Biosurfactant Enhanced Soil Bioremediation and Associated Microbial Community Analysis

by

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ABSTRACT

The nutrient and rhamnolipid biosurfactant enhanced soil bioremediation of petroleum hydrocarbons (PHCs) and the associated microbial communities were investigated in this thesis. A systematic factorial design was conducted, and a response surface reduced quadratic model was developed to determine the effects of the nutrients and two surfactants (i.e., rhamnolipids and Tween 80) in the degradation of PHCs within 36 days. A significant effect as a result of nutrient addition and a 92.3% removal of PHCs was achieved by applying rhamnolipids at a concentration of 150 mg/kg and a 1000 μL nutrient solution in every 30 g of soil. Rhamnolipids resulted in higher metabolic activities of indigenous soil microorganisms to assimilate hydrocarbons than Tween 80 based on kinetic investigation. Using phospholipid fatty acid (PLFA) analysis, three different total biomass transformation patterns were observed when the soils were treated by natural attenuation, by rhamnolipid aided bioremediation or Tween 80 aided bioremediation, respectively. As indicated by PLFA biomarkers of the Gram-negative bacterial populations (cy17:0, cy19:0, 16:1ω7c and 18:1ω7c), Gram-negative bacteria are closely correlated with the total amount of soil biomass and are the sources of hydrocarbon-degrading microorganisms. The physiological status of the indigenous microorganisms was also evaluated using PLFA compositional characteristics to indicate environmental stress and elucidate biodegradation mechanisms linked to different soil treatments.

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CHAPTER 1

INTRODUCTION

1.1 Background

Soil contamination is a global environmental problem caused by improper disposal of industrial discharge, mining tailings, waste disposal, and stockpiles, and it leads to damage to human health and lose of economics (Mao et al., 2015). Tabak et al. (2005) indicated that toxic organics, heavy metals, and radionuclides are the major soil contaminants. Petroleum hydrocarbons (PHCs) are widespread toxic organic pollutants in soil habitats and have raised global concerns due to their negative impacts on all forms of life (Li et al., 2007). There are many forms of PHC contamination caused by various anthropogenic activities, including inappropriate transportation processes, oil wells leakages, improper disposal of petroleum wastes and accidental oil spills (Moldes et al., 2011).

Thermal, chemical and physical methods have been developed to treat the PHCs contaminated soil (Frick et al., 1999). However, significant expenses and difficult site restoration process are required in these methods (Lundstedt et al., 2003). Compared with the numerous other remediation technologies for treating hydrocarbon contaminated soils, bioremediation is an attractive and promising approach because of its advantages such as its simple maintenance, low cost, low environmental impact, and applicability over large areas and a wide variety of organic contaminants (Megharaj et al. 2010). Bundy et al. (2004) indicated that, some biological materials like bacteria, fungi algae with vermicompost, and animal and plant compost behave as expected during the bioremediation.

During bioremediation, PHCs will possibly serve as organic carbon sources in most environments, and this will lead to the enrichment of oil-degrading microbial populations (Margesin et al. 2000). Among a wide variety of bioremediation processes, natural

biodegradation is limited in its ability to remove contaminants and has a low natural rate of decontamination. The stimulation of indigenous oil-degrading microorganisms is often beneficial and the appropriate addition of nitrogen (N) and phosphorous (P) based-nutrients could readily improve the degradation competence of the native microbial consortia. Arslan et al. (2014) proved that nutrients help to degrade hydrocarbon contaminated soil effectively. Tahseen et al. (2016) proved that nutrients were consumed in abundance when the contents of N, P, K are 71%, 61% and 47% respectively.

A concern of biodegradation to be stressed is the low bioavailability when PHCs exist in the deep pores of soils and are poorly accessible for hydrocarbon degraders. Under such circumstances, microbial populations can produce oil dispersive compounds like biosurfactants that could accelerate the degradation of toxic compounds (Pacwa-Płociniczak et al. 2014; Ron and Rosenberg 2002). In this regard, bioremediation enhanced by direct supplementation of biosurfactants exhibits great potential to improve the mobility and bioavailability of PHCs and its subsequent biodegradation.

Biosurfactants are functional amphiphilic compounds, either produced on the cell surface or secreted extra-cellularly by a variety of microorganisms, and they reduce surface and interfacial tensions (Cai et al., 2014). Biosurfactants may enhance PHC bioremediation by active interaction with the cell surface to increase the hydrophobicity of the surface, allowing hydrophobic substrates to bind more easily to bacterial cells (Dias et al., 2012; Rahman et al., 2003). Because surfactants are a group of amphiphilic chemicals consist of both hydrophilic and hydrophobic parts in the molecular structure simultaneously. The unique molecular structure of surfactant allows to enhance the water solubility of soil

contaminants, especially for the hydrophobic organic compounds (Mao et al., 2015). For example, rhamnolipids was found effectively remove a hydrocarbon mixture from soil and their removal rate was dependent on the type of hydrocarbon removed and the concentration of the surfactant used (Scheibenbogen et al., 1994; Burd & Ward, 1996; Ammami et al., 2015). Although many studies have investigated the effects of nutrient and biosurfactant addition on PHC degradation (Cameotra and Singh, 2008; Nikolopoulou et al., 2013; Szulc et al., 2014), the factors were usually investigated through a simple one-factor-at-a-time (OFAT) approach. This approach typically exhibits less accuracy in estimating the optimal conditions and provides limited information on factor interactions when compared with designed experiments (Vasilev et al., 2014). In addition, precise knowledge was still needed to study the long-term and short-term effects of chemical surfactants and biosurfactants in enhancing soil remediation (Makkar and Rockne, 2003). Thus, a systematic design with factors including the type and dose of surfactants coupled with nutrient in PHC bioremediation will provide interesting information to elucidate the interactions of the factors and the fate of the contaminants.

Soil microbial communities play an important role in the biodegradation of PHCs. The contaminants together with the addition of nutrients and biosurfactants will significantly influence the composition and activities of indigenous microorganisms in soil, thus affect microbes that survive and function under those conditions (Mrozik and Piotrowska-Seget, 2010). Natural soil microorganisms are very sensitive to any ecosystem perturbation (Nikolopoulou et al., 2013), and their rapid alteration in structure and biomass is considered the best indicator of soil pollution (Wang et al., 2016). Knowledge of microbial

communities in the overall biodegradation process is important for determining the potential for bioremediation, transformation, or the persistence of pollutants (Pratt et al., 2012; Wang et al., 2013). However, there is a lack of research concerning the microbial community and the associated biological mechanisms during the biosurfactants enhanced bioremediation.

Phospholipid fatty acid (PLFA) analysis, a rapid, inexpensive, sensitive, and reproducible tool for assessing soil structure (Frostegård et al., 2011; García-Orenes et al., 2013; Ławniczak et al., 2013), was used to evaluate the performance of microorganisms during PHC bioremediation. PLFA analysis is based on the extraction and quantification of phospholipids from whole microorganisms in the sample, so it provide a relatively precise results(Kato et al., 2005). Specific PLFA patterns revealed robust information on microbial community structures, their physiological and nutritional status, and the viable biomass of the microbial population in soil (Frostegård et al., 2011). PLFA analysis has been widely used to determine differences in microbial community structure on soil over various environmental factors, such as soil pore size (Ruamps et al., 2011), soil water availability (Ruamps et al., 2011), spatial patterns in marine sediments (Fischer et al., 2010), and spatial covariation in polluted soil (Torneman et al., 2008). It was also used in associated community composition studies on PAH contaminated riverbank sediment (Pratt et al., 2012), nutrient-stimulated (Hammer et al., 2011) and chemical surfactant-enhanced bioremediation of hydrocarbon-contaminated soil (Lai et al., 2009; Mair et al., 2013). Therefore, the PLFA approach is more promising in its application for microbial

community analysis and microbial dynamic investigation over the biodegradation process, and this has received limited focus in the literature.

1.2 Objective

The objective of this thesis is to investigate the microbial communities in PHC-contaminated soil supplemented with nutrients and a rhamnolipid to track biodegradation mechanisms and provide potential biodegradable internal information through PLFA analysis. A chemical surfactant, Tween 80 (TW80), was also used for comparison purpose, due to its high solubilization capacities and the ability to remove PHCs from the soil(Alcántara et al., 2008). Design of experiments (DOE) was applied to conduct the biostimulation under various conditions. Soil samples from a PHC contaminated site in Goose Bay, Newfoundland, was applied. The main tasks entailed:

1) conducting a factorial design to systematically study the factors of surfactant type, surfactant concentration, and nutrient concentration on enhanced bioremediation of PHC contaminated soil;

2) evaluating performance of biosurfactant (i.e., rhamnolipid) and chemical surfactant (i.e., Tween-80) enhanced bioremediation systems;

3) adopting a PLFA based approach for microbial community analysis during the enhanced bioremediation; and

4) investigating the microbial dynamic over the biodegradation process under multiple scenarios (types of surfactants, time, and levels of nutrients).

1.3 Thesis Structure

This thesis consists of five chapters, shown in Figure 1.1. Chapter 1 introduces the research background, knowledge gaps, research objective, and thesis structure. Chapter 2 summarizes literature of relevant topics including technologies for remediation of PHC contaminated soil, biosurfactants-enhanced bioremediation, and approaches for monitoring microbial communities. Chapter 3 introduces the methodologies used for achieving the research goals. Chapter 4 states the results of all the experiments and conducts associated result discussions. Chapter 5 concludes the research and includes recommendations for future studies.

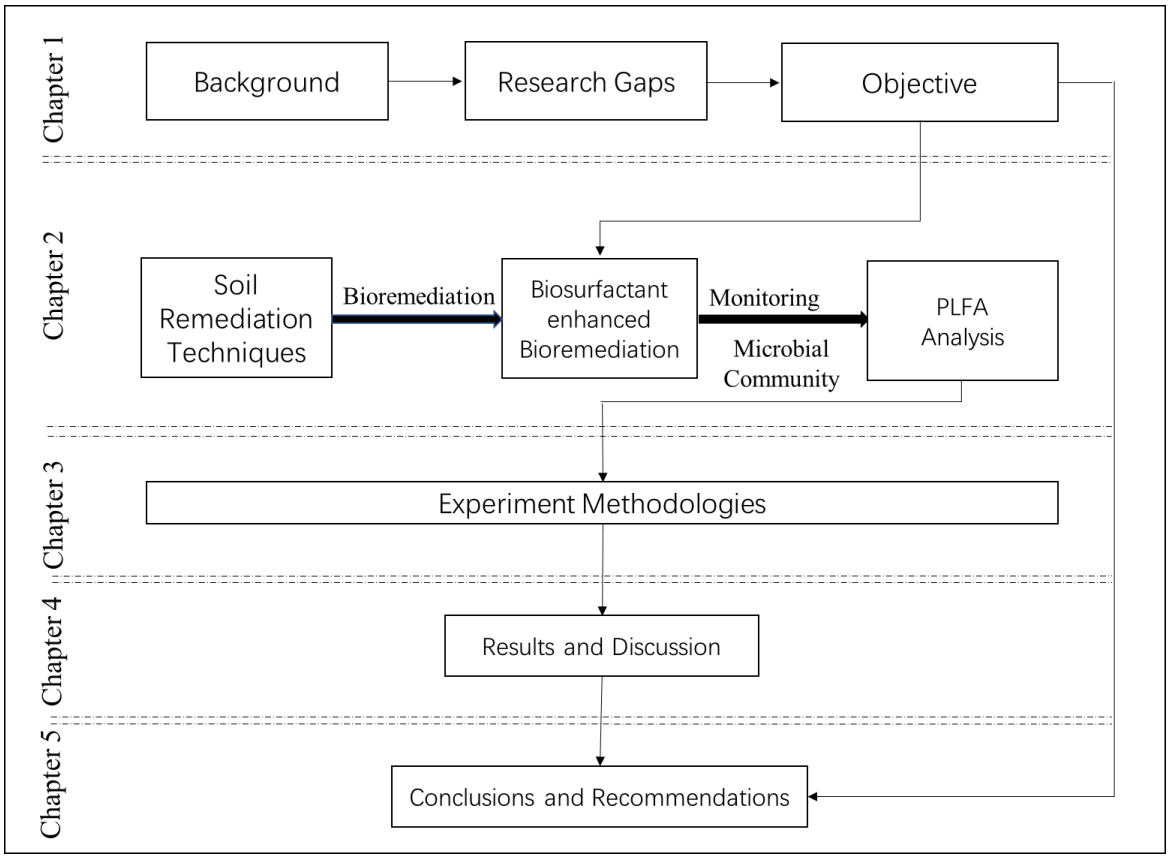


Figure 1. 1 Thesis structure

CHAPTER 2

LITERATURE REVIEW

2.1 Technologies for PHC Contaminated Soil Remediation

2.1.1 Physical, Chemical and Biological Technologies

Physical, chemical, and biological techniques have been developed to treat PHC-contaminated soil. Researchers found that there was no single technique to restore a contaminated site, owing to the different types of contaminants (Azubuike et al., 2016). Dispersants, solvents extractions, and chemical oxidation are popular chemical technologies. Among biological technologies adopted thus far are bioremediation, phytoremediation, rhizoremediation, bio-augmentation, plant-assisted bioremediation, chemical oxidation coupled with bioremediation, and land-farming. Each strategy has its own pros and cons and is discussed in the following section.

Physical technologies adopted for petroleum hydrocarbon-contaminated lands consist of in situ and ex-situ techniques. In situ techniques include such as soil aeration, whereas ex-situ techniques involve methods like shifting of contaminated soil to a chemical treatment unit, e.g., solvent/water extraction and/or a thermal treatment unit, a low temperature thermal unit or a high temperature thermal unit (Gomes et al., 2013). Thermal treatment is a method used to burn contaminated soil without use of complicated materials or equipment. Although physical techniques are easily handled and can be applied to all kinds of oils, they can only be used with other techniques and may cause atmospheric pollution with incomplete combustion (Wang et al., 2010). The advantages and disadvantages of physical remediation technologies are shown in Table 2.1.

Table 2.1 Advantages and disadvantages of physical remediation technologies

Advantages	**Disadvantages**
Can be applied for all petroleum hydrocarbon contaminated soil	Secondary pollution may be caused by thermal desorption and incineration
Simple to handle	Expensive cost of installation if required
Request less time than other technologies	Cannot be used independently.

Chemical technologies for remediating PHC contaminated soil include such as the use of dispersants, encapsulation, and chemical oxidation. Solvent extraction is an ex-situ technique widely used but is associated with the high project cost and the production of secondary pollution (Berset et al., 1999). There are several kinds of chemical oxidants, for example, Fenton's reagent, hydrogen peroxide, permanganate of sodium and potassium, and ozone. The choice depends on the hydrogeological condition of the site. Zhao et al. (2015) found that, when the dosage of dispersant was increased, the solubility of all PAHs enhanced linearly. However, a lab study showed that chemical dispersants may have negative effects on microbial biomass and activities in soil (Pietroski et al., 2015). Thus, the use of dispersants can bring adverse effects to humans and the environment. The characteristics of chemical techniques are displayed in Table 2.2.

Bioremediation is the process of using biological methods to reduce the toxicity and concentrations to an innocuous level (Mueller et al., 1996). According to the definition, it utilizes bacteria, fungi or plants to degrade environmental hazards. These microorganisms may be native or isolated from elsewhere and brought to the polluted site (Vidali, 2001). Azubuike et al. (2016) reintroduced some terms related to bioremediation. He pointed out that "biodegradation" is an alternative form of "bioremediation." The former is a term that applies to a process belonging to the latter. In this thesis, bioremediation refers to the process of degrading, detoxifying, and transforming PHCs. Owing to the eco-friendly and cost effect characteristics, bioremediation techniques have therefore been widely used.

Table 2.2 Advantages and disadvantages of chemical remediation technologies

Advantages	**Disadvantages**
Can be applied for an in-situ treatment	High installation and handling cost
To takes only a couple of weeks or months degrade the contaminants	Chemicals like dispersants may influence human's health and pollute the environment
Some techniques (e.g., oxidation of contaminant)	Oxidant may not get contact with contaminants due to low permeability soils

Compared with the physical and chemical methods, biological technologies are the most cost-effective and environmentally friendly alternatives. (Gargouri et al., 2014; Fuentes et al., 2014; Pizarro et al., 2014). Atlas and Bartha (1998) found that the concentration of hydrocarbon pollutants could be decreased effectively by using adapted microbial communities. Therefore, hose most effective oil degraders can be used to speed up the bioremediation of PHC-polluted sites (Atlas and Bartha, 1998). Natural attenuation, biostimulation, and bioaugmentation are the main technologies of biological remediation strategies for the remediation of soils affected by different types of pollutants. Natural attenuation uses the autochthonous microorganisms to degrade pollutants, avoiding breaking the original ecological habitats (Couto et al., 2010). However, it takes a long time to complete this degradation. Verginelli & Baciocchi (2013) discussed the relatively slow biodegradation rate of PAHs, on the order of 0.0001–0.001 d^{-1}. Bioaugmentation is a method that promotes the biodegradability of contaminants by adding exogenous bacteria, whereas biostimulation requires additional nutrients or substrates to stimulate the degradation of native microorganisms as in situ engineered bioremediation processes (Azubuike et al., 2016). These processes aim to improve the microbial population to a certain level to achieve effective and high efficient biodegradation (Atlas and Bartha, 1992). In the past 20 years, bioremediation techniques have been regarded as environmental friendly methods to restore contaminated soil effectively at low cost (Azubuike et al., 2016).

2.1.2 Enhanced Soil Bioremediation

Bioaugmentation

Bioaugmentation through addition of oil-degrading microorganisms to a bioremediation system has been used since the 1970s. This method can successfully decrease the lag period, and the seed microorganisms can degrade petroleum components, maintain genetic viability and stability in storage, survive hostile environments, and compete with indigenous microorganisms (Cai et al., 2010). This treatment is more suitable for soils contaminated by compounds requiring long-term acclimation or adaptation of microorganisms. However, it may be difficult to deliver the exogenous microorganisms to the desired sites (Mrozik and Piotrowska-Seget, 2010).

The bioaugmentation approach involves inoculation of endogenous or genetically engineered microorganisms with desired degradation capability into soil. It has the potential to enhance the biodegradability of toxic contaminants (Andreolli et al., 2015). There are many advantages to using imported bacteria, because some may not be effective when applied in different regions. However, this approach should only be used where indigenous microbial populations cannot degrade potential substrates in complex mixtures, like petroleum (Leahy and Colwell, 1990). Silva et al. (2009) investigated the degradation behavior of PAHs using an undefined culture obtained from PAH-contaminated soil. They bioaugmented the culture with three PAH-degrading strains. The results from the carbon-limited chemostat cultures indicated that the soil culture efficiently degraded the PAHs, but no significant enhancement of PAHs biodegradation has been observed from bioaugmentation. Herwijnen et al. (2006) used a PAH-degrading bacterial consortium, enriched from mangrove sediments, to investigate the effect of bioaugmentation on the removal of a mixture of PAHs. The degradation percentages of three PAHs were not

significantly different from natural attenuation, possibly because of the inhibitory effect caused by autochthonous microbes on the enriched consortium, during PAH biodegradation. Thus, we propose that bioaugmentation should be implemented for soils in which microorganisms cannot biodegrade from these compounds, even after the failure of bio-stimulation and bio-attenuation (Fantroussi and Agathos 2005; Adams et al., 2015).

Biostimulation

Indigenous microbial communities are important for the success of bioremediation treatments (Martin et al., 2012). Even for the application of bioaugmentation, the use of native microflora is preferred, because these microorganisms typically have a better ability to adapt to target specific pollutants than exogenous microorganisms (Venkata Mohan et al., 2008).

Biostimulation is one of the natural remediation treatment that helps to improve the biodegradability of organic pollutants in the soil (Tyagi et al., 2011). Biostimulation involves the environment in the modification of the stimulated bacteria capable of bioremediation, which can be realized by adding multiple forms of electron-acceptors and limiting nutrients such as carbon, oxygen, nitrogen, and phosphorus. Reinhard (2017) described biostimulation as the addition of oxygen, nutrients, and other electron acceptors and donors to a coordinated site to enhance the population or activity of natural remediation which naturally generate microorganisms available for bioremediation. There are various factors that can limit hydrocarbon biodegradation in the soil, such as soil properties, contaminant presence, oxygen, moisture, temperature, pH, and nutrients (Atagana, 2008; Al Sulaimani, 2010; Bundy et al., 2002). Fan et al. (2014) showed that remediation via

inoculating yeast removed 83% of PHCs in 180 days, whereas an experiment with the indigenous microorganisms alone removed 61%. The use of native microflora is preferred, because these microorganisms typically have a better ability to adapt to target specific pollutants than exogenous microorganisms (Venkata Mohan et al., 2008). Thus, the best performance can be approached by using microorganisms that are already present in the soil and by increasing their abundance. With the increase of a specific microbial community and nutrient addition, this approach reduces cleanup time substantially. Margesin et al. (2000) believed that biostimulation can be regarded as an appropriate remediation when the indigenous microorganisms are well-adjusted to their own environment.

Biostimulation is an approach that reduces the toxicity to harmless compounds using natural biological activity. Additionally, it requires relatively low-cost and low-technology techniques, which makes it widely accepted. It will not always be suitable, however, because its effects are limited on various contaminants, the time scales involved are relatively long, and the achievable residual contaminant levels may not always be appropriate. Thus, to overcome these disadvantages, improvements have been developed to enhance biostimulation, such as adding nutrients and biosurfactants.

2.2 Biosurfactant enhanced Bioremediation

In most field studies, enhanced biodegradation always focuses on the stimulation of microorganisms present in the contaminated area and the promotion of the bioavailability of the contaminants. Hydrocarbon-degrading microorganisms are ubiquitous in most ecosystems, and indigenous microorganisms with degrading potential are well-adjusted to

their own environment. Thus, the contaminants may serve as organic carbon sources in most environments, leading to an enrichment of oil-degrading microbial populations (Margesin et al., 2000). The stimulation of indigenous oil-degrading microorganisms is often beneficial, and the appropriate addition of nitrogen- and phosphorous-based nutrients will readily improve degradation competence of the native microbial consortia. Another important concern for biodegradation is the bioavailability issue that occurs when pollutants exist in deep soil pores. They are poorly accessible by hydrocarbon degraders. Under such circumstances, microbial populations can produce oil-dispersive compounds, like biosurfactants, that could accelerate the degradation of toxic compounds (Ron and Rosenberg, 2002; Pacwa-Płociniczak et al., 2014). In this regard, bioremediation, enhanced by the direct supplementation of surfactants, exhibits great potential for improving the mobility and bioavailability of hydrocarbons and subsequent biodegradation processes.

2.2.1 Biosurfactants

Biosurfactants are functional amphiphilic compounds produced by a variety of microorganisms, either produced on the cell surface or secreted extracellularly to reduce surface and interfacial tensions. Biosurfactants contain both hydrophobic and hydrophilic groups that confer the ability to accumulate between fluid phases (Shown in Figure 2.1). Specifically, biosurfactants can decrease the surface tension between water and oil phases and improve the solubility of PHCs in liquid. Thus, biosurfactants can reduce the surface and interfacial tension at the surface and interface, respectively (Md, F., 2012). So as to increase the extraction of hydrophobic groups in soil (Silva et al., 2014; Mao et al., 2015).

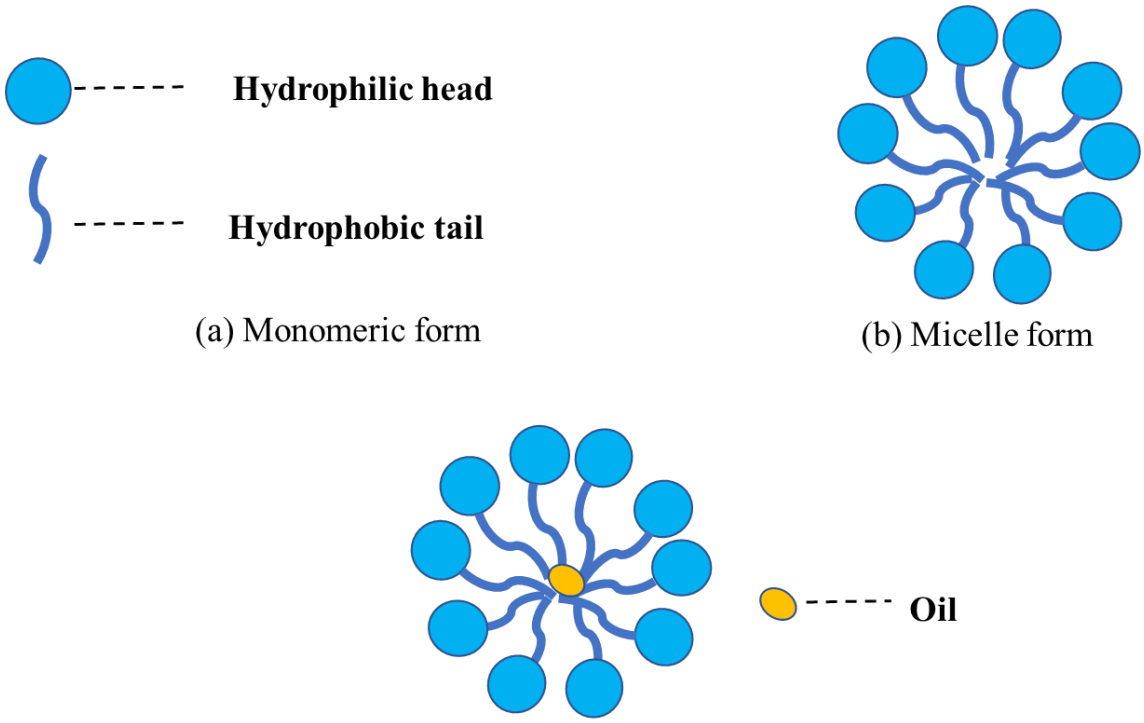


Figure 2. 1 Diagram of a biosurfactant molecule

Biosurfactants are regarded as a substitute for chemical surfactants because of the existing requirements of industries (Banat et al., 2010). Surface-active biomolecules, prepared by microorganisms, have unique characteristics and become optimal alternatives for chemical surfactants (Geys et al., 2014). Compared with recalcitrant chemical surfactants, biosurfactants are not harmful to the environment because of their high biodegradability. Moreover, Biosurfactants have distinct biological functions, such as antifungal activity, antibiotic activity, antiviral activity, insecticidal activity, and immunomodulation (Fracchia et al., 2012). Therefore, biosurfactants have broad potential for application, including in biological control of pests in cancer treatment, pharmaceuticals, medicine (Awada et al., 2011), wound-healing (Piljac et al., 2008; Stipcevic et al., 2006), and in environmental engineering such as biosurfactant-enhanced bioremediation and microbial-enhanced oil recovery (Souza et al., 2014). As Muthusamy et al. (2008) have summarized, due to the production of biosurfactants by various microorganisms, as well as various properties of biosurfactants, such as their biomedical and therapeutic properties, the production of cheap alternative substrates has recently been studied.

2.2.2 Classification of Biosurfactants

Muthusamy (2008) reported that biosurfactants can be categorized mainly by their chemical composition and microbial origin. Low-molecular mass molecules and high-molecular mass polymers are the two classes of biosurfactants. According to Rosenberg and Ron (1999), the major classes of low-mass surfactants include glycolipids, lipopeptides, and phospholipids, whereas high-mass surfactants include polymeric and particulate surfactants. Specifically, biosurfactants are classified per their chemical configuration as

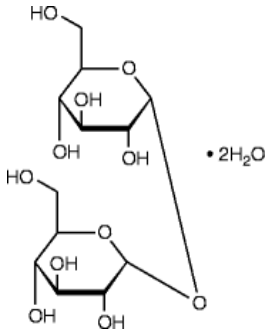
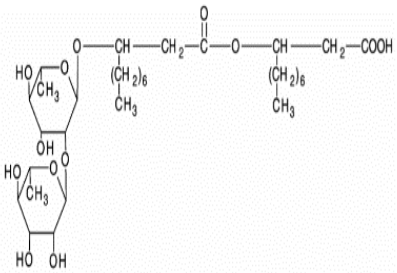
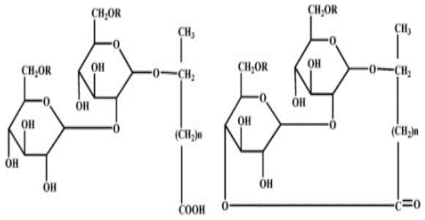
glycolipids, lipopeptides, lipopolysaccharides, or oligosaccharides and are produced by diverse bacterial genera, including glycolipids (e.g., rhamnolipids, sophorolipids, trehalose lipids), lipopeptides, lipoproteins (e.g., peptide, viscosin, serrawettin, surfactin, subtilisin, gramicidin, polymyxin), fatty acids, neutral lipids, phospholipids (e.g., fatty acids, neutral lipids, phospholipids), polymeric surfactants (e.g., emulsan, biodispersan, liposan, carbohydrate-lipid-protein, mannan-lipid-protein), and particulate surfactants (Franzetti et al., 2010). A brief discussion of each class of biosurfactants is shown in Table 2.3.

2.2.3 Application of Biosurfactant-enhanced Soil Bioremediation

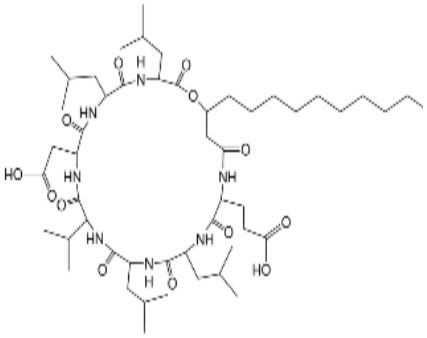
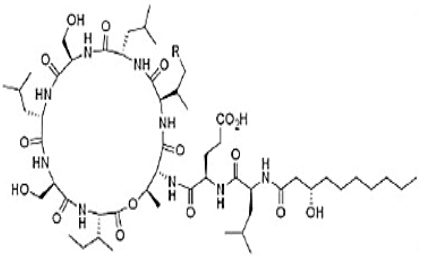
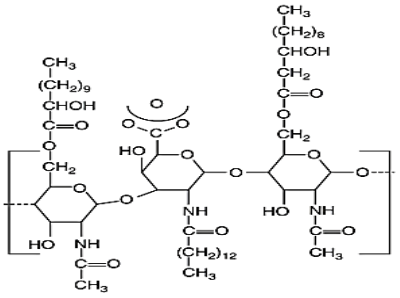
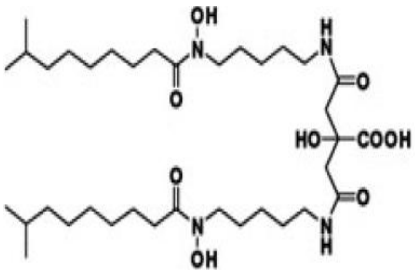
In recent years, more attention has been directed towards biosurfactants, owing to their various functional properties and diverse synthetic capabilities of microbes (Muthusamy et al., 2008). These properties provide a faster degradation rate and a more ecologically safe application. Souza (2014) indicated that, compared with chemical surfactants, biosurfactants have more benefits, such as higher biodegradability, lower toxicity, biocompatibility, and a strong adaptive ability in extreme conditions. Biosurfactants have various structures that apply to many environmental conditions with robust physico-chemical properties. When surfactant concentration further increases, the threshold surfactant concentration at which micelles begin to form is termed the critical micelle concentration (CMC) (Mao et al., 2015). Therefore, surfactants at a low concentration mainly accumulate at solid–liquid or liquid–liquid interface in the form of monomers. With increasing concentrations, surfactant molecules gradually replace the interfacial solvent like water, resulting in a lower polarity of the aqueous-phase and decreased surface tension (Mao et al., 2015). Biosurfactants aggregate and form micelles in aqueous solutions when

the concentration is higher than the critical micelle concentration. In this case, the solution's properties, including electrical conductivity, density, viscosity, osmotic pressure, and surface tension, change sharply (Ayatollahi & Zerafat, 2012). Mulligan (2005) indicated that an effective surfactant can lower the surface tension of water from 72 to 35 mN/m and the interfacial tension of water/hexadecane from 40 to 1 mN/m. Lai et al. (2009) investigated the effect of alternative nutrients on biodegradation of crude oil contaminated beach sand and the ability of rhamnolipids to further stimulate biodegradation in laboratory microcosms. The results suggested that the presence of biosurfactant could possibly have contributed to utilization of lipophilic nutrients (Lai et al. 2009).

Table 2.3 Classification of biosurfactants

Head group	Biosurfactants	Molecular formula	Applications and references
Glycolipids	Trehalolipids		Enhance the bioavailability of hydrocarbons Eg: Rhodococcus, tuberculosis, erythropolis, mycobacterium (Kuyukina et al., 2015)
	Rhamnolipids		Improve of the degradation of hydrocarbons; remove metals from soil Eg: Pseudomonas sp. (Irfan-Maqsood & Seddiq-Shams, 2014)
	Sophorolipids		Structures of free-acid (left) and lactonic (right) forms of palmitic and stearic acid sophorolipids. Enhance oil recovery; remove heavy metals from sediments; remove hydrocabons from muds Eg:Torulopsis bombicola

(Zhang et al., 2017).

Lipopeptides	Surfactin		Enhance the biodgradation of hydrocarbons and chlorinated pesticides; remove heavy metals from contaminated site Eg: Bacillus subtilis (Liu et al., 2012)
	Viscosin		Improve oil recovery (Khattari et al., 2015).
Polymeric	Emulsan		Stabilize the hydrocarbon emulsions (Chamanrokh et al., 2010)
Siderophore	Flavolipids		Chelate the ferric iron (Bodour et al., 2004)

Hisatsuka et al. (1971) found that rhamnolipids decrease the surface tension of water to 26 mN/m and the interfacial tension of water/hexadecane to <1 mN/m. Generally, biosurfactants are more effective and efficient than chemical surfactants, which means fewer biosurfactants can also obtain a maximum decrease in surface tension (Muthusamy et al., 2008).

Based on these advantages, biosurfactants have great potential for application in PHC-contaminated soil remediation. Chebbi et al. (2017) conducted an experiment with a Pseudomonas genus and found that 80% of phenanthrene degraded under the condition of an original concentration of 200 mg/l, after 30 days of incubation at 37 °C and 180 rpm. Based on forty-seven bacterial isolates, Achromobacter, Bacillus, Citrobacter, Lysinibacillus, Ochrobactrum, and Pseudomonas were discovered by Joy et al. (2017). They found that Achromobacter sp. PS1 degraded to 46.32% of 2% (w/v) crude oil with a 70.77 % and 77.17% reduction in peak area of aliphatic and aromatic fractions, respectively, in the same study. Another study from China shows that lipopeptides, produced by Pseudomonas sp. WJ6, have 92.46% of heavy-oil washing efficiency (Xia et al., 2014).

Biosurfactants can not only improve desorption of contaminants including petroleum hydrocarbons from soil, but also enhance solubilization in the liquid phase and the nonpolar compound concentration in an aqueous solution (Souza et al., 2014). Most biosurfactants are either neutral or charged negatively because of the presence of sulfate groups, phosphates, or carboxylates (Prabakaran et al., 2014). Additionally, their excellent tolerance of extreme conditions, such as high temperatures and high salt concentrations, makes them attractive components for many industrial products (Banat et al., 2010). Table

2.4 shows the representative strains of microorganisms for enhanced site bioremediation reported in the past 5 years.

2.2.4 Limitations of Biosurfactant Enhanced Bioremediation

Although the contaminants, with the addition of nutrients and biosurfactants, will significantly influence the composition and activities of indigenous microorganisms in soil. The role of biosurfactants is to promote the distribution of contaminants into the aqueous phase in order to increase the contaminants' bioavailability. There is a lack of awareness concerning of the microbial community and the associated biological mechanisms operating during the contributing to biosurfactant-enhanced bioremediation. Natural soil microorganisms are very sensitive to any ecosystem perturbation (Nikolopoulou et al., 2013), and their rapid alteration in structure and biomass is considered one of the best indicators of soil pollution (Wang et al., 2016). Knowledge of microbial communities in the overall biodegradation process is important for determining the potential for bioremediation, transformation, or persistence of pollutants (Pratt et al., 2012; Wang et al., 2013). To fill in this gap, this study made an effort to monitor microbial communities during the process of bioremediation.

Table 2.4 Example microorganisms used for treating PHC contaminated soil in recent studies

Microorganism	Contaminants	References
Pseudomonas sp. P-1 strain (rhamnolipid)	Fractions A5 and P3 of crude oil, and hexadecane	(Pacwa-Płociniczak et al., 2014)
P. aeruginosa #112 (rhamnolipid)	Crude oil	(Gudiña et al., 2015)
Rhamnolipid	PHCS	(Brown et al., 2017)
Bacillus Licheniformis ATHE9 Bacillus Mojavensis ATHE13	PAH	(Eskandari et al., 2017)
Bacillus subtilis CN2 (Lipopeptide)	PAH	(Bezza et al., 2015)
Bacillus amyloliquefaciens An6	Diesel oil	(Ayed et al., 2015)
Bacillus cereus, Bacillus subtilis, Escherichia coli, Staphylococcus aureus and Pseudomonas aeruginosa.	Petroleum hydrocarbon	(Shekhar et al., 2015)
Rhodococcus	cyclohexane	(Auffret et al.,2015)

2.3 Microbial Community Analysis during Bioremediation

Soil microbial communities play an important role in the biodegradation of PHCs. Contaminants, along with the addition of nutrients and biosurfactants, will significantly influence the composition and activities of indigenous microorganisms in soil, thus affect microbes that survive and function under those conditions (Dias et al., 2012). Natural soil microorganisms are very sensitive to any ecosystem perturbation, and their rapid alteration in structure and biomass is considered as one of the best indicators of soil pollution. However, there is a lack of information about the microbial community and the associated biological mechanisms during biosurfactant-enhanced bioremediation.

Concerning natural microbial communities, a large amount of genetic information has not been discovered. Balzer et al. (2010) demonstrated that culturable bacteria show a minor fraction of the total bacterial population present. Therefore, it is important to focus on both the culturable and the nonculturable bacteria from different environments. Diversity studies are also important for comparing samples (Fakruddin et al., 2013).

As per the definition, biodiversity refers to the range of distinct kinds of organisms and their relative abundance in a composite or community. Based on the works of Wei et al. (2018), and in accordance with information theory, diversity is also conceptualized as the quantity and distribution information in a composite or a community. Biodiversity is explicitly indicated by the levels of species (i.e., genetics), number of species, and community (i.e., ecological) diversity (Beres, 2005). According to Rastogi & Sani, (2011), in the natural microbial community, considerable genetic information remains to be discovered. Cultured bacteria only account for a small part of the current total number of

bacteria. Thus, it is significant to conduct continuous work on cultivable and uncultured bacteria from different environments. Moreover, the study of diversity is also of relative significance for comparing samples.

Abiotic and biotic factors refer to two types, which could exert influence on microbial diversity. According to Amić & Tadić, (2018), Abiotic factors consist of physical and chemical factors, like water use efficiency, salinity, aerobic/anoxic conditions, temperature, pH value, pressure, chemical pollution, heavy metals, pesticides, and antibiotics. Biotic factors include plasmid, phage, auxiliary type of DNA transposons that affect genetic characteristics, and host phenotypes in most cases. Thereby, they influence microbial diversity to a larger extent.

2.3.1 Methods for microbial community analysis

Plate counts

The most conventional approaches to detect microbial diversity via plate counts include selectivity, differential tablets, and subsequent viable counts. These methods not only have fast and inexpensive characteristics, but they also offer evidence about the activities for microbial communities and the cultivatable interbreeding segments. There are difficulties restricting the application of these approaches, such as removing bacteria or spores from soil particles or biofilm, choosing appropriate growth medium (Tabacchioni et al., 2000), and providing the exact growing situations (temperature, pH and light). It is impossible to cultivate numerous bacteria and fungi species via currently accessible technology.

Sole carbon source utilization (SCSU)

SCSU, is considered a community-level physiological profile system, such as a biochemistry identification system (API and Biolog, 1991). This tool originally developed and identified pure bacterial cultures at the level of species based on extensive surveys of their metabolic characteristics. SCSU is considered a method to use 96 commercially accessible microtiter plates, which are generally composed of 95 unlikely origins of carbon, nutrients, and a tetrazolium dye. It examines the functions of the microbial community, analyzes related data through multivariate technology, and compares the metabolic capacity of the community (Fakruddin & Mannan, 2013). The carbon substrate's oxidation changes along with the declining dye. Differentiation exists while utilizing a sole carbon source, which was used to differentiate bacteria over 50 years ago (Crotty et al., 2011). Nevertheless, because microbial communities include rapidly-growing and slow-growing organisms, slow growers may not be contained in the analysis. Additionally, during incubation, the secondary metabolites may also grow. SCSU has the advantage of possessing the ability to distinguish microbial communities, provide ease of use, ensure repeatability, and describe the generation of large amounts of data about the metabolic characteristics of communities (Ghatak & Ansar, 2017). The automated microbial identification system, Biolog, was developed generally based on aerobic metabolic activities. It has contributed to our comprehension of the use of carbon source (Bochner, 1989). The community-level method, which is considered efficient in evaluating the utilization modes belonging to the elements of a sole carbon is now being employed to research the dynamics of microbial communities. In fact, SCSU only limits the cultivable

part of the applied microbial communities (Huang et al., 2012), remaining advantageous to the rapid growth of microorganisms (Yao et al., 2000), having a sensibility to the inoculation density and reflecting the potential metabolic diversity, rather than the metabolic diversity in situ (Huang et al., 2012). Accordingly, there exist doubts over the accuracy of the information.

Nucleic acid hybridization

Based on Roh et al. (2010), nucleic acid hybridization of a specificity probe was proven to be an efficient qualitative and quantitative tool in molecular bacterial ecology. These hybridization techniques can be conducted on extracted DNA or RNA samples or in situ. Fluorescence in situ hybridization (FISH) is one of the most widespread methods of DNA hybridization. FISH (Cho et al., 2010) can be used to determine the spatial distribution of bacterial communities in various environments. The lack of sensitivity to nucleic acid hybridization directly extracted from environmental samples is the most obvious disadvantage of nucleic acid hybridization.

Polymerase chain reaction (PCR)

PCR, a form of 16S rRNA analysis, is employed to magnify a DNA sample for the sake of convenient analysis. Data on DNA's exposure to a thermostable polymerase is available, and DNA undergoes the processes of repeated cycles of template strand denaturation, oligonucleotide primer annealing, and polymerization of the template-primer duplex. The main point of PCR is using oligonucleotide primers generated to complement the desired gene or genetic area. During PCR, the DNA of double strands will be separated into a single strand when it is heated, per denaturation.

Fluorescent in situ hybridization (FISH)

FISH is regarded as a method that belongs to the cytogenetic type and it was created by biomedical experts during the early 1980s (Langer-Safer and Levine, 1982). It facilitates in situ phylogenetic recognition and count of individual microbial cells through whole-cell hybridization with ribosomal RNA-targeted oligonucleotide probes. Thus, they are covalently mono-labelled with molecules that are dyed fluorescent (Moraru & Allers, 2014)).

Although it is of vital importance in microbiology, the method of FISH is limited in many aspects. Frequently found in FISH analysis are low intensity signals and fluorescence. Other problems of FISH include those produced by utilizing rRNA as the molecule targeted for the nucleic acid probes. FISH cannot offer information on the physiology, and generally, a bacterium's common physiological activity fails to be indicated from the content of cellular rRNA (Morgenroth et al., 2000).

Lipid analysis

PLFA and fatty acid methyl esters (FAME) are lipid biomarkers that are not cultured. In their analysis, the property and dispersal of different membrane lipids are employed to establish profiles of phylogeny and metabolic activity for a micro-biological community. Membrane lipids are material that offer information on the origin of the organisms. Microbes change the membranes' lipid constitution in reaction to different environmental conditions. For instance, the content of unsaturated fatty acids will be added to enhance membrane fluidity as a response to cold temperatures (Casanueva et al., 2010; Li et al.,

2012). Thus, membrane lipids can offer information on the physiological conditions possessed by a given microbe or community (Willers et al., 2015).

Analyzing the PLFA of organisms is an efficient way to check the entire microbial community structure, because different subsets of the community possess different PLFA patterns (Jonathan et al., 2016). As a proof of principle, PLFAs provide three important attributes of microbial communities: viable biomass, microbial community structure, and physiological status (Igboji, 2015). PLFA analysis has many advantages. For example, it is repeatable, quantitative, and inexpensive, and it characterize large microbial communities rapidly. Chemically, phospholipids consist of a glycerol linked to one fatty acyl side-chain that varies in composition (i.e., length, alkyl branches, substituent, double bonds) between eukaryotes and prokaryotes, as well as between many prokaryotic groups (Joergensen and Wichem, 2008). This property makes PLFAs useful as biomarkers to determine the presence and abundance of broad functional microbial groups such as fungi, gram positive and negative bacteria (Chowdhury & Dick, 2012).

PLFA and FAME both have the advantages of being quick and inexpensive to execute. GC/MS equipment is generally utilized in most chemistry-related laboratories, and the money spent on operating individual samples can be ignored, notwithstanding the initial cost of purchasing equipment. Operating periods as short as 24 hours. have been noted for running almost 100 samples (Buyer, 2002). Moreover, PLFA profile analysis has a superior competitive advantage over conventional measures (i.e., culturable method) in studying the microbial community structure of soil, because it occupies a relatively lager percentage of the soil microbial community (Prayogo et al., 2014). The effectiveness of the rapid and

inexpensive identification of cultured isolates by PLFA and FAME has been employed to widely characterize community members, initially recognized by non-specific mechanisms of analysis (Green & Scow, 2000). For example, one researcher recognized a microorganism of interest by in situ hybridization, employing probes created from 16S denaturing gradient gel electrophoresis analysis. Then, they were separated, cultured, and subordinated to overall PLFA analysis (Macnaughton et al., 1999). Whereas such a method usually cannot be used to detect individual strains or microbial species, it can be used to detect changes in the general composition of the community. Therefore, lipid analysis provides a quantitative alternative to community structure, which is not dependent on microbial culture and does not require potential selection. According to Huberet al. (2007), this describes the microbial community on the basis of functional group affinity instead of having the specificity of identifying the members of a microbial population. In ecological studies, PLFA has become the most commonly applied.

2.3.2 PLFA Analysis

Fatty Acid

Phospholipids are composed of a single-molecule glycerol, with two OH groups belonging to glycerol, bonded to two chains of fatty acid and an OH group that is bonded to a phosphate group（Frostegård & Bååth, 1996). Therefore, the lipids are not symmetric, possessing hydrophilic and hydrophobic areas. Moreover, within the membrane, they produce two layers where the hydrophilic sides grow to the outer surface of the membrane, whereas the hydrophobic sides are buried in the inner surface (Figure 2.2).

Fatty acids are regarded as the main composition of cellular membrane for all living cells (Simons et al., 2010). There are two types of fatty acids: saturated and unsaturated. Unsaturated fatty acid occur when the carbon-carbon double bonds are found in the long aliphatic chain. Otherwise, it is a saturated fatty acid. A cis or trans configuration can be separated by the position of two carbon atoms bound to either side of the double bond (Figure 2.3). Viable microbes possess a membrane with fatty acids composing its phospholipids. However, this cannot be found in stored products or in cells that are not alive. Lipids generally account for no more than 5% of the net weight of dry bacteria and are diversified both in structure and in function (Kosa & Ragauskas, 2011). The findings obtained from precipitates and soils with base material (Saldarriaga et al., 2018) reveal that fast changes in the pattern of micro-biological samples can be identified by changing PLFA patterns, showing that PLFA analysis is a method fit for recognizing rapid changes in living creatures. Whereas PLFAs have great structural diversity, coupled with high biological specificity, there are few fatty acids that can be applied to more than one group of organisms. Branched fatty acids are biomarkers for Gram-positive bacteria and are present in some anaerobic Gram-negative sulfate-reducing bacteria. They are of the genera Cytophaga and Flavobacterium (Haack et al., 2001).

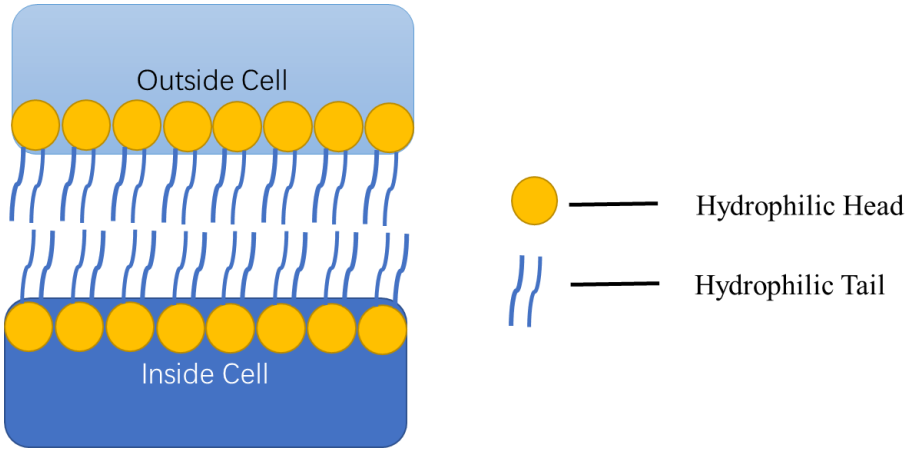


Figure 2.2 Phospholipids bilayer of membrane

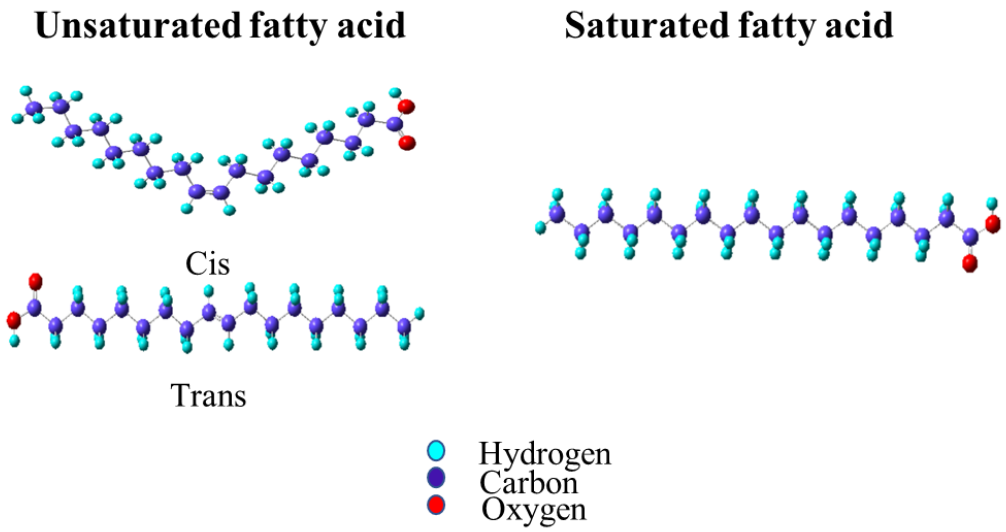


Figure 2.3 Diagram of fatty acids

There are two approaches to determine the fatty acid. First, a purely cultured lipid is analyzed to compare profiles or to build a database to help describe the polyphasic taxonomy of organic entities. Commercial databases are now accessible displaying all-cell fatty acid profiles of the saponification type for describing the taxonomy of purely cultured bacteria (Rikalovic et al., 2013). Second, lipids being analyzed within the micro-biological communities, whether straight from environment samples or from the cultivated communities found in the laboratory, are used by the community profile to compare changes obtained in the micro-biological biomass and structure of the community (Ibekwe et al., 2001). All changes obtained for the community structure can then be explained based on the database for purely cultured organisms and can obtain biological synthetic pathways (e.g., isomerization, and cyclopropyl ring formation).

There are several fatty acid extraction techniques: the microbial identification system (MIDI), simple PLFA extraction, and extended PLFA extraction (Pratt et al., 2012; Watzinger, 2015; Kaur et al., 2005). These have all been used in recent research.

PLFA extraction methods

The most widely employed method of drawing and separating fatty acids drawn from phospholipids is the one recommended by Dyer and Bligh, and altered by White (Bligh & Dyer, 1959; White, 1979). Large quantities of soils, ranging from 2g to 25g dry net weight were mixed or put in a mixed buffer fluid. The water solution of the soil was deducted from the mixture. Methanol and chloroform (2.5: 5.0: 2.5 v/v/v) was also mixed for 2 hrs. Distilled water and chloroform of the same volume were added and the two different stages were permitted to separate overnight. Then, the chloroform phase decreased by evaporating,

and then separated. It was kept at a temperature of –20°C for further use. The lipids were then separated into neutral, glyco- and phospho- (or polar) lipids. This separation employed silicic acid columns, eluted with acetone, chloroform and methanol in succession. Then, phospholipids underwent methylation and the resultant PL-FAME was split. Quantification was made by GC. PLFA profiles obtained from soil samples provide quick and reliable measuring for characterizing the numerically dominant part of soil micro-biological communities, containing uncultivatable organisms.

This sort of measurement is efficient and has been employed for various types of soil for analyzing the microbiological communities (Frostegard and Baath, 1996) and for overall micro-biological biomass assessment. Part of the alteration discovered in PLFA modes was limited generally to one or a few signature fatty acids. However, because of the restricted quantity of the widely-scattered fatty acids gained from this process, they were regarded as alterations undergone in the percentage accounted for by some key groups of organic matter in the soil samples (Fakruddin & Mannan, 2013). As for the soil's PLFA, analysis could then be deemed an efficient and accurate method for the restricted recognition of entire changes in the microbiological community pattern.

PLFA aided microbial community analysis

Analyzing the PLFA of organisms is an efficient way to check the entire microbial community structure, because different subsets of the community possess different PLFA patterns (Fakruddin & Mannan, 2013). Although this usually cannot be used to detect individual strains or microbial species, it can be used to detect changes in the general composition of the community. Interference factors that are generally linked to studying

microbial communities of soil, such as sieving, storing, and incubating temperature, were studied by Klug and Petersen (Klug and Petersen, 1994). They found an obvious connection between increased temperature (25°C) and improved the overall situation of the PLFA fraction. Some alterations could be explained by referring to the temperature adjustment mechanisms or as reactions to stressful situations, including a decreased level of unsaturation, improved generation of cyclopropyl fatty acids, and an improved percentage of the sub-chain fatty acids, i15:0 and i17:0 over a15 :0 and a17: 0, in succession. A decline of the overall quantity of PLFAs was also shown. Helgason et al. (2010) demonstrated that alterations in lipid profiles revealed shifts in the constitution of microbial community and the functions linked to soil warmth. The richness of PLFA biomarkers for Gram-negative bacteria and Gram-positive bacteria were found to have a significant difference at different temperatures. Therefore, lipid analysis provided a quantitative alternative to the community structure that is not dependent on microbial culture and does not require potential selection. PLFAs are useful biomarkers or signatures for fingerprinting the soil microbial community because of the relative abundance of certain PLFAs, which differ considerably among the specific groups of microorganisms (Kujur & Patel, 2014). Rodriguez et al. (2010), described the microbial community on the basis of functional group affinity instead of having the specificity of identifying members of the microbial population. In ecological studies, lipids have become the most commonly applied marker component used to determine a microbial communities' composition (Drenovsky et al., 2010). Blood lipid profiles possibly change because of changes in the physiological state of the existing population or the actual changes in the community structure. Estimates of such "signatures" may offer instructive insights into community structures, nutritional

status, and activities. PLFA analysis is usually employed in soil studies with the aim of distinguishing between bacterial and fungal biomass (Rousk et al., 2010). Due to the frequent treatment of hop plants with copper-based fungicides, a large amount of copper has accumulated in soil where they are cultivated. This has resulted in the creation of a microbial community with markedly different PLFA patterns compared to communities associated with grassland soil or crop-rotation soils (Kaiser et al., 2010). Based on (Rillig & Thies, 2012), the ratio of fungi and bacteria (F:B) can be determined by calculating the value of the sum of the molar percentage of fungal fatty acid markers (i.e., saprophyte and mycorrhizal fungi) divided by the sum of the molar percentage of bacterial fatty acid markers. The increase of F:B suggests an increase in the concentration of fungi in the microbial community. The fluctuation of F:B is related to environmental processes, including nutrient cycling, organic decomposition, and carbon fixation (Romaniuk et al., 2011; Hogberg et al., 2013; Bragazza et al., 2015). It is common to compare the influence of agricultural practices on soil microbial communities (Bailey et al., 2002b; De Vries et al., 2006; Romaniuk et al., 2011; Zhang et al., 2012).

PLFA analysis, a rapid, inexpensive, sensitive, and sustainable tool for assessing soil structure (Frostegård et al., 2011; García-Orenes et al., 2013; Ławniczak et al., 2013), was used to evaluate the performance of microorganisms during PHC bioremediation. Specific PLFA patterns revealed robust information on microbial community's structures and physiological and nutritional status, as well as the viable biomass of the microbial soil population (Frostegård et al., 2011). PLFA analysis has been widely used to determine differences in microbial community structures in soil under various environmental factors,

such as soil pore size (Ruamps et al., 2011), soil water availability (Ruamps et al., 2011), spatial patterns in marine sediments (Fischer et al., 2010), and spatial covariation in polluted soil (Torneman et al., 2008). It was also used in associated community composition studies on PAH-contaminated riverbank sediment (Pratt et al., 2012), nutrient-stimulated (Hammer et al., 2011) and chemical surfactant-enhanced bioremediation of hydrocarbon-contaminated soil (Lai et al., 2009; Mair et al., 2013). According to Mahmoudi et al. (2013), the majority of microorganisms in environmental samples cannot be cultured in laboratory culture media. Moreover, PLFAs and high-throughput sequencing technology provide different opportunities to study microorganism cultures. A PLFA map, as a reliable tracer for microbial environmental stress under different treatment conditions, can demonstrate advanced taxonomic groups of soil microorganisms, such as bacteria, fungi, actinomycetes, and protozoa (Pratt et al., 2012; Silva et al., 2010). Additionally, detailed soil bacterial diversity and in situ community structures can be revealed from the high-throughput sequencing of the 16S rRNA gene. Furthermore, with an accuracy of 82–95% metagenome, based on the community system development survey, the calculation method can be used to accurately predict a 16S rRNA sequence of bacterial community gene families (Langille et al., 2013).

Therefore, the PLFA approach are applicable for tracking microbial communities and microbial dynamics during biosurfactant-enhanced bioremediation, the study of which previously has been very limited.

2.4 Summary

Removing PHCs from contaminated soil has been a challenging task. Traditional physical and chemical approaches, such as landfills, have been frequently used. However, these solutions are destructive and unsustainable. Contrastingly, during bioremediation, microbial populations can produce oil dispersive compounds, like biosurfactants that can accelerate the degradation of toxic compounds without environmental destruction (Ron and Rosenberg, 2002; Pacwa-Płociniczak et al., 2014). In this regard, bioremediation, enhanced by direct supplementation of biosurfactant, exhibits great potential for improving the mobility and bioavailability of PHCs and their subsequent biodegradation. Although surfactants have been approved that show strong performance in biodegradation, the approach typically exhibits less accuracy in estimating the optimal conditions and provides limited information on factor interactions when compared with designed experiments (Vasilev et al., 2014). Additionally, precise knowledge is still needed to study the long-term and short-term effects of chemical surfactants and biosurfactants for enhancing soil remediation (Makkar and Rockne, 2003).

Concerns about how soil microbial communities work in the biodegradation of PHCs have been growing. Thus, Section 2.3 reviewed methods of monitoring microbial communities. PLFA analysis has been widely used to determine differences in the microbial community structure of soil under various environmental factors, such as soil pore size (Ruamps et al., 2011), soil water availability (Ruamps et al., 2011), spatial patterns in marine sediments (Fischer et al., 2010), and spatial covariation in polluted soil (Torneman et al., 2008). In this thesis, PHC-contaminated soil was supplemented with nutrients and a biosurfactant to

enhance bioremediation. The design of experiments (DOE) based system optimization was conducted and the PLFA based microbial community analysis was achieved.

CHAPTER 3

METHODOLOGY

[1]

The contents of Chapters 3 and 4 are based and expanded on the following paper:

(1) Li, X., Fan, F., Zhang, B., Zhang, K., & Chen, B. (2018). Biosurfactant enhanced soil bioremediation of petroleum hydrocarbons: Design of experiments (DOE) based system optimization and phospholipid fatty acid (PLFA) based microbial community analysis. International Biodeterioration & Biodegradation.

*Xixi Li, Fuqiang Fan and Kedong Zhang conceived of the presented idea. Xixi Li carried out the experiments. Xixi Li and Fuqiang Fan developed the theory, performed the computations, and contributed to the final version of the manuscript. Dr. Baiyu Zhang and Dr. Bing Chen encouraged and supervised the findings of this work.

3.1 PHC Contaminated Soil and Its Characterization

PHC impacted soil used throughout the experiments was collected from a farm that was a part of a petroleum supply storage system in the province of Newfoundland and Labrador, Canada. The average daily temperatures in the farm range from -18.1°C (January) to 15.5°C (July) and the average precipitation is 949 mm. The soil was characterized by the material of peat, fine-to-medium grained sand and discontinuous silt layers.

The moisture content of the soil was measured by a gravimetric method from 5 g sample, in which the crucible with samples was dried at 105 °C in the oven overnight until a constant weight was achieved. The dried samples in crucible were further placed into muffle-furnace at 550 °C for 4 hr and the weight of the ignited sample was used to calculate soil organic matter content. The C/N ratio was obtained from measuring total carbon and nitrogen contents by the LECO TruSpec CN Determinator (LECO Corporation, St. Joseph, MI) after a drying process in the oven (105 °C for 24 hr). A bench top pH meter (EL20, Mettler Toledo) was used to measure the soil pH in a mixture with a soil: water ratio of 1:2 (weight/volume). The electrical conductivity (EC) was measured by bench top EC meter (Orion Star A222 and A322, Thermo Scientific).

3.2 Experimental Design and Kinetic Settings of PHCs Degradation

Design of experiment (DOE) was firstly proposed by Fisher (1937) for analyzing the influence of water and rain on crop production. A set of experiments using orthogonal arrays was developed. Compared with traditional experiment design, DOE focuses on the effect of variation on the process characteristics (Ross, 1996). DOE can be used to modify

the collocation in bioremediation. Mohan et al. (2007) used Taguchi orthogonal array (OA) to evaluate the influence of eight biotic and abiotic factors including humic substance concentration, substrate-loading rate, soil microflora load, slurry phase dissolved oxygen, soil water ratio, slurry phase pH, temperature, and application of bioaugmentation. The substrate-loading rate was recognized to have representative significant influence on the bioremediation process. Kazemi et al. (2016) design an experiment to investigate the effects of multiple factors and their interactions on the performance of a municipal solid waste (MSW) composting process. Final C/N, germination index (GI) and especially the enzyme activities were determined as the significant factors that influence the municipal solid waste (MSW) bioremediation through composting. A DOE based methodology was adopted in this study to investigate the effects of multiple factors and their interactions on the performance of bioremediation of PHC. The impact of four factors, type of surfactant, concentration of surfactants, concentration of nutrient and degradation time were investigated.

To identify the quantity of these factors, literature review on previous studies and a lots pre-experiments have been learned and conducted. Szulc et al. (2013) and Tahseen et al. (2016) provided the optimized concentration of rhamnolipid based on a set of experiments. A general factorial design was used to systematically investigate the factors of surfactant type, surfactant concentration and nutrient concentration on biostimulation. Rhamnolipids and Tween 80 were used to represent BS and synthetic surfactant, respectively, and the concentrations (0, 50 and 150 mg/kg) applied to the soil were referred from previous studies (Nikolopoulou et al., 2013; Szulc et al., 2014). The nutrient solution containing

$NaNO_3$ and K_2HPO_4 at 10 g/l and 2 g/ L, respectively, was applied to the soil at three levels (0, 400 and 1000 μL for each run) to support the growth of hydrocarbon metabolizing bacteria (Leys et al., 2005; Moldes et al., 2011). PHCs of each run were analyzed after 0, 3, 8, 17, 26 and 36 days of incubation. Apart from the 108 general factorial runs (2 × 3 × 3 × 6) Table 1, an extra of 36 duplicates was set to test experimental stability and yield pure errors in the analysis of variance (ANOVA) test. For each run, a 30 g of soil was independently added to a semi-open beaker and was stirred weekly to maintain aerobic conditions at ambient temperature. The sterilized and non-sterilized control were set to investigate the degradation kinetic of PHCs. Experiments with increased amounts of rhamnolipids and nutrients were conducted to further investigate the degradation potential of the microorganisms.

Table 3.1 Experimental design
(Factor A: Type of surfactant (2 levels); Factor B: concentration of surfactants (3 levels); Factor C: Concentration of nutrient (3 levels); Factor D: Time (4 levels)

Std	Run	Factor A	Factor B	Factor C	Factor D
1	14	Rhamnolipid	0	0	0
2	16	Tween 80	0	0	0
3	22	Rhamnolipid	50	0	0
4	71	Tween 80	50	0	0
5	20	Rhamnolipid	150	0	0
6	66	Tween 80	150	0	0
7	28	Rhamnolipid	0	400	0
8	51	Tween 80	0	400	0
9	29	Rhamnolipid	50	400	0
10	64	Tween 80	50	400	0
11	36	Rhamnolipid	150	400	0
12	38	Tween 80	150	400	0
13	49	Rhamnolipid	0	1000	0
14	37	Tween 80	0	1000	0
15	48	Rhamnolipid	50	1000	0
16	43	Tween 80	50	1000	0
17	42	Rhamnolipid	150	1000	0
18	47	Tween 80	150	1000	0
19	34	Rhamnolipid	0	0	3
20	70	Tween 80	0	0	3
21	19	Rhamnolipid	50	0	3
22	65	Tween 80	50	0	3
23	30	Rhamnolipid	150	0	3
24	39	Tween 80	150	0	3
25	9	Rhamnolipid	0	400	3
26	15	Tween 80	0	400	3
27	55	Rhamnolipid	50	400	3

28	44	Tween 80	50	400	3
29	53	Rhamnolipid	150	400	3
30	2	Tween 80	150	400	3
31	60	Rhamnolipid	0	1000	3
32	23	Tween 80	0	1000	3
33	27	Rhamnolipid	50	1000	3
34	63	Tween 80	50	1000	3
35	57	Rhamnolipid	150	1000	3
36	40	Tween 80	150	1000	3
37	5	Rhamnolipid	0	0	8
38	58	Tween 80	0	0	8
39	8	Rhamnolipid	50	0	8
40	62	Tween 80	50	0	8
41	25	Rhamnolipid	150	0	8
42	18	Tween 80	150	0	8
43	69	Rhamnolipid	0	400	8
44	32	Tween 80	0	400	8
45	33	Rhamnolipid	50	400	8
46	31	Tween 80	50	400	8
47	56	Rhamnolipid	150	400	8
48	11	Tween 80	150	400	8
49	24	Rhamnolipid	0	1000	8
50	10	Tween 80	0	1000	8
51	7	Rhamnolipid	50	1000	8
52	6	Tween 80	50	1000	8
53	41	Rhamnolipid	150	1000	8
54	67	Tween 80	150	1000	8
55	4	Rhamnolipid	0	0	17
56	46	Tween 80	0	0	17
57	72	Rhamnolipid	50	0	17
58	52	Tween 80	50	0	17

59	35	Rhamnolipid	150	0	17
60	1	Tween 80	150	0	17
61	3	Rhamnolipid	0	400	17
62	21	Tween 80	0	400	17
63	50	Rhamnolipid	50	400	17
64	61	Tween 80	50	400	17
65	68	Rhamnolipid	150	400	17
66	45	Tween 80	150	400	17
67	13	Rhamnolipid	0	1000	17
68	17	Tween 80	0	1000	17
69	26	Rhamnolipid	50	1000	17
70	54	Tween 80	50	1000	17
71	12	Rhamnolipid	150	1000	17
72	59	Tween 80	150	1000	17
73	88	Rhamnolipid	0	0	26
74	78	Tween 80	0	0	26
75	97	Rhamnolipid	50	0	26
76	105	Tween 80	50	0	26
77	91	Rhamnolipid	150	0	26
78	86	Tween 80	150	0	26
79	107	Rhamnolipid	0	400	26
80	84	Tween 80	0	400	26
81	98	Rhamnolipid	50	400	26
82	99	Tween 80	50	400	26
83	104	Rhamnolipid	150	400	26
84	79	Tween 80	150	400	26
85	106	Rhamnolipid	0	1000	26
86	77	Tween 80	0	1000	26
87	76	Rhamnolipid	50	1000	26
88	87	Tween 80	50	1000	26
89	75	Rhamnolipid	150	1000	26

90	73	Tween 80	150	1000	26
91	74	Rhamnolipid	0	0	36
92	82	Tween 80	0	0	36
93	72	Rhamnolipid	50	0	36
94	92	Tween 80	50	0	36
95	95	Rhamnolipid	150	0	36
96	94	Tween 80	150	0	36
97	102	Rhamnolipid	0	400	36
98	83	Tween 80	0	400	36
99	101	Rhamnolipid	50	400	36
100	95	Tween 80	50	400	36
101	90	Rhamnolipid	150	400	36
102	93	Tween 80	150	400	36
103	80	Rhamnolipid	0	1000	36
104	90	Tween 80	0	1000	36
1 05	81	Rhamnolipid	50	1000	36
106	103	Tween 80	50	1000	36
107	89	Rhamnolipid	150	1000	36
108	85	Tween 80	150	1000	36

The degradation kinetic of PHCs were achieved by several settings. Firstly, the sterilized control was set through sterilizing soil in an autoclave (at 120 °C for 45 mins) to block the influence of the microbial activity and evaluate the influence of non-bionic factors in the biodegradation of hydrocarbon-contaminated soil. The no-sterilized control with contaminants but in the absence of nutrients and biosurfactant was also set as a comparison to evaluate the natural activities of indigenous microorganisms on the PHCs removal. All the sterilized and non-sterilized control samples were prepared in triplicate and analyzed after 0, 3, 8, 17, 26 and 36 days of incubation. Additionally, the overall PHCs biodegradation behavior in soil stimulated by rhamnolipids and Tween 80 at final concentrations of 50 mg/kg and 150mg/kg were investigated. The results were obtained from the averages of treatments with different nutrient solutions from the factorial design.

Besides, experiments with increased amounts of rhamnolipids and nutrients were conducted to further investigate the degradation potential of the microorganisms over the studied time period. In brief, the Nutrient Double was achieved by spraying 2000 µL nutrient solution while maintaining 150 mg/kg rhamnolipids in the initial soil treatment. The Rhamnolipids Double was achieved by adding 300 mg/kg rhamnolipids while spraying 1000 µL nutrient solution in the initial soil treatment. PHCs were analyzed for each run after 0, 3, 8, 17, 26 and 36 days of incubation and all runs in this experimental setting were prepared in triplicate.

Based on the degradation results of PHCs, soil samples for PLFA analysis were collected on day 0, 17 and 54 to indicate the initial, thriving and decaying status of microbial activities. Samples from no-sterilized control as well as those amended by Tween 80 and

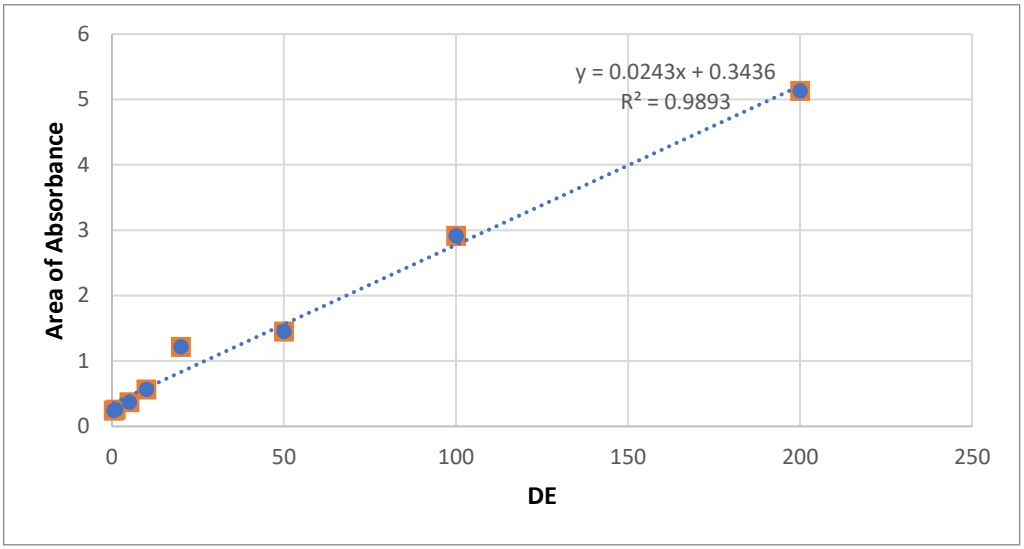
rhamnolipids with 400 µL nutrient solution added were subjected to PLFA profiling. All the PLFA analyses were carried out in triplicate during the degradation period.

3.3 PHCs Analysis

Quantification of the PHCs in soils was conducted by gas chromatography/mass spectrometry (GC-MS). Each individual set in the experimental design with 30 g soil was employed as an independent sample to ensure the accuracy of the PHCs determination. An aliquot 50 ul of surrogate hexadecane-d34 at 2000 ppm was firstly spiked into the sample in order to examine the methodological recovery. Soil samples were dried with anhydrous Na_2SO_4 and extracted with a 30 ml solvent of dichloromethane (DCM) and hexane (1:1). Subsequently, the mixture was subjected to intermittent ultra-sonication (15s on/15s off pulses) for 3 minutes at the maximum power of an ultrasonic instrument. The soil sample was extracted three times using the same amount of extraction solvent. All the extraction solvents were filtrated, collected and then concentrated to 10 mL via rotary evaporator in 35 °C water bath. The internal standard nonadecane-d40 was further added and yielded a concentration of 20 ppm in the final solution before GC-MS analysis.

The PHCs solution was transferred into amber vials and analyzed on a GC-MS system (Agilent 7890A GC system coupled with a 5975C MSD) interfaced with an Agilent 7693 auto-sampler. Data acquisition, processing and evaluation from the full scan mode (range 50-500 m/z) were carried out using Agilent Chem Station Software Version 2.01. PHCs were separated on a 30 m × 250 µm (internal diameter, i.d.) × 0.25µm DB-5MS UI fused silica capillary column. An electronic pressure control (9.07 psi) was utilized to maintain a constant carrier gas (Helium of ultrahigh purity) flow of 1.2 mL/min throughout the oven

program. Sample injections (2μL) were conducted using a split/splitless injector (single tapered inlet liner, pulsed-splitless mode) at 200 °C under a pulse pressure of 25 psi. The initial oven temperature was set to 55 °C, followed by a temperature ramp of 7 °C/min up to 265 °C, a rump of 15°C/min to 295 °C, and finally to 300°C with a running time of 40 min. The standard calibration curves of C_{14}, C_{18}, and C_{22} are shown in Fig. 3.1.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R² = 0.9893
Area of Absorbance
DE
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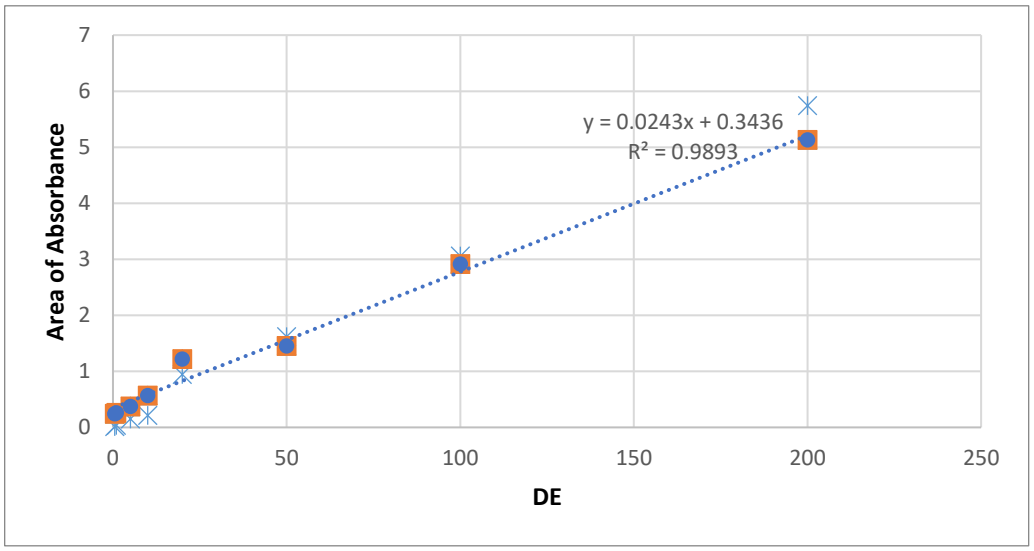
1
2
3
4
5
6
0
50
100
150
200
250

(a)


y = 0.0243x + 0.3436
R² = 0.9893
Area of Absorbance
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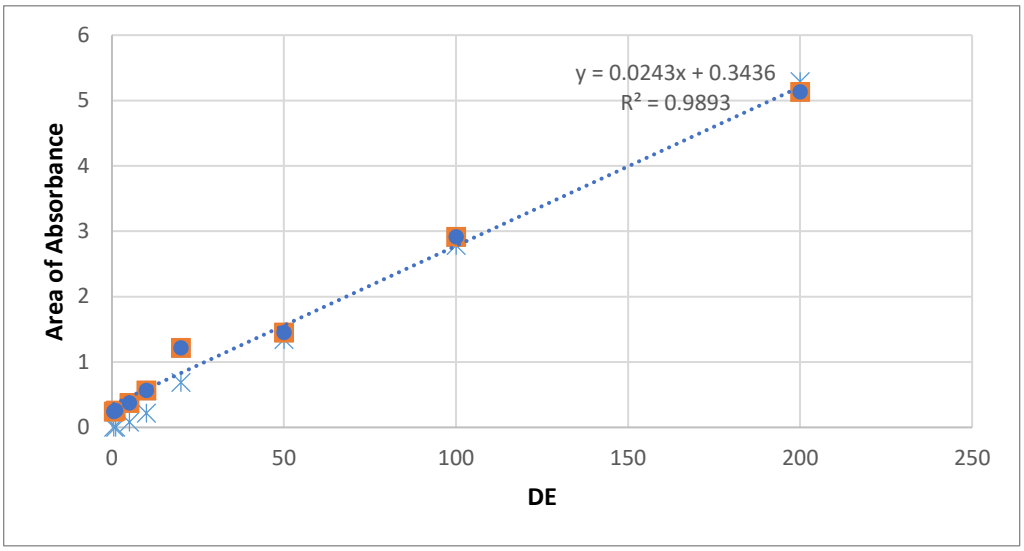
(b)



(c)

Figure 3.1 Standard calibration curves for (a) C_{14} analysis (b) C_{18} analysis (c) C_{22} analysis.

3.4 Microbial PLFA Identification

PLFA analysis was conducted following a modified Bligh and Dyer extraction method (Fang and Findlay, 1996). Briefly, total lipids were extracted from 5 g soil sample using 9.5 ml of one-phase extraction solvent (methanol: DCM: 125 mM phosphate buffer at pH 7.4 = 2:1:0.8). The extraction mixture was allowed to stand overnight in the dark at -20 ^{o}C and followed by a partitioning with the addition of DCM and water to yield a ratio of DCM: methanol: water of 1: 1: 0.9. The lower organic phase was transferred and collected. The extraction was performed three times to ensure high extraction efficiency. The lipids were then fractionated into neutral lipids, glycolipids and phospholipids using 4 mL of DCM, 4 mL of acetone and 10 mL of methanol on a home-made solid phase extraction (SPE) tube (miniature champagne column) packed with 0.1 g silica gel, respectively. The fraction of phospholipids was evaporated to dryness, redissolved in 1 mL of methanol: toluene (1:1, v/v) and transesterified into fatty acid methyl esters (FAMEs) by mild alkaline methanolysis. The FAMEs solution was finally concentrated to 400 μL with the addition of methyl ester internal standards 14:1ω5c and 21:0 to each sample before GC-MS quantitative analysis (Ziegler et al., 2013). Phospholipid standard C19:0 PC (1,2-dinonadecanoyl-sn-glycero-3-phosphocholine; Avanti Polar Lipids) was spiked in the beginning to determine phospholipid recovery and four blanks spiked with C19:0 PC were performed for process control. Phospholipids of all samples were determined with three replicates.

Quantification and identification of FAMEs were referred from several standards: Bacterial Acid Methyl Esters CP Mixture, FIM-FAME-7 Mixture, 10Me16:0, and 16:1ω7t were from Matreya LLC (Pleasant Gap, Pennsylvania, USA); 18:1ω7t was from Sigma-Aldrich (Oakville, Ontario, Canada). PLFA nomenclature follows the form A:BωC, where A indicates the total number of C atoms, B indicates the number of double bonds and C refers to the location of double bonds in the fatty acid molecule. The geometric isomers cis and trans are indicated by the suffixes c and t, respectively. Prefixes i and a are given for iso- and anteiso-branched FAMEs, while Me and cy refer to methyl groups and cyclopropane groups, respectively.

Certain PLFAs were used as biomarkers to track relative differences in the activities of broadly separated functional groups relevant to PHC degradation (Li et al., 2007; Zelles, 1999). Specially, the fatty acids i15:0, a15:0, 15:0, i16:0, 16:1ω7c, 17:0, i17:0, cy17:0, 18:1ω7c and cy19:0 were used to represent bacterial biomass (bacterial PLFAs), and 18:2ω6,9c was chosen to indicate fungal biomass (fungal PLFA). The PLFAs of bacterial origin were further classified as Gram-positive bacterial PLFAs (i-C15:0， a-C15:0, i-C16:0, and i-C17:0) and Gram-negative bacterial PLFAs (cy17:0, cy19:0, 16:1ω7c and 18:1ω7c). Phospholipid fatty acids not assigned as biomarkers were included in total PLFA yields (total biomass).

Besides, PLFA compositional patterns including isomerization of cis to trans unsaturated fatty acids, the distribution of saturated, monounsaturated and cyclopropyl PLFAs can also be employed to track at least partly physiological change or stress responses in contaminated environments. The physiological or nutritional stress in bacterial

communities may also be indicated by the ratio of cyclopropyl PLFAs and their monounsaturated precursor fatty acids (Fischer et al., 2010; Zelles, 1999). In our studies, the ratios of saturated (SAT) and monounsaturated (MONO) PLFAs (Gómez-Brandón et al., 2011; Moore-Kucera and Dick, 2008) in conjunction with (cy17:0+cy19:0)/ (16:1ω7c+18:1ω7c) were used as indicators of physiological or nutritional stress in bacterial communities.

3.5 Statistical Analysis

Triplicate samples (in PLFA analysis, PHCs experimental controls and enhanced nutrient & rhamnolipids study) were prepared and analyzed to ensure the reproducibility of results, and the error bars in the plotted data stand for the standard deviations of the mean values of triplicate samples. Principal component analysis (PCA; SPSS 18.0) was conducted to analyze patterns of intercorrelations among variables of PLFA species measured. The PLFA data were orthogonally transformed into a new coordinate system and principal components (PCs) with the greatest variances (80.4% and 10.0%, respectively) were used as coordinates.

Correlations between the measured parameters (surfactant type, surfactant concentration, nutrient concentration and time) were analyzed by Design-Expert 8.0. A response surface reduced quadratic model was developed to provide efficient estimates of the effects and investigate the weights and interactions of them. The abundance of PHCs was transformed to a natural logarithmic scale to reduce the effect of outlying observations. An ANOVA of the response surface reduced quadratic model was conducted to analyze significant

differences among the means of each PHCs abundance group. Differences were considered statistically significant at $p < 0.05$.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Soil Characterization

The soil texture, as well as the content of organic matter, moisture, C/N ratio, pH, EC, and the PHC concentrations, were given in Table 4.1 (supplementary material). The standard deviations from triplicate tests were all below 10%. The soil investigated was a mixture of clay, sand and silt with low permeability. The relatively high moisture of 26.6% was due to the influence of offshore wet weather and onshore runoff distribution. From the crucible tests, the organic matter accounted for 31.2% and its enrichment was attributed to the presence of peat in soil. Most agricultural soils of Atlantic Canada are naturally acidic (Harmsen et al., 2007) due to the severe leaching of elements (e.g. Ca, Mg) from the surface soil caused by high precipitation. The soil pH detected was in the range of 5.4–5.8, and the EC was 653.6 mS/cm with a C/N ratio about 16.4. The results of PHC analysis indicated the contamination was mainly attributed to petroleum diesel. The above-mentioned parameters entail appropriate set-up design factors to allow the sufficient development of the microbial population with robust enzymatic activities.

Table 4.1 Physicochemical characteristics of the soil employed in the experiments

Properties	units	value
Moisture	%	26.6
Organic matter	%	31.2
Texture		clay-sand-silt
C/N ratio		16.4
pH		5.4-5.8
EC	mS/cm	653.6
C10-C16	μg/g	2077
C16-C20	μg/g	258
C20-C24	μg/g	301

4.2 System Analysis

Enhanced bioremediation is directly associated with two factors that may limit biodegradation of petroleum pollutants on site. One is the catabolic potential of microorganisms used for biodegradation and the other is the bioavailability of the contaminants. The effects of surfactant type, surfactant concentration and nutrient concentration on the removal of PHCs by indigenous microorganisms were involved in the time-based general factorial design. The ANOVA results from for PHC microbial remediation are presented in Table 4.2. The Model F-value is as high as 161.85, which implies the model is significant and there is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The experimental data were fitted to a response surface reduced quadratic model to describe the weights of each factor on the system. The final equation in terms of coded factors was obtained as:

Ln (PHCs concentration) = 6.69+0.032×A-0.035×B-0.24×C-
0.89×D+0.032×A×C+0.054×A×D-0.25×C×D+0.053×C2+0.10×D2

where A is surfactant type, B is surfactant concentration, C is nutrient, and D is time. Detailed description of the response surface reduced quadratic model was provided in Appendix C.

The model had a satisfactory coefficient of determination (R2 = 0.8924), which revealed that more than 91% of the variations could be explained by the independent parameters listed above. Also, the "Pred R-Squared" is in reasonable agreement with the "Adj R-Squared" (data not shown). These results indicated that our experimental results agreed well with the values predicted by the model. According to our model, the lowest PHC

concentration was 201.88 mg/kg (92.3% removal of PHCs), and it was achieved by applying rhamnolipids at a concentration of 150 mg/kg and nutrient solution at 1000 µL. Therefore, we initially concluded that the application of rhamnolipids exhibited better performance than Tween 80 in PHC degradation under sufficient nutrient conditions.

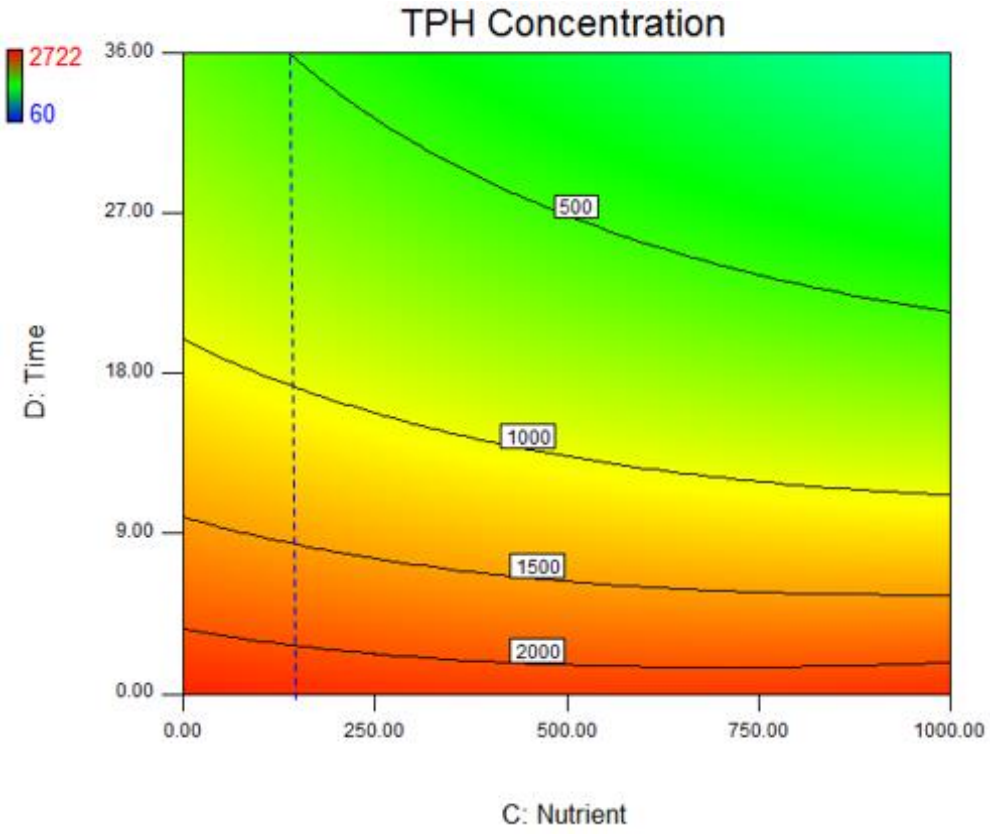
Table 4.2 Analysis of variance (ANOVA) for response surface reduced quadratic model

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F
Model	63.75545	9	7.083939	123.4815	< 0.0001
A-Surfactant type	0.138315	1	0.138315	2.410999	0.1228
B-Surfactant concentration	0.120281	1	0.120281	2.096639	0.1500
C-Nutrient	5.056945	1	5.056945	88.14861	< 0.0001
D-Time	56.39806	1	56.39806	983.0857	< 0.0001
AC	0.099129	1	0.099129	1.727938	0.1909
AD	0.206962	1	0.206962	3.607595	0.0597
CD	3.015944	1	3.015944	52.57152	< 0.0001
C^2	0.081806	1	0.081806	1.425979	0.2345
D^2	0.223352	1	0.223352	3.893296	0.0505
Residual	7.687366	134	0.057368		
Lack of Fit	6.238746	98	0.063661	1.582046	0.0602
Pure Error	1.448621	36	0.040239		
Cor Total	71.44281	143			

The biostimulation of indigenous soil microorganisms is influenced by a series of environmental factors such as nutrient availability, oxygen content, water, pH, and temperature (Dias et al., 2012). The results indicated the nutrient addition, which is of great importance to microbial assimilation and dissimilation processes (Maki et al., 2003), contributed significantly to the biodegradation of PHCs. Nitrogen (N) is found in all amino acids, proteins, and enzymes, while phosphorus (P) is involved in energy trapping and transfer as adenosine triphosphate. Specially, the nutrients or fertilizer use may be essential in some environments with insufficient nutrient levels. Our results of significant nutrient bioremediation enhancement indirectly indicate the soils are infertile due to the leaching of nutrients by high precipitation in Atlantic area (Harmsen et al., 2007). Walworth et al. (1997) evaluated the relationship between soil water content and microbial response to soil nitrogen (N) in petroleum-contaminated soils and mentioned ineffective nutrient stimulation of degradation can be correlated with low water content in soil. Soil texture and organic matter are the key factors in soil to determine soil water holding capacity. Moreover, soil organic matter rich in nutrients such as nitrogen (N), phosphorus (P) can be an important sink and source of nutrients (McMurtrie et al., 2001). Consequently, adding appropriate organic (peat or muck) soils or other organic bulking agents (such as manure, vegetable wastes, etc) were recommended in practical large-scale remediation acts (Naseri et al., 2014) to change soil physical properties and increase soil organic matter.

The interactions of independent variables from the quadratic model were shown in Fig. 4.1. The results indicate that rhamnolipid treatment on contaminated soil facilitated the biodegradation more than the treatment of Tween 80 during the long-term investigation

(day 36) under same nutritional conditions (Fig. 4.1a & 4.1b). Less time or lower amount of nutrient is required to achieve the same degree of PHC degradation using the treatment of rhamnolipids than Tween 80, although the difference is not obvious during the initial 17 days. Response surface plots of Fig. 4.1c & 4.1d indicated that the concentrations of PHCs in soil decreased rapidly in the first stage (10 days), but the degradation rates of PHCs gradually slowed down after the period. Under the two circumstances, the nutrient concentrations were both found to be positively correlated with the PHC removal while the surfactant type only slightly affected the final PHC response. In general, a bit lower PHC level was obtained from the treatment of rhamnolipids than the identical treatment of Tween 80 on contaminated soil. The lowest predictions of PHC concentration from rhamnolipids and Tween 80 treatment on soil are 201.9 mg/kg (92.3% removal of PHCs) and 255.7 mg/kg (90.3% removal of PHCs), respectively. The lowest values are both obtained from a biodegradation of 36 days with 1000 μL nutrient solution and 150 mg/kg surfactant initially added. The biofriendly and biocompatible rhamnolipids better promoted metabolic activities of the population than Tween 80 in contaminant biodegradation.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36.00
27.00
18.00
9.00
0.00
D: Time
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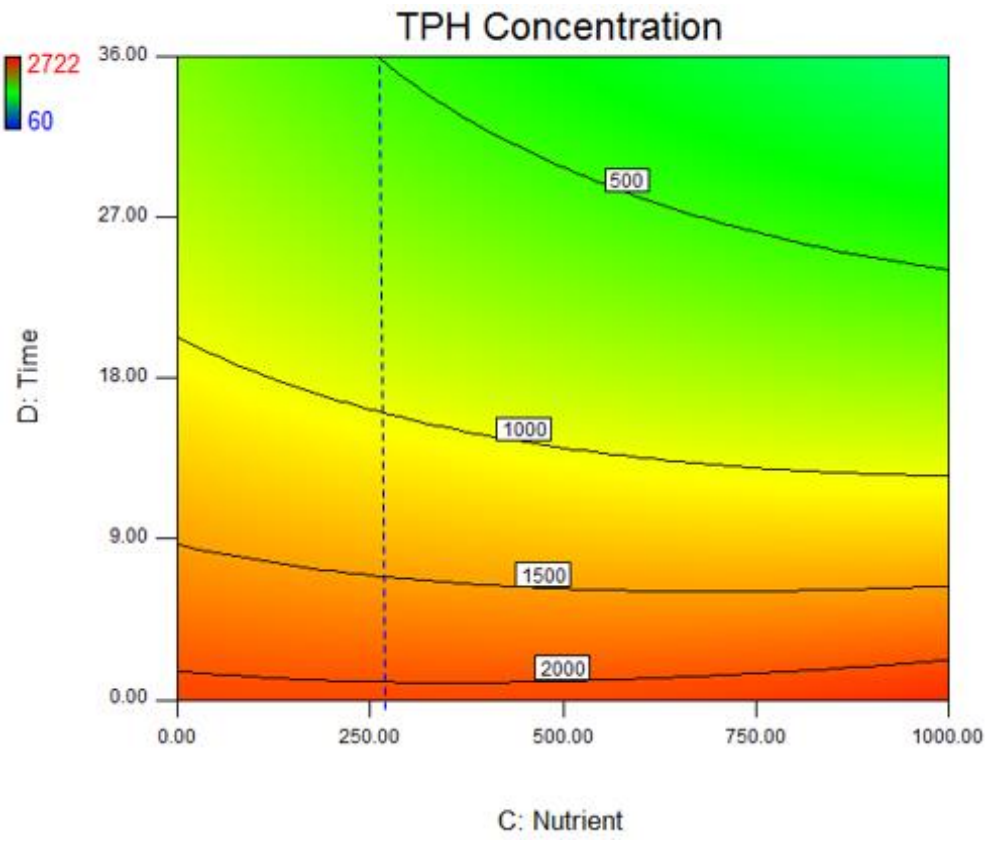
1000
1500
2000
0.00
250.00
500.00
750.00
1000.00
C: Nutrient

(a)


TPH Concentration
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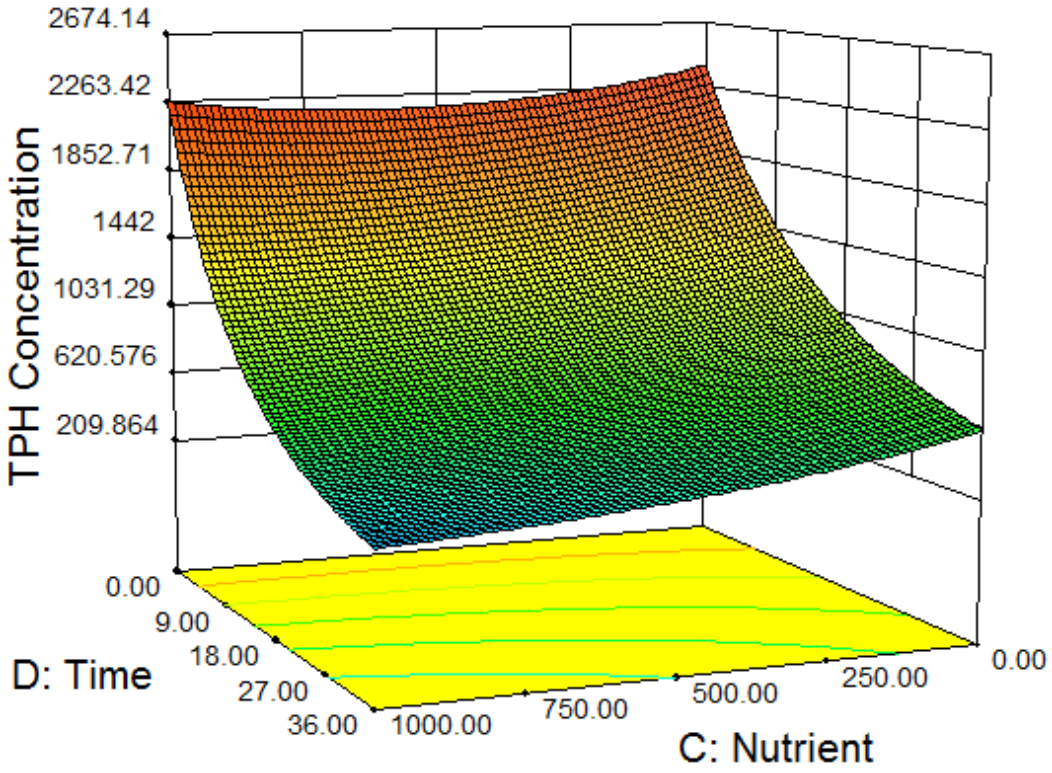
C: Nutrient

(b)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TPH Concentration
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D: Time
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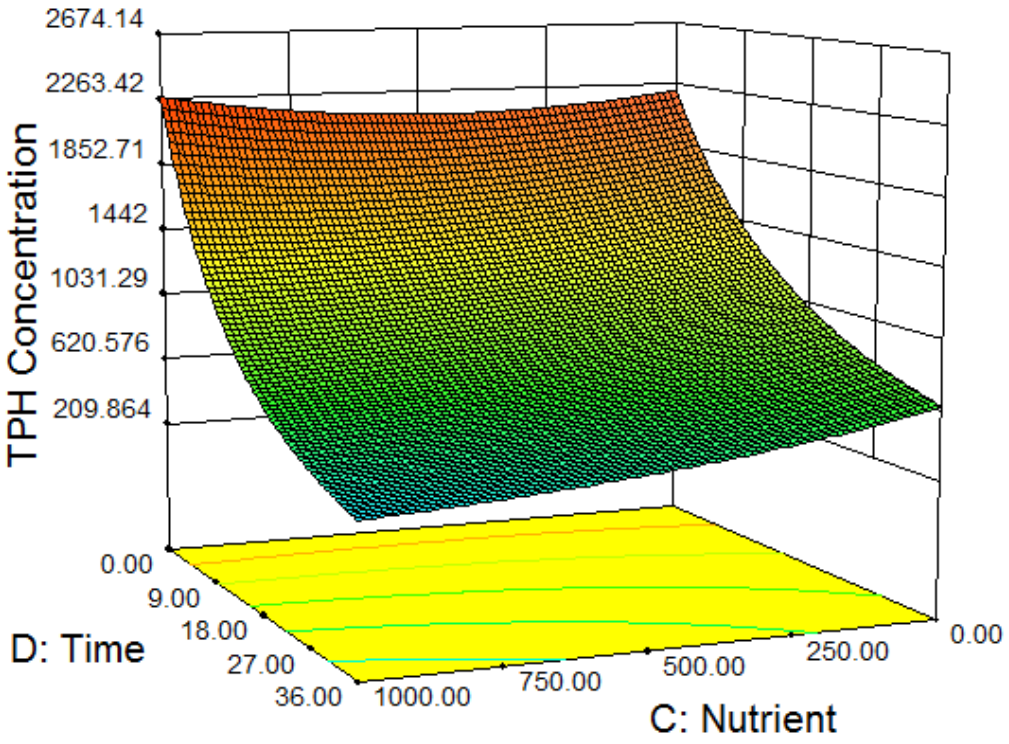
C: Nutrient

(c)



(d)

Figure 4.1 Response surface plots depicting the interactions of independent variables (a) rhamnolipids at 75 mg/kg (b) Tween 80 at 75 mg/kg (c) rhamnolipids at 150 mg/kg (d) Tween 80 at at 150 mg/kg.

(TPH concentration decreased continuously during the 36 days; When the addition of nutrient increased, TPH concentration decreased faster.)

4.3 Kinetics of Enhanced Biodegradation

Fig. 4.2 shows the kinetic behavior of PHC consumption during the bioremediation experiments after the supplementation of rhamnolipids (150 mg/kg) and nutrient (1000 μL solution) for 36 days. The equations of fitting curves for Sterilized control, Non-sterilized control and Biosurfactant & nutrient are

$y = -0.2331x^2 - 29.891x + 2637.2$, $y = 2.2945x + \frac{29631.85}{x + 11.3884}$ and $y = \frac{11813.42}{x + 4.2284}$,

respectively. X represents time and y represents the concentration of PHCs, indicating that PHCs in all above groups were degraded constantly in this period. PHC concentration maintained in a relatively high value after the treatment of sterilization. However, a dramatic removal of PHCs was observed from all the different soil treatments in the initial 7 days. It's highly possible that soil PHCs volatilized quickly at an early stage (<7 days), but the volatilization of PHCs was inhibited due to the appreciable PHC distribution below the surface and their deep binding to soil components (Clair et al., 2003). As revealed by the results, a different type of equation fitted the PHC concentration change under sterilized conditions. Once the hydrocarbons are present or absorbed in pores smaller than the size of microorganisms, the limited bioavailability restricts the PHC degradation (Clair et al., 2003). The results suggested that although a considerable 91.4 % removal of PHCs (227.4 mg/kg at day 36) was observed, certain hydrophobic contaminants can persist in the soil matrix for a long period of time (>36 days). Apparently, during long-term residence in soil, organic contaminants can form stronger bonds with soil by adsorption and partitioning or can be incorporated in structural micropores (Moyo et al., 2014; Zhang et al., 2014).

In all the cases, the differences in biodegradation extent between natural/enhanced treatment and the sterilized control were always significant. The biodegradation occurred very fast once microorganisms adapted to the environment. Accordingly, the enhanced bioremediation by biosurfactant and nutrient will timely reduce the adverse environmental impact of pollutants, and possibly avoid any further movement or diffusion of pollutants to another matrix (Moldes et al., 2011). Similar results were achieved by Thavasi et al. (2011) during the bioremediation of contaminated sites with crude oil in laboratory scale microcosm experiment. During room temperature investigations, 75% of the crude oil was biodegraded in a period of 168 h and biosurfactant alone proved to be capable of promoting biodegradation to a large extent when soil moisture was below 100% (Thavasi et al., 2011).

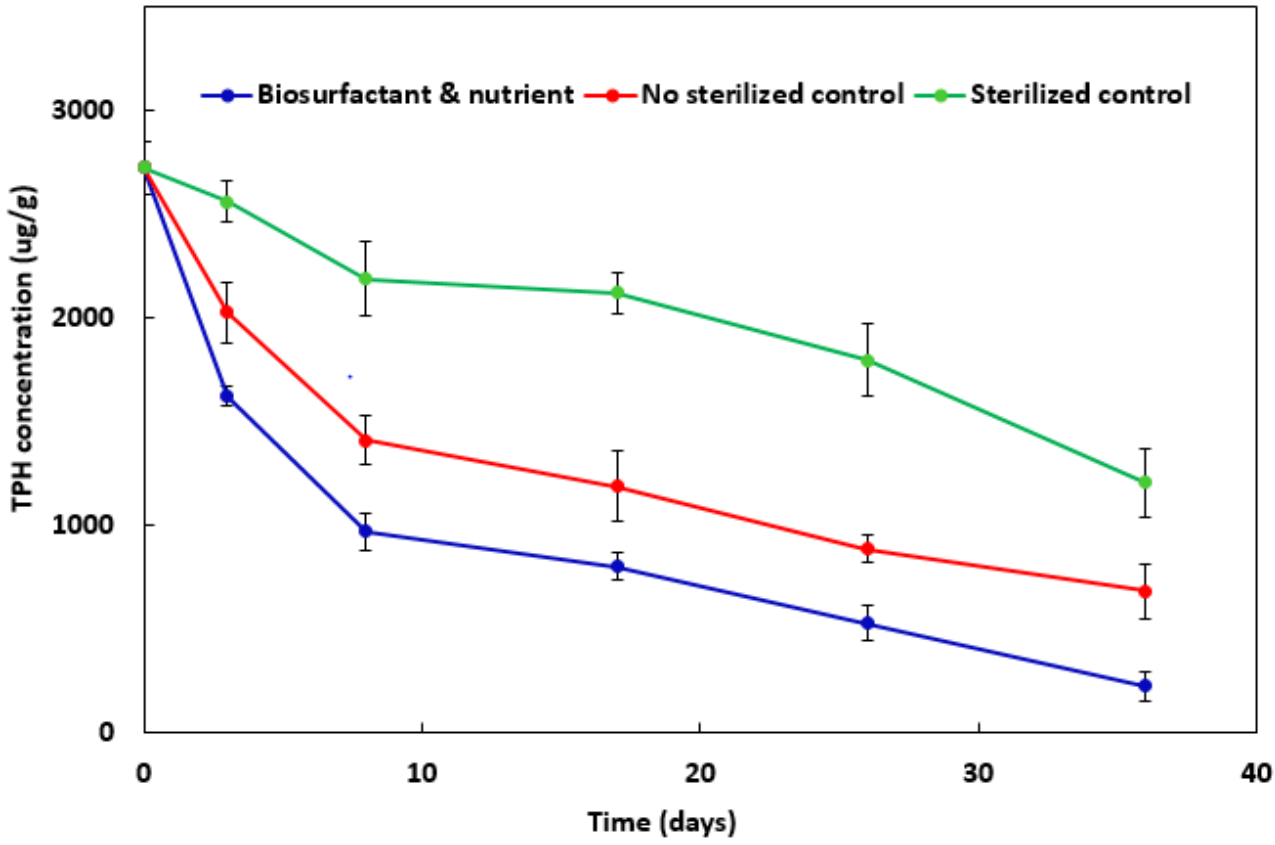


Figure 4.2 Kinetics of the PHCs biodegradation in soil stimulated by rhamnolipids and nutrient. Data are presented by mean values of three replicates with standard deviations as errors.

In order to evaluate the microbial biodegradation potential, two batches of experiments were set while maintaining the highest levels of rhamnolipids or nutrient in general factorial design but doubling the amount of the other factor. The results in Fig. 4.3 showed the extra rhamnolipid addition beyond 150 mg/kg (300 mg/kg) accelerated the reducing of the final PHC concentration from 227.4 mg/kg (91.4% removal of PHCs) to 131.2 mg/kg (95.0% removal of PHCs). In contrast, no further increase in the extent of PHC biodegradation (238.4 mg/kg at day 36) through increasing the nutrient concentration beyond 1000 uL (2000 uL) was observed. The results are in accordance with the conclusions proposed by Maki et al. (2003) and Singh and Lin (2009) that fertilization stimulated abilities of indigenous microorganisms mainly during the initial stages of degradation. In fact, excessive nutrient concentrations can also inhibit the biodegradation activity and the negative effects of high nutrient levels on the biodegradation of hydrocarbons were also reported (Chaillan et al., 2006; Chaîneau et al., 2005; Singh et al., 2014). An excess of nutrient amendment may have the potential to stimulate the activities of other microbes other than hydrocarbon-degrading microorganisms.

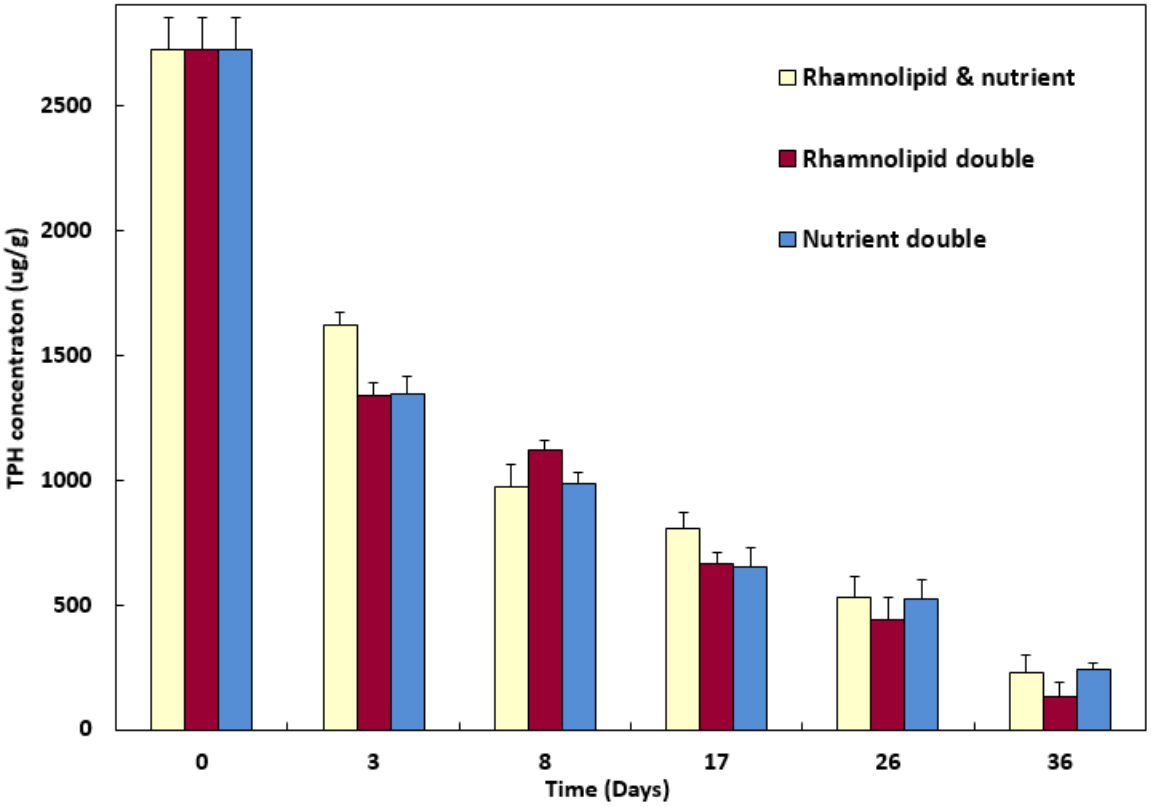


Figure 4.3 The biodegradation behavior of PHC by the supplementation of increased amounts of rhamnolipids and nutrients to investigate the degradation potential of the microorganisms.

On the other hand, the increased amendment of rhamnolipids increased the assimilation of PHCs, especially after 17 days of incubation (Fig. 4.3). The observed further decrease of PHCs was attributed to the increment of the bioavailability of hydrophobic contaminants to hydrocarbon-degrading microorganisms. As stated above, although the application of rhamnolipids at 50 mg/kg and 150 mg/kg did not show overall obvious variance in the PHC removal within the study period, the further elevated addition of nutrient accelerated the assimilation of PHCs in each level of rhamnolipid application.

4.4 PLFA Profiles of Soil Microbial Community

From the results of long-term hydrocarbon biodegradation, most of the PHCs were consumed within 54 days. The observed PHC concentrations from long-term treatments by natural attenuation, biostimulation by rhamnolipids and Tween 80 are 188.1 mg/kg, 34.6 mg/kg, and 34.5 mg/kg, respectively, which correspond with 92.9%, 98.7%, and 98.7% of PHCs removal. Day 54 was thus used to represent microbial activities in the final stage of PHC biodegradation. According to all the PHC degradation results, PLFA patterns on Day 0, Day 17th and the end of the experiment (Day 54th) were determined for the assessment and interpretation of microbial viability and vitality. The obtained PLFA profiles from different experimental runs could represent soil microbial community structure at the initial stage, the most active stage, and the last stage of PHC degradation, respectively. A total of 22 different PLFA biomarkers were identified from the soils contaminated by PHCs although their patterns varied under different soil treatments. Major fatty acids identified in the soil samples include 14:0, 16:0, 18:1ω9c, the cis and trans isomers of 16:1ω7, 18:1ω7 and two cyclopropane fatty acids, cy17:0 and cy19:0. Fatty acids i15:0 and a15:0 were also

found in appreciable amounts from the unstimulated soil structures, and these PLFAs accounted for 77.6%-88.9% of the total PLFAs present in each sample. Compared with other studies, the distinguishing feature in the PLFA profiles was the significant coexistence of cyclopropyl PLFAs cy17:0 and cy19:0 with their monoenoic precursors of 16:1ω7 and 18:1ω7 (Li et al., 2007; Main et al., 2015). High amounts of cy17:0 and cy19:0 may be resulted from the microbial stress response to harsh environments in the North Atlantic region. Small amounts of fatty acids with carbon numbers of 12, 13, 17, 18, and 20 as well as were also detected. To achieve a consistent and reliable interpretation, only 11 of the PLFA indicators were selected to represent bacterial or fungal groups in the soil (Frostegård et al., 2011; Wixon and Balser, 2013). For instance, 10Me16:0 were widely accepted as Actinomycetes in soil samples (Kong et al., 2011; Moore-Kucera and Dick, 2008), but it was also used as sulfate-reducing biomarker (Desulfobacter) in anaerobic environments (Córdova-Kreylos et al., 2006; Pratt et al., 2012). Fatty acid 18:1ω9c may serve as ambiguous indicators for fungal biomarker (Barreiro et al., 2015; Kong et al., 2011; Lazcano et al., 2013) or bacterial biomarker (Covino et al., 2016; Helfrich et al., 2015).

Overall, the total microbial biomass, estimated as total PLFAs, ranged from 27.0 to 40.0 nmol/g during the initial stage of soil remediation. The amounts of PLFAs reached an appreciable abundance of 45.3-52.0 nmol/g at day 17, but varied to 12.0-52.1 nmol/g at day 54 for all the samples with different treatments. The PCA performed with the whole PLFA data set of the soil samples collected at different sampling times (0, 17, and 54 days after the treatments) revealed that the PC1 and PC2 factors contained 80.4% and 10.0% of the total variance, respectively (Fig. 4.4). PC1 revealed major differences (80.4%) in the

microbial communities and differentiated all the samples, especially for those time-oriented samples taken from natural attenuation and rhamnolipid enhanced soil remediation. It was observed that PLFA indicators 18:1ω7c, 16:1ω7, 16:1ω7t, cy17:0, and cy19:0 for Gram-negative bacteria, 18:2ω6,9c for as Fungi, as well as some general PLFAs 14:0, 16:0, 18:0 and 20:0 showed significant differences in PC1 loadings. These PLFAs are seen as major indicators to differentiate microbial structures among different PHC degradation stages. These PLFAs carrying positive loadings on component 1 were present in greater abundance in all samples on Day 17 and in soil samples treated by Tween 80 on Day 54. Component 2 mainly separated samples taken from various periods of Tween 80-enhanced soil remediation. The major differences can be indicated by PLFAs, 18:1ω7c, cy17:0, i16:0, i15:0 and a15:0, which include both Gram-negative (18:1ω7c and cy17:0) and Gram-positive bacteria (i15:0, a15:0 and i16:0) indicators. Bacterial PLFA indicator 2OH 14:0 (Willers et al., 2015; Zaady et al., 2010) and PLFAs characteristic of gram-positive bacteria (i15:0, a15:0 and i16:0), which carried high positive loadings on component 2, were enriched in soil samples treated by Tween 80 on Day 0.

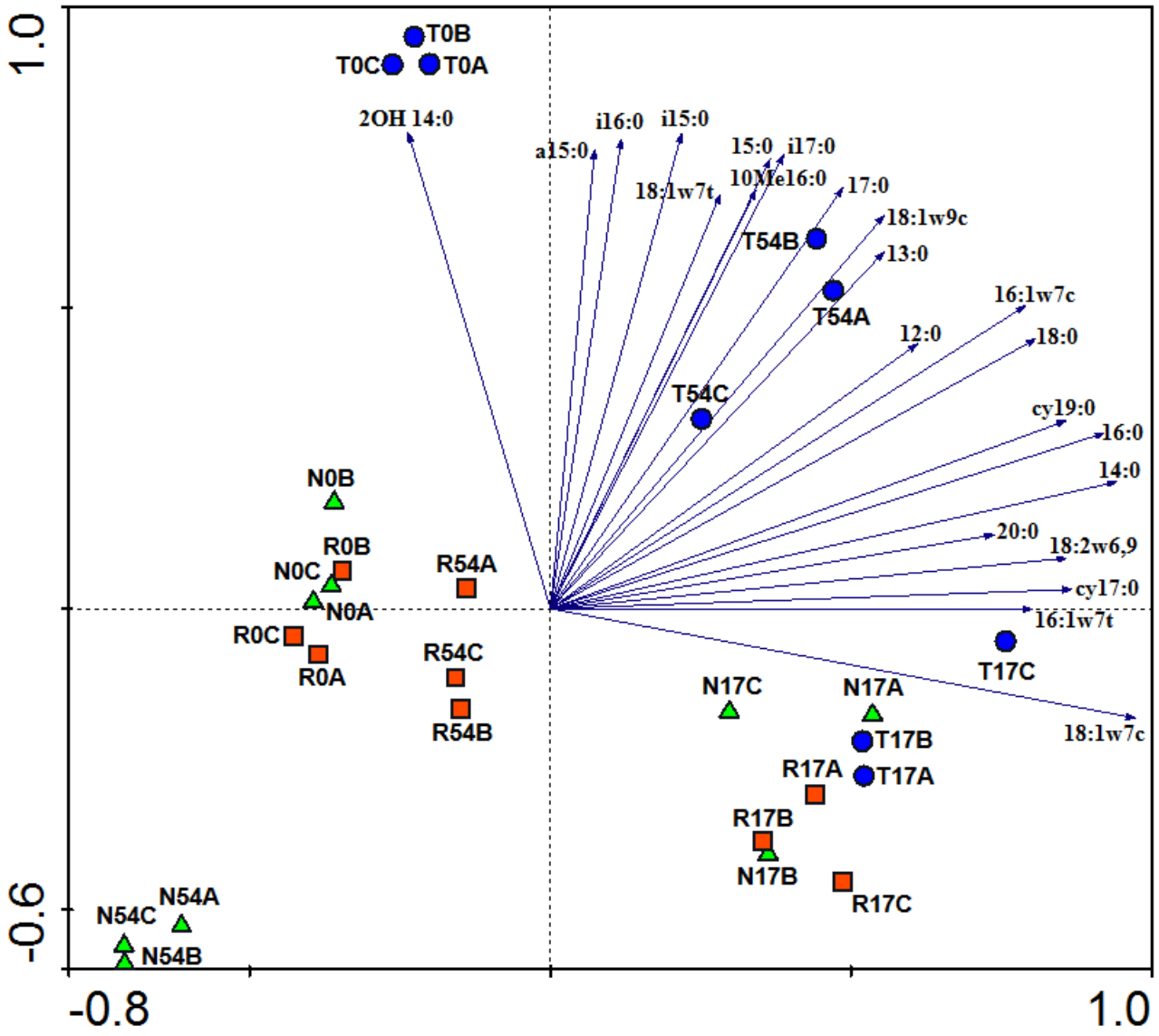


Figure 4.4 Principal component analysis (PCA) plot of the soil microbial community structure with loadings of separate PLFAs along the sample distribution patterns. Samples are denoted by the treatments of soil (N for natural attenuation, R for rhamnolipids, and T for Tween 80) coupled with the sampling days.

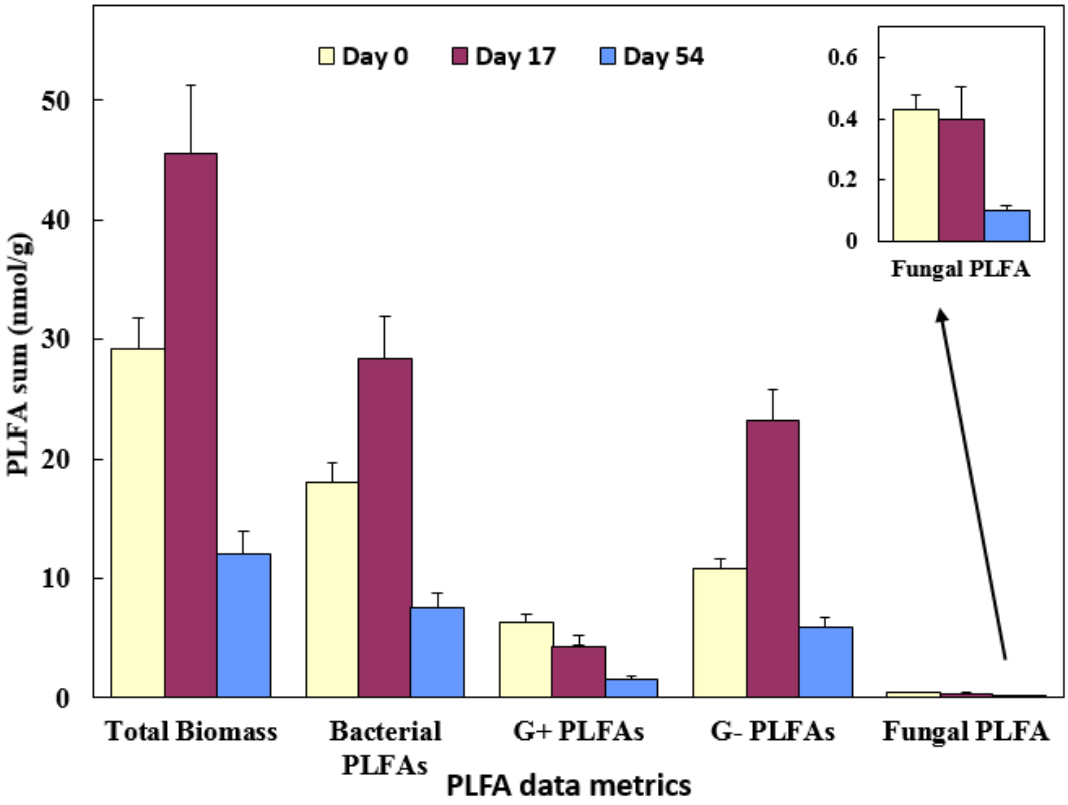
(The distribution (near or far) of the samples in the new coordinate system represented their respective correlation and their correlation with the PLFA species.)

4.5 Microbial Community Structure Shifts and Dynamic Change

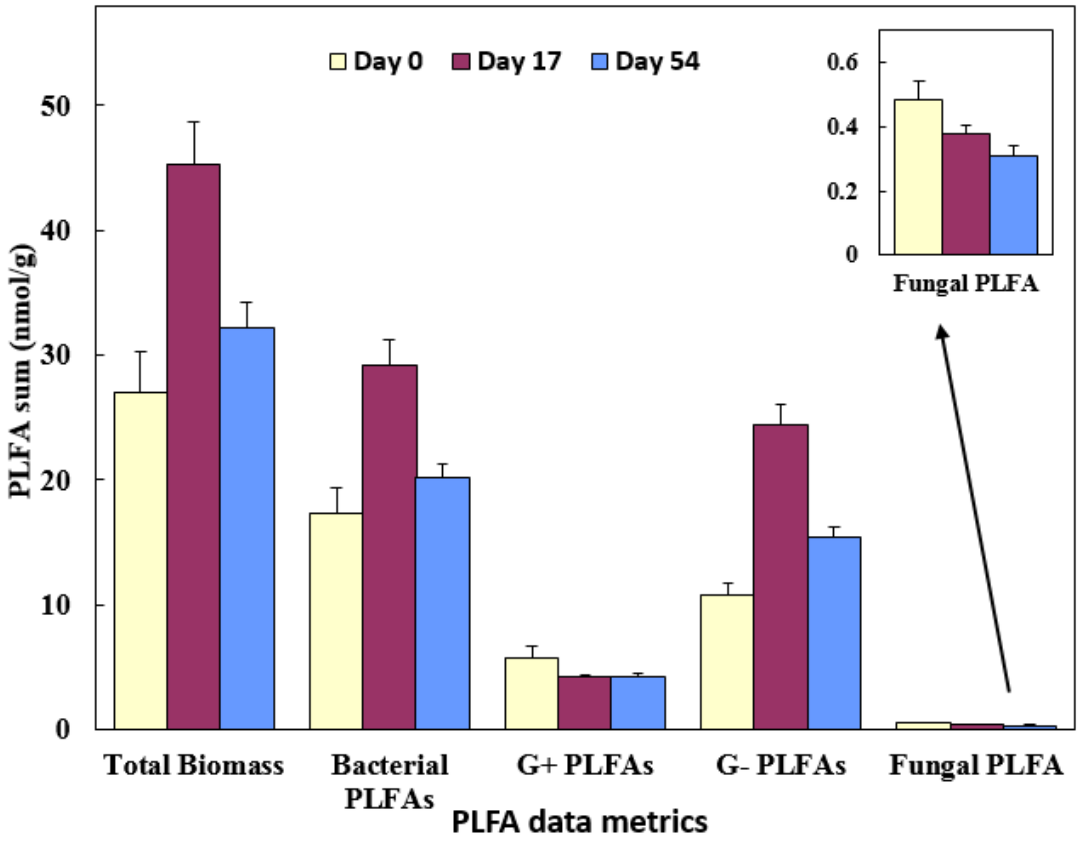
Amounts of PLFAs specific for total soil biomass, total bacterial biomass, Gram-positive and Gram-negative bacteria as well as soil fungi over the three sampling periods were shown in Fig. 4.5. Three different total biomass transformation patterns over the study period were observed when the soils were treated by natural attenuation, enhanced by rhamnolipids and Tween 80, respectively. The total soil biomass raised from the initial level to a higher level after 17 days and then it dramatically dropped to a much lower amount at the end in the process of natural attenuation. In contrast, the total soil biomass went through the similar trend to a high point after 17 days, but it eventually decreased to a level higher than the initial amount during the rhamnolipid-enhanced soil treatment. Interestingly, although the total PLFA amount from Tween 80-enhanced soil treatment exhibited the raising period like the other two, the microbial biomass maintained high yields as much as it was on Day 17. Consequently, it can be speculated that the soil employed in this study has microbial biomass not inhibited by the presence of PHCs and some indigenous heterotrophic microorganisms were stimulated by nutrients and surfactants injection to degrade PHCs.

Notably, bacterial PLFA biomass and PLFA amounts of Gram-negative bacteria were closely correlated with the amount of total soil biomass and experienced the same transformation patterns over the three periods as the total soil biomass. The significant correlation between PLFAs specific for the Gram-negative bacterial populations and total microbial biomass clearly revealed that they are the hydrocarbon-degrading populations during all soil treatments. On the other hand, no raised PLFA yields from Gram-positive

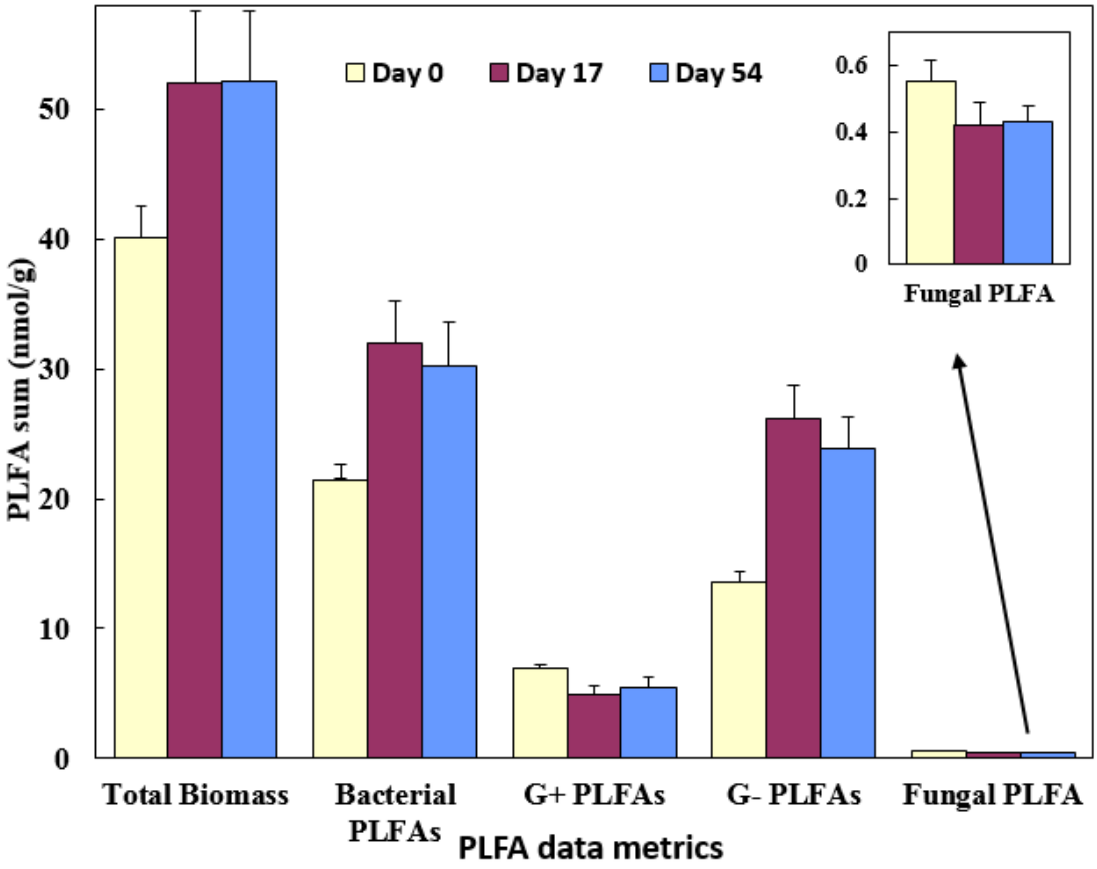
bacteria and Fungi were observed across the study period from all the soil treatments. The two specific microbial groups are assumed not actively involved in the PHC biodegradation activities and PHCs incurred environmental stress on them. Margesin et al. (2007) monitored the changes in microbial community composition and activity during biostimulation treatments of diesel oil contaminated soil and found Gram-negative bacterial community significantly increased. Similarly, Gram-positive population was not significantly affected by PHC content or biostimulation treatment (Margesin et al., 2007). Saturated PLFAs 15:0 and 17:0 were reported to increase in a consortium of oil degrading marine bacteria cultivated by light petroleum (Aries et al., 2001), however, no significant increase of their concentrations was observed in all of the current soil treatments. The increase of even-numbered monounsaturated PLFAs 16:1ω7, 18:1ω9c and 18:1ω7 in rhamnolipid treated soil was accorded with the understanding that the removal of hydrocarbon contaminants is accompanied by the increase of monounsaturated PLFAs (Main et al., 2015).


Day 0
Day 17
Day 54
PLFA sum (nmol/g)
0
10
20
30
40
50
0
0.2
0.4
0.6
Fungal PLFA
Total Biomass
Bacterial PLFAs
G+ PLFAs
G- PLFAs
Fungal PLFA
PLFA data metrics

(a)


Day 0
Day 17
Day 54
PLFA sum (nmol/g)
0
10
20
30
40
50
Total Biomass
Bacterial PLFAs
G+ PLFAs
G- PLFAs
Fungal PLFA
PLFA data metrics
0
0.2
0.4
0.6
Fungal PLFA

(b)



(c)

Figure 4.5 Total PLFA biomass and sum of bacterial and fungal PLFA concentrations over the contaminated soil treated with natural attenuation (a) and biostimulation of rhamnolipids (b) and Tween 80 (c). Error bars are derived from standard deviations of samples (n=3).

It was noticed from close clustering in Fig. 4.5 (N0 and R0 series) that although the soils were applied with different treatments in bioremediation practice, all the time zero points were supposed to have similar PLFA patterns. Soils initially treated with Tween 80 resulted in relatively different, especially in the differences of 2OH 14:0. PC 2 (10.0% of the variance) tended to separate soil samples collected at time 0. Fig. 4.6 showed the whole picture on distribution of PLFA biomarkers of original soil and soils treated with rhamnolipids, Tween 80 on Day 0. The results indicated that although generally consistent results were obtained from all the PLFA compositions, the biggest differences of PLFA concentrations between the three soils lied in the variances in three PLFAs 2OH 14:0, 16:1ω7c and 18:1ω9c. However, soil samples with the addition of Tween 80 exhibited relatively higher values of PLFAs with regard to the other supplement treatments, especially for three fatty acids listed above. This is attributed to the inherent analytical bias using PLFA profiling, which involves the transesterification of phospholipids into FAMEs. The methylation reaction (transesterification) is applicable to various types of lipids, which involves the cleaving of the ester bond by an alcohol and form new esters (Anastopoulos et al., 2009). Tween 80 containing polyoxyethylene groups and oleate groups could be extracted and further involved in the methylation reaction. The hydroxyl groups and oleate groups of Tween 80 are believed to contribute the extra increase of PLFA abundance.

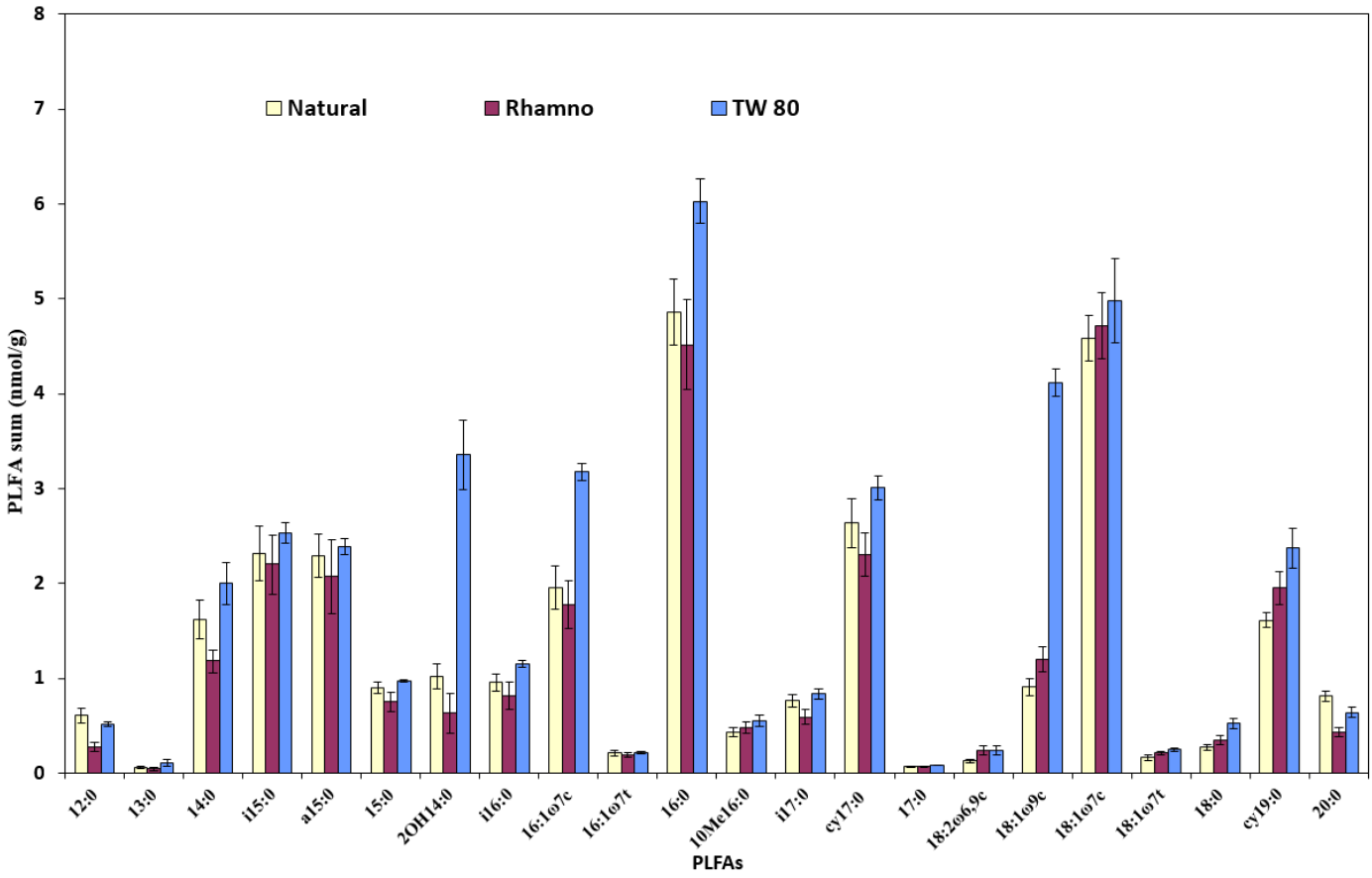


Figure 4.6 Distribution of PLFA biomarkers of original soil and soils treated with rhamnolipids, Tween 80 on Day 0 from the hydrocarbon-contaminated site.

Nonetheless, the analytical bias for certain PLFAs caused by the presence of Tween 80 was not permanent during the study period. As revealed by Fig. 4.7, although significant difference of the three fatty acids was observed from the Time 0, the variations of 2OH 14:0 and 16:1ω7c were eliminated after 17 days. Referred from the total biomass and bacterial PLFAs transformation patterns in this period (Fig. 4.7), the results were believed to represent bacterial compositions due to the elimination of determination interference caused by properties of Tween 80. The high abundance of 18:1ω9c on Day 17, however, still indicated that certain amounts of FAMEs were derived from Tween 80 itself but microbial sources. After 54 days, the amount of 18:1ω9c decreased while the total PLFA biomass maintained the same level as Day 17, an obvious sign that Tween 80 were degraded to some extent accompanied by long-term PHC degradation. his is supported by differential total biomass patterns on Day 54 and the fact that the 18:1ω9c concentration increased from the initial value when they are stimulated. In summary, although certain biases of PLFA species were involved in the profiling of microbial community structure due to the characteristic properties of Tween 80, the analytical error is a minor factor in evaluating the effectiveness of each microbial group. The results were in accordance with Nielsen and Petersen (2000) that the PLFA yields from non-microbial sources accounted for no more than 5-10% of the total amounts. Moreover, the concentration of Tween 80 in soil decreased over time to a final ignorable level so that its impact on PLFA analysis became limited.

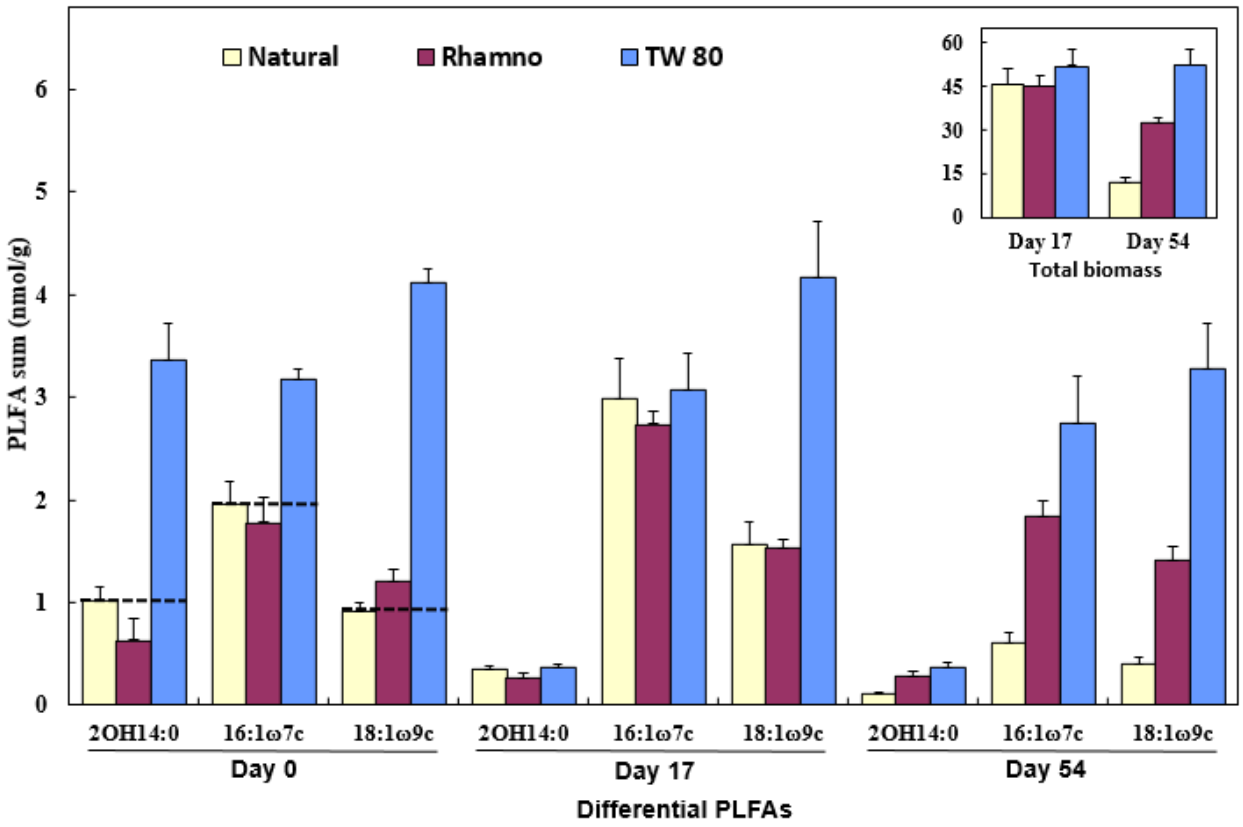
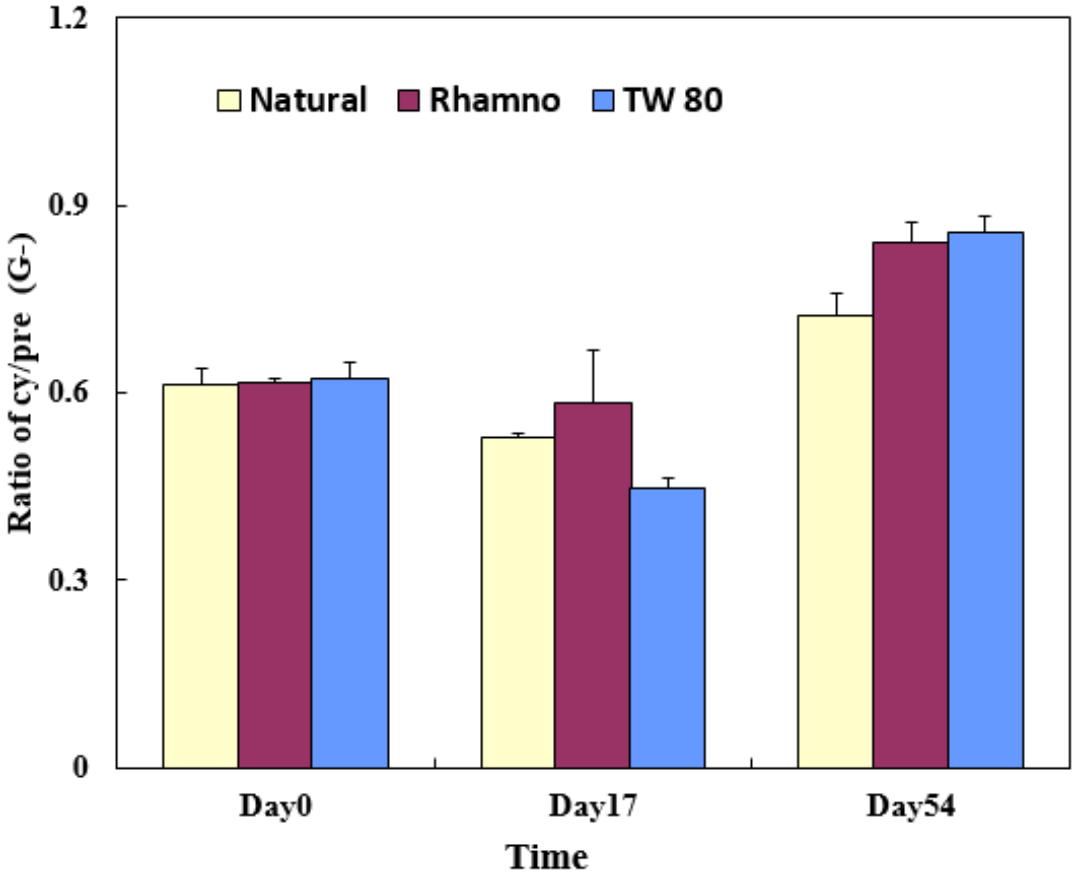
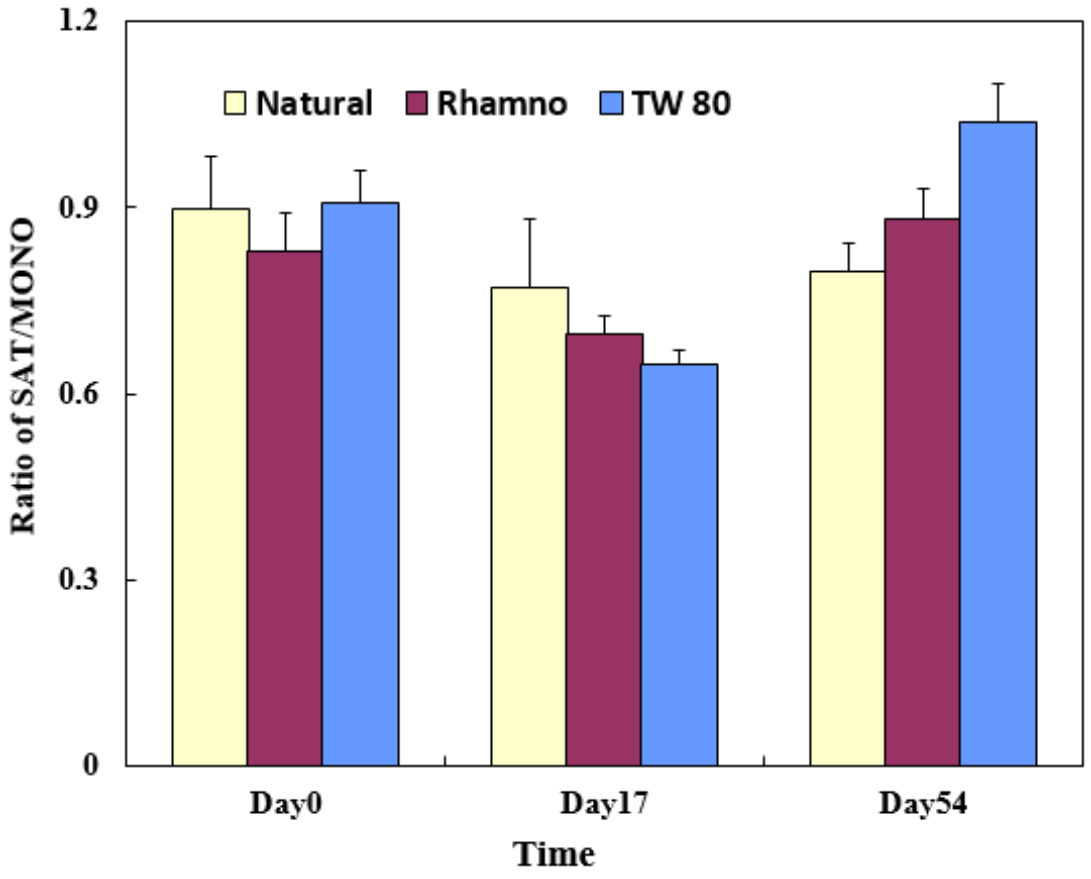


Figure 4.7 The determination bias changes of three fatty acids yielded from PLFA analysis due to the addition of Tween 80 into the contaminated soil over time

The PLFA compositional characteristic is an important indicator of the change of membrane fatty acid composition to study microbial adaptive reaction against the environment alteration. Physiological status was determined using and the ratios of cyclopropyl PLFAs to their monoenoic precursors (cy/pre) and the ratios of SAT to MONO (S/M) PLFAs (Fig. 4.8). The cy/pre ratios of all the soils treated by three incubation methods showed a decreasing tendency at Day 17, but the values were significantly higher at Day 54. Although a heating event with ample nutrition may induce the re-growth of bacteria and increase the cy/pre ratio under nonstressful conditions (Bárcenas-Moreno et al., 2011), this ratio would typically increase with other stresses caused by insufficient nutrients, low pH, pesticide use (Wixon and Balser, 2013), water (Moore-Kucera and Dick, 2008) and other factors. From our results, the microorganisms experienced the starvation during the last stage of biodegradation and the physiological status of the microbes was correspondingly reflected. The similar conclusions could be deduced from the results indicated by S/M ratios (Fig. 4.8b). The physiological change or stress responses from the indigenous microorganisms were closely correlated with the degradation stage of the contaminants and the availability of nutrients.


1.2
0.9
0.6
0.3
0
Natural
Rhamno
TW 80
Ratio of cy/pre (G-)
Day0
Day17
Day54
Time

(a)



(b)

Figure 4.8 Physiological stress status changes of the indigenous microorganisms in the environment indicated by cyclopropyl PLFAs to their precursors (a) and saturated to monounsaturated PLFAs (b) ratio over time

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

In this thesis, an enhanced PHC biodegradation system was investigated and the DOE methodology was adopted. PLFA analysis elucidated soil microbial community, shifts and dynamic change of the microbial community structure, and associated biodegradation mechanisms during natural attenuation, and rhamnolipids or Tween 80 amended bioremediation. The study integrated PLFA analysis with biosurfactant-enhanced soil bioremediation, which was extremely limited in the literature. The results provide valuable guidance in understanding bacterial communities and further biosurfactant-enhanced bioremediation practices. The key research findings were stated below:

(1) A DOE methodology was applied to investigate the effects of multiple factors and their interactions on the performance of bioremediation of PHC-contaminated soil. The impact of four factors including the type of surfactant, concentration of surfactants, concentration of nutrients and degradation time were investigated. The Model F-value was as high as 161.85, which implied that the model is significant, and there is only a 0.01% chance that a "Model F-Value" this high could occur due to noise. Values of "Prob > F" less than 0.0500 indicated that the model terms are significant.

(2) The enhanced PHC bioremediation with addition of surfactants and nutrients were conducted. Results indicated that the enhanced biodegradation is of great importance to microbial assimilation and dissimilation. The lowest predictions of PHC concentration from rhamnolipids and Tween 80 treatment in soil were 201.9 mg/kg (92.3% removal of PHCs) and 255.7 mg/kg (90.3% removal of PHCs), respectively. The nutrient concentrations were found to be positively correlated with the PHC removal. The lowest

values are both obtained from a biodegradation of 36 days with 1000 µL nutrient solution and 150 mg/kg surfactant initially added. The biofriendly and biocompatible rhamnolipids better promoted metabolic activities of the population than Tween 80 in contaminant biodegradation.

(3) PLFA based microbial community analysis was conducted. A total of 22 different PLFA biomarkers were identified from the soils contaminated by PHCs, although their patterns vary under different soil treatments. Major fatty acids identified in the five pseudomonad strains grown on natural toluene include 16:0, 14:0, 18:1ω9c, the cis and trans isomers of 16:1ω7, 18:1ω7 and two cyclopropane fatty acids, cy17:0 and cy19:0. Fatty acids i15:0 and a15:0 were also found in appreciable amounts from the unstimulated soil structures, and these PLFAs accounted for 77.6%-88.9% of the total PLFAs present in each sample. Results indicated that although generally consistent results were obtained from all the PLFA compositions, the biggest differences of PLFA concentrations between the three soils lay in the variances in the three PLFAs 2OH 14:0, 16:1ω7c and 18:1ω9c. From our results, microorganisms experienced starvation during the last stage of biodegradation and the physiological status of the microbes was correspondingly reflected.

The results obtained during the laboratory studies suggest that biological treatment with the supplementation of nutrients and rhamnolipids on hydrocarbon contaminated soil enhanced the biodegradation rate. It can also be concluded that the nutrient addition contributed significantly to the biodegradation of PHCs due to the essential needs of the contaminated soil with insufficient nutrient levels. Compared with the biosurfactant, Tween 80 as a synthetic surfactant exhibited lower removal efficiency of PHCs over long

period of soil remediation. The extra addition of nutrients showed an insignificant improvement of PHCs biodegradation but the increased amendment of rhamnolipids increased the assimilation of hydrocarbons by promoting the bioavailability of contaminants. The biodegradation mechanism from the PLFA analysis revealed that microorganisms have different transformation patterns when the soils were treated by natural attenuation, enhanced by rhamnolipids and Tween 80, respectively. The Gram-negative bacterial populations are closely correlated with the amount of total soil biomass and are responsible for degrading PHCs. Their physiological status accessed from the PLFAs' ratios of cy/pre and S/M indicated different environmental stress at different degradation stages. Results concerning the PHCs biodegradation and relevant microbiological information provide valuable guidance for the practical treatment of hydrocarbon-contaminated soil by biosurfactant-enhanced bioremediation.

5.2 Recommendations for Future Research

(1) A general factorial design was used to systematically investigate the factors of surfactant type, surfactant concentration and nutrient concentration on biostimulation. It helps to analyze the interaction between different factors. However, the condition achieves the highest removal rate may not be the most optimal one. So further efforts can be made on integrating numerical simulation and system optimization tools with experimentation of soil bioremediation systems to further investigate the system mechanisms and achieve a better system operation.

(2) The rhamnolipid used in this experiment was a commercial biosurfactant. If the biosurfactant was applied to a large area in-situ, it will cost a lot. Therefore, future studies should use biosurfactant that was carried out from lab.

(3) Soil environment such as temperature, nutrition concentration, and water content may influence the degradation of PHCs. Soil samples in this study was conducted under a room temperature around 18 °C, whereas in some natural environment (i.e. Goose Bay) they appear to be about 0 °C. Temperatures should be evaluated as another factor on the transport and fate of PHCs in future.

(4) Besides the environmental effects, the population of microorganisms with ability of degrading PHCs also affects the PHCs degradation rates. Such microorganisms should be added into contaminated soil with adequate quantities.

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