosms. From an average initial TPH concentration of 2929 ± 160 mg/kg at day 0, the amended system reached 1650 ± 71 mg/kg at day 65 (43.67% reduction) and 1900 ± 141 mg/kg (35.13%) at day 80; while at day 80, 1 CMC systems reached 1767 ± 208 mg/kg (39.67%), 4 CMC reached 1750 ± 71 mg/kg (40.25%), and 30 CMC reached 1867 ± 153 mg/kg (36.26%). The 1 CMC and 4 CMC systems demonstrated very similar trends throughout the 80 days. After day 14 the overall trend of TPH reduction for the 30 CMC systems also changed to more closely resemble the trends of 1 and 4 CMC. This result implies that Biopile B soil, as opposed to Biopile A, does not seem to benefit from the addition of biosurfactants. Quite the contrary, it seems like the rhamnolipids exhibited a slight inhibitory effect.

Interestingly, the addition of BH media had a much bigger impact for the Biopile B case as opposed to the Biopile A soil. As seen in Figures 1 and 2 for Biopile A, there was no significant difference between the nutrient lacking and nutrient dosed systems, both among the controls and treated systems. Comparatively for Biopile B, the unamended and amended controls in Figure 4 demonstrate a clear and large difference in terms of TPH reduction.

Taking a closer look at the three control systems in Figure 4, the unamended control exhibits nearly the same trajectory as the killed control until day 45, after which a moderate decrease in TPH is noted. Being unamended, with only distilled sterile water added, it could be also considered a natural attenuation case. Closely matching the unamended system is the highest dosage of biosurfactant, the 100 CMC rhamnolipid system, with almost identical endpoints (2300 mg/kg and 2233 ± 321 mg/kg, for unamended and 100 CMC respectively), seen in Figure 3. While the three lower doses of rhamnolipid showed an average TPH reduction of 39%, the highest dose was most definitely inhibitory. It could be concluded that after a certain threshold of rhamnolipid addition, any possible benefit was negated and

TPH reduction began to be hindered. The exact threshold is unknown, but seems to lie between 30 CMC and 100 CMC (600 mg/L and 2000 mg/L).

There are a number of possible reasons to explain why the lower rhamnolipid doses had little improvement compared to plain BH media addition, and why the highest rhamnolipid dose had an inhibitory effect. Within studies investigating rhamnolipids and oil biodegradation efficiency amongst 218 microbial consortia, the addition of rhamnolipids was found to have equally increased, decreased and had no effect on the biodegradation extent (Owsianiak et al., 2009).

For instance, Zhang and Miller (1994 & 1995) observed that the addition of rhamnolipids stimulated the uptake and biodegradation of hexadecane and octadecane for some species; but for other species inhibited octadecane biodegradation. Likewise, Arino et al. (2008) found rhamnolipid producing species enhanced PAH biodegradation efficiency, while inhibiting the growth of other species.

The difference in effect between species is likely due to the roles biosurfactants play in biological processes, which include bacterial toxicity/pathogenesis, motility, changing cell surface properties, biofilm formation, cellular differentiation, bacterial cell signaling, use as a possible carbon source, protection against toxic compounds, and accessing substrate/nutrients (Christova and Stoineva, 2014; Cameotra and Makkar 2004; Kitamoto et al. 2002; Lang 2002; Van Hamme et al. 2006).

Some species require rhamnolipids to uptake hydrocarbons at all; Koch et al. (1991) observed that mutants unable to produce rhamnolipids were also unable to grow or utilize hexadecane; the addition of rhamnolipids restored their ability to do so. For *Bacillus*, in fact, lipopeptides have been shown to function as signal molecules for coordinated growth and differentiation (Raaijmakers et al., 2010).

On the other hand, rhamnolipids are reported as having antimicrobial activity against several bacterial, yeast, and fungal species (Haba et al. 2003; Benincasa et al., 2004). In natural habitats, biosurfactants

are used to gain a competitive advantage in interactions with other microorganisms and against nematodes and protozoan predators. For example, when tested in vitro, biosurfactants produced by *Pseudomonas* and *Bacillus* species demonstrated growth-inhibition and lysis against a range of competitive microorganisms, including viruses, mycoplasmas, bacteria, fungi and oomycetes (Raaijmakers et al., 2010; Van Hamme, 2004).

The use of surfactants to enhance motility is vital in allowing bacteria to travel from depleted environments towards nitrogen and phosphorus rich environments. This has been especially observed in the case of rhamnolipids, and one of the reasons why rhamnolipid production is boosted in nitrogen limited cases. Rahmnolipids can also increase nutrient uptake for specific groups of bacteria. Additionally, while some microorganisms evolved to survive by using rhamnolipids, other non-biosurfactant producing microorganisms employed coexistence with the biosurfactant-producing bacteria to survive (Christova and Stoineva, 2014; Raaijmakers et al., 2010).

Generally, biosurfactants are all synthesized from the same basic metabolic components: amino acids, sugars, fatty acids and lipids. The resulting biosurfactant compound is thus also rich in sugars, lipids and amino acids. As a result, biosurfactants can be used by microorganisms as a form of 'nutrient storage'. This is especially supported by the fact that biosurfactant production can be induced by limiting access to nutrients (nitrogen, phosphorous or iron) (Amézcua-Vega et al. 2007; Soberón- Chávez et al. 2005; Teichmann et al. 2007). In bioremediation cases, there is a possibility that the biosurfactants might then be used as a preferred carbon source instead of the contaminants; this has been observed in several studies (Chrzanowski et al., 2012; Ławniczak et al., 2013; Van Hamme & Urban, 2009). In some other cases, however, using the biosurfactants as a carbon source could be beneficial to bioremediation by giving a boost and increasing the biomass of hydrocarbon degraders (Hickey et al. 2007).

Thus for our particular results among the Biopile B microcosms, it could be that:

a) There were sufficient biosurfactant levels to begin with, produced by the indigenous microorganisms, and thus any extra added rhamnolipids had little effect.

b) The microorganisms preferentially degraded and utilized the biosurfactants as a carbon source compared to the hydrocarbon contaminants.

c) The rhamnolipids stimulated preferential growth of particular microbial groups over others (i.e. not hydrocarbon degraders, or degraders of specific hydrocarbon compounds not present in the soil).

It should be noted that in natural habitats and in biopiles, microorganisms do not act in isolation; the biodegradation processes are accomplished by mixed cultures. Various specialized groups of microorganisms compete and work together in consortia. Currently within the literature, studies focused on examining the influence of rhamnolipids on environmental consortia are limited (Chrzanowski et al., 2012).

2.3.3 Microbial Community Analysis

Changes in the microbiological community were tracked over time For the Biopile B microcosms. DNA samples were analyzed using high-throughput Next Generation Sequencing (Illumina MiSeq) for 16S rRNA. The community shifts at high doses of biosurfactants are especially interesting, exhibiting clear succession with time.

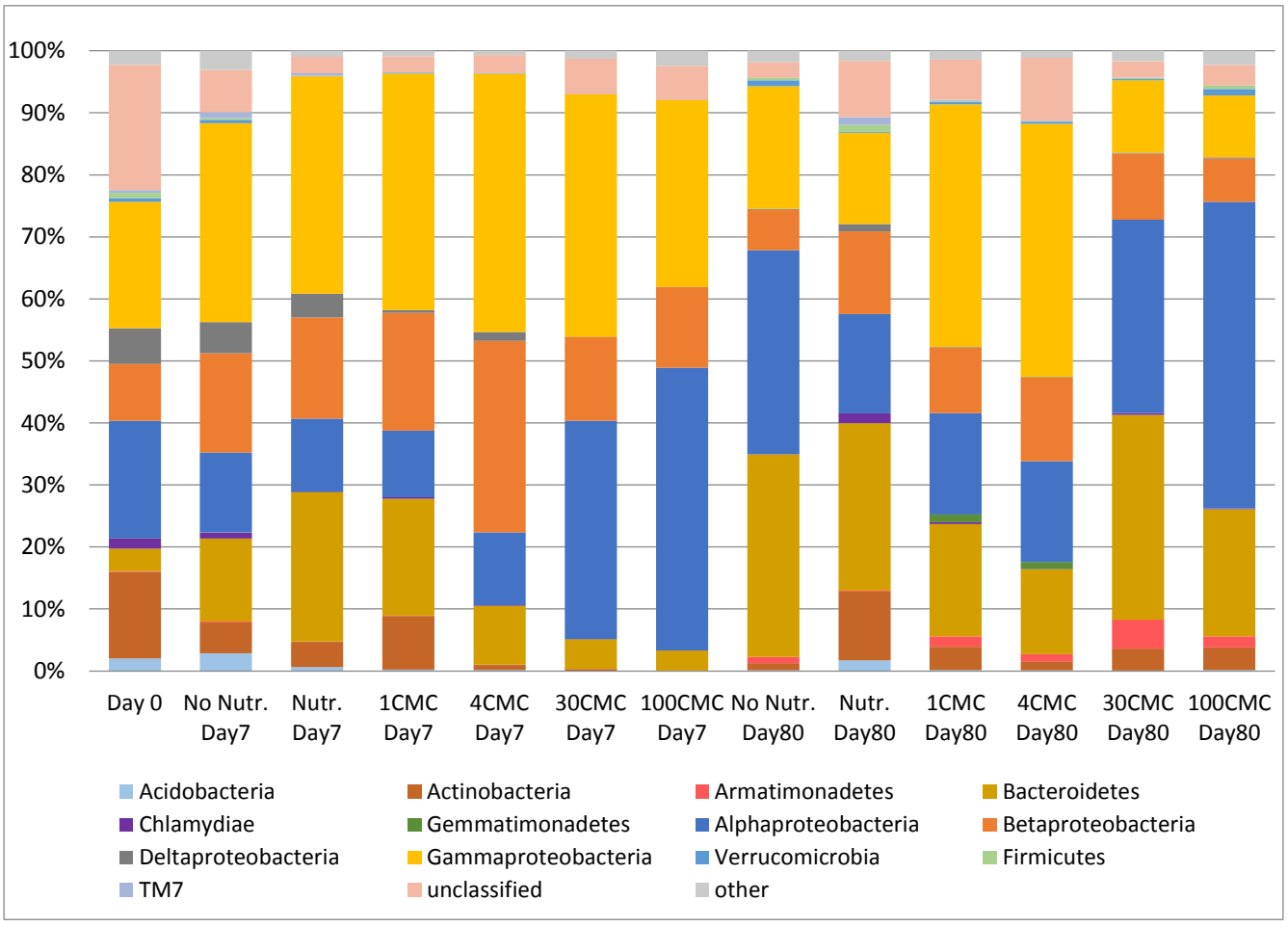


Figure 6 : Relative abundance of microbial community groups for Biopile B Systems

Figure 6 displays a summary of these shifts. *Gammaproteobacteria, Alphaproteobacteria, Betaproteobacteria, Bacteroidetes,* and *Actinobacteria* were the major bacterial phyla throughout, totaling 66.1% of the relative abundance on day 0, and 79.4- 95.3% the subsequent days. *Proteobacteria* was the dominant bacterial phylum, ranging from, at its lowest, 45% of the community in Nutr. Day 80,

up to 88.8% in 100 CMC Day 7. *Proteobacteria* are a common dominant phylum in soil samples, encompassing an enormous diversity of species, and playing an important role in carbon, nitrogen and sulphur cycles (Militon et al., 2010; Sutton et al., 2013). This phylum, especially the *Gamma* class, is especially important in hydrocarbon-polluted soil microbial communities and contains a number of hydrocarbon degraders (Militon et al., 2010; Kim and Crowley, 2007). Other hydrocarbon degrading bacteria include species among the *Actinobacteria, Firmicutes, Bacteroidetes* and *Chlamydiae* phyla, all of which were detected in the Biopile B soil (Prince et al., 2010). The current study showed that besides the above named phyla, there were also *Acidobacteria, Armatimonadetes, Gemmatimonadetes, Deltaproteobacteria, Verrucomicrobia,* and TM7 bacterial phyla observed in the contaminated soil. These minor groups were present in relative abundances totaling <0.1% to 11.4%. It was noted that at the end of the treatment at 80 days the biosurfactant systems all had a small, but noticeable, increase of *Armatimonadetes* (1.2-4.7%), which is not particularly known for hydrocarbon degraders. Unfortunately information on the ecology of *Armatimonadetes* strains is currently lacking, but they are estimated as being engaged with the degradation of plant material and polysaccharide-based compounds (Lee et al., 2014).

The dominance of *Proteobacteria* found among all the systems is typical of temperate and cold-region hydrocarbon-contaminated soil, especially during the early stages of biodegradation. *Proteobacteria* groups, particularly the *Gamma* subclass, generally increase in soil environments when given access and/or are contaminated with hydrocarbon substrates. This dominance decreases over time as biodegradation eventually reaches its plateau, which is a pattern that was also seen in Figure 6 (Greer et al., 2010).

Labbé et al. (2007) found the relative abundance of *Alphaproteobacteria* was larger in pristine over contaminated soils, while *Beta* and *Gamma* subclasses were exclusive to hydrocarbon- contaminated soils, and thus enriched after contamination. *Betaproteobacteria* are widespread soil bacteria, with

many isolates being capable of aerobic hydrocarbon degradation (aromatic, chloroaromatic, nitroaromatic, and aminoaromatic compounds), although degradation ability tends to be limited to specific hydrocarbon subsets for specific strains of each genus (Parales, 2010). However *Actinobacteria*, also capable of degrading various hydrocarbon compounds, was found to be more or less independent of contamination (Kampfer, 2010; Lo Giudice et al., 2010).

As this was aged contaminated soil, *Beta* and *Gamma* subclasses were already present at the beginning of the experiments. Relative abundance of the *Beta* subclass fluctuated only slightly during the biodegradation process; increasing slightly by day 7 (3.89- 9.86% higher), and then decreasing again by day 80. The sole exception was 4 CMC at Day 7, which reported a larger spike in *Beta* abundance (21.74% higher, or 3.37 times bigger than the original *Beta* abundance). As mentioned, the *Gamma* subclass increased during the early stages (11.70-21.23% higher), and then generally decreased as biodegradation plateaued (0.59-10.42% lower, with two exceptions). There were two exceptions that displayed a *Gamma* increase at Day 80; the 1 CMC and 4 CMC systems. This could potentially mean that the 1 and 4 CMC systems were still undergoing biodegradation and had not yet reached their final endpoints of improved TPH reduction; however without more data points this is only speculation.

Of all the biosurfactant-treated systems, 1 CMC and 4 CMC had very similar community compositions for day 80. These two systems were also the only systems to have any noticeable abundance (1.3 and 1.2%) of a *Gemmatimonadetes* population. It could be inferred that a small dosage of rhamnolipids led to the right conditions for increased *Gemmatimonadetes* – alternatively, the day 80 relative abundance of the 1 and 4 CMC systems could be a 'snapshot' of this experiment's typical microbial community before biodegradation had begun to plateau. Earlier data points of the other systems would have to be consulted to explore this potential hypothesis.

Interestingly, the nutrient (amended) and 30 CMC systems, though both showed comparable TPH reductions to 1 CMC and 4 CMC systems, had very different community compositions. The nutrient

(amended) case had the highest diversity at the phyla level at day 80 of all the systems, while 30 CMC at day 80 was closer to the no nutrient (unamended) case than any of the other systems. The 100 CMC system, the second lowest performing case in TPH reduction, had the lowest bacterial diversity at day 7 and second lowest bacterial diversity at day 80. The no nutrient (unamended) case was comparable in terms of lowered diversity. As expected, the least diverse no nutrient and 100 CMC systems had similar low TPH reductions. At day 80 the more diverse nutrient case was the only system to return to noticeable *acidobacteria, firmicutes*, TM7, and *chlamydiae* populations (1.7, 1.1, 1.2, 1.6%, respectively), and the only one to have any noticeable abundance of the *deltaproteobacteria* left (1.2%). Not only was it more diverse, but it could also be considered the most effective; looking at the general trend over the full 80 days, the nutrient (amended) case seemed to have the best overall performance in TPH reduction.

Taking a closer look at the least diverse microbial community distribution at day 7 100 CMC, it is noted that the *Proteobacteria* dominate, at 88.8% relative abundance, 45.6% of which is the *Alpha* subclass. However, this is closely matched by the 30 CMC at 87.9% *Proteobacteria*, 35.2% *Alpha* subclass. Although the other systems also experienced dominant *Proteobacteria* abundance at this early stage, their *Alpha* abundances were not so drastic, actually decreasing from 19% at day 0 to 10.7-12.9% at day 7, and stabilizing at 16.0-16.4% by day 80. They also had greater abundances of the other phyla, particularly at day 7. *Alphaproteobacteria* seemed to become enriched, increasing in relative abundance for the higher rhamnolipid doses. Perhaps the larger rhamnolipid doses of 30 and 100 CMC supressed and inhibited growth of the other phyla, or gave the *Alpha* subclass an advantage.

In addition to very similar relative abundance distributions at Day 7, the 30 and 100 CMC cases also had very similar TPH concentrations at Day 7. Their TPH levels began to diverge around day 14. Intriguingly, this was mirrored by their abundance distributions. By day 80, 30 CMC showed the community had begun to “recover” – the *Alpha* subclass decreased to 31.2% and the other phyla had increased,

resulting in a somewhat more diverse microbial community. The 100 CMC system demonstrated an overall *Proteobacteria* dominance decrease to 66.6%, while the *Alpha* subclass actually increased to 49.4%. The no nutrient system, similar to 100 CMC in TPH levels, qualitatively demonstrated a similar pattern with an increase of the Alpha subclass up to 32.8% by day 80.

Interestingly, rhamnolipids are most commonly produced by *Pseudomonas aeruginosa* (*Gammaproteobacteria*). All known rhamnolipid producing strains have been found in the phyla *Proteobacteria* (especially in the *Beta, Gamma* and *Delta* subclasses)*, Firmicutes* (gram-positives), and *Actinobacteria* (Kiran et al., 2016; Leitermann et al., 2010; Perfumo et al. 2010). Perhaps the members of the *Alpha* subclass present in the soil were more resistant to rhamnolipid antibacterial activity; alternatively they may have been also utilizing the rhamnolipids as a carbon source. Following this train of thought, it is possible that by day 14 enough rhamnolipids were degraded in the 30 CMC systems for them to begin performing like the 1 and 4 CMC systems, and allowing TPH degradation to increase.

This is especially interesting considering that higher biosurfactant doses lead presumably to a greater solubilization of entrapped hydrocarbons, but within the literature *Alphaproteobacteria* tend to decrease in relative abundance when exposed to a spike in hydrocarbons, as compared to *Gammaproteobacteria* which tends to increase (Lo Giudice et al., 2010).

2.3.4 Pure Culture Isolation

From Biopile B soil ten cultures were isolated as described in the Methods & Materials (Section 2.2.6). Flask cultures used BH media and sterile Bakken crude oil as the sole carbon source, while the agar plates used R-2A as the culture medium. The appearance and genus of the isolates are enumerated in Table 1. Note that isolates #1 and #4 are likely the same species, though not yet confirmed. The rest seem to be different individual species and/ or strains. Photos (Figures A8-A19) and greater details of potential species (Table A2) can be found in the Appendix.

Table 1 : Genus of Culture Isolates

	1	2	3	4	5
Appearance:	Small white	Red/pink	Red/orange	Creamy translucent	Tiny white
Phylum:	*Proteobacteria*	*Actinobacteria*	*Actinobacteria*	*Proteobacteria*	*Proteobacteria*
Class:	*Gamma Proteobacteria*	*Actinobacteria*	*Actinobacteria*	*Gamma Proteobacteria*	*Gamma Proteobacteria*
Genus:	*Pseudomonas*	*Gordonia*	*Gordonia*	*Pseudomonas*	*Pseudomonas*
Species:	*Pseudomonas sp.*	*Gordonia sp.*	*Gordonia sp.*	*Pseudomonas sp.*	*Pseudomonas sp.*

	6	7	8	9	10
Appearance:	Red	White bumpy	Yellow	White smooth	Filamentous
Phylum:	*Firmicutes*	*Firmicutes*	*Proteobacteria*	*Proteobacteria*	*Actinobacteria*
Class:	*Bacilli*	*Bacilli*	*Alpha Proteobacteria*	*Gamma Proteobacteria*	*Actinobacteria*
Genus:	*Bacillus*	*Bacillus*	*Sphingomonas*	*Pseudomonas*	*Nocardia*
Species:	*Bacillus sp.*	*Bacillus sp.*	*Sphingomonas sp.*	*Pseudomonas sp.*	*Nocardia sp.*

Soil bacteria populations of hydrocarbon degraders are generally dominated by the genera *Pseudomonas, Bacillus, Sphingomonas* and *Actinobacteria* (Perfumo et al. 2010). Unsurprisingly, these were the exact genera of the pure cultures isolated from Biopile soil B. Isolates #1, 4, 5, 9 were *Pseudomonas ssp.*; #2 & 3 were *Gordonia ssp.*; #10 was a *Nocardia sp.*; #6 & 7 were *Bacillus ssp.*; and #8 was a *Sphingomonas sp*.

Pseudomonas species are well known as hydrocarbon degraders; they are also capable of metabolizing a wide variety of xenobiotic and naturally occurring compounds. Research on the metabolic capability of *P. putida* KT2440, for instance, has found that it is able to grow in minimal medium using any of the following as carbon and energy sources: benzoate, 4-hydroxybenzoate, benzylamine, phenylacetate, phenylalanine, tyrosine, phenylethylamine, phenylhexanoate, phenylheptanoate, penyloctanoate, coniferyl alcohol, 4-coumarate, 4-hydroxyphenylpropionate, ferulate, caffeate, vanillate, nicotinate, and quinate (Palleroni et al., 2010). Additionally, although rhamnolipids are most commonly produced by *P. aeruginosa*, other *Pseudomonas* species have also been identified as rhamnolipid producers, notably *P.*

chlororaphis, P. putida, P. oleovorans, P. cruciviae, P. fluorescens, P. boreopolis, P. stutzeri, and *P. pseudomallei* (Leitermann et al., 2010).

The *Nocardia* and *Gordonia* genera both belong to the *Nocardiaceae* family of the *Actinobacteria* class. Several species of the genus *Gordonia* are known for degrading different hydrocarbons and they are sometimes also additionally involved with removing sulphur or nitrogen from petroleum. Like most hydrocarbon degraders, they are capable of producing biosurfactants; the type depends on the strain and the substrate. Hydrocarbon degraders capable of desulfurization include *Gordonia alkanivorans, Gordonia amicalis, Gordonia nitida,* and *Gordonia desulfuricans; Gordonia namibiensis* is capable of nitrogen removal. Likewise, a number of *Nocardia spp.* have been observed hydrocarbon degraders at oil-contaminated sites undergoing bioremediation. Some of these include *N. otitiscaviarum, N. hydrocarbonoxydans, N. uniformis, N. simplex, N. asteroids, N. transvalensis,* and *N. cyriacigeorgica* (Kampfer, 2010).

The *Bacillus* genus contains a number of hydrocarbon degraders, some of which are able to grow at relatively high salinities. *Bacilli* are generally producers of lipopeptides (surfactin, iturin and fengycin); the exact type produced again depending on strain and substrate. For example, two strains of *B. subtilis* grown on different substrates, starch and hydrocarbons, were found to produce iturin and surfactin, respectively (Perfumo et al. 2010). Meanwhile though *Bacillus cereus* is capable of producing rhamnolipids, rhamnolipids are antimicrobial against *Bacillus subtilis* (Leitermann et al., 2010; Wecke et al., 2011).

Members of the *Sphingomonas* genus are gram-negative, aerobic, non-sporulating, rod-shaped organisms belonging to the class of *Alphaproteobacteria*. The isolate found (#8) had the deep yellow color common to many *Sphingomonas* colonies. They are well known for degrading a range of mono- and polycyclic aromatic hydrocarbons (PAHs), but are also being identified in increasing numbers within

non-contaminated soil. Many, though not all, sphingomonads secrete sphingan exopolysaccharide (EPS) biosurfactants (Kertesz and Kawasaki, 2010).

Most of the isolates were found able to grow in liquid cultures of minimal BH media dosed with 600mg/L (30 CMC) sterilized rhamnolipids as the sole carbon source. The growth of the cultures for isolates #1–9 was monitored by measuring the absorbance, or optical density, of samples in a spectrophotometer at a 600nm wavelength (OD_{600}). *Nocardia sp.* #10 was strongly adhered to the bottom of the tube and not included in the growth curve. As seen in Figure 7, all of the isolates except for *Bacillus sp.* #6 showed growth in the minimal media, reaching a peak and stationary phase. Note that sterile BH media with biosurfactants had negligible OD_{600} compared against DI water. The decrease in OD after peak noted in some of the curves was the death phase of the bacterial population. Because the Lac Mégantic isolates were relatively slow growing, data points were collected until 290 hours had passed, and so captured the death phase as well.

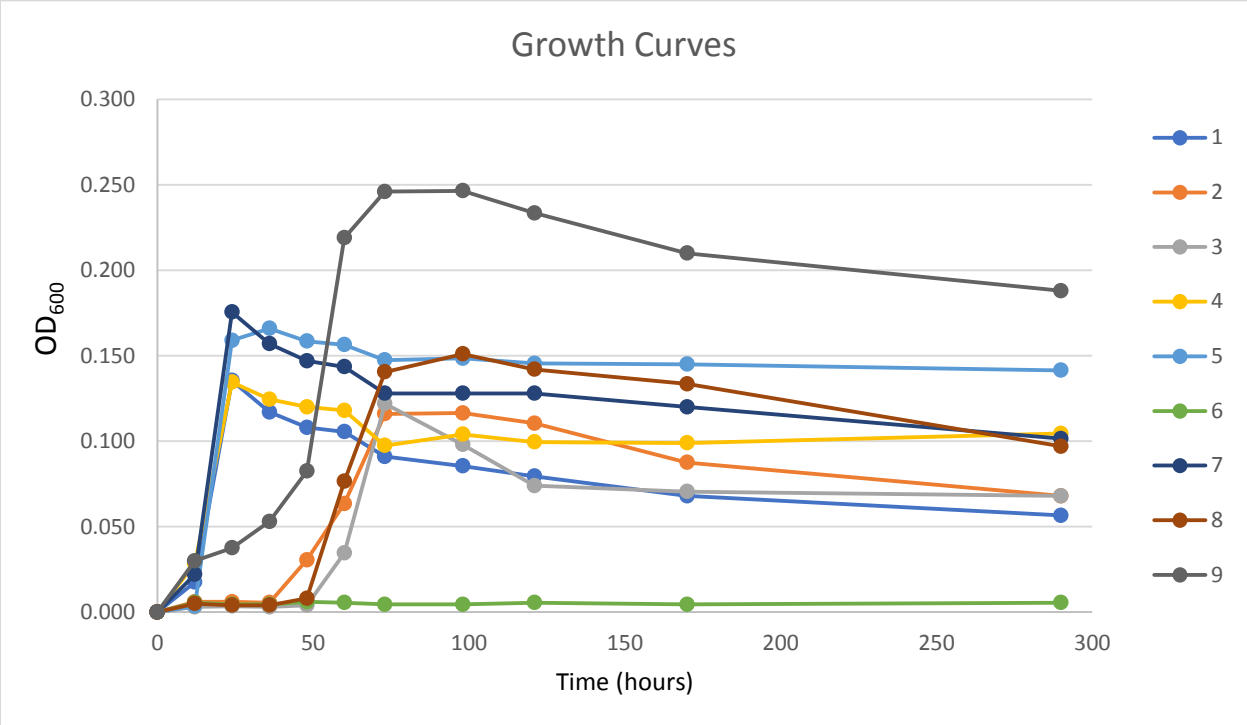


Figure 7 : OD_{600} growth curves of isolates using rhamnolipids as sole carbon source

The decrease of OD_{600} during the death phase is primarily caused by cells undergoing lysis; when cell membranes are damaged, the cell contents leak out, decreasing the refractive index, and therefore OD_{600}. Surviving cells can use the released nutrients; in some cases, especially during starvation, cells do this on purpose, killing and "cannibalising" sibling cells to feed on their nutrients (Mytilinaios et al., 2012; Rice and Bayles, 2008). Furthermore, as carbon is depleted in media with a single carbon source, cells can also reduce in volume, and filaments divide into smaller cells (Stevenson et al., 2016).

The confirmed growth of the isolates using rhamnolipids as the sole carbon source suggests that there may have been preferential degradation of biosurfactants over residual hydrocarbons in the weathered soils. This could be a possible explanation particularly for the community data points where *Alphaproteobacteria* (including *Sphingomonas*) increased with higher rhamnolipid concentrations while at the same time displaying lower TPH biodegradation rates.

2.4 Conclusions

Biosurfactant application to microcosms with biopile soil A resulted in higher total petroleum hydrocarbon (TPH) reductions for rhamnolipids, sophorolipids and surfactin. However, there was not a significant difference between the systems with and those without nutrients. For the longer term microcosm experiments using biopile soil B, the controls and the four different concentrations of rhamnolipids produced variable results. A large difference was noted between the control systems with and without nutrients. The control system with nutrients produced one of the best overall reduction trends, and the system without nutrients had one of the lowest. The low and medium biosurfactant dose systems of 1, 4 and 30 CMC had comparable performances to the control with nutrient, though not necessarily better results. The highest biosurfactant dose, 100 CMC, had very low TPH reduction, with a similar trend to the control without nutrients. The different results between biopile soil A and B, as well as between the different concentrations, were most likely due to the biosurfactant influence on the

microbial community during the process of biodegradation. Data from the microbial community analysis of the biopile soil B microcosms demonstrated that *Proteobacteria* dominated overall throughout the 80 days, though the *Gamma* subclass tended to increase early and then decrease as biodegradation plateaued. Generally, with a few exceptions, higher microbial diversity was found in the better performing TPH reducing systems. The *Alpha* subclass seemed to become enriched and increase in relative abundance with higher rhamnolipid doses. Hydrocarbon degrading bacteria isolated from the biopile B soil were found capable of utilizing rhamnolipids as a sole carbon source. The possibility exists that the biosurfactants were preferentially degraded over hydrocarbons within the soil B biodegradation systems.

2.5 Acknowledgements

Assistance for the project from the following are gratefully acknowledged: financial support from Hydro-Quebec through their Masters in Engineering Award (part of the McGill Masters Top-Up Award); financial support from Fonds de recherche Nature et technologies (bourse de maitrise en recherche) for a fellowship; Natural Sciences and Engineering Research Council as well as other industrial and institutional partners for their financial support for the project; Englobe for providing the soil samples and their research collaboration, Maxxam Analytique for their analytical assistance, and the research collaboration of Prof. Rosa Galvez and her team at Université Laval .

2.6 Literature Cited

Adrion, A., Nakamura, J., Shea, D., & Aitken, M.D. (2016). "Screening Nonionic Surfactants for Enhanced Biodegradation of Polycyclic Aromatic Hydrocarbons Remaining in Soil After Conventional Biological Treatment." *Environmental Science & Technology*, **50**(7), 3838−3845.

Akbari, A. and S. Ghoshal (2014). "Pilot-scale bioremediation of a petroleum hydrocarbon-contaminated clayey soil from a sub-Arctic site." *Journal of Hazardous Materials* **280**: 595-602.

Akbari, A. and S. Ghoshal (2015). "Bioaccessible Porosity in Soil Aggregates and Implications for Biodegradation of High Molecular Weight Petroleum Compounds." *Environmental Science & Technology* **49**(24): 14368-14375.

Alfred, DeGray, Edwards, Hedrick, Klemme, Rogers,... Hodge. (1963). "Proposed Procedures for Microbiological Examination of Fuels." Society for Industrial Microbiology (SIM) Committee on Microbiological Deterioration of Fuels: Special Publications, **1**:1-35.

Amézcua-Vega, C., Poggi-Varaldo, H.M., Esparza-Garcia, F., Rios-Leal, E., & Rodriguez-Vazquez, R. (2007). "Effect of culture conditions on fatty acids composition of a biosurfactant produced by *Candida ingens* and changes of surface tension of culture media." *Bioresource Technology* **98**(1):237–240.

Arima, K., Kakinuma, A., and Tamura, G. (1968). "Surfactin, a crystalline peptide lipid surfactant produced by *Bacillus subtilis*: Isolation, characterization and its inhibition of fibrin clot formation." *Biochemical and Biophysical Research Communications*, **31**(3):488–494.

Arino, S., Marchal, R., and Vandecasteele, J.P. (2008). "Involvement of a rhamnolipid-producing strain of *Pseudomonas aeruginosa* in the degradation of polycyclic aromatic hydrocarbons by a bacterial community." *Journal of Applied Microbiology* **84**(5):769–776

Ayed, H.B., Jemil, N., Maalej, H., Bayoudh, A., Hmidet, N., Nasri, M. (2015). "Enhancement of solubilization and biodegradation of diesel oil by biosurfactant from *Bacillus amyloliquefaciens* An6." *International Biodeterioration & Biodegradation*, **99**: 8-14.

Benincasa, M., Abalos, A., Oliveira, I., and Manresa, A. (2004). "Chemical structure, surface properties and biological activities of the biosurfactant produced by *Pseudomonas aeruginosa* LBI from soapstock." Antonie Van Leeuwenhoek International Journal G, **85**(1):1–8.

Bezza, F.A., and Nkhalambayausi-Chirwa, E.M. (2015). "Desorption kinetics of polycyclic aromatic hydrocarbons (PAHs) from contaminated soil and the effect of biosurfactant supplementation on the rapidly desorbing fractions." *Biotechnology & Biotechnological Equipment*, **29**(4): 680-688.

Bordoloi, N. K. and Konwar, B. K. (2009). "Bacterial biosurfactant in enhancing solubility and metabolism of petroleum hydrocarbons." *Journal of Hazardous Materials* **170**(1): 495-505.

Bushnell, L. D., and Haas, H. F. (1941). "The Utilization of Certain Hydrocarbons by Microorganisms". *Journal of Bacteriology*, **41**(5): 22.

Cappello, S., Crisari, A., Hassanshahian, M., Genovese, M., Santisi, S. & Yakimov, M. (2012). “Effect of a Bioemulsificant Exopolysaccharide (EPS_{2003}) on Abundance and Vitality of Marine Bacteria.” *Water, Air and Soil Pollution*, **223**(7): 3903-3909.

Centre d’expertise en analyse environnementale du Québec CEAEQ, (2016). « Détermination des hydrocarbures pétroliers (C10 à C50): dosage par chromatographie en phase gazeuse couplée à un étecteur àionisation de flamme. » In MA. 400-HYD. 1.1. Rev. 3; Ministère du Développement durable, de l’Énvironnement, et Lutte contre les changements climatiques du Québec,; p 17.

Chang, W., Akbari, A., Snelgrove, J., Frigon, D., & Ghoshal, S. (2013). "Biodegradation of petroleum hydrocarbons in contaminated clayey soils from a sub-arctic site: The role of aggregate size and microstructure." *Chemosphere* **91**(11): 1620-1626.

Chikere, C. B., Surridge, K., Okpokwasili, G. C., Cloete, T. E., (2012). "Dynamics of indigenous bacterial communities associated with crude oil degradation in soil microcosms during nutrient-enhanced bioremediation." *Waste Management & Research* **30**(3): 225-236.

Chrzanowski, Ł., Ławniczak, Ł., & Czaczyk, K. (2012). “Why do microorganisms produce rhamnolipids?” *World Journal of Microbiology and Biotechnology*, **28**(2): 401-419.

Congiu, E., Parsons, J.R., & Ortega-Calvo, J.J. (2015). “Dual partitioning and attachment effects of rhamnolipid on pyrene biodegradation under bioavailability restrictions.” *Environmental Pollution*, **205**: 378-384.

Das, K., and Mukherjee, A.K. (2007). “Differential utilization of pyrene as the sole source of carbon by *Bacillus subtilis* and *Pseudomonas aeruginosa* strains: role of biosurfactants in enhancing bioavailability.” *Journal of Applied Microbiology,* **102**(1):195–203.

de la Cueva, S. C., Rodriguez, C. H., Cruz, N. O. S., Contreras, J. A. R., & Miranda, J. L. (2016). "Changes in Bacterial Populations During Bioremediation of Soil Contaminated with Petroleum Hydrocarbons." *Water, Air, and Soil Pollution* **227**(3): 12.

Dhamodharan, D. & Jayapriya, J. (2015). “Integrated Approach for Polycyclic Aromatic Hydrocarbon Solubilization from the Soil Matrix to Enhance Bioremediation” *Bioremediation Journal* **19** (4): 287-295.

Dhanarajan G. and Sen R. (2014). “Amphiphilic Molecules of Microbial Origin” in *Biosurfactants: research trends and applications.* Ed. Mulligan, Catherine N., Ackmez Mudhoo, and Sanjay K. Sharma. Boca Raton, FL: CRC Press.

Galvez-Cloutier, R., Guesdon, G., & Fonchain, A. (2014). "Lac-Mégantic: Analysis of environmental emergency, trials and evaluation of impacts." *Canadian Journal of Civil Engineering* **41**(6): 531-539.

Greer, C.W., Whyte, L.G., and Niederberger, T.D. (2010). “Microbial Communities in Hydrocarbon-Contaminated Temperate, Tropical, Alpine, and Polar Soils.” *Handbook of Hydrocarbon and Lipid Microbiology* **51**: 2313-2328.

Haba, E., Pinazo, A., Jauregui, O., Espuny, M.J., Infante, M.R., and Manresa, A. (2003). "Physicochemical characterization and antimicrobial properties of rhamnolipids produced by *Pseudomonas aeruginosa* 47T2 NCBIM 40044." *Biotechnology and Bioengineering* **81**(3):316–322.

Hazra, C., Kundu, D., & Chaudhari, A., (2012). "Biosurfactant-assisted bioaugmentation in bioremediation". *Microorganisms in Environmental Management*. Springer, Netherlands, pp.631–664.

Kampfer, P. (2010). "Actinobacteria." *Handbook of Hydrocarbon and Lipid Microbiology* **16**: 1819-1838.

Kavitha, V., Mandal, A.B., & Gnanamani, A. (2014). "Microbial biosurfactant mediated removal and/or solubilization of crude oil contamination from soil and aqueous phase: An approach with *Bacillus licheniformis* MTCC 5514." *International Biodeterioration & Biodegradation*, **94**: 24-30.

Kertesz, M. A., and Kawasaki, A. (2010). "Hydrocarbon-Degrading Sphingomonads: Sphingomonas, Sphingobium, Novosphingobium, and Sphingopyxis." *Handbook of Hydrocarbon and Lipid Microbiology* **2**: 1693-1705.

Kim, J.S., and Crowley, D.E. (2007). "Microbial diversity in natural asphalts of the Rancho La Brea Tar Pits." *Applied and environmental microbiology* **73**(14): 4579-4591.

Kim, S.-J., and Kwon, K. (2010). "Marine, Hydrocarbon- Degrading Alphaproteobacteria." *Handbook of Hydrocarbon and Lipid Microbiology* **3**: 1707-1714.

Kiran, G. S., Ninawe, A.S., Lipton, A.N., Pandian, V., and Selvin, J. (2016). "Rhamnolipid biosurfactants: evolutionary implications, applications and future prospects from untapped marine resource." Critical Reviews in Biotechnology **36**(3): 399-415.

Koch, A.K., Kappeli, O., Feichter, A., Reiser, J. (1991). "Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants." *Journal of Bacteriology* **173**(13):4212–4219.

Labbé, D., Margesin, R., Schinner, F., Whyte, L.G., Greer, and C.W. (2007). "Comparative phylogenetic analysis of microbial communities in pristine and hydrocarbon contaminated Alpine soils." FEMS Microbiology Ecology **59**(2): 466–475.

Ławniczak, Ł., Marecik, R., and Chrzanowski, Ł. (2013). "Contributions of biosurfactants to natural or induced bioremediation." *Applied Microbiology and Biotechnology*, **97**(6): 2327-2339.

Lee, K., Dunfield, P., and Stott, M. (2014). "The Phylum Armatimonadetes." *The Prokaryotes – Other Major Lineages of Bacteria and the Archaea* **32**: 447-458.

Leitermann, F., Walter, V., Syldatk, C., and Hausmann R. (2010). "Rhamnolipids." *Handbook of Hydrocarbon and Lipid Microbiology* **49**: 3037-3051.

Li, F., Zhu, L., and Zhang, D. (2014). "Effect of surfactant on phenanthrene metabolic kinetics by *Citrobacter* sp. SA01." *Journal of Environmental Sciences*, **26**(11): 2298-2306.

Lo Giudice, A., Bruni, V., De Domenico, M., and Michaud, L. (2010). “Psychrophiles - Cold-Adapted Hydrocarbon-Degrading Microorganisms.” *Handbook of Hydrocarbon and Lipid Microbiology* **22**: 1897-1921.

Maier, R.M. and Soberón-Chávez, G. (2000). “*Pseudomonas aeruginosa* rhamnolipids: Biosynthesis and potential applications.” *Applied Microbiology and Biotechnology*, **54**(5):625–633.

Malavenda, R., Rizzo, C., Michaud, L., Gerce, B., Bruni, V., Syldatk, C., Hausmann, R., & Lo Giudice, A. (2015). "Biosurfactant production by Arctic and Antarctic bacteria growing on hydrocarbons." *Polar Biology* **38**(10): 1565-1574.

Martienssen, M. & Schirmer, M.. (2007). “Use of Surfactants to Improve the Biological Degradation of Petroleum Hydrocarbons in a Field Site Study.” *Environmental Technology*, **28**(5): 573-582.

McKew, B.A., Coulon, F., Osborn, A.M., Timmis, K.N., and McGenity, T.J. (2007). “Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary.” Environmental Microbiology **9**(1): 165–176.

Mejia-Avendano, S., Munoz, G., Duy, S. V., Desrosiers, M., Benoit, P., Sauve, S., & Liu, J. (2017). "Novel Fluoroalkylated Surfactants in Soils Following Firefighting Foam Deployment During the Lac-Megantic Railway Accident." *Environmental Science & Technology* **51**(15): 8313-8323.

Militon, C., Boucher, D., Vachelard, C., Perchet, G., Barra, V., Troquet, J., Peyretaillade, E., and Peyret, P. (2010). “Bacterial community changes during bioremediation of aliphatic hydrocarbon-contaminated soil.” *FEMS Microbiology Ecology* **74**(3): 669-681.

Ministère du Développement durable, Environnement, Faune et Parcs (MDDEFP) (2014). « Tableau-Synthèse : Estimation au 22 juin 2014 des quantités de pétrole brut léger impliquées dans l’accident à Lac-Mégantic » http://www.mddefp.gouv.qc.ca/lac-megantic/tableau_synthese/2014078-tableau-synthese-petrole.pdf

Mulligan, Catherine N., Ackmez Mudhoo, and Sanjay K. Sharma. (2014). *Biosurfactants: research trends and applications.* Boca Raton, FL: CRC Press.

Mulligan, C.N., Yong, R.N., and Gibbs, B.F. (2001). “Surfactant-enhanced remediation of contaminated soil: A review.” *Engineering Geology* **60**(1-4): 371-380.

Mytilinaios, I., Salih, M., Schofield, H.K., and Lambert, R.J.W. (2012). "Growth curve prediction from optical density data." *International Journal of Food Microbiology* **154**(3): 169-176.

Nikolopoulou, M. and Kalogerakis, N. (2008). "Enhanced bioremediation of crude oil utilizing lipophilic fertilizers combined with biosurfactants and molasses." *Marine Pollution Bulletin* **56**(11): 1855-1861.

Owsianiak, M., Szulc, A., Chrzanowski, Ł., Cyplik, P., Bogacki, M., Olejnik-Schmidt, A.K., and Heipieper, H.J. (2009). “Biodegradation and surfactant-mediated biodegradation of diesel fuel by 218 microbial consortia are not correlated to cell surface hydrophobicity.” *Applied Microbiology and Biotechnology* **84**(3):545–553.

Pacwa-Plociniczak, M., Plaza, G. A., Piotrowska-Seget, Z., and Cameotra, S. S. (2011). "Environmental Applications of Biosurfactants: Recent Advances." *International Journal of Molecular Sciences* **12**(1): 633-654.

Palleroni, N.J., Pieper, D.H., and Moore, E.R.B. (2010). "Microbiology of Hydrocarbon-Degrading Pseudomonas." *Handbook of Hydrocarbon and Lipid Microbiology* 1**2**: 1787-1798.

Pamp, S.J., and Tolker-Nielsen, T. (2007). "Multiple roles of biosurfactants in structural biofilm development by *Pseudomonas aeruginosa*." *Journal of Bacteriology* **189**(6):2531–2539.

Parales, R.E. (2010). "Hydrocarbon Degradation by Betaproteobacteria." *Handbook of Hydrocarbon and Lipid Microbiology* **4**: 1715-1724.

Pekin, G., Vardar-Sukan, F., and Kosaric, N. (2005). "Production of sophorolipids from *Candida bombicola* ATCC 22214 using Turkish corn oil and honey." *Engineering in Life Sciences*, **5**(4):357–362.

Perfumo, A., Smyth, T.J.P., Marchant, R., and Banat, I.M. (2010). "Production and Roles of Biosurfactants and Bioemulsifiers in Accessing Hydrophobic Substrates." *Handbook of Hydrocarbon and Lipid Microbiology* **47**: 1501-1512.

Pignatello, J.J., "Bioavailability of Contaminants in Soil" in Singh, A., Kuhad, R. C., & Ward, O. P. (2009). *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 35-64

Prince, R.C., Gramain, A., McGenity, T.J. (2010) "Prokaryotic hydrocarbon degraders." *Handbook of Hydrocarbon and Lipid Microbiology* **19**: 1669-1692.

Raaijmakers, J.M., De Bruijn, I., Nybroe, O., and Ongena, M. (2010). "Natural functions of lipopeptides from Bacillus and Pseudomonas more than surfactants and antibiotics." *FEMS Microbiology Reviews*, **34**(6): 1037-1062.

Rice, K.C. and Bayles, K.W. (2008). "Molecular control of bacterial death and lysis." *Microbiology and Molecular Biology Reviews* **72**(1): 85-109.

Singh, A., Kuhad, R.C., and Ward, O.P. (2009). "Biological Remediation of Soil: An Overview of Global Market and Available Technologies" in Singh, A., Kuhad, R. C., & Ward, O. P. (2009). *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 1-17

Singh, A., & Ward, O. P. (2004)."Biotechnology and Bioremediation - An Overview" in Singh, A., & Ward, O. P. *Biodegradation and Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 1-17

Singh, A., Van Hamme, J.D., Ward, O.P., (2007). "Surfactants in microbiology and biotechnology : part 2. Application aspects." *Biotechnology Advances* **25**(1): 99–121.

Souza, E.C., Vessoni-Penna, T.C., and Oliveira, R.P.D. (2014). "Biosurfactant-enhanced hydrocarbon bioremediation: An overview." *International Biodeterioration & Biodegradation* **89**: 88-94.

Stevenson, K., McVey, A.F., Clark, I.B.N., Swain, P.S., and Pilizota, T. (2016). "General calibration of microbial growth in microplate readers." *Scientific Reports* **6**(38828): 1-7.

Surridge, A.K.J., Wehner, F.C., and Cloete, T.E. (2009). "Bioremediation of Polluted Soil" in Singh, A., Kuhad, R. C., & Ward, O. P. (2009). *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp.103-117

Sutton, N.B., Maphosa, F., Morillo, J.A., Al-Soud, W.A., Langenhoff, A.A, Grotenhuis, T., Rijnaarts, H.H., and Smidt, H. (2013). "Impact of long-term diesel contamination on soil microbial community structure." *Applied and Environmental Microbiology* **79**(2): 619-630.

Van Hamme, J.D. (2004). "Bioavailability and Biodegradation of Organic Pollutants - A Microbial Perspective" in Singh, A., & Ward, O. P. *Biodegradation and Bioremediation*. Springer-Verlag Berlin Heidelberg. pp. 37-56

Van Hamme, J.D., Singh, A., and Ward, O.P. (2006). "Physiological aspects. Part I in a series of papers devoted to surfactants in microbiology and biotechnology." *Biotechnology Advances* **24**(6):604–620.

Van Hamme, J.D., and Urban, J., (2009). "Biosurfactants in Bioremediation" in Singh, A., Kuhad, R. C., & Ward, O. P. *Advances in Applied Bioremediation*. Springer-Verlag Berlin Heidelberg. pp.73-86

Vinas, M., Sabaté, J., Espuny, M.J., and Solanas, A.M. (2005). "Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil." Applied Environmental Microbiology **71**(11): 7008–7018.

Vipulanandan, C., & Ren, X.P. (2000). "Enhanced solubility and biodegradation of naphthalene with biosurfactant." *Journal of Environmental Engineering – ASCE*, **126**(7): 629-634.

Wecke T., Bauer T., Harth H., Mader U., and Mascher T. (2011). "The rhamnolipid stress response of *Bacillus subtilis*." FEMS Microbiology Letters **323**(2):113-23.

Xia, W., Du, Z., Cui, Q., Dong, H., Wang, F., He, P., and Tang, Y. (2014). "Biosurfactant produced by novel *Pseudomonas sp.* WJ6 with biodegradation of n-alkanes and polycyclic aromatic hydrocarbons." *Journal of Hazardous Materials* **276**: 489–498.

Zhang, Y., and Miller, R.M. (1994). "Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane." *Applied and Environmental Microbiology* **60**(6):2101–2106.

Zhang, Y., and Miller, R.M. (1995). "Effect of rhamnolipid (biosufactant) structure on solubilization and biodegradation of n-alkanes." *Applied and Environmental Microbiology* **61**(6):2247–2251.

Zhu, H., and Aitken, M.D., (2010). "Surfactant-enhanced desorption and biodegradation of polycyclic aromatic hydrocarbons in contaminated soil". *Environmental Science & Technology* **44**(19): 7260–7265.

Chapter 3 – Contribution to New Knowledge

Both the total petroleum hydrocarbon mineralization and the microbial community analysis data provide useful insights towards future research and bioremediation feasibility assessment.

1) Efforts to design biosurfactant enhanced bioremediation systems for hydrocarbon contaminated soils should be done on a case by case basis. Even two soils taken from the same site but at different sampling locations may respond differently. While biosurfactants may improve TPH reduction for a soil from one location, it is not necessarily the same case for another soil from the same original site but different location.

2) In certain bioremediation cases, it could be more beneficial not to add biosurfactants to achieve the highest TPH reduction. However, more research will be required to determine the exact mechanisms that lead to degradation inhibition, although it is likely linked to the effect on the microbial community.

3) In the presence of biosurfactants, the community of microorganisms changed and shifted over time. Although rhamnolipids are primarily produced by *Pseudomonas aeruginosa (Gammaproteobacteria)*, high doses seemed to enrich *Alphaproteobacteria* populations of the environmental consortia. Systems with larger *Alphaproteobacteria* populations also tended to have lower degradation rates, though there were exceptions. Further research is needed to investigate this aspect, and it would be cautious to avoid drawing definite and generalized assumptions.

4) Finally, potential lines of future inquiry were found. The possibility was opened that within the microcosms of environmentally aged soils the biosurfactants were being preferentially degraded over the hydrocarbons for the soil B biodegradation systems. It would be interesting to directly assess and compare rhamnolipid versus hydrocarbon degradation, either with pure culture isolates or in mixed community microcosms.

Appendix

Biopile A TPH Chromatograms

The following are representative samples; all of the chromatograms have not been included.

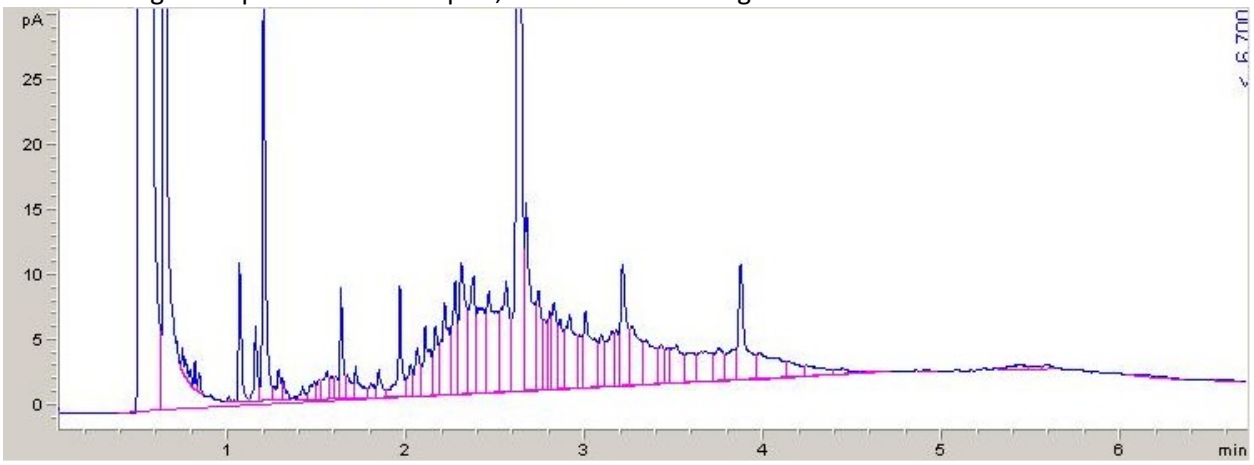


Figure A1 : Rhamnolipid w/o nutrient – unaltered baseline

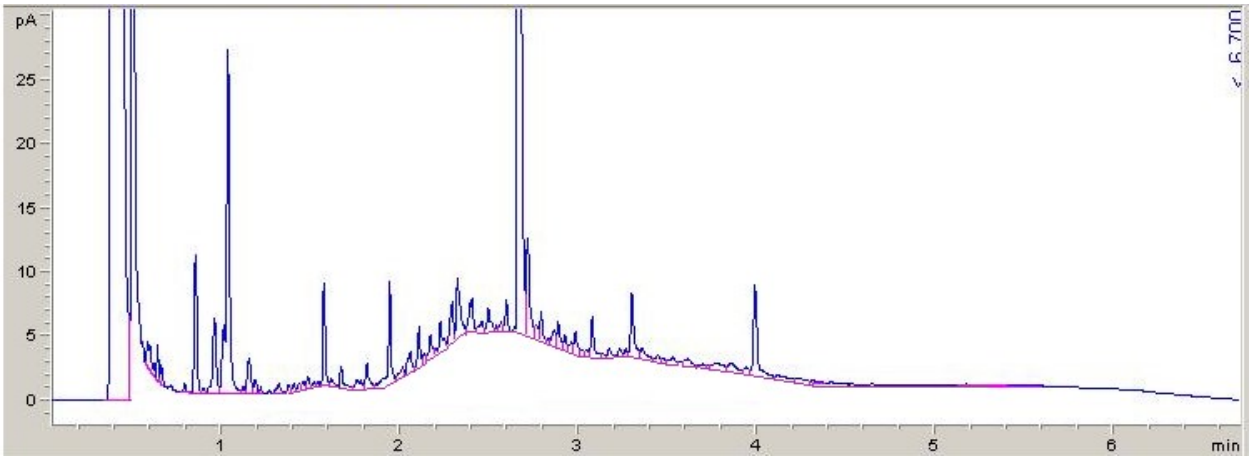


Figure A2 : Rhamnolipid w/o nutrient – resolved peaks

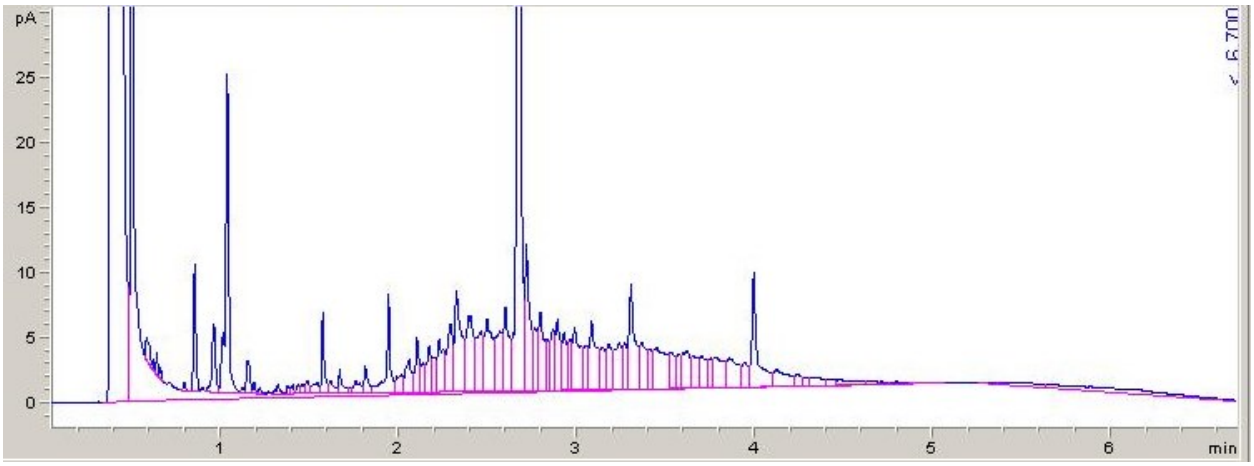


Figure A3 : Rhamnolipid w/ nutrient – unaltered baseline

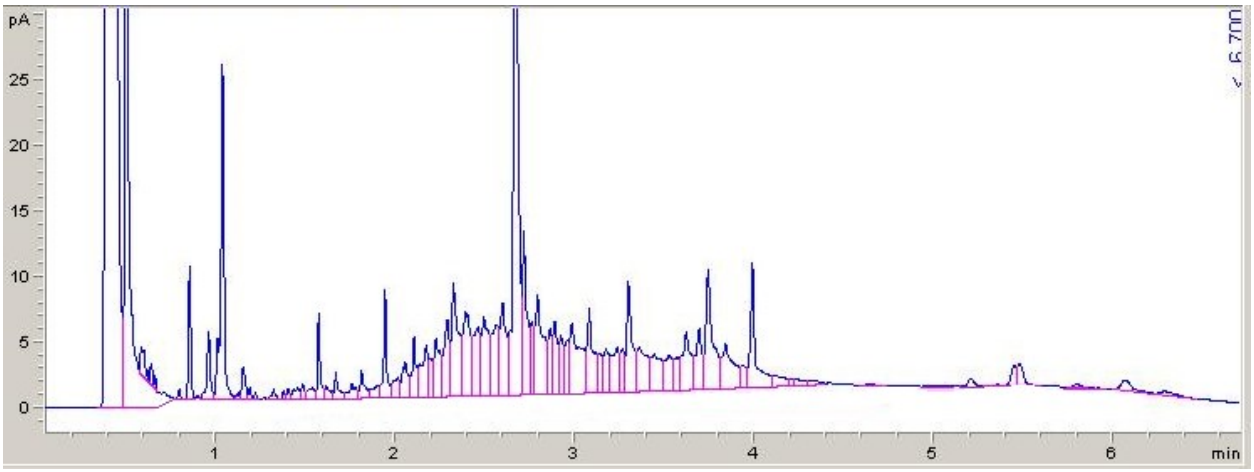


Figure A4 : Sophorolipid w/ nutrient – unaltered baseline

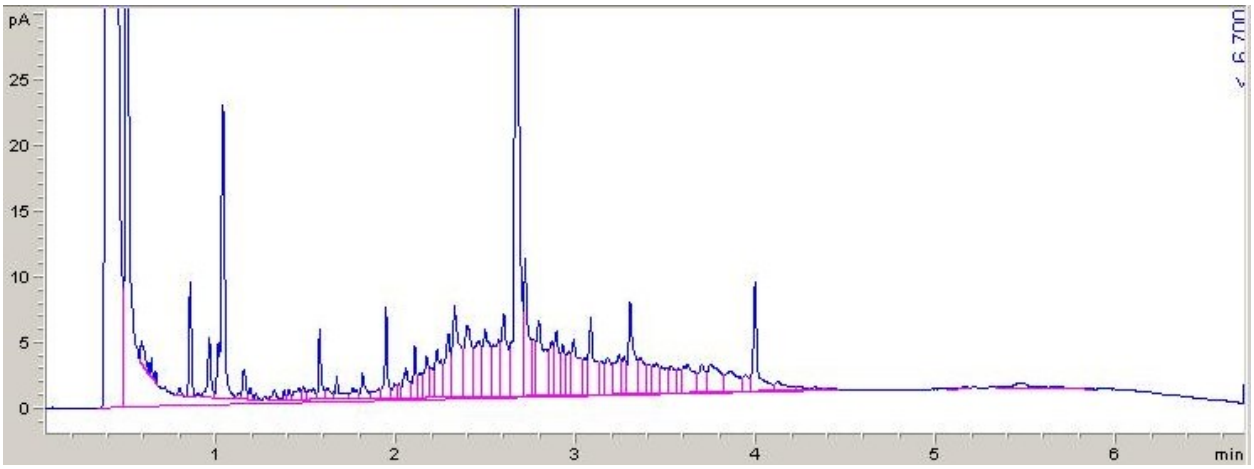


Figure A5 : Surfactin w/ nutrient – unaltered baseline

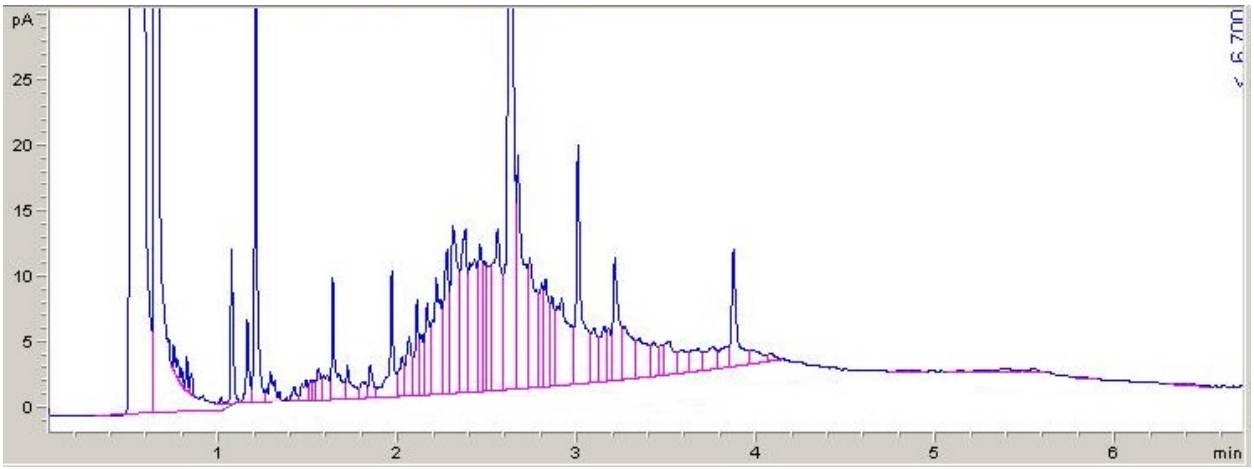


Figure A6 : Unamended control – unaltered baseline

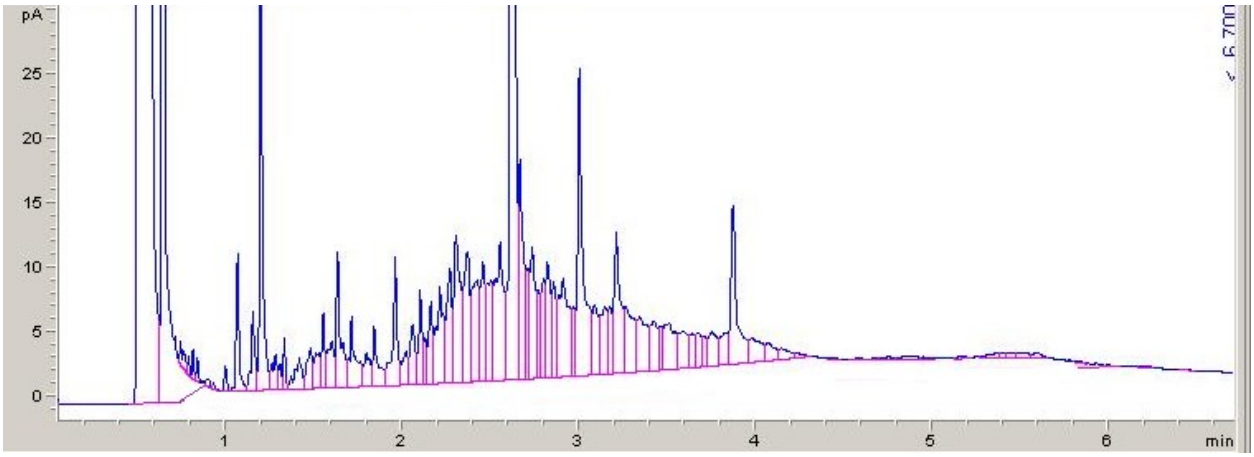


Figure A7 : Amended control – unaltered baseline

Table A1 : Biopile A soil t-test results

	P-value (One-tailed)	Statistically significant difference
Unamended Control vs Amended Control	0.371	No
Unamended Control vs Rhamnolipids w/o Nutrients	0.042	Yes
Unamended Control vs Rhamnolipids w/ Nutrients	0.049	Yes
Unamended Control vs Sophorolipids w/ Nutrients	0.032	Yes
Unamended Control vs Surfactin w/ Nutrients	0.017	Yes
Rhamnolipids w/o Nutrients vs Rhamnolipids w/ Nutrients	1.000	No

Table A2 : Potential Species of Isolates

	1*	2	3	4*
Appearance:	Small white	Red/pink	Red/orange	Creamy translucent
Phylum:	*Proteobacteria*	*Actinobacteria*	*Actinobacteria*	*Proteobacteria*
Class:	*Gamma Proteobacteria*	*Actinobacteria*	*Actinobacteria*	*Gamma Proteobacteria*
Genus:	*Pseudomonas*	*Gordonia*	*Gordonia*	*Pseudomonas*
Possible Species:	*Pseudomonas sp.*	*Gordonia sp.*	*Gordonia sp.*	*Pseudomonas sp.*
	P. mandelii	*G. amicalis*	*G. amicalis*	*P. mandelii*
	P. arsenicoxydans	*G. rubripertincta*	*G. rubripertincta*	*P. arsenicoxydans*
	P. ficuserectae	*G. alkanivorans*	*G. alkanivorans*	*P. ficuserectae*
	P. fluorescens	*G. namibiensis*	*G. namibiensis*	*P. fluorescens*
	P. prosekii	*G. westfalica*	*G. westfalica*	*P. prosekii*
	P. lini	*G. neofelifaecis*		*P. lini*
	P. monteilii			*P. monteilii*

	5	6	7	8
Appearance:	Tiny white	Red	White bumpy	Yellow
Phylum:	*Proteobacteria*	*Firmicutes*	*Firmicutes*	*Proteobacteria*
Class:	*Gamma Proteobacteria*	*Bacilli*	*Bacilli*	*Alphaproteobacteria*
Genus:	*Pseudomonas*	*Bacillus*	*Bacillus*	*Sphingomonas*
Possible Species:	*Pseudomonas sp.*	*Bacillus sp.*	*Bacillus sp.*	*Sphingomonas sp.*
	P. umsongensis	*B. aryabhattai*	*B. thuringiensis*	*Sphing. abikonense*
	P. migulae	*B. megaterium*	*B. cereus*	*Sphing. lactosutens*
	P. putida		*B. subtilis*	*Sphing. olei*
	P. baetica		*B. toyonensis*	*Sphing. soli*
	P. mandelii			*Sphing. rhizovicinum*

	9	10
Appearance:	White smooth	Filamentous
Phylum:	*Proteobacteria*	*Actinobacteria*
Class:	*Gamma Proteobacteria*	*Actinobacteria*
Genus:	*Pseudomonas*	*Nocardia*
Possible Species:	*Pseudomonas sp.*	*Nocardia sp.*
	Pseudomonas sp.A3	*N. asteroides*
	P. mandelii	*N. neocaledoniensis*
	P. arsenicoxydans	*N. thailandica*
	P. lini	*N. abscessus*
	P. ficuserectae	*N. sungurluensis*
	P. monteilii	*N. cyriacigeorgica*
	P. fluorescens	*N. rhizosphaerihabitans*
	P. prosekii	

Isolates Culture Photos

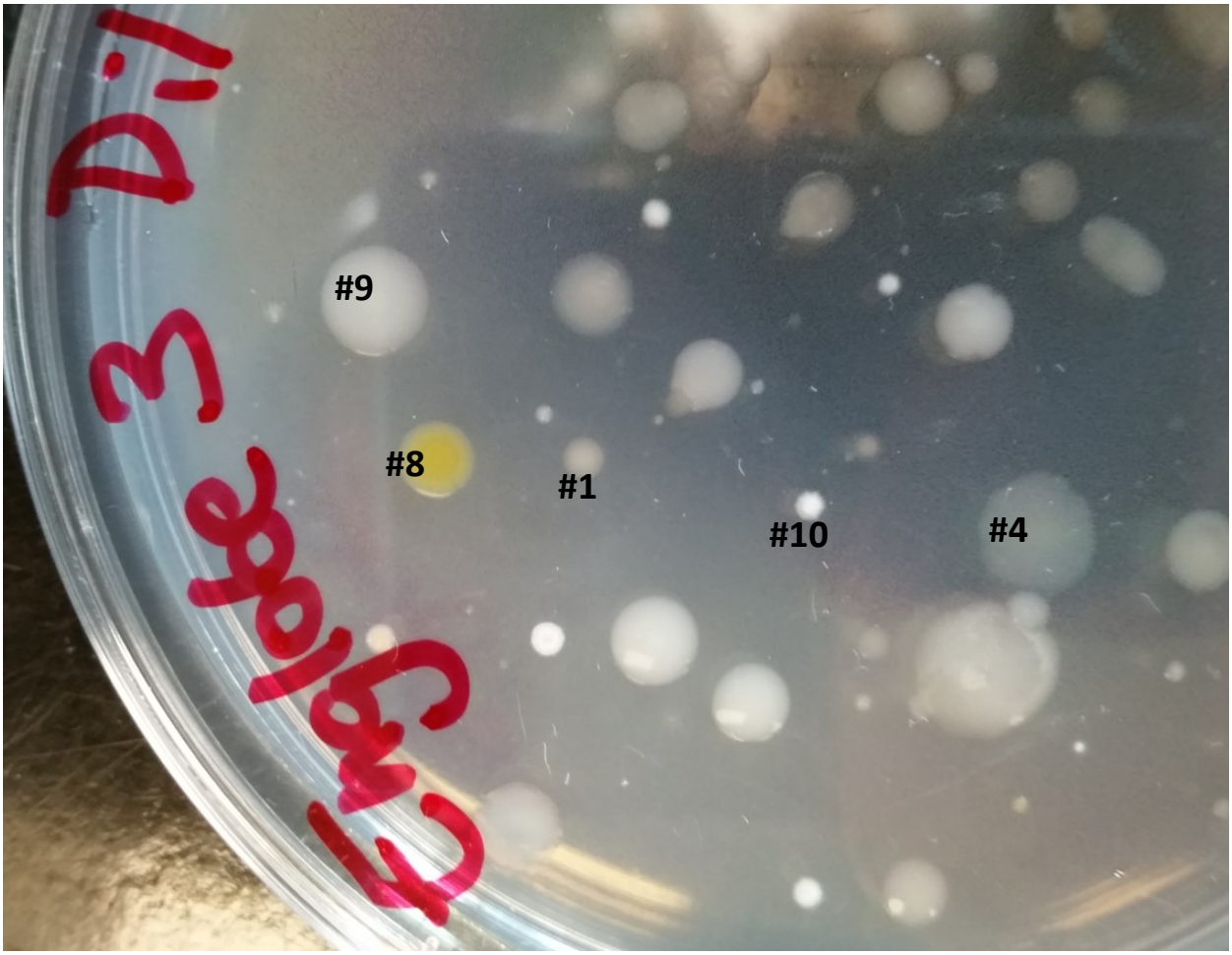


Figure A8 : Mixed culture plate of hydrocarbon degraders

Figure A9: Isolate #1 Plate and Close-up [Appearance: Small white]

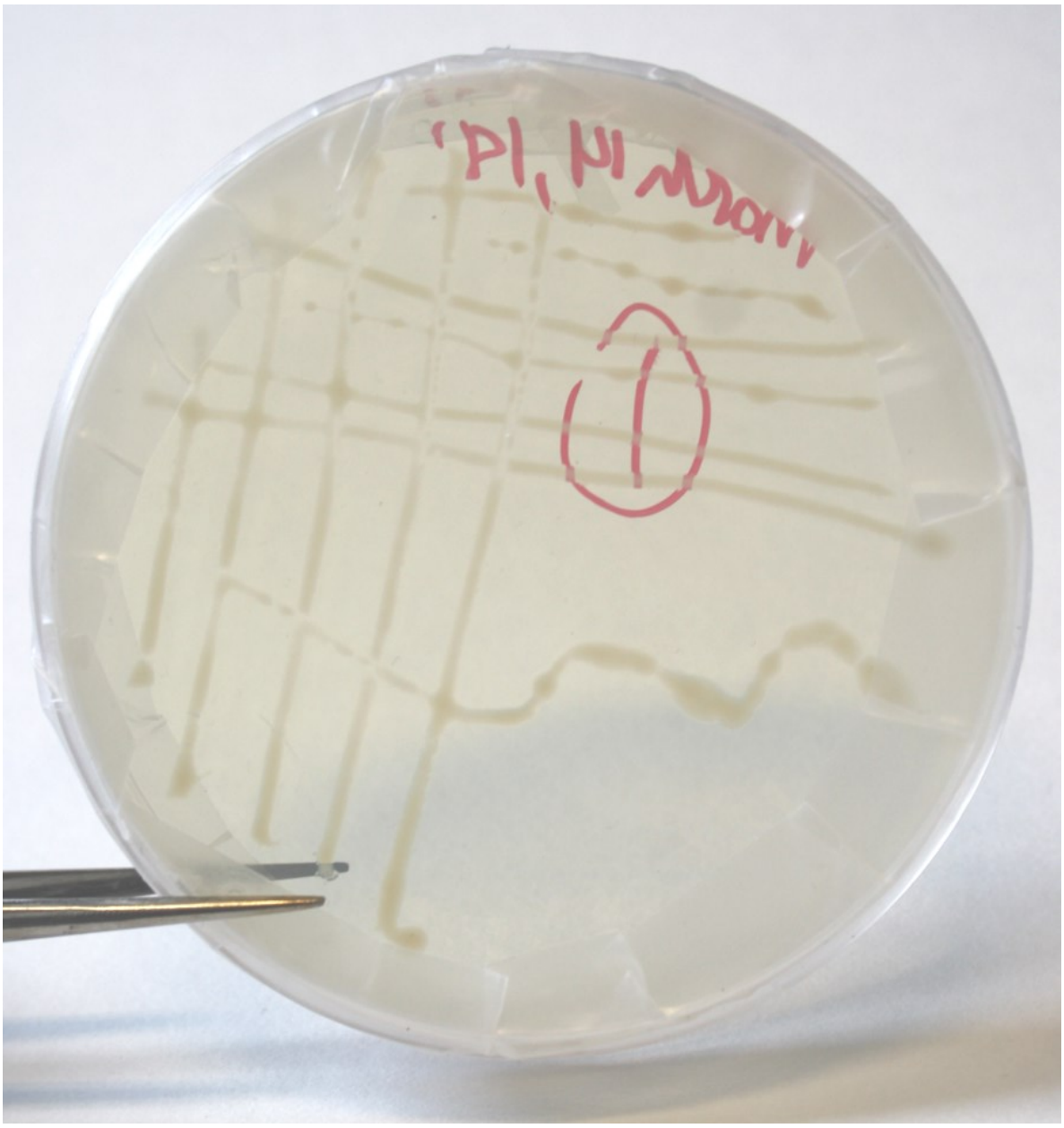
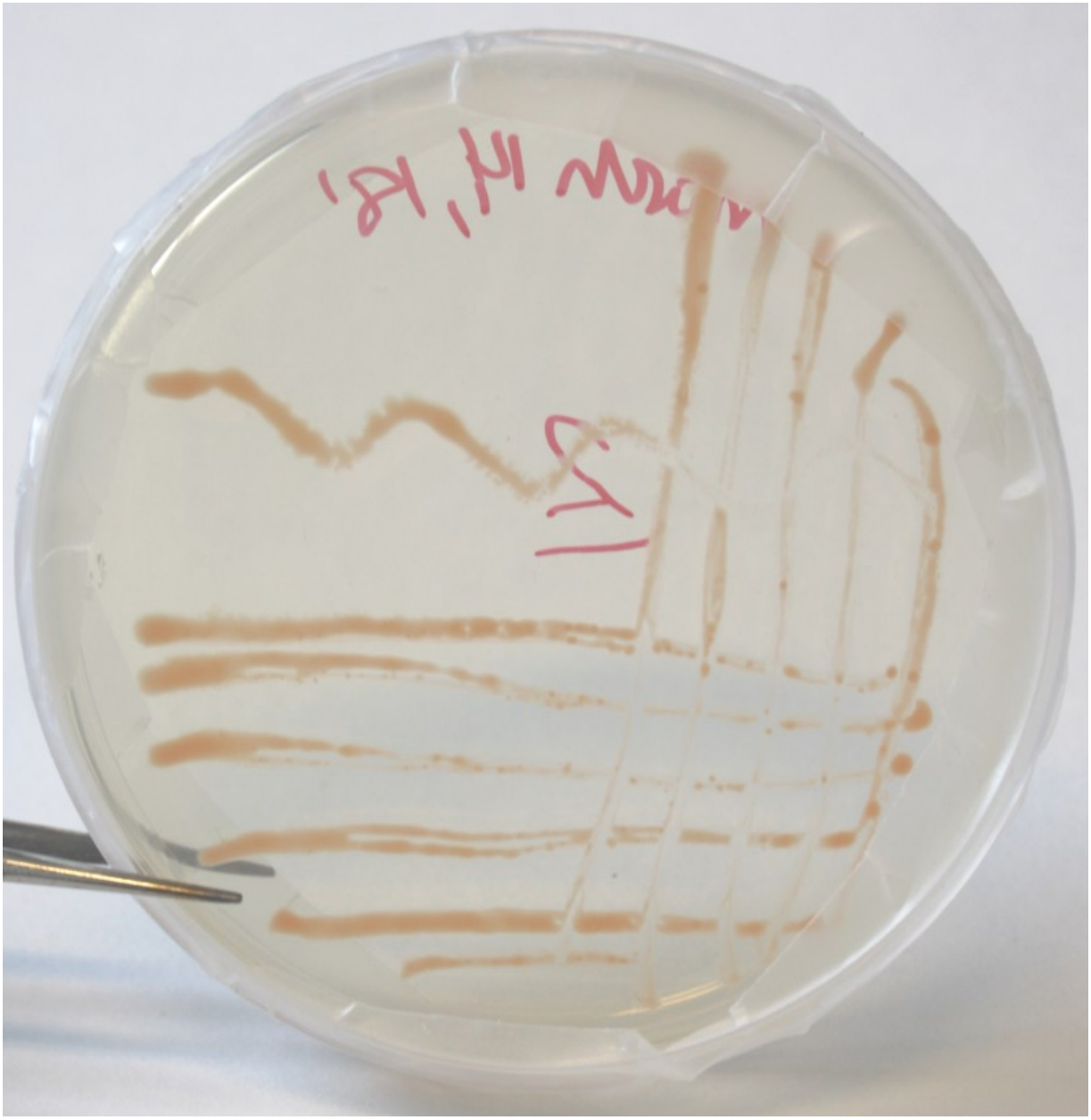


Figure A10: Isolate #2 Plate and Close-up [Appearance: Red/pink]



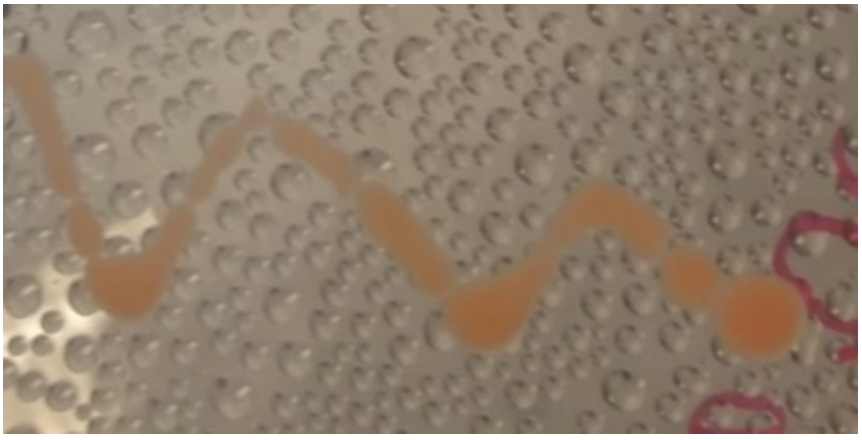


Figure A11: Isolate #3 Plate and Close-up [Appearance: Red/orange]

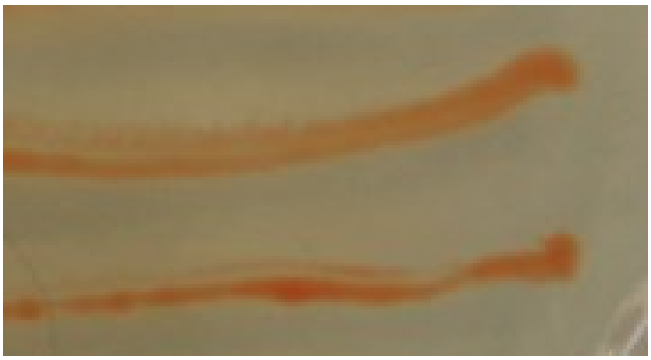
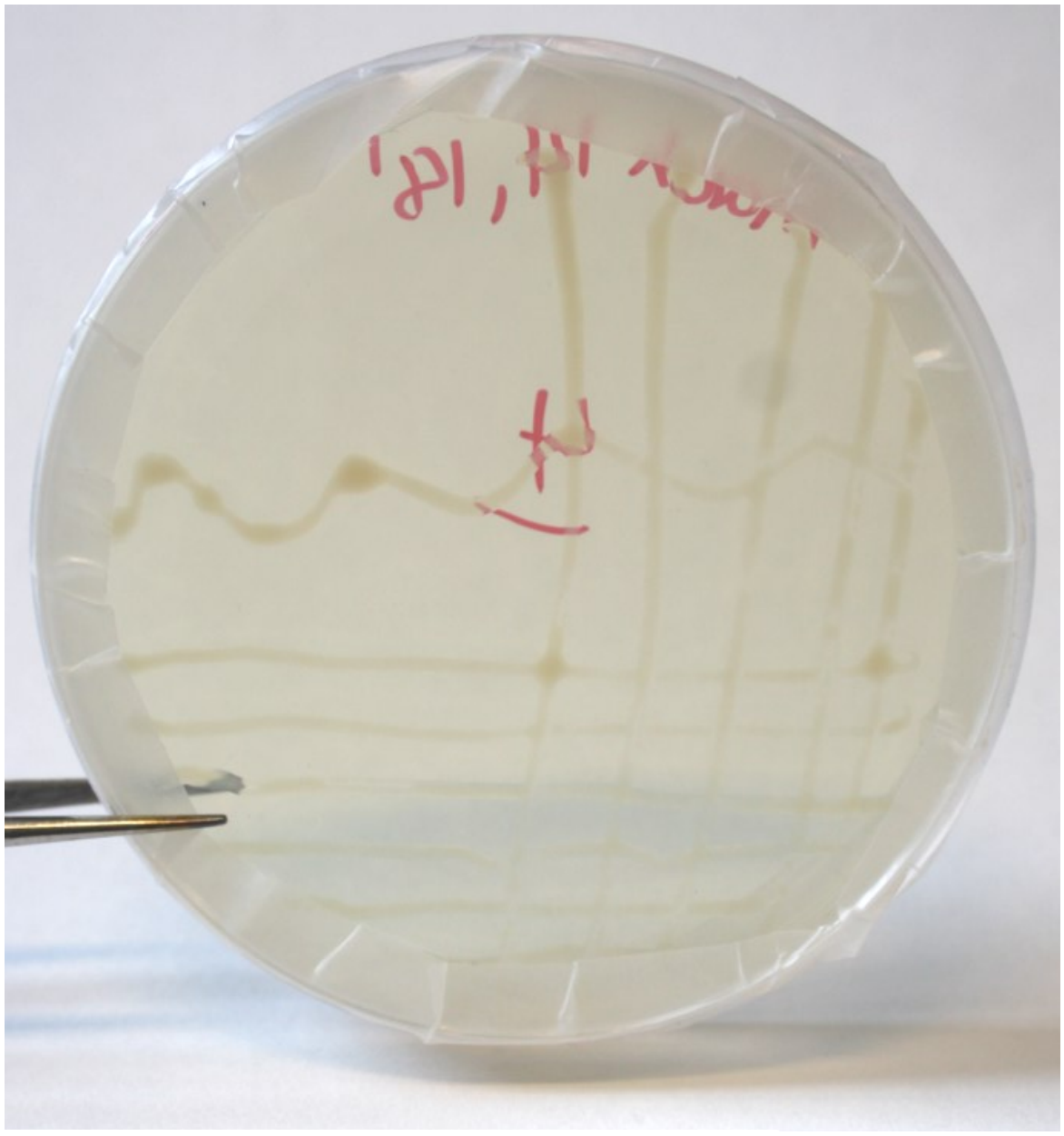


Figure A12: Isolate #4 Plate and Close-up [Appearance: Creamy translucent]



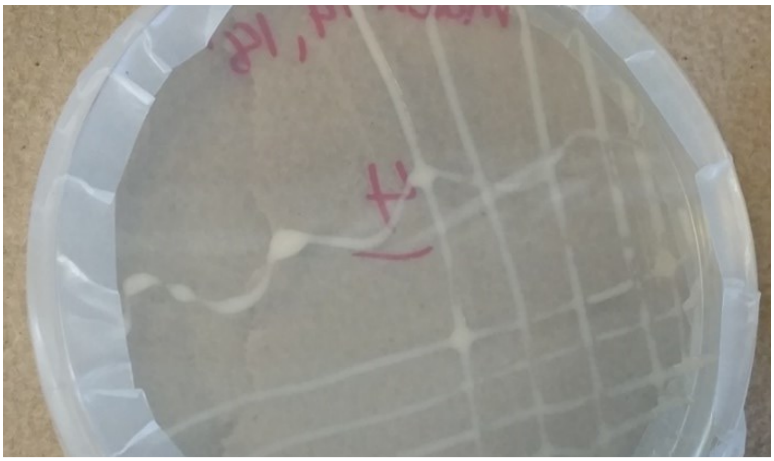
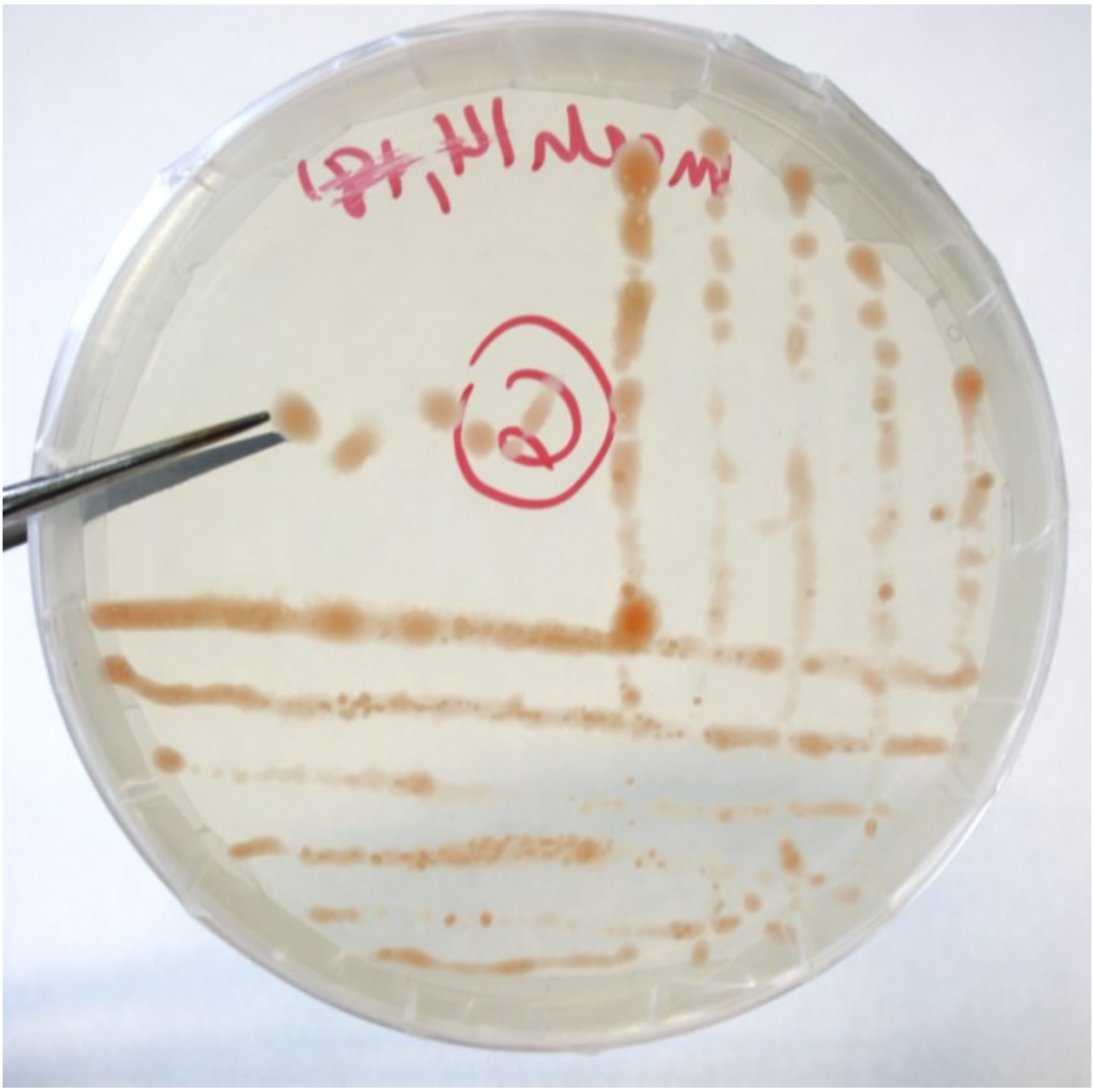
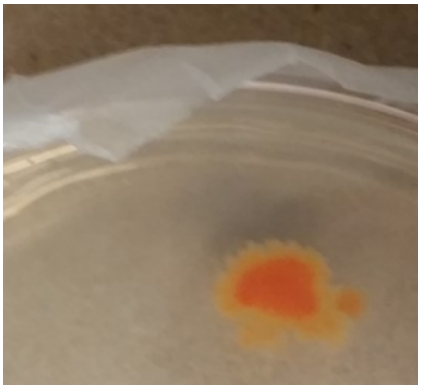


Figure A13: Isolate #5 Plate and Close-up [Appearance: Tiny white]

Figure A14: Isolate #6 Plate and Close-up [Appearance: Red]





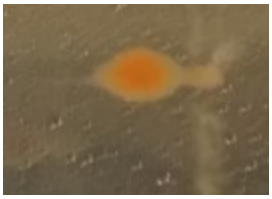
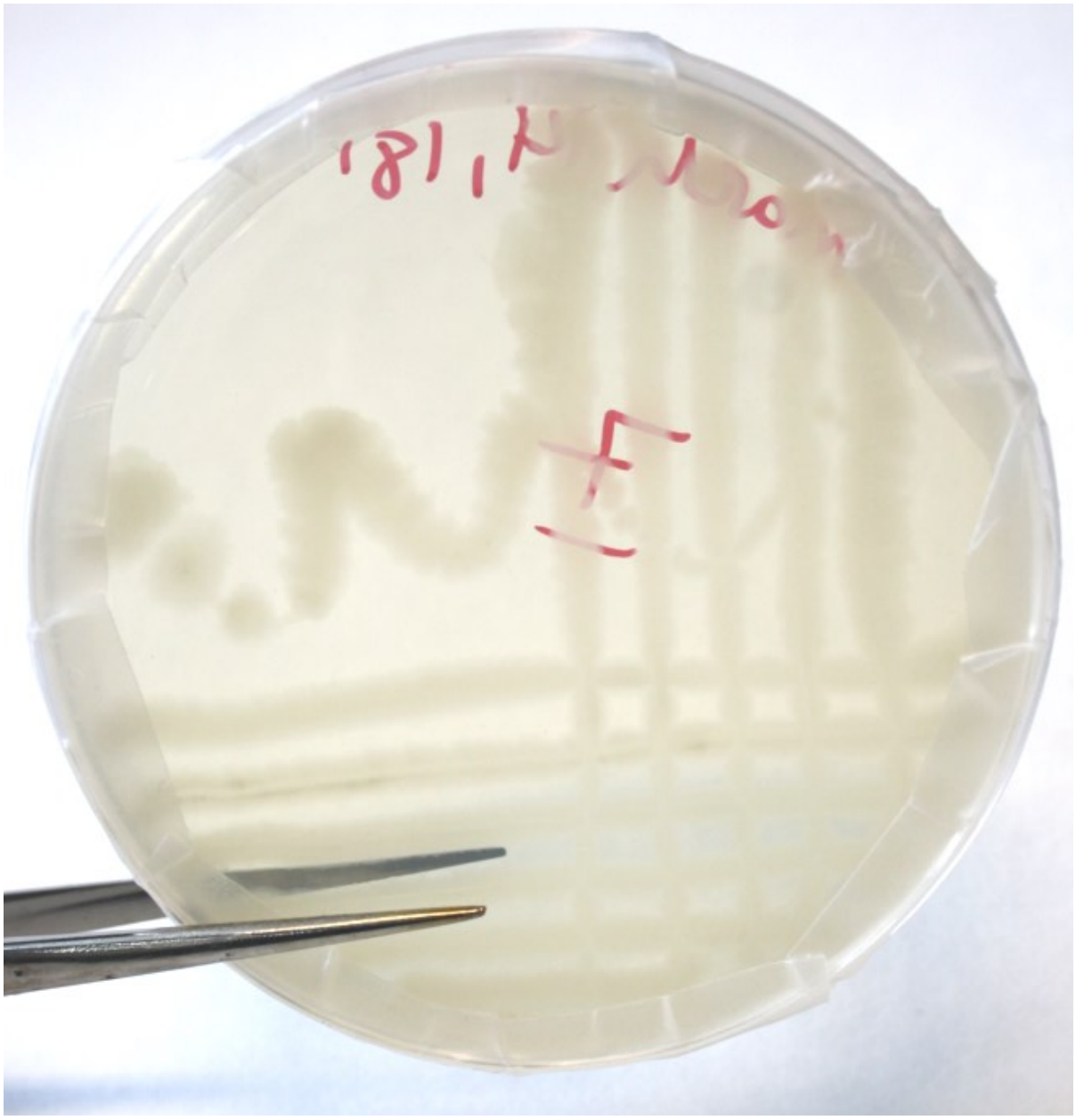


Figure A15: Isolate #7 Plate and Close-up [Appearance: White bumpy]



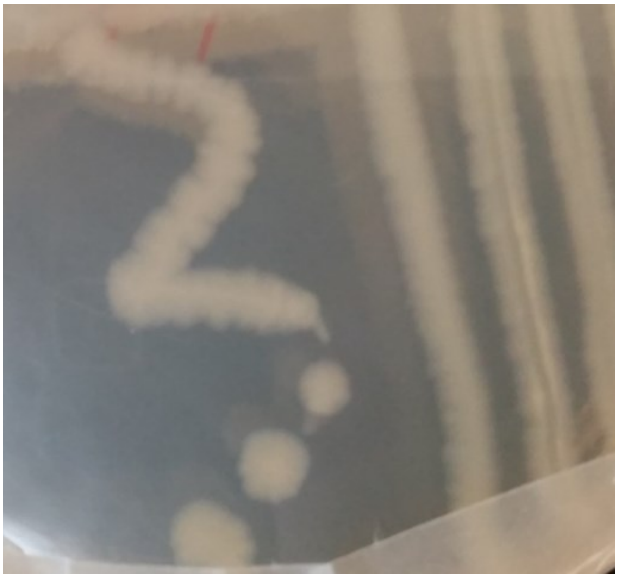
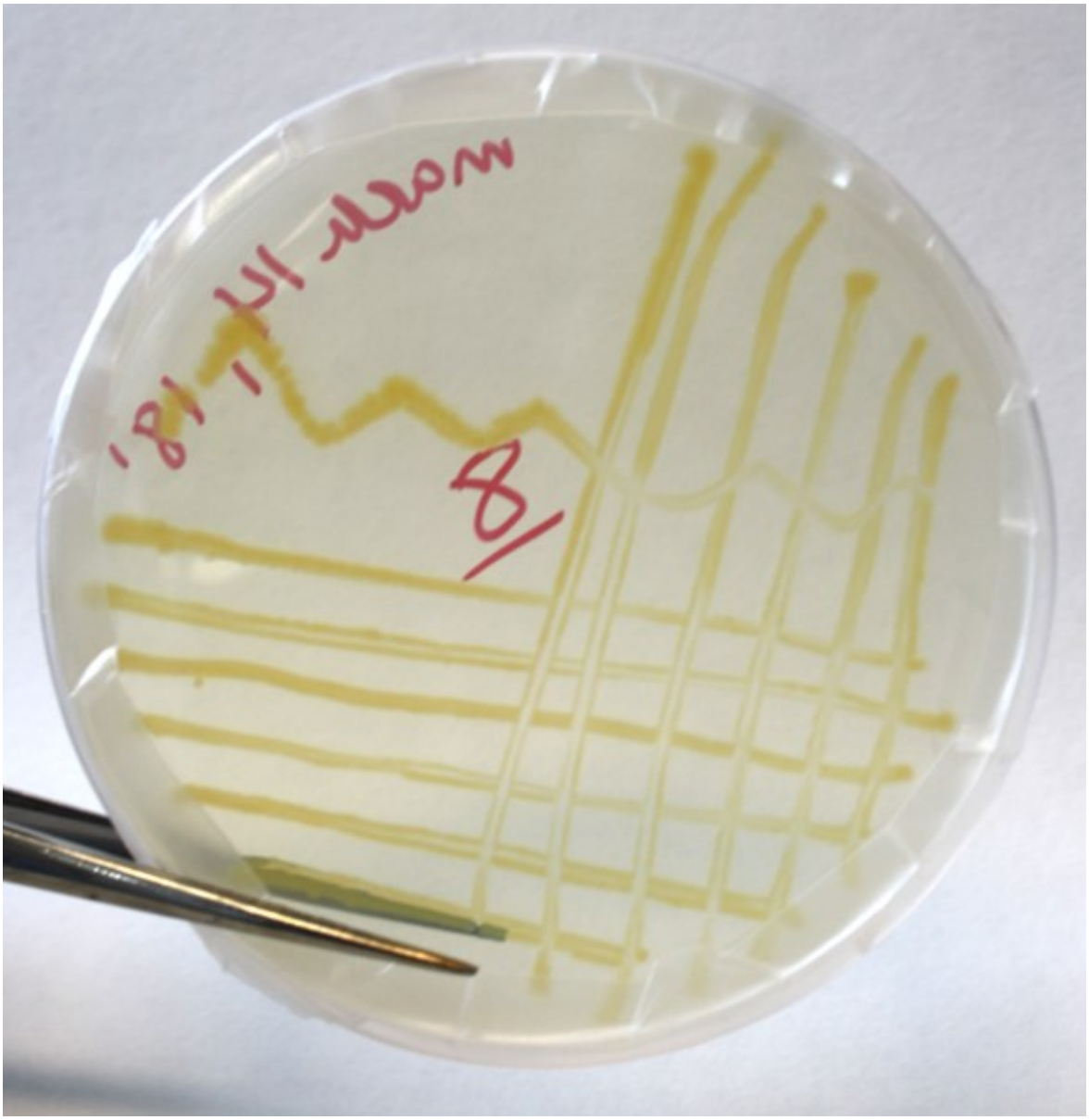


Figure A16: Isolate #8 Plate and Close-up [Appearance: Yellow]



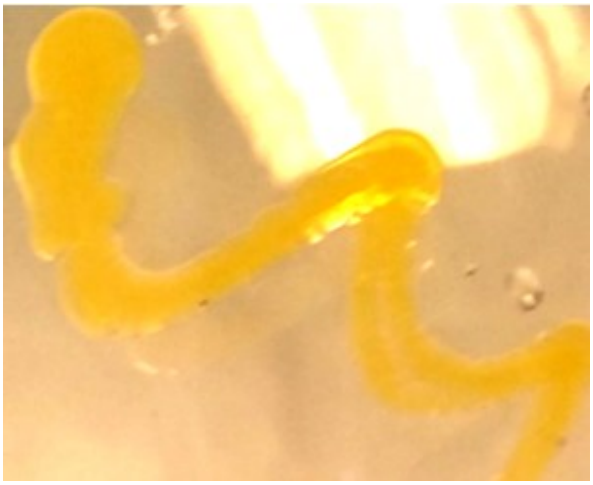


Figure A17: Isolate #9 Plate and Close-up [Appearance: White smooth]

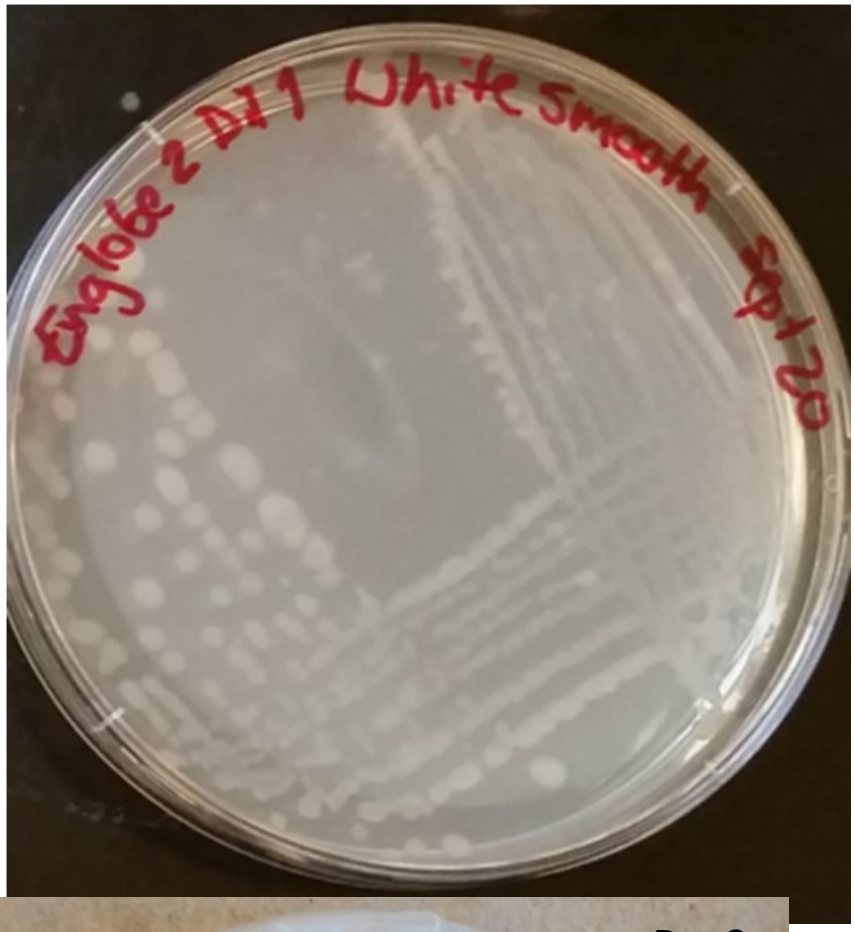
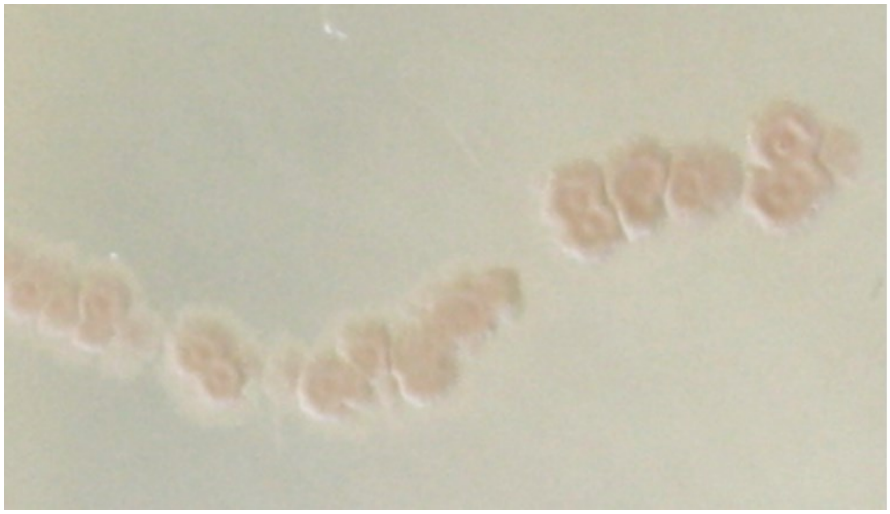


Figure A18: Isolate #10 Plate [Appearance: Filamentous]

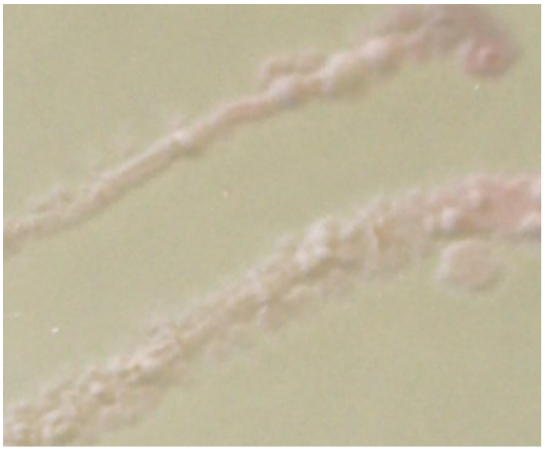
Figure A19: Isolate #10 Close-ups

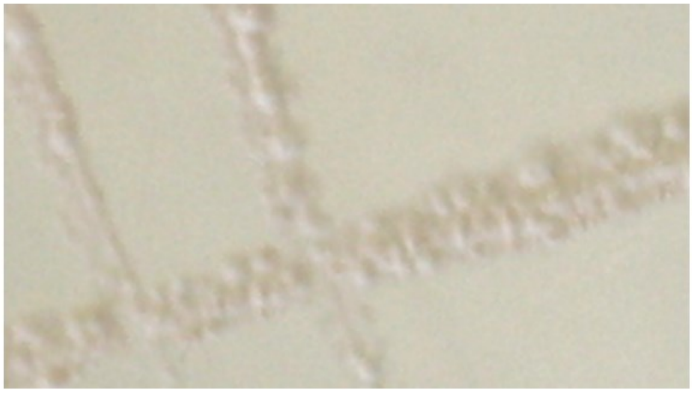


Top side Close-ups

Note the very crusty & bumpy 'cottage cheese' texture.

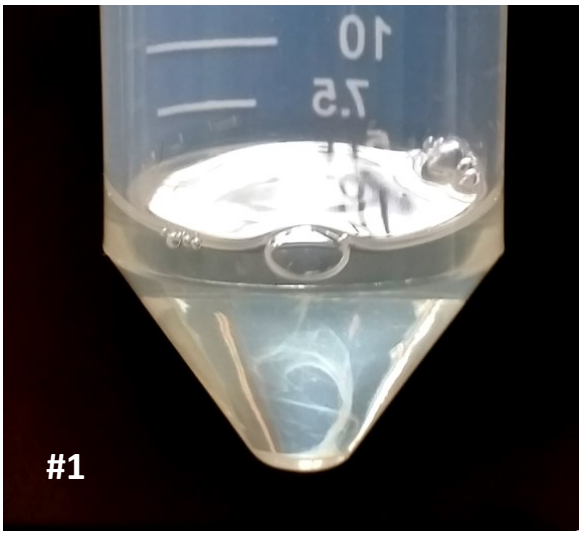
Seems to be non-motile. Becomes embedded within the agar as it grows. The white crusty parts come off in flakes.

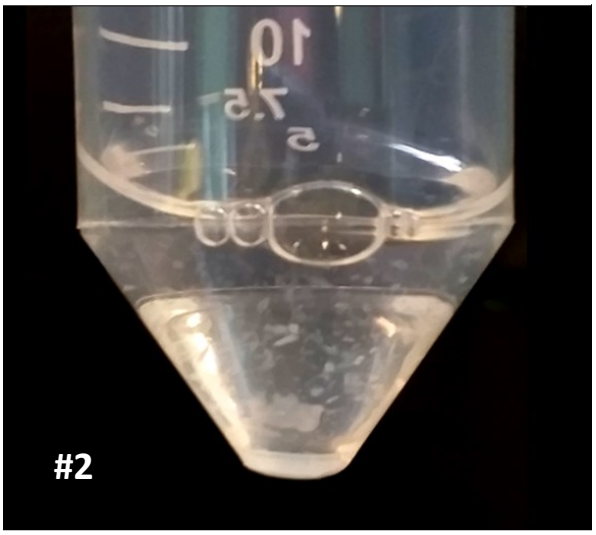


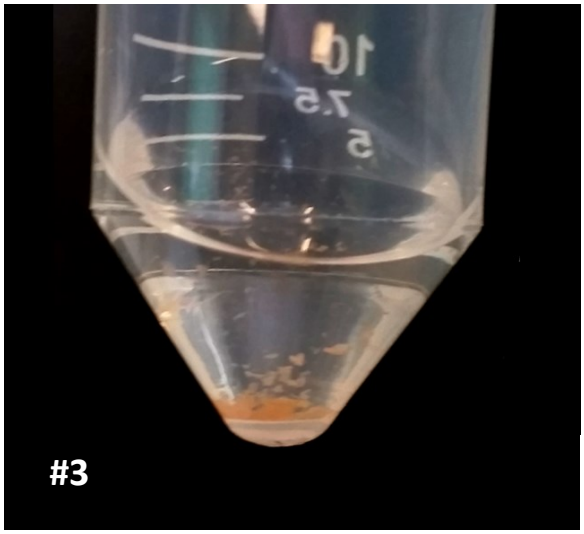


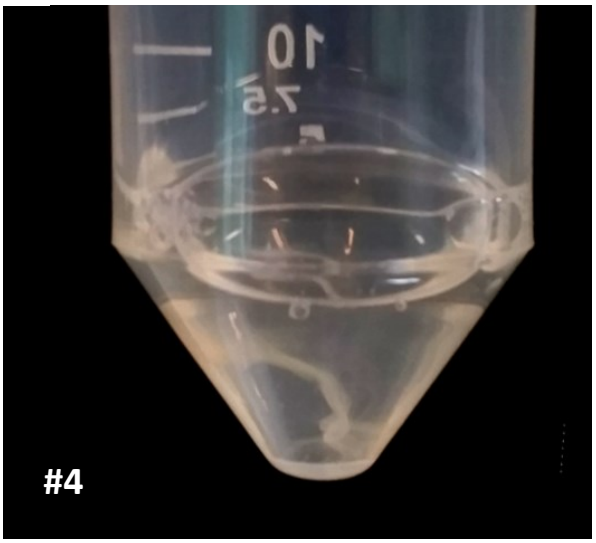
Second plate (bottom side)

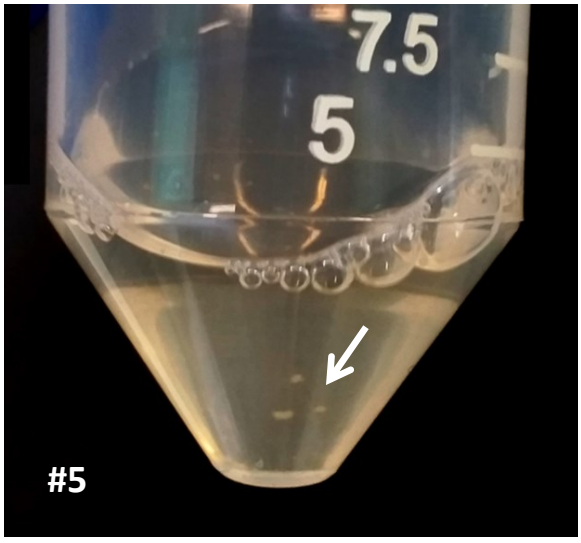
Figures A20: Isolate growth in minimal Bushnell Haas media with rhamnolipids

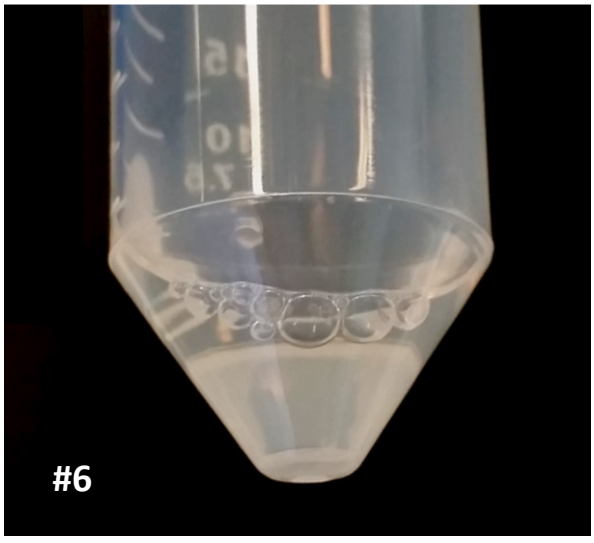


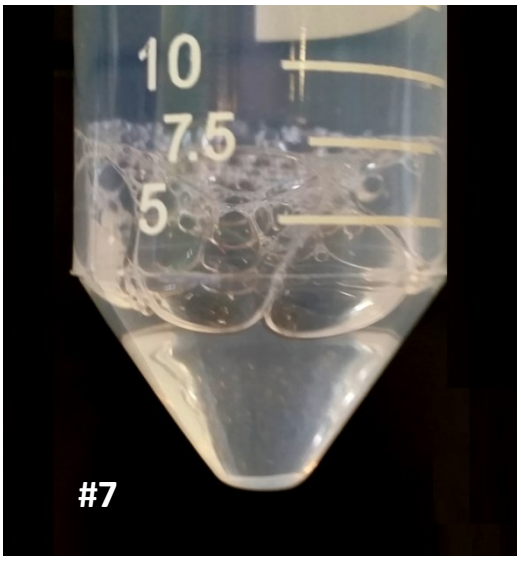


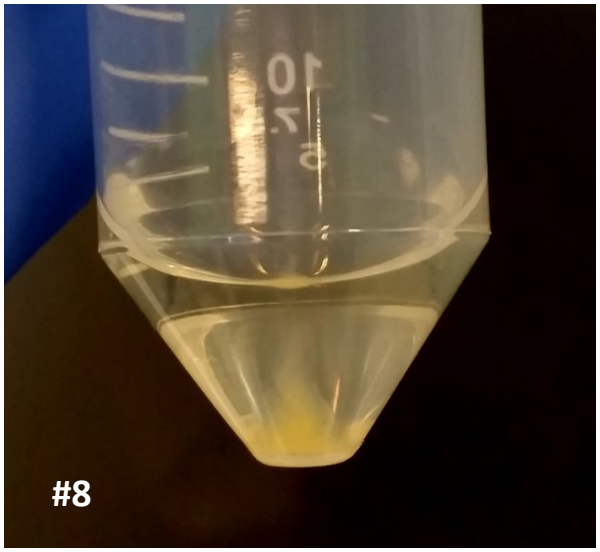


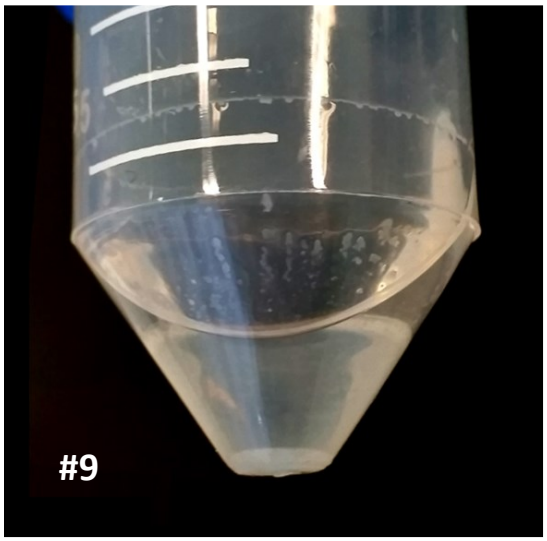


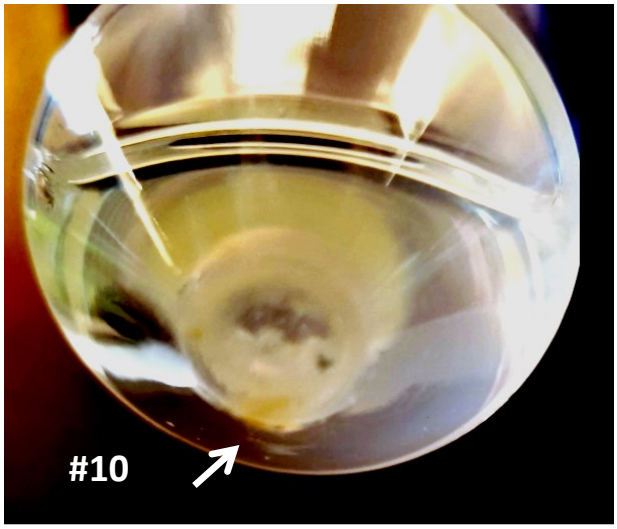





10
7.5
5
#7


#8


#9


#10