Dispersion and Bacterial Degradation of Weathered Diesel, Biodiesel and Light Crude Oil in Seawater by Sophorolipid Biosurfactant

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ABSTRACT

Dispersion and Bacterial Degradation of Weathered Diesel, Biodiesel and Light Crude Oil in Seawater by Sophorolipid Biosurfactant

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The significant surface activity, low toxicity, biodegradability and stability over broad ranges of salinity, temperature, and pH make the sophorolipid biosurfactant an alternative to the toxic chemically-based dispersants for oil cleanup from marine environments. The potential application of sophorolipid biosurfactant for dispersion and bacterial degradation of weathered biodiesel (BD), diesel (D) and light crude (L) oil in artificial seawater was studied. The mixtures of artificial seawater (salinities of 0-30 ppt, pH of 6-8, temperatures of 8ºC, 22ºC and 35ºC), -weathered diesel, biodiesel, and light crude oil and various concentrations and quantities of sophorolipid were prepared, shaken (150 rpm, 20 min) and analyzed according to the swirling flask dispersant effectiveness flask method. The biodegradation experiment was conducted by incubation (100 rpm, room temperature, 28 days) of mixtures of seawater (30 ppt), weathered diesel, biodiesel and light crude oil and sophorolipid. The oil dispersion increased 1.5-fold as the sophorolipid concentration doubled. For example, nearly 16 ± 0.5%, 12 ± 0.5% and 27 ± 0.5% of the diesel, light crude oil and biodiesel were dispersed, respectively, with 80 mg/L of sophorolipid. The dispersion of diesel, biodiesel, and light crude oil increased 1.3 (BD), 1.4 (L) and 1.5-fold (D) as the quantities of the sophorolipid in the seawater doubled. The mechanisms involved in the oil dispersion at higher concentration and quantity of sophorolipid were the incorporation of oil droplets in the micelles and the formation of small oil droplets. No oil dispersion was formed at the minimum level of agitation (0% at 0 rpm), regardless of the sophorolipid concentrations, and the dispersion occurred only when the mixing increased to maximum (150 rpm). The stability of dispersed oil was influenced by the level of mixing so that the oil dispersion reduced to <8% due to resurfacing process. The dispersion of oil doubled as the salinity increased from 0 to 10 ppt, and gradually increased at the higher salinities (10 to 30 ppt). The effectiveness of sophorolipid at the higher salinities was due to the better surface

activity of sophorolipid at solutions with a higher content of NaCl salt, the effect of "salting out", and the lower solubilization of sophorolipid. The biodiesel, diesel, and light crude oil dispersion increased with increasing temperature from 8ºC to 22ºC and 35°C, with the exception of biodiesel that had a reduced dispersion at 35ºC. The low effectiveness of sophorolipid at temperatures of 8 and 35ºC (in the case of biodiesel) can be attributed to the significant changes in the viscosity and density of biodiesel, diesel, and light crude oil. The presence of active oil-degrading bacteria in the weathered biodiesel, diesel, and light crude oil was confirmed by a significant natural biodegradation (42%), microbial growth and characterization of bacteria by 16S rRNA pyrosequencing technique. The dominant bacteria (e.g., dominant phyla) were *Firmicutes, Actinobacteria, Actinobacteria*, and *Proteobacteria*. The bacteria seem to uptake the hydrocarbons through the changes in the cell surface hydrophobicities based on the available hydrocarbons in the system. The high level of oil biodegradation in the presence of sophorolipid (46%) was due to the increase in the solubilization and dispersion of diesel, biodiesel and light crude oil by sophorolipid biosurfactant. The present study showed that the dispersion of oil by the sophorolipid biosurfactant was influenced by the quantity and concentration of sophorolipid, mixing, temperature and salinity. However, the seawater pH had an insignificant effect on the oil dispersion by sophorolipid. This research suggests the positive effect of sophorolipid biosurfactant on tested oil dispersion under the studied conditions. However, the oil biodegradation was not significantly stimulated with the sophorolipid.

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LIST OF ABBREVIATIONS

BD	Biodiesel
CMC	Critical Micelle Concentration
D	Diesel
E	Effectiveness
FID	Flame Ionization Detection
GC	Gas Chromatography
Hx	n-Hexane
IFT	Interfacial Tension
L	Light crude oil
ppt	Part per thousands
R^2	Coefficient of determination
SLs	Sophorolipids
ST	Surface Tension

Chapter 1: INTRODUCTION

1.1. Oil Contamination of Marine Environments and Chemical Treatments

Oil spills are one of the pollutants introduced to the aquatic environments (Liu et al., 2015; McKew et al., 2007). Organic pollutants such as crude oil and petroleum products have different chemical compositions (e.g., hydrocarbons, nitrogen, oxygen and sulfur) (Riser-Roberts, 1992). This is due to the variation in the origin of petroleum (e.g., sources of raw materials) and the methods of manifesting (Fingas, 2011b; Neumann et al., 1981). Based on their hydrocarbons, petroleum products categorize as (i) saturated or aliphatic, (ii) aromatic, (iii) asphaltic or polar (Riser-Roberts, 1992). Spilled oil lasts for a long time (Fingas, 2011a) before cleaning up by the natural abiological and biological processes (known as "weathering processes") (Hollebone, 2011).

Evaporation is the main weathering process, which occurs immediately following the spill (Hollebone, 2011). In this process, the lighter fractions (e.g., the low molecular weight compounds) of oil evaporate into the atmosphere (Hollebone, 2011). The rate, however, controls by the composition of the oil, its surface area and physical properties, wind velocity, air and sea temperatures, sea energy, and the intensity of solar radiation (Hollebone, 2011).

Natural dispersion is another process that occurs following the oil spill (Fingas, 2011c). Dispersion of oil depends on the type of oil and sea energy level (Fingas, 2011c). For example, the oil droplets smaller than 20 μm readily disperse into the water, while larger droplets (≤ 50 μm) will disperse for just a few seconds (Fingas, 2011c). In addition, the possibility of dispersion of the oils (such as heavy oil) with high content of high molecular weight compounds such as resins and asphaltenes is much lower than the oils (such as diesel fuel) with high level of low molecular weight saturates compounds and lower amount of asphaltenes (Fingas, 2011c). The sea state is also an important factor in the stability of dispersed oil (Fingas, 2011c). The dispersion of oil droplet decreases if the mixing is insufficient (Fingas, 2011c).

Natural biodegradation is a process by which microorganisms metabolize hydrocarbons and transfer them to the oxidized compounds (Fingas, 2011c). The biodegradation process has lower

intrusive effects on the contaminated environments (Whang et al., 2008). However, the effectiveness of this process generally is influenced by bioavailability of hydrocarbons to microorganisms and environmental conditions such as pH, temperature, mixing, oxygen level, and water salinity (Fingas, 2011c). The availability of hydrocarbons is the main parameter in biodegradation process (Fingas, 2011c; USEPA, 1993). The rate of biodegradation increases at the water-oil interface. This is because more oil droplets are available to the microorganisms (USEPA, 1993).

The biodegradation of oils that contain the low amount of the saturate compounds and high amount of asphaltenes is almost impossible, while the oils with the high level of saturates are more biodegradable (Fingas, 2011c; Wang et al., 2011). Thus, natural biodegradation is time-consuming and complete removal of petroleum compounds with this method is virtually impossible (Fingas, 2011c). The effectiveness of biodegradation can improve by (1) stimulation of indigenous microorganisms through addition of nutrients, enzymes, and additives (known as biostimulation), (2) introduction of particular species of naturally occurring oil-degrading microorganisms (known as seeding) or genetically modified microorganisms with oil-degrading properties (known as bioaugmentation) (USEPA, 1993).

The natural processes remove some compounds of the spilled oil, but complete removal of most compounds is either impossible or very time-consuming (Fingas, 2011c). Therefore, physical, chemical and biological strategies have been developed to decrease the negative impacts of oil spills on the marine systems through accelerating the natural removal of spilled oil (Clayton et al., 1993a). One of the reasons that chemical dispersants have extensively been used was to increase the dispersion and consequently the biodegradation of spilled oil in the oil-contaminated water (Fingas, 2011d). The chemical dispersants are designed to interact with both water (through their hydrophilic or water-like part in their molecular structures) and oil molecules (through the hydrophobic or water-repellent part) (Clayton et al., 1993a). This influences the properties of both water and spilled oil (e.g., decrease the surface and interfacial tensions), form oil droplets and encapsulate the oil droplets in the “micellar” aggregates (Clayton et al., 1993a). These processes make the oil droplets more accessible to indigenous oil-degrading microorganisms (Clayton et al., 1993a; Lessard and DeMarco, 2000). Success in the spilled oil cleanup by chemical materials such as dispersants increased the worldwide acceptance of

dispersants as one of the oil cleanup methods from marine environments (Lessard and DeMarco, 2000). However, surfactants (main components of the dispersants) were found to be resistant to biodegradation (Rosen and Kunjappu, 2012). Thus, attempts have been made to increase their biodegradation through changes in their formulations such as changes in the linearity and the positions of hydrophobic groups (Rosen, 2004a). The modifications improved the biodegradability of surfactants but increased the surfactants toxicities (Rosen, 2004a). Studies showed that dispersants had negative effects on the marine environment (Albers, 1979; Ramachandran et al., 2004; Swedmark et al., 1973). In addition, the dispersion effectiveness of dispersants is also influenced by the environmental factors such as the type of oil, sea energy and weather conditions (Colcomb et al., 2005; Fingas et al., 1995; Trudel et al., 2005; Trudel et al., 2010). For example, chemical dispersants effectiveness is changed at high and low salinities. This is because, some chemical dispersants are formulated to be applicable to high salinities (e.g., 30‰), mainly because the salinity of most saline waters (e.g., oceans) is around 30‰ (Blondina et al., 1999; Chandrasekar et al., 2006; National Research Council, 1989). Therefore, when such dispersants are used for oil dispersion at low salinities, their effectiveness reduces as a result of an increase in their solubility (due to the changes in the hydrophilic-lipophilic balance, HLB) (Belk et al., 1989; Canevari, 1985). On the other hand, the dispersants that are formulated for the application in low salinity (e.g., ethoxylated surfactants) are less effective at high salinities. This is because the water solubility of such dispersants reduces due to the salting out effect (National Research Council, 1989). Apart from the effect of temperature on the spilled oil and seawater rheological properties (e.g., viscosity), the dispersants effectiveness also is influenced by the temperature (Fingas et al., 1991; Wells and Harris, 1979). The solubility and adsorption rates of dispersants and the interaction of dispersants with oil change with temperature (Fingas, 2011e). For example, low temperature influences the HLB of the ethoxylated surfactants and increases the water solubility of these surfactants (Canevari, 1985). The seawater pH also affects dispersant effectiveness (Rosen, 2004b). The reasons proposed for the effect of pH on effectiveness is the changes in the chemical structure of dispersants (e.g., ionization), especially dispersants with ionic based surfactants (Rosen, 2004b).

Variations in the results obtained from the laboratory studies on the dispersants effectiveness under different environmental conditions have generated debates regarding the application of

chemical dispersants for oil spill control (Chapman et al., 2007). The inconsistency in the laboratory results is because different methodologies are used by investigators to study the effectiveness of dispersants (Fingas et al., 1990). Moreover, the effects of environmental factors such as the weathering processes were not considered in the laboratory studies (Fingas et al., 1990). For example, a study conducted by Fingas et al., (1990) showed that the high effectiveness of the laboratory tests with the chemical dispersants is because the dispersants were used on the fresh oils which contain more water soluble compounds (Fingas et al., 1990). While the dispersion effectiveness of less than 10% was obtained when a chemical dispersant (Corexit 9527) was used for the dispersion of weathered Endicott, Hibernia, Prudhoe bay oils (Fingas et al., 1990). Moreover, a higher dispersion was obtained by the premixation method (mixing the dispersants with spilled oil before the application) than that of the dropwise method (one drop of the dispersant is dropped on the spilled oil without prior mixing) (Fingas et al., 1990). These are some of the reasons that the field tests of oil dispersion with dispersants show lower effectiveness than that of the laboratory tests (Fingas et al., 1990). Given the low biodegradability and toxicity and low effectiveness of chemical dispersants at real oil spill situation, the use of chemical dispersants is not the preferred method for the removal or control of spilled oil in many parts of the world (Chapman et al., 2007).

1.2. Biosurfactants and Potential Environmental Applications

Some microbial species under different fermentation conditions produced compounds known as biosurfactants (Mulligan, 2005). The physicochemical structures of biosurfactants are strongly influenced by the producing species and growth conditions (e.g., pH, temperature, and source of carbon) (Abouseoud et al., 2008; Eswari et al., 2013; Kosaric and Sukan, 1993).

Biosurfactant molecular structures are formed from both “hydrophilic and hydrophobic parts” (Mulligan, 2005). Depending on their hydrophilic head group, biosurfactants classify as cationic, anionic, and neutral (Mulligan, 2005) and based on their molecular structure, they classify as low and high molecular weight biosurfactants (Ron and Rosenberg, 2002). The high molecular weight biosurfactants (also known as bioemulsifiers) efficiently cover the oil droplets and reduce the oil coalescence (Mulligan, 2005; Ron and Rosenberg, 2002). The low molecular weight biosurfactants such as rhamnolipids can lower the surface tension to 29 mN/m (Zhang and

Miller, 1992), which is as effective as the chemical dispersants such as Corexit 7664 (surface tension of 31.1 mN/m at 20°C) (Singer et al., 1995). This is because, as with chemical dispersants, when biosurfactants are added to the water, they adsorb at the surface of water (Muthusamy et al., 2008). This reduces the system free energy/surface tension. Reduction of surface tension (ST) continues to the point ("critical micelle concentration" (CMC)) that beyond this point the surface tension of the system does not change significantly. Beyond this point, the additional biosurfactant starts forming aggregates known as "micelles" (Muthusamy et al., 2008). In addition to surface tension, some other properties of the aqueous solutions change (Rosen, 2004c). The most common properties are turbidity, density, and conductivity (Morrison and Ross, 2002). The CMC can be determined from the plot of a physical property such as surface tension as a function of surfactant concentration (Morrison and Ross, 2002). The value of the CMC in an aqueous system can be affected by the type of surfactants, temperature and the presence of electrolytes in the solution (e.g., Na^{+}) (Rosen, 2004c).

Biosurfactants are used in cleaning, cosmetics, pharmaceuticals and agricultural products (Gudiña et al., 2013; Hirata et al., 2009b; Kitamoto et al., 2002; Sajna et al., 2013; Vaughn et al., 2014). However, no full scale applications of biosurfactants were reported for the treatment of environmental contaminants to date. Successful applications of biosurfactants in those industries encouraged researchers to study the possible uses of biosurfactants in the removal or control of environmental pollutants. For example, Nikolopoulou and Kalogerakis (2008) reported an increase in the bioremediation of crude oil due to the application of rhamnolipid biosurfactant in combination with natural additives (Nikolopoulou and Kalogerakis, 2008). Moreover, enhancement in oil spill treatment in water and soil by biosurfactants produced by *Gordonia* sp. strain JE-1058 (Saeki et al., 2009), surfactin (synthesized by *Bacillus subtilis* ATCC 21332) and rhamnolipid (synthesized by *Pseudomonas aeruginosa* J4) (Whang et al., 2008), remediation of tetrachloroethylene (PCE), and trichloroethylene (TCE) contaminated aquifers by rhamnolipid and surfactin are examples of successful applications of biosurfactants (Albino and Nambi, 2009). Studies conducted by Dagnew (2004) and Kim et al. (2015) showed that rhamnolipid biosurfactant enhanced oil biodegradation (from 10 to 82%) and inhibited biofilm formation on reverse osmosis (RO) membranes, respectively. The literature suggests that the biosurfactants

can be considered as one of the alternatives to chemical dispersants for treatments of oil contaminated marine environments.

1.3. Effect of Biosurfactants on Oil Biodegradation

Biological treatments are effective when the oil-degrading microorganisms are present in the oil-contaminated sites (Das and Chandran, 2010), the environmental conditions are optimal (Leahy and Colwell, 1990) and the hydrocarbons (HCs) are available to microorganisms (Das and Chandran, 2010; Okafor, 2011; Zhang and Miller, 1992). The HCs' availability depends on the cell surface properties of microorganisms and the solubility properties of hydrocarbons in the aqueous environments (Ward, 2010; Zhang and Miller, 1992). Microorganisms uptake the oil droplets through interaction with droplets if they have hydrophobic cell surface properties (Ward, 2010). Moreover, they interact with the solubilized parts of oil in the aqueous phase or the oil droplets that encapsulated in the micellar aggregates (Ward, 2010). As most hydrocarbons are not readily water-soluble, their uptake by the microbial cells is very limited (Ward, 2010; Zhang and Miller, 1992). Biosurfactant production is one way that helps some microorganisms such as *Pseudomonas* species uptake hydrocarbons (Bouchez-Naïtali and Vandecasteele, 2008; Sekelsky and Shreve, 1999; Tzintzun-Camacho et al., 2012). Bioavailability of the hydrocarbons was improved through the application of extracellular or pure biosurfactants (Ward, 2010; Zhang and Miller, 1992). Extracellular biosurfactants increase oil bioavailability through (i) modifications of the microbial cell surface properties (Kaczorek, 2012; Zhang and Miller, 1994), (ii) encapsulation of the oil droplets in the micelles and (iii) providing a greater surface area for microorganisms (Franzetti et al., 2010; Ron and Rosenberg, 2002; Rosenberg, 1993).

1.4. Sophorolipid Biosurfactant

Sophorolipids are a class of glycolipids (compounds containing a carbohydrate head and lipid tail) and synthesized by *Candida* species (non-pathogenic) (Gorin et al., 1961; Hirata et al., 2009b; Van Bogaert et al., 2007). Sophorolipid molecules are comprised of hydrophilic (e.g., dimeric sugar sophorose) and hydrophobic (e.g., a C16 to C18 hydroxylated fatty acid) parts (Van Bogaert et al., 2007). The structure and properties of sophorolipids vary with the environmental conditions and species of *Candida*. However, they are classified as lactonized and

acidic forms (Van Bogaert and Soetaert, 2010). The acidic sophorolipid forms when the carboxylic end of the fatty acid is not bound, and the lactonic sophorolipid forms when the carboxylic end of the fatty acid is esterified at the 4′ to form a lactone ring (Van Bogaert and Soetaert, 2010). The lactonic form shows better surface activity, while the acidic sophorolipid has more foaming and solubility properties (Van Bogaert et al., 2007). The detergency and oil in water emulsion stabilizing properties of sophorolipids are due to their hydrophilic-lipophilic balance (HLB of 10 to 13) (Van Bogaert et al., 2007). Figure 1.1 shows the molecular structure of two common types of sophorolipid biosurfactants.

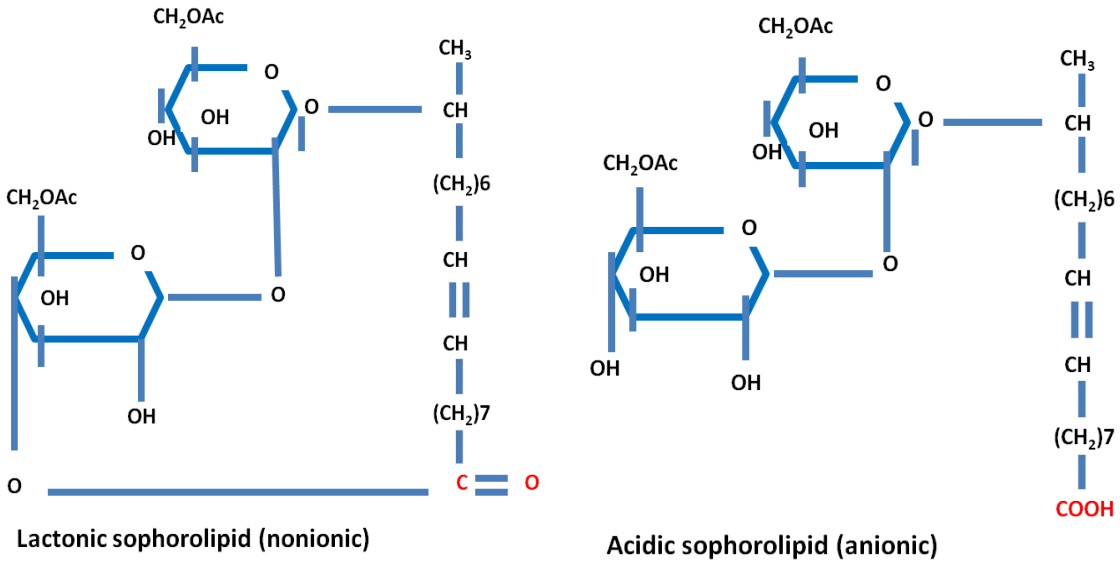

Figure 1.1. The lactonic (A) and acidic (B) sophorolipid biosurfactants (adapted from Hirata et al., 2009).

Production from non-pathogenic yeast species, significant surface activity (a property that is very important in oil dispersion application) and low sensitivity to salinity and temperature (Daverey and Pakshirajan, 2010) make the sophorolipid biosurfactants very attractive for industrial and environmental applications (Van Bogaert et al., 2007). The studies that focused on the biodegradability and ecotoxicity of sophorolipids showed that these biosurfactants are more biodegradable and have lower toxic effects than the chemical surfactants (Develter and Lauryssen, 2010; Hirata et al., 2009b; Poremba et al., 1991; Renkin, 2003). For example, a comparison of the toxicity (following the "MTT assay with normal human epidermal

keratinocytes (Kurabo)" and biodegradability ("through biochemical oxygen demand (BOD) test") of sophorolipids with chemical and biological surfactants such as surfactin (SF), Arthrofactin (AF), Sodium laurate (SP), Pluronic L31 (BPL31), Pluronic L64 (BPL64), Sodium dodecyl sulfate (SDS), Polyoxyethylene lauryl (AE) showed that after the Pluronic L31, which exhibited the lowest cytotoxicity, the sophorolipids had the lowest cytotoxicity among the tested surfactants (Hirata et al., 2009b). The biodegradation of sophorolipids was also slightly lower than the sodium laurate but higher than the BPL31, LAS, and AE (Hirata et al., 2009b). In a study, the chronic toxic effects of sophorolipid concentrations on *Daphnia magna* following the "no observed effect concentration test (NOEC)" were examined. The chronic toxicity was reported based on the quantity of survived young *Daphnia* in comparison to the parent *Daphnia* over a period of three weeks. Results showed that sophorolipid biosurfactant had an insignificant chronic toxic effect (e.g., 11.3 mg/l) (Develter and Lauryssen, 2010). One of the known challenges in the application of sophorolipids (SLs) is the instability of SLs at pH values higher than 7-7.5 at which the chemical structures of sophorolipid biosurfactants may permanently change (Van Bogaert and Soetaert, 2010). However, a study conducted by Inoue et al. (1980) reported that the hydrolysis of acetyl and ester bonds in sophorolipids occur at pH 9-10 and room temperature and/or at pH neutral and high temperature or during the long term storage to the point that the lactonic sophorolipid structure changes (Inoue et al., 1980), but the "glycosyl ether bond" that link the "hydroxy fatty acid" to "sophorose sugar" is insensitive to the pH and temperature changes (Inoue et al., 1980). Thus, the SLs maintain their main structures at such conditions.

1.5. Problem Statement

There are several reasons that make it almost impossible to investigate the effects of dispersants on the aquatic environment. First, it takes time to observe the effects of such reagents on the environment and second, the effects may not be recognized with the current knowledge and technologies. For example, although the primary studies reported the successful and harmless applications of dispersants (Lessard and DeMarco, 2000), recent studies revealed that the dispersants and chemically dispersed-oils are toxic to the marine life (Chapman et al., 2007; Rosen, 2004a; Zhang et al., 2013). Moreover, the chemical dispersants' performance are influenced by the environmental factors such as the type and compositions of spilled oil, salinity,

pH, temperature, and sea state (Clayton et al., 1993b; Fingas, 2011e; Jones et al., 1978; Li et al., 2010).

Biosurfactants are less influenced by the environmental factors and are less toxic than chemical surfactants (Desai and Banat, 1997; Zhang et al., 2004). Despite the increasing industrial applications of biosurfactants (Develter and Lauryssen, 2010; Lourith and Kanlayavattanakul, 2009; Nitschke and Costa, 2007) and the promising results obtained from the studies on the applicability of rhamnolipid biosurfactant, produced by *Pseudomonas,* and surfactin biosurfactant, produced by *Bacillus* for the control of environmental contaminants (Lin, 1996; Mulligan, 2005, 2009) their environmental applications have not yet been initiated. This is due to (i) the focus on assessing a few types of biosurfactants such as rhamnolipid and surfactin (Mulligan, 2005, 2009; Perfumo et al., 2010) and (ii) limited studies on the potential environmental applications of biosurfactants (e.g., for oil spill control).

On the other hand, although, the role of indigenous oil-degrading microorganisms in the hydrocarbon biodegradation and the effect of chemical dispersants on such microorganisms in the oil-impacted environments has been well studied (Kostka et al., 2011; Lin et al., 2009; Yakimov et al., 2007), the role of indigenous microbial communities present in the spilled oil on the oil biodegradation has rarely been studied.

1.6. Objectives of the Research Proposal

Sophorolipid biosurfactants have been known since the 1960s (Gorin et al., 1961). Sophorolipids have properties (e.g., surface activity (Ashby et al., 2008), low sensitivity to salinity and temperature (Daverey and Pakshirajan, 2010), low toxicity and high biodegradability (Hirata et al., 2009b) and high yield of production (Daniel et al., 1998; Daverey and Pakshirajan, 2009; Pekin et al., 2005)) that make them a potential product for dispersion of spilled oil in seawater. In this study, the effectiveness of various concentrations and quantities of sophorolipid on the dispersion of biodiesel, diesel and light crude oil with different chemical structures were investigated. As most spilled oils go through the weathering processes shortly after the spill, the diesel, biodiesel and light crude oil were artificially weathered to simulate the real oil spill situation. The effect of environmental factors such as mixing, salinity, temperature and pH on the

effectiveness of sophorolipid was examined. Moreover, oil biodegradation with the indigenous microbial communities in the spilled oil was studied. The contributions of such microorganisms in the hydrocarbon degradation under laboratory conditions, the mechanisms of hydrocarbon uptake with and without sophorolipid biosurfactant were also studied.

The specific objectives of the present research were as follows:

I. To evaluate (a) the sophorolipid biosurfactant effectiveness on the dispersion of weathered diesel, biodiesel and light crude oil under various sophorolipid concentrations and quantities and settling times and (b) to determine the effects of salinity, temperature and pH on the oil dispersion by the sophorolipid biosurfactant.
II. To characterize the microbial communities naturally present in the weathered diesel, biodiesel and light crude oil and to determine their contribution in the oil biodegradation with and without sophorolipid biosurfactant.
III. To determine the relationship between the dispersion and biodegradation of weathered diesel, biodiesel and light crude oil in the presence and absence of the biosurfactant sophorolipid.

1.7. Thesis Organization

This thesis has been prepared in a manuscript-based format. The thesis consists of six chapters including the introduction and literature review, three chapters that cover the results of this study, general conclusions and the reference section.

Chapter 1 briefly reviews the literature on the oil spill, chemical cleanup method and new cleanup products and the important challenges with each method.

Chapter 2 (Objective I-a) describes the applicability of sophorolipid biosurfactant, one of the less studied products, for dispersion of diesel, biodiesel and light crude oil in seawater under the laboratory conditions according to the swirling flask dispersant effectiveness technique. The technique slightly was modified to represent the real effectiveness of sophorolipid in oil dispersion. For example, the sophorolipid and the diesel, biodiesel and light crude oil were not premixed and the diesel, biodiesel and light crude oil were artificially weathered to minimize the

effect of volatile compounds in the dispersion effectiveness. The effectiveness of sophorolipid biosurfactant in the oil dispersion (the amount of oil that dispersed in the artificial seawater) was quantified at the different sophorolipid concentrations and quantities and settling times. Further examination was performed to determine the mechanisms involved in the oil dispersion. The focus was on the variations in the surface and interfacial tensions and the oil droplet properties.

Chapter 3 (objective I-b) describes the effect of common environmental factors such as seawater salinity, temperature and pH on the oil dispersion by the sophorolipid biosurfactant. Further examinations were done to determine the mechanisms involved in the oil dispersion under the environmental conditions.

Chapter 4 (objectives II and III) describes the biodegradation experiment that was conducted to (1) determine the presence of bacteria capable of consuming the weathered biodiesel, diesel and light crude oil, (2) determine the biodegradation (oil removal percentages) by the bacteria, and (3) determine the mechanisms of oil uptake by microorganisms. Moreover, biodegradation experiments were conducted with and without sophorolipid biosurfactant to determine the relationship between the dispersion and biodegradation of weathered diesel, biodiesel and light crude oil in the presence and absence of sophorolipid.

Chapter 5 summarizes the overall results obtained from this study.

Chapter 6 represents the supporting information in this dissertation.

Articles in preparation: Chapters 2-4 are manuscripts prepared for submission to the peer-reviewed journals. The author of the current thesis is the primary author of the manuscripts. The second contributing author, Dr. Catherine N. Mulligan, provided advice on the experimental sections and interpretation of the results and revised the content of the articles.

The detailed description of each author's contributions to the manuscripts is as follows,

Manuscript 1: Prepared for submission to the journal of Environmental Science and Technology.

Title: Sophorolipid Biosurfactant Dispersed Weathered Biodiesel, Diesel and Light Crude Oil in Seawater

N. Saborimanesh: designed, conducted the dispersion experiments and analyzed the data, and was the primary author of the manuscript.

C. N. Mulligan: obtained the funding for the work, provided advice on the experimental sections and interpretation of the results and revised the content of the manuscript.

Manuscript 2: Prepared for submission to the journal of Environmental Science and Technology.

Title: Effect of Salinity, Temperature and pH on Biodiesel, Diesel and Light Crude Oil Dispersion by Sophorolipid Biosurfactant

N. Saborimanesh: designed, conducted the dispersion experiments and analyzed the data, and was the primary author of the manuscript.

C. N. Mulligan: obtained the funding for the work, provided advice on the experimental sections and interpretation of the results and revised the content of the manuscript.

Manuscript 3: Published in the Journal of Bioremediation and Biodegradation.

Saborimanesh, N., Mulligan, C.N. (2015), Effect of Sophorolipid Biosurfactant on Oil Biodegradation by the Natural Oil-Degrading Bacteria on the Weathered Biodiesel, Diesel and Light Crude Oil. Journal of Bioremediation and Biodegradation, 6: 314. doi:10.4172/2155-6199.1000314

N. Saborimanesh: designed, conducted the biodegradation experiments and microbial verifications and analyzed the data, and was the primary author of the manuscript.

C. N. Mulligan: obtained the funding for the work, provided advice on the experimental sections and interpretation of the results and revised the content of the manuscript.

1.8. Contributions to Knowledge

Chemical dispersants have been widely used to accelerate the natural removal of spilled oil through oil dispersion and biodegradation. However, recent studies revealed the deleterious effects of the dispersants and the chemically dispersed oils on marine life. As attempts for developing the less intrusive chemical dispersants have not been promising, investigations on the

potential application of compounds with high surface activity, low toxicity and stability under natural marine environmental conditions would help the cleanup agencies to eventually replace the toxic chemical dispersants with more environmentally friendly compounds. Microbially produced surface-active compounds are used in the food, cleaning, cosmetics and pharmaceutical products. However, the biosurfactants have not been used for removal or control of contaminants from the environment. This is due to extensive attention to the limited biosurfactants, focus on the applicability of biosurfactants for removal of single compounds (e.g., octadecane) and oil (e.g., diesel fuel) under limited environmental conditions (e.g., only temperature) (Shreve et al., 1995; Whang et al., 2008; Zhang and Miller, 1992, 1995).

On the other hand, the effect of chemical or microbial biosurfactants on oil biodegradation was usually studied by the isolated microorganisms from the oil-contaminated soil or marine environments (Campo et al., 2013; Lindstrom and Braddock, 2002). Therefore, the effect of indigenous microbial communities in the spilled oil in the oil biodegradation was not understood. Moreover, the biodegradation studies were conducted by the initially enriched microorganisms (Campo et al., 2013; Lindstrom and Braddock, 2002). Using enriched microorganisms is limited by the fact that the growth rate of microorganisms and consequently the oil biodegradation rate are higher than the growth of non-enriched indigenous oil-degrading cultures. In addition, the microbial enrichment may influence the dominance of the microbial species. This would make the prediction of biodegradation difficult in a real oil spill situation.

This study attempted to consider the limitations in the previous studies with the following specific contributions.

- Application of a biosurfactant (sophorolipid) with hydrophobic nature, high surface activity, stability at high salinities and temperatures and pH up to 7.5.
- Modification of the methodology to simulate the effectiveness of sophorolipid in the real spill condition. Generally, the oil becomes weathered (e.g., loses the volatile compounds) soon after the spill occurs. Therefore, the diesel, biodiesel and light crude oil in this study were artificially weathered to (i) simulate the real oil spill conditions and (ii) to prevent the

overestimating of the effectiveness of sophorolipid biosurfactant. In addition, unlike many laboratory studies where the dispersant and oil were initially premixed, in this study, the biosurfactant and diesel, biodiesel and light crude oil were not premixed. This method increased the reliability of results and reduced the complexity of prediction of sophorolipid effectiveness in real spill conditions.

- Determination of oil dispersion by the sophorolipid under a broad range of salinities, temperatures and pH, two settling times, sophorolipid concentrations and quantities provided useful information about the effect of each factor on the sophorolipid effectiveness.
- Demonstrating that the spill conditions such as mixing and cold temperature and low salinity (0 ppt) have significant effects on the natural oil dispersion and the oil dispersion by the sophorolipid.
- Demonstrating that the sophorolipid was effectively stable at the pHs common to the most marine environments.
- Demonstrating the significant role of the indigenous oil-degrading communities in the diesel, biodiesel and light crude oil in the oil biodegradation.
- Determining the indigenous oil-degrading communities in the diesel, biodiesel and light crude oil by the traditional technique (e.g., plate culturing) and advanced molecular techniques (e.g., 16S rRNA gene PCR amplicon pyrosequencing).
- Demonstrating the positive effect of sophorolipid biosurfactant on oil dispersion and biodegradation.

Chapter 2: Sophorolipid Biosurfactant Dispersed Weathered Biodiesel, Diesel and Light Crude Oil in Sea Water

Connecting text: The unique properties such as good surface activity, low toxicity, biodegradability and easy production of sophorolipid biosurfactants not only provided considerable opportunities in the food, pharmaceutical and cosmetics industries, it also makes them an alternative to dispersants for removal of spilled oil from the marine environment. In this chapter, the sophorolipid biosurfactant effectiveness for removal of spilled oil was examined through dispersion experiments. In addition, the dispersion effectiveness was examined as a function of sophorolipid concentration and quantity and mixing energy or settling time to determine the role of each factor in the oil dispersion. Results of this chapter provide useful information regarding the applicability of sophorolipid biosurfactant for oil spill removal and the factors that influence its effectiveness. It also provides the fundamental information for the oil cleanup agencies to consider the sophorolipid as one of the replacements to the toxic chemical dispersants in real oil spill situations.

ABSTRACT

This study investigates the application of sophorolipid biosurfactant for dispersion of weathered biodiesel, diesel and light crude oil in the seawater following the swirling flask dispersant effectiveness method with modifications. Results showed that the dispersion of biodiesel, diesel and light crude oil increased as the sophorolipid concentration increased. Nearly 27%, 16% and 12% of the biodiesel, diesel and light crude oil were dispersed, respectively, with the 80 mg/L of sophorolipid. The dispersion of biodiesel, diesel and light crude oil increased from 12%, 6%, and 5% to 13.5%, 7%, and 8% and finally to 19%, 12.5% and 10%, respectively, as the ratios of sophorolipid (20 mg/L) to oil increased from 1:2, to 1:1 and 2:1. No oil dispersion was observed in the seawater with no mixing, regardless of the sophorolipid concentration and negligible oil dispersion was observed when a 10 min settling time was applied following the dispersion process due to the oil resurfacing process. This study showed the capability of sophorolipid in the weathered biodiesel, diesel and light crude oil dispersion due to the significant surface and interfacial tension reduction and solubilization of oil droplets in the micelles.

2.1 Introduction

Oil spills are one way petroleum hydrocarbons enter aquatic environments (Liu et al., 2015; McKew et al., 2007). Depending on the components of the spilled oil, both direct and indirect effects of oil spill on the human health and aquatic systems have been recognized (e.g., through drinking oil-contaminated water (Gross et al., 2013), skin contact (Solomon and Janssen, 2010), and consumption of contaminated seafood (Solomon and Janssen, 2010)). The harmful effects of spilled oils are attributed to their producing compounds such as benzene, toluene, and xylene (Kirkeleit et al., 2008; Smith, 2010; Solomon and Janssen, 2010). These compounds are known to cause health problems (Kirkeleit et al., 2008; Smith, 2010; Solomon and Janssen, 2010). Therefore, chemical dispersants have been used to (i) reduce the movement of spilled oil toward the sensitive places and (ii) accelerate the natural oil removal processes (e.g., biodegradation) through increase in the dispersion of spilled oil in the aquatic systems (Epstein et al., 2000; Lessard and DeMarco, 2000). Success in control of the spilled oil by dispersants increased the worldwide acceptance of dispersants as one of the oil cleanup methods from marine environments (Lessard and DeMarco, 2000). In the latest applications, nearly 7192 m^3 of Corexit 9527 and Corexit 9500 were used to control the spilled oil in the Gulf of Mexico (Gray et al., 2014).

Although, the primary studies reported the successful and harmless applications of dispersants (Lessard and DeMarco, 2000), the recent laboratory and field studies showed that even a short period of contact of the aquatic life with dispersants or dispersed oil can negatively influence the activities of aquatic organisms such as mangroves, sea grasses, and coral reefs (Baca et al., 2005; Goodbody-Gringley et al., 2013; Ramachandran et al., 2004; Shafir et al., 2007; Swedmark et al., 1973; Wise et al., 2014). Knowing the deleterious effects of the spilled oil and chemical dispersants on the aquatic environments and the low effectiveness of the dispersants under different environmental conditions (Clayton et al., 1993b; Fingas, 2011e; Jones et al., 1978; Li et al., 2010; Moles et al., 2002) have directed researchers to investigate the potential applications of safer and environmentally friendly materials such as biologically-based dispersants or biosurfactants (Chen et al., 2013; Dagnew, 2004; Holakoo, 2001; Vipulanandan and Ren, 2000; Yu et al., 2011; Zhang and Miller, 1992).

Laboratory studies suggest that the applications of biosurfactant can control the damaging effects of spilled oil. For example, oil droplets disperse due to the amphipathic nature of biosurfactants (Vipulanandan and Ren, 2000; Zhang and Miller, 1992). This process can reduce the spread of oil from reaching to the sensitive areas and increases the biodegradation of spilled oil through an increase in the bioavailability of oil to oil-degrading microorganisms (Chen et al., 2013; Dagnew, 2004; Holakoo, 2001; Vipulanandan and Ren, 2000; Yu et al., 2011; Zhang and Miller, 1992). Unlike the chemical dispersants, the biosurfactants are less toxic (Hirata et al., 2009b; Renkin, 2003) and are less influenced by salinity, pH and temperature (Daverey and Pakshirajan, 2010).

Despite the increasing industrial applications of biosurfactants (Develter and Lauryssen, 2010; Lourith and Kanlayavattanakul, 2009; Nitschke and Costa, 2007) and the promising results of studies on the applicability of rhamnolipid biosurfactant, produced by *Pseudomonas,* and surfactin biosurfactant, produced by *Bacillus* for the control of environmental contaminants (Lin, 1996; Mulligan, 2005, 2009) their environmental applications have not yet been initiated. This is due to the focus on assessing a few types of biosurfactants such as rhamnolipid and surfactin (Mulligan, 2005, 2009; Perfumo et al., 2010) and limited studies on the potential applications of biosurfactants for oil spill removal. Therefore, the aim of this research was to study the effectiveness of a less studied biosurfactant (sophorolipids) for the dispersion of spilled oil in the seawater.

Sophorolipid biosurfactants have been known since the 1960s (Gorin et al., 1961). Sophorolipids are synthesized by *Candida* species (Van Bogaert et al., 2007) from waste substrates (Daniel et al., 1998; Pekin et al., 2005; Shah et al., 2007; Wadekar et al., 2012). The surface active properties, low toxicity, and biodegradability of sophorolipids were confirmed through studies (Develter and Lauryssen, 2010; Hirata et al., 2009b; Renkin, 2003). Sophorolipids showed effectiveness in various salinities and temperatures (Daverey and Pakshirajan, 2010). Therefore, it was hypothesized that the sophorolipids can provide considerable opportunities as a replacement to the chemical dispersants for the treatment of different oils such as weathered biodiesel, diesel, and light crude oil in the seawater. The effectiveness of sophorolipid on the weathered biodiesel, diesel, and light crude oil was examined as a function of sophorolipid concentration and quantity and mixing or settling time to determine the role of each factor in the

oil dispersion. Results of this study can provide useful information regarding the applicability of sophorolipid biosurfactant for oil spill removal and the factors that influence its effectiveness. It also provides the fundamental information for the oil cleanup agencies to consider the sophorolipid as one of the replacements to the toxic chemical dispersants in real oil spill situations.

2.2 Materials and Methods

2.2.1 Sophorolipid Biosurfactant, Fuels and Synthetic Seawater

The sophorolipid biosurfactant used in this study was purchased from a company in Belgium (ECOVER, N.V, SL18, 41%). Biodiesel, diesel and light crude oil were supplied by the local companies (Rothsay Biodiesel Company, Petro-Canada, Montreal, Canada). The fresh diesel, biodiesel and light crude oil were weathered ((Wang et al., 1998) with modifications) to simulate the natural oil spill conditions. Therefore, known amounts of the fresh diesel (D), biodiesel (BD) and light crude oil (L) were weighted and poured in the clean Petri dishes and left for three days (72 h) under a fume hood. Following the weathering process, the weathered diesel, biodiesel, and light crude oil were sterilized (syringe filter, 0.22 µm, Fisher Scientific or autoclaved at 121°C for 20 min, due to the high viscosity of the light crude oil, it could not be syringe filtered). The weathered oils were transferred to amber vials (80 ml). The biodiesel was kept at 4°C (to reduce further oxidation) and brought to room temperature before using. Various sophorolipid solutions and synthetic seawater stock solutions were prepared using deionized water. The stock solutions of (i) nitrogen and phosphate ($Na_2HPO_4.2H_2O$ (18.40 g/L) and KNO_3 (76.30 g/L)), (ii) $MgSO_4.7H_2O$ (22.5 g/L), (iii) $CaCl_2.2H_2O$ (27.50 g/L), (iv) $FeCl_2.6H_2O$ (0.25 g/L), (v) trace elements ($MnSO_4.H_2O$ (30.2 mg/L), H_3BO_3 (57.2 mg/L), $ZnSO_4.7H_2O$ (42.8 mg/L) and $(NH_4)_6Mo_7(O_2)_4$ (34.7 mg/L)) were separately prepared, autoclaved (121°C, 20 min) and stored at room temperature (USEPA, 2011). The synthetic seawater was prepared by dissolving the NaCl salt (30 g), nitrogen and phosphate (N&P) solution (10 mL), three main solutions (2 mL each) and trace elements solution (2 mL) in deionised water (1L) (USEPA, 2011). The pH adjustment was done using HCl (1%) or NaOH (0.1 N).

2.2.2 Effect of Sophorolipid Concentration and Settling Time on Oil Dispersion

The swirling flask dispersant effectiveness test (USEPA, 2011) with modifications was followed to determine the diesel (D), biodiesel (BD), and light crude oil (L) dispersion by various concentrations of sophorolipid biosurfactant at settling times of 0 and 10 min. Dispersion treatments included control (representing the natural oil dispersion) and biodispersion (dispersion by the sophorolipid solutions). The biodispersion treatment samples contained the synthetic seawater, weathered diesel, biodiesel and light crude oil and sophorolipid solutions. The control samples comprised of only the synthetic seawater and weathered diesel, biodiesel and light crude oil. Each treatment included sample preparation, the dispersant test, dispersed oil sampling, solvent extraction and total petroleum hydrocarbons (TPHs) analysis. The experiment was carried out at room temperature (22 ± 1ºC). Three or more identical samples were prepared for the treatments (the same amount of seawater, weathered oil and sophorolipid solution). Triplicate samples were separately hexane-extracted and separately analyzed by a gas chromatograph (GC-FID). The reproducibility of treatments was evaluated by the relative standard deviation value (RSD). Treatments that achieved the RSD ≤ 15% were accepted, otherwise dispersion experiments (e.g., control and biodispersion treatments) were repeated until the RSD ≤ 15% was achieved.

Dispersion Experiment: The artificial seawater (100 mL), one drop of the weathered diesel, biodiesel and light crude oil (0.1 mL) and sophorolipid solutions (0.1 mL) were added, respectively, to the two sets (for control and biodispersion treatments) of Erlenmeyer flasks (250 mL) and shaken on an orbital shaker for exactly 20 min at 150 rpm. The samples were taken from the mixtures through disposable syringes (30 mL) at settling times of 0 and 10 minutes (USEPA, 2011).

Solvent Extraction: The dispersion samples were thoroughly vortexed (10 sec, 3000 rpm) and transferred to separatory funnels (60 or 120 mL). Pure n-hexane (5 ml, 95%, Sigma-Aldrich) was added to the funnels, thoroughly mixed (15 sec) and were settled for at least 3 min or until two layers were separated. The solvent layer was collected in the amber vials (40 mL) and after three subsequent extractions, the solvent layer was increased to 20 mL with the hexane. The remaining

traces of water were removed by the addition of sodium sulfate, anhydrous (2 g, ≥ 99%, Fisher Scientific) (USEPA, 2011).

Chemical Analysis: Analysis of the dispersed oil was performed by a CP-3800 VARIAN gas chromatograph-Flame Ionization Detector (GC/FID). The GC (with a DB-5 fused silica column) information according to the manufacturer is as follows; 30 m long, 0.25 mm inner diameter, 0.25 µm film thickness with -60ºC to 325ºC temperature limits. A method was created according to the literature (Toxics Cleanup Program, 1997) and the manufacturer recommendations with modifications. The carrier gas (helium), make-up, hydrogen and airflows were adjusted to 5, 28, 30, and 300 mL/min, respectively. The splitless injection mode was selected according to the manufacturer recommendations. The middle injector and detector temperatures increased to 250ºC. The column oven temperature was adjusted to 50°C (hold for 2 min) and increased to 250ºC (hold for 6 min at 8ºC/min, total run time of 33 min). The dispersed oil concentration was determined from the total peak area corresponding to the total petroleum hydrocarbons (TPHs, retention times of 3 to 33 min). The dispersion percentage was calculated as ($C_{disersed\ oil)}$/ C_{in}) ×100, where C_{in} and $C_{dispersed\ oil}$ are the total concentration of oil and the concentration of dispersed-oil in the seawater, respectively.

2.2.3 Effect of Sophorolipid Quantity on Oil Dispersion

Samples (n: 3) were prepared by the addition of 30 mL of seawater (30 ppt, pH 7.2 ± 0.01), the weathered diesel, biodiesel and light crude oil (0.1 mL) and three sophorolipid to oil ratios (SOR) including SOR of 2:1 (0.2 mL of SL, which was the maximum sophorolipid to oil ratio used in the study and 0.1 ml of oil), 1:1 (0.1 mL of SL and oil) and 1:2 (0.05 mL of SL and 0.1 ml of oil) to the centrifuge tubes. The mixtures were thoroughly vortexed (3000 rpm, 1 min), transferred to the separatory funnel (60 or 120 mL) and settled for 1 min to minimize the effect of sampling errors. A 10 mL dispersion layer was drained from the funnel and extracted by n-hexane (10 mL). The solvent layer was increased to 20 mL with the hexane.The extracted dispersed-biodiesel and diesel were analysed (3X) by a UV-VIS spectrometer (Perkin-Elmer Lambda 40) at 250 nm and the extracted dispersed-light crude oil was analysed at 400 nm.

2.2.4 CMC Measurement of Sophorolipid Biosurfactant

Critical micelle concentration (CMC) is a concentration of a surfactant that the surface tension of a solution (e.g., water) reaches its minimum and the external addition of the surfactant has insignificant influence on the solution's surface tension. After this concentration the added surfactant, which contains monomers with both hydrophobic and hydrophilic parts in their molecules, form structures known as micelle (Rosen, 2004b). In this study, the CMC of the sophorolipid was determined from the surface tension measurements (Du Nouy tensiometer, Fisher Scientific, Model 21) of several sophorolipid dilutions in synthetic seawater (30 ppt) at room temperature.

The tensiometer was calibrated following the manufacturer's recommendations. The apparent surface tension of tap water was measured as the control due to the similarities in its chemicals with the seawater. The surface tension of SL solution was measured and the measurement continued until insignificant changes in the surface tension were observed. The true surface tension values in mN/m were calculated from the Equation 2.1 (Hollebone, 2011). The results presented in this study are based on the mean true ST values (3-12 readings) obtained from a minimum of one to maximum of four surface tension measurements.

$$\delta = \delta app\left(0.7250 + \sqrt{\frac{(1.452 \times \delta app)}{C^2(D-d)} + 0.04534 - \frac{1.679}{R/r}}\right) \qquad \textbf{Eq. 2.1}$$

Where δ, δ*app*, *R, r, C, D* and *d* are the true surface/interfacial tension, the apparent surface/interfacial tension, the radii of the Du Nouy tensiometer and the wire of the ring, the circumference of the ring, and the densities of the lower and the upper phases, respectively (Hollebone, 2011).

The true surface tension values corresponding to SL concentrations (in logarithmic scale, base: 10) of 20, 25, 30, and 35 mg/L (set-1), and 40-90 mg/L (set-2), were separately plotted and the regressions (linear) of two data sets were plotted. The CMC and the minimum surface tension

(the point (x,y) of intersection for two linear regressions) were found from the two regression equations.

2.2.5 Interfacial Tension Measurements

The oil-water interfacial tension (IFT) measurements were performed with and without sophorolipid as follows. First, the tensiometer (Du Nouy tensiometer, Fisher Scientific, Model 21) was calibrated with both tap water and artificial seawater (30 ppt). Then the artificial seawater (16 mL) was added to the Pyrex Petri dish (d: 22 mm) followed by the addition of weathered diesel, biodiesel, and light crude oil and the mixtures were gently mixed. The tensiometer ring was adjusted at the interface, between the seawater and weathered oil, and left to stand for 5 min. The sophorolipid solution was added to the mixture and mixed gently and the ring was adjusted on the interface (30 sec). The ring was slowly raised to the level that the seawater and hydrocarbon (e.g., diesel) layer was ruptured. The apparent interfacial tension of the mixture was recorded as mN/m (Hollebone, 2011). The true interfacial tension was calculated from the Zuidema and Waters correction (Eq. 2.1) (Hollebone, 2011).

2.2.6 Microscopic Observation

Light microscopy was performed to visualize the dispersed oil droplets properties (e.g., quantity) formed in the treatments with and without sophorolipid using an Olympus BX51 microscope (Civil Engineering laboratory, McGill University). Samples were prepared in the same way that prepared for the dispersion effectiveness experiment. Briefly, the seawater (100 mL), biodiesel, diesel, and light crude oil (0.1 mL each) and two concentrations of sophorolipid solutions (below and above the CMC) were added to the Erlenmeyer flasks (250 mL) and shaken at 150 rpm for 20 min. Samples (1 mL) were taken from the aqueous phase (using a Pasteur pipette) and poured on three clean slides. The slides were covered by cover slides and sealed by nail polish. The slides were observed under the microscope at various magnifications (e.g., 10, 20, 40, 100X). Multiple images of each sample were captured and analysed using the default image analysis program and the best images were presented in this study.

2.3 Results and Discussion

Dispersant effectiveness depends on the oil and dispersant type, dispersant-to-oil ratio (DOR), mixing energy, and even dispersant application method (Blondina et al., 1997). Therefore, the potential application of the sophorolipid biosurfactant for the dispersion of weathered biodiesel, diesel, and light crude oil was investigated as the function of sophorolipid concentration and quantity and settling time.

2.3.1 Surface Tension of Sophorolipid in Seawater

Figure 2.1 shows the surface tension (ST) of dilutions of sophorolipid in the artificial seawater (30 ppt). The surface tension of 50.8 ± 1.30 mN/m (n:9) was obtained at a sophorolipid solution (final concentration of 20 mg/l), while at concentrations of ≥40 mg/l, the surface tension reduced to ~34 mN/m. The CMC of sophorolipid was determined as 38 mg/L (based on the mean of 1-4 ST measurements). The surface tension and the CMC values obtained in this study are in agreement with the previous studies that reported the surface tensions of 34 mN/m (Mulligan et al., 2001), 32.1-34.2 mN/m (Develter and Lauryssen, 2010) and 35-36 mN/m (Ashby et al., 2008) and the CMC values of 35 mg/L, 140 mg/L, 200 mg/L (Ashby et al., 2008) and 800 mg/L (Ashby et al., 2008; Mulligan et al., 2001). The variations in the ST and CMC values can be due to the influence of the materials and conditions that were applied in the sophorolipid production (Ashby et al., 2008; Daverey and Pakshirajan, 2010), the purity of sophorolipids (SL) (Ashby et al., 2008), the solution that was used for surfactant dilution (e.g., distilled water (Joshi-Navare et al., 2013), buffer solution (Hirata et al., 2009a)) and the type of sophorolipids (e.g., regarding "the alkyl ester chain length" (Zhang et al., 2004) or "the lactonic to acidic ratio" (Hirata et al., 2009a)). For example, a CMC value of 59.4 mg/l was determined from the SLs synthesized by *C. bombicola ("*grown on sugarcane molasses, yeast extract, urea and soybean oil"), while much lower CMCs were determined when the SLs was synthesized on the "deproteinized whey and oleic acid" (Daverey and Pakshirajan, 2010).

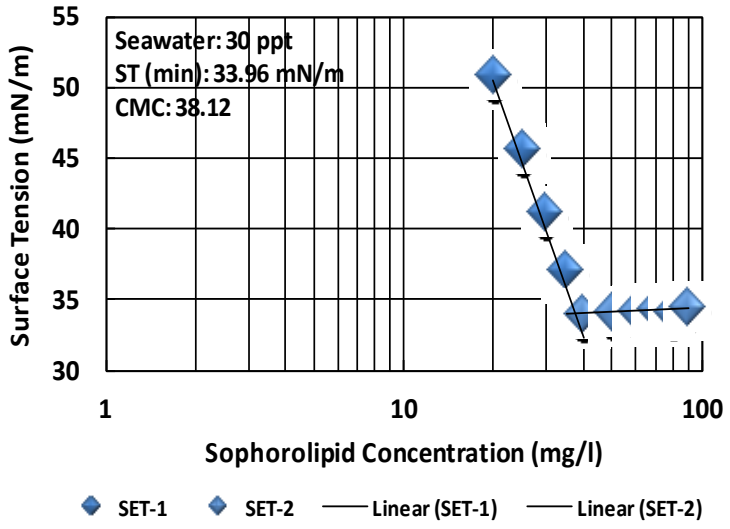

Figure 2.1. Surface tension values of sophorolipid in artificial seawater (salinity of 30 ppt) at room temperature using a De Nouy tensiometer**.**

2.3.2 Dispersion Effectiveness as a Function of Sophorolipid Concentration

Figure 2.2 shows the correlation between the sophorolipid concentration and the weathered diesel, biodiesel and light crude oil. A direct relationship was achieved between the oil dispersion and the sophorolipid concentrations at low SL concentration (e.g., ≤ 40) and the higher concentrations of hydrocarbons were dispersed as the concentrations of sophorolipid increased in the mixtures of artificial seawater-hydrocarbons. For example, the dispersion of weathered diesel, biodiesel and light crude oil increased to 5 ± 1%, 6 ± 0.2% and 5 ± 1%, respectively, as a 5 mg/L of sophorolipid was added to the system. The dispersion of weathered diesel, light crude oil, and biodiesel increased further to 16 ± 1%, 12 ± 0.5% and 27 ± 1.5%, respectively, as the oils were treated with an 80 mg/L of SL. However, the levels of diesel and light crude oil dispersion were not similar to the biodiesel and the biodiesel dispersion was higher than those the diesel and light crude oil by the sophorolipid biosurfactant. A direct relationship between the solubilization of hydrocarbons with the chemical and biological dispersants was reported by investigators (Vipulanandan and Ren, 2000; Zhang and Miller, 1992).

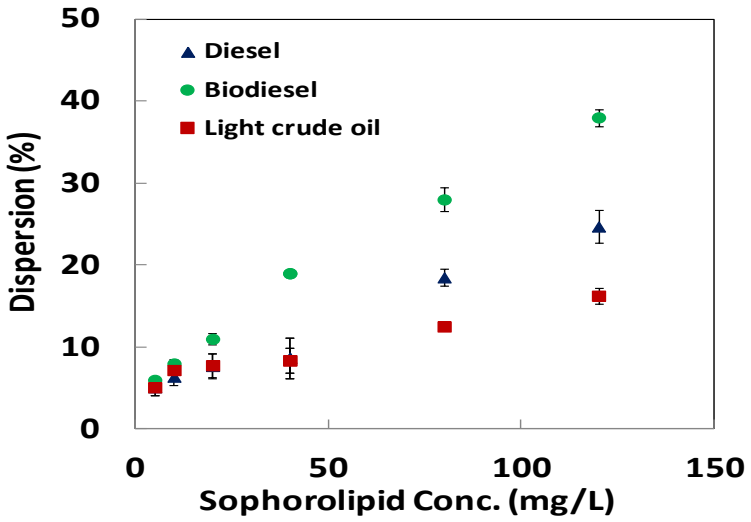

Figure 2.2. Dispersion effectiveness as a function of sophorolipid concentration and hydrocarbon type.

Figure 2.3 shows the reduction in the interfacial tension (IFT) of solutions of artificial seawater and weathered biodiesel, diesel and light crude oil with and without sophorolipid biosurfactant. Results showed a lower IFT of seawater-biodiesel, diesel and light crude oil mixtures in the presence of an 80 mg/l of sophorolipid. The initial interfacial tensions of 15, 28 and 26 mN/m were obtained for the mixtures of weathered biodiesel, diesel and light crude oil at seawater (salinity of 30 ppt, 22 ± 1ºC) without the SL. These data suggested the presence of some surface active compounds (e.g., amphipathic molecules) in the biodiesel, diesel and light crude oil, which explains the slight natural dispersion of the tested oil in the control samples (Allen et al., 1999; Fingas, 2011b). When an 80 mg/l of sophorolipid was applied to the same oil-seawater mixtures, the interfacial tension of the weathered diesel, biodiesel and light crude oil decreased from 28, 15, and 26 mN/m to less than 3, 1.5 and 3 mN/m, respectively, which is in agreement with the previous studies on the variation in the interfacial tensions of oils with dispersants (Clifford et al., 2007; Khelifa et al., 2007; Khelifa and So, 2009; Kirby et al., 2015; Reichert and Walker, 2013; Riehm and McCormick, 2014).

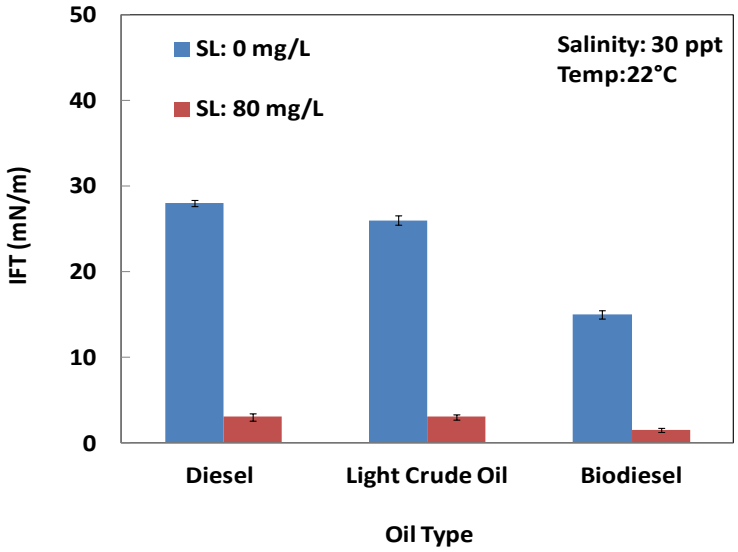

Figure 2.3. Effect of sophorolipid (SL) concentration on the interfacial tension (IFT) of weathered diesel (D), biodiesel (BD), and light crude oil (L) at 22 ± 1ºC using a De Nouy tensiometer.

The visualization (optical microscopy) of the dispersed oils at two concentrations of sophorolipid, 30 and 40 mg/L, showed an increase in the quantities of oil droplets at a slightly higher concentration (Figures 2.4.A and 2.4.B). The same behavior was also observed with biodiesel and light crude oil in which few oil droplets were formed without sophorolipid and the quantities of oil droplets increased when the concentration of sophorolipid was increased.

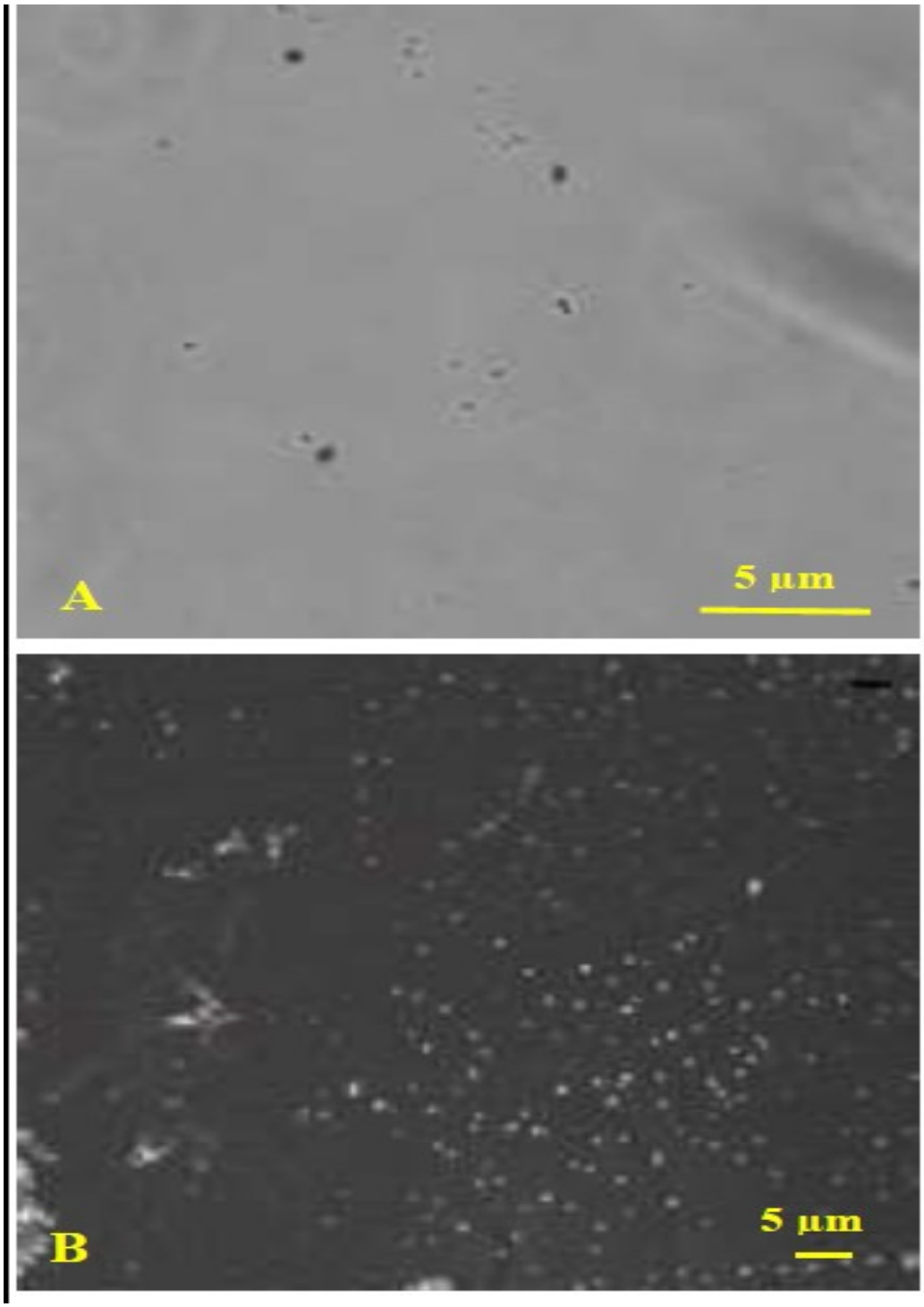

Figure 2.4. Comparison of the quantities of dispersed diesel droplets by sophorolipid biosurfactant; A) 30 mg/L and B) 40 mg/L with a light microscope (magnification: 10X). The scale bars represent 5 µm.

In general, studies showed that the high surface activity of dispersants is one of the main factors in the oil dispersion (Vipulanandan and Ren, 2000; Zhang and Miller, 1992). This is because, the dispersants with the surface active properties (due to the presence of hydrophobic and hydrophilic parts in their molecular structures) can decrease the surface tension of solutions (Rosen, 2004a)) and initiates the faster formation of aggregates and micelles above the critical micelle concentration (Attwood and Florence, 2008a) and consequently, increase the dispersion of insoluble or less soluble compounds through encapsulation in micelles (Seo and Bishop, 2007; Zhang and Miller, 1992). The decrease in the surface tension to ~34 mN/m following the application of sophorolipid and reduction in the interfacial tensions of diesel, biodiesel, and light crude oil in the presence of SL highlighted the surface activity of sophorolipid biosurfactant. The oil dispersion in the presence of sufficient mixing (150 rpm, 20 min) and low concentrations of sophorolipid can be due to the effect of SL on the surface and interfacial tensions and the oil dispersion at high SL concentrations can be due to encapsulation of the weathered oils in the micelles (Clifford et al., 2007; Zhang and Miller, 1992). The development of more oil droplets at high SL concentration can be due to the decrease in the interfacial tensions between the seawater and the diesel, biodiesel and light crude oil (Belore et al., 2009). That is, the sophorolipid possibility formed a stronger film around the oil droplets which resisted the formation of bigger oil droplets (as the result of resurfacing) for a longer time (Rosen, 2004e).

The differences in the dispersion of weathered diesel, biodiesel and light crude oil may be due to (i) the differences in the compositions (e.g., the presence of amphipathic and or polar compounds) and (ii) the properties (e.g., density and kinematic viscosity and interfacial tension) (Clayton et al., 1993b). For example, the results of the interfacial tensions measurements (in the absence of SL) showed that the IFT of weathered biodiesel was 15 mN/m (due to the surface active properties of biodiesel (Allen et al., 1999)), while the IFTs of diesel (28 mN/m) and light crude oil (26 mN/m) were higher. Surface activity is directly influenced by the molecular structure (Shu et al., 2008). Biodiesels are composed of fatty acids (e.g., fatty acid methyl ester, FAME). FAME has surface active properties as the result of their amphipathic structure (Allen et al., 1999). The amphipathic molecules composed of a hydrophilic head group and a hydrophobic tail. According to Shu et al. (2008) the biodiesels show a higher surface activities if their fatty acid hydrocarbon chain is longer or more unsaturated bands is present in their molecular

structures (Shu et al., 2008). The amphipathic structure of biodiesel may have influenced the dispersion of biodiesel in the present of SL. Moreover, studies showed that the compositions of petroleum oil impact the oil dispersion (Effects et al., 2005; Fingas, 2011d). For example, the oils that have the high molecular weight compounds such as asphaltenes (e.g., crude oil) are less disperse (possibly due to increasing in the oil viscosity) than the oils with the high amount of low molecular compounds such as saturates (e.g., diesel fuel) (Fingas, 2011d). Moreover, when the low molecular compounds of oil evaporate (e.g., due to the natural weathering processes), the viscosity, density, and surface and interfacial tensions of oil also change (Hollebone, 2011). Oil physicochemical properties are influenced by the weathering process so that the weathered oils showed lower dispensability than those of fresh oils (Fingas et al., 1990; Hollebone, 2011).

2.3.3 Dispersion Effectiveness as a Function of Sophorolipid Quantity

Figure 2.5 shows the effect of SOR of 2:1, 1:1 and 1:2 on the weathered biodiesel, diesel, and light crude oil dispersion. The effect of sophorolipid quantity on the oil dispersion varied with the ratio of sophorolipid to oil. For example, at a final sophorolipid concentration of 20 mg/L and three sophorolipid to oil ratios of 2:1 (the maximum sophorolipid to oil ratio), 1:1 and 1:2 (the minimum sophorolipid to oil ratio), different extents of oil dispersion were obtained. The lowest oil dispersion among the three ratios was achieved when the amount of sophorolipid was 50% of the weathered oil (e.g., SOR of 1:2). The oil dispersion increased as equal amounts of the sophorolipid and oil (e.g., SOR of 1:1) were added to the seawater and increased further when the ratio of sophorolipid to oil doubled (e.g., SOR of 2:1). Similar results were also obtained in the dispersion of oils with chemical dispersants (Clayton et al., 1993a; Fingas, 2011e; Fingas et al., 1991; Ghurye et al., May 2014; Khelifa et al., 2007). The higher oil dispersion in the samples with the higher amount of sophorolipid biosurfactant seems to be slightly due to the influence of sophorolipid biosurfactant on the seawater-diesel, biodiesel, and light crude oil interfacial tensions. When the quantity of SL was 50% of the oil, the interfacial tensions of seawater-biodiesel reduced from 15 mN/m to 2 and when the sophorolipid to oil ratio increased to 100% (SOR 1:1), the IFT reduced further to 1.5 at. However, the IFT values of the oil-seawater at SOR 2:1 were not detected due to the tensiometer detection limits. A study conducted by Fingas et al. (1991) showed that the dispersion effectiveness of 42%, 25%, 20% and 5% achieved, respectively, when the ratio of Corexit 9527 to oil changed from 1:5 to 1:10, 1:20 and 1:30. A

similar effect was also observed in the dispersion effectiveness of Corexit CRX-8 and the dispersion effectiveness decreased from 85% to 65%, 45%, and 13% as the quantity of dispersant changed from 1:5 to 1:40. A detailed study conducted by Khelifa et al. (2007) on the variations in the oil viscosity, interfacial tension and oil droplets size distributions as a function of dispersant to oil ratio showed that the oil viscosity, the interfacial tension and the oil droplets size distribution significantly changed with the dispersant to oil ratios (DORs). For example, when the oils with low viscosities (e.g., Alaska North Slope, viscosity of 17 mPa s) was dispersed with a high amount of a viscous dispersant (Corexit 9500, viscosity of 92.9 mPa s) at the dispersant to oil ratios of 0 to 1:5, the viscosities of crude oils increased nearly 20-40%. The interfacial tensions of oils also decreased as the low quantity of dispersants (e.g., DOR 1:500 of Corexit 9527) applied to the oil-aqueous systems, but much higher interfacial tension reduction was achieved as the quantities of dispersants increased. Moreover, the higher quantities of smaller oil droplets were formed at the higher quantities of dispersants (up to DOR 1:20) (Khelifa et al., 2007).

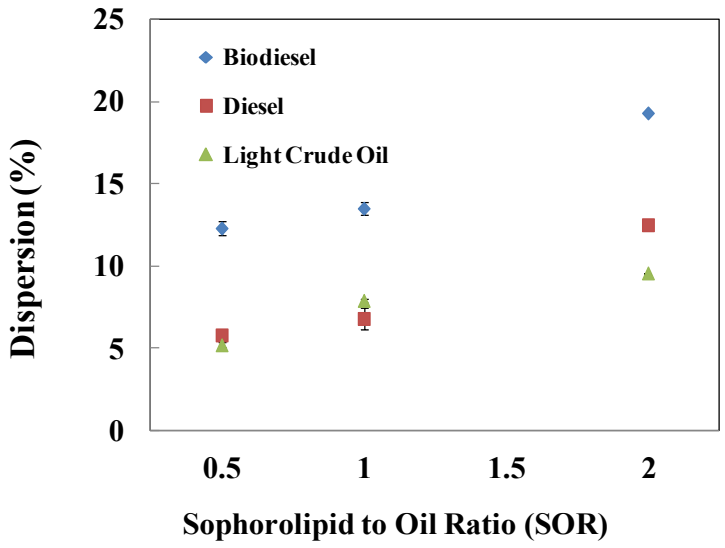

Figure 2.5. Dispersion effectiveness as a function of sophorolipid (20 mg/L) to oil (0.1 ml) ratios of 2:1 (the maximum sophorolipid to oil ratio), 1:1 and 1:2 (the minimum sophorolipid to oil ratio).

2.3.4 Dispersion Effectiveness as a Function of Settling Time

Figure 2.6 shows the stability of dispersed diesel, biodiesel and light crude oil by the sophorolipid solutions at settling times of 0 and 10 minutes. Analysis of the dispersed oil at two mixing levels of 0 and 150 rpm showed that the dispersed oil was formed only when the mixing of 150 rpm was provided. No dispersion was formed at the minimum level of agitation (0 rpm), regardless of the sophorolipid concentration, and the dispersion occurred only when the mixing increased to 150 rpm. In addition, results showed that the mixing influenced not only the formation of dispersed oil, but it also influenced the stability of dispersed oil. The dispersion effectiveness with even the high concentrations of sophorolipid solutions was considerably reduced as the mixing was reduced from 150 rpm to 0 rpm. For example, the dispersion of weathered biodiesel, diesel and light crude oil reduced from nearly 27%, 16%, and 12% with the sophorolipid biosurfactant (80 mg/L) at 0 min settling time to less than 2% (light crude oil and diesel) and 8% (biodiesel), respectively, at the settling times of 10 minutes. However, the visual examinations of samples after stopping the mixing showed that the resurfaced oil did not form a uniform oil slick as observed right after the addition of diesel, biodiesel, and light crude oil to the surface of seawater. Although different dispersants and experimental methods were used in the studies conducted by Fingas et al. (1996) and Sterling Jr. et al. (2004), a considerable decrease in the dispersion effectiveness was also observed by the investigators following the settling time. Similarly, better dispersant effectiveness was reported at the higher level of mixing or shorter settling times (Clayton et al., 1993b; Delvigne, 1985). Belore et al. (2009) also showed that at higher mixing, the formation of smaller oil droplets increased. Based on the results obtained from the oil droplet size distribution as a function of settling time, Sterling Jr. et al. (2004) suggested that at longer settling times bigger oil droplets were formed because more oil droplets coalesced.

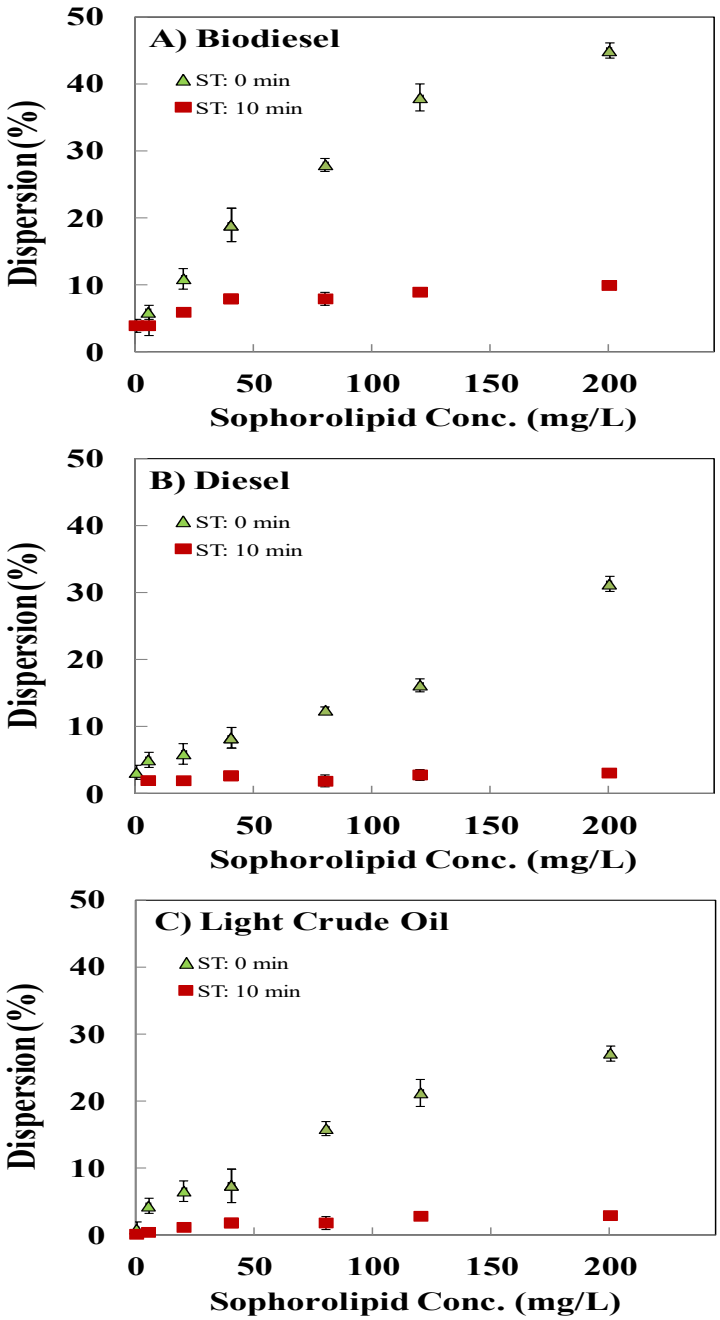

Figure 2.6. Variations of the weathered A) biodiesel, B) diesel and C) light crude oil dispersion as a function of sophorolipid concentration and settling time (ST).

2.4 Conclusions

The potential application of sophorolipid biosurfactant for the dispersion of weathered biodiesel, diesel, and light crude oil was investigated as the function of sophorolipid concentration and quantity and settling time. This study showed that the sophorolipid biosurfactant dispersed the

weathered biodiesel, diesel and light crude oil in the solutions with the salinity of 30 ppt due to the high surface activity of sophorolipid (~34 mN/m) and changes in the oil interfacial tension and encapsulation of oil droplets in the micelles.

The sophorolipid effectively dispersed the weathered biodiesel followed by the diesel and light crude oil. The effect of different components of oil on dispersion by SL was not investigated in this study. However, it seems that the differences in the oil dispersion by the SL can also be due to differences in the physicochemical properties of the biodiesel, diesel, and light crude oil. For example, the biodiesel had a lower interfacial tension than the diesel and light crude oil. Studies showed that the presence/absence of low molecular weight n-alkanes and the high molecular weight compounds such as resins, asphaltenes and waxes (Fingas, 2011d; Moles et al., 2002) also influence the oil dispersion by dispersants (Fingas, 2011e).

Although the higher percentages of fresh oil dispersion by the chemical dispersants were reported by investigators, the higher effectiveness may be because in many of the studies the dispersants and the oils were initially premixed. The premixing of the oil-dispersants found to result in the higher oil dispersion effectiveness (Blondina et al., 1997; Fingas et al., 1990), while in this study, attempts were made to simulate the real oil spill situation and thus not only the oils were initially weathered, the oils and the sophorolipid biosurfactant were not premixed. A detailed study conducted by Moles et al., (2002) showed that a dispersion of ≥10% was achieved in seawater (32 ppt and 22°C), regardless of the Corexit 9500 and Corexit 9527 (chemical dispersants) concentrations (Moles et al., 2002), while an 80 mg/L of SL dispersed approximately 27% of the weathered biodiesel, 16% of the weathered diesel and 12% of the weathered light crude oil, respectively, in the seawater (30 ppt, 22°C, 0 min settling time). Another example is a 16% dispersion of the spilled oil in the Gulf of Mexico by the Corexit 9527 and Corexit 9500 (Gray et al., 2014). This study suggests the sophorolipid biosurfactant had the properties for the dispersion of the weathered diesel, biodiesel and light crude oil in the seawater under the tested conditions. However, detailed investigations will be needed to determine the sophorolipids effectiveness under various environmental conditions.

Chapter 3: Effect of Salinity, Temperature, and pH on Biodiesel, Diesel and Light Crude Oil Dispersion by Sophorolipid Biosurfactant

Connecting text:

Laboratory and field studies showed that a variety of chemical and environmental factors affect the dispersion of oil by chemical and biological dispersants. In the previous chapter, the applicability of sophorolipid biosurfactant for the dispersion of weathered diesel, biodiesel and light crude oil from the synthetic seawater as the function of sophorolipid concentration and quantity and settling time was studied. In this chapter, attempts have been made to examine the dispersion of diesel, biodiesel and light crude oil by sophorolipid under different salinities, temperatures, and pHs. The results of this study provide useful information on the environmental conditions for the application of sophorolipid for dispersion of weathered biodiesel, diesel, and light crude oil in seawater.

ABSTRACT

The effectiveness of sophorolipid biosurfactant for dispersion of weathered biodiesel, diesel and light crude oil at salinities of 0-30 ppt, temperatures of 8ºC, 22°C and 35ºC and pH 6-8 was studied following the swirling flask dispersant effectiveness test with modifications. Results showed that the dispersion of biodiesel, diesel, and light crude oil by sophorolipid was influenced by the salinity and doubled as the salinity increased from 0 to 10 ppt and further increased at the salinity of 20-30 ppt. The higher oil dispersion at higher salinities was due to the higher surface activity and formation of the stronger interfacial film. The oils dispersion increased as the temperature increased from 8°C to 22°C. However, the dispersion of biodiesel decreased, while the dispersion of diesel and light crude oil increased further at 35ºC. The low dispersibility of diesel, biodiesel and light crude oil at 8ºC and 35ºC can be due to changes in the properties of diesel, biodiesel and light crude oil such as viscosity and density. The dispersion was not influenced by the changes in the pH. This study showed that the stability of biodispersed diesel, biodiesel and light crude oil was lower at the salinity of 0 ppt and temperature of 8ºC, but the average dispersions of 27% (biodiesel), 15% (diesel) and 12% (light oil) were obtained at higher salinities and temperatures.

3.1 Introduction

Dispersants lead to the formation of emulsions of oil in water that lasts for a period of time (Fingas, 2011e). Sophorolipid biosurfactants are biodegradable, less toxic (Develter and Lauryssen, 2010; Hirata et al., 2009b; Poremba et al., 1991; Renkin, 2003), hydrophobic biological compounds that produced by *Candida* species (Van Bogaert and Soetaert, 2010). Due to their molecular structures (amphipathic), they significantly decrease the surface tension of solutions (e.g., to 34 mN/m) (Van Bogaert and Soetaert, 2010). As with chemical dispersants, the sophorolipid monomers absorb simultaneously at the oil and water interface and cover the oil droplet and prevent their coalescence (Van Bogaert and Soetaert, 2010). However, the environmental conditions such as salinity, temperature, pH and dispersant compositions influence the interfacial films (Fingas, 2011e). The relationship between dispersant effectiveness and salinity at fresh and saline waters was previously studied (Belk et al., 1989). Laboratory studies conducted by Belk et al., (1989) and Clayton et al., (1993) showed that the oil dispersion

increases when salinity increases (Belk et al., 1989; Clayton et al., 1993b). The proposed mechanisms are (i) the "salting in/out" effect (Mackay et al., 1984), at which dispersants are forced to interact with oil molecules than the surrounding water, and (ii) increase in the electrostatic forces (Clayton et al., 1993b; Fingas et al., 1991; Mackay et al., 1984). The properties of dispersants/surfactants can change as the result of salting in/out effect. The changes may result in an increase (known as salting in) or decrease (known as salting out) in the solubility of surfactants (e.g., nonionic surfactants) (Schott, 1997). In general, because of the amphipathic nature of surfactants, the hydrophilic part (polar head groups) of surfactants interacts with water molecules. When the salt concentration is increased, due to stronger attraction between the ions (e.g., Na+) and water molecules, the quantity of water molecules which can interact with the polar head groups of the surfactants decrease ("head group dehydration") (Anton et al., 2007; Saberi et al., 2014). Therefore, the water-polar head group interactions reduced (Anton et al., 2007). High salinity doesn't always lead to an increase in the dispersion stability (Sterling Jr et al., 2004). Sterling et al. (2004) determined the extent of crude oil coalescence by chemical dispersants at different salinities. They measured the zeta potential of systems of low to high salinities (10, 30, 50 ppt) and pH values of 4-10 and noticed that the oil coalescence did not reduce at high salinity. They concluded that although the zeta potential of the system was lower at higher salinities (e.g., decreased from -3 mV to -10 mV), the decrease in the zeta potential did not lead to the higher stability of the dispersed crude oil. The higher salinity was influential in the formation of dispersed oil (due to the salting out effect) than the stability of dispersed oil.

Studies showed that dispersant effectiveness also changes with temperature because the properties of dispersants vary with temperature (Fingas et al., 1991; Jones et al., 1978; Li et al., 2010). For example, the solubility and adsorption rates of dispersants and the interaction of dispersants with oil change with temperature (Fingas, 2011e). The physical properties (e.g., viscosity) of spilled oil and the surrounding water also change with temperature (Chandrasekar et al., 2006; Clayton et al., 1993b). However, Nguyen et al. (2010) showed that the emulsions produced by the mixtures of sophorolipid and rhamnolipid biosurfactants in combination with chemical surfactants slightly changed with temperature (10ºC, 20ºC, and 40ºC) and salinities (0.9% and 4% w/v).

pH is another important factor that influences the dispersion formation and stability of oil due to the presence of compounds that have acidic and basic functional groups. Studies by Strassner (1968) showed that the pH influenced the emulsion formation and stability by influencing the "film and oil wetting properties" of the asphaltene and resin parts of the tested oil. The solution pH also affects dispersant effectiveness. The reasons proposed for the effect of pH on effectiveness is the changes in the chemical structure of dispersants, especially dispersants with ionic based surfactants (Rosen, 2004b). A study conducted by Albino and Nambi (2009) showed that rhamnolipid biosurfactant was less water soluble at lower pH, thus was less dissociated in the water. The pH also affects the dispersant micellar interactions (Baccile et al., 2012; Shin et al., 2004). Baccile et al. (2012) studied the micellar behavior of acidic sophorolipid biosurfactant at different pH values. This study showed that, at acidic pH, the micellar formation and behavior were only controlled by the sophorolipid concentration and the micelles dominated in the system. At higher pH values, the micelles were dissociated and monomers with negative charges were released to the system. At basic pH, all the sophorolipid micelles were dissociated to the point that the monomers dominated in the system. Shine et al. (2004) study also showed that the phenanthrene solubility by rhamnolipid biosurfactant was maximized at pH of 4.5-5.5 due to the development of pH-dependent micellar aggregates.

The performance of chemical dispersants under different chemical and environmental factors was studied in detail and the influence of the dispersants by the environmental factors has been confirmed (Clayton et al., 1993b; Li et al., 2010; Moles et al., 2002; Shin et al., 2004). Moreover, it has been found that sophorolipid biosurfactants keep their high surface activities at a broad range of salinity and temperature and pH (Daverey and Pakshirajan, 2010). The emulsifying property and surface activity of sophorolipid biosurfactants have previously been evaluated at different pHs and salinities for use in detergency application, pretreatment of synthetic dairy wastewater and biodegradation enhancement (Chandran and Das, 2012; Daverey and Pakshirajan, 2011; Kang et al., 2010). However, the interactions of sophorolipids with hydrocarbons in seawater for removal of spilled oil have not been studied under the environmental conditions. In our previous study (Chapter 2), the effectiveness of sophorolipid in the dispersion of biodiesel, diesel, and light crude oil in seawater was confirmed. The aim of this study was to determine the sophorolipid dispersion effectiveness under different salinities,

temperatures, and pHs. Results of this study will help to predict the weathered diesel, biodiesel, and light crude oil dispersion by the sophorolipid biosurfactant under actual oil spill conditions.

3.2 Materials and Methods

3.2.1 Sophorolipid Biosurfactant, Fuels and Synthetic Seawater

The sophorolipid biosurfactant used in this study was purchased from a company in Belgium (ECOVER, N.V, SL18, 41%). Biodiesel, diesel and light crude oil were supplied by the local companies (Rothsay Biodiesel Company, Petro-Canada, Montreal, Canada). The fresh diesel, biodiesel, and light crude oil were weathered ((Wang et al., 1998) with modifications) to simulate the natural oil spill conditions. Therefore, known amounts of fresh diesel, biodiesel, and light crude oil were weighted and poured in the clean Petri dishes and left for three days (72 h) under a fume hood. Following the weathering process, the weathered oils were sterilized (syringe filter, 0.22 µm, Fisher Scientific or autoclaved at 121°C for 20 min, due to the high viscosity of the light crude oil, it could not be syringe filtered). The weathered oils were transferred to amber vials (80 ml). The Biodiesel was kept at 4°C (to prevent further oxidation) and brought to room temperature before using. Various sophorolipid solutions and synthetic seawater stock solutions were prepared using deionised water. The stock solutions of (i) nitrogen and phosphate ($Na_2HPO_4.2H_2O$ (18.40 g/L) and KNO_3 (76.30 g/L)), (ii) $MgSO_4.7H_2O$ (22.5 g/L), (iii) $CaCl_2.2H_2O$ (27.50 g/L), (iv) $FeCl_2.6H_2O$ (0.25 g/L), (v) trace elements ($MnSO_4.H_2O$ (30.2 mg/L), H_3BO_3 (57.2 mg/L), $ZnSO_4.7H_2O$ (42.8 mg/L) and $(NH_4)_6Mo_7(O_2)_4$ (34.7 mg/L)) were separately prepared, autoclaved (121°C, 20 min) and stored at room temperature (USEPA, 2011). The synthetic seawater was prepared by dissolving the NaCl salt (0, 10, 20, and 30 g), nitrogen and phosphate (N&P) solution (10 mL), three main solutions (2 mL each) and trace elements solution (2 mL) in deionised water (1L) (USEPA, 2011). The pH adjustment was done using HCl (1%) or NaOH (0.1 N).

3.2.2 Measurement of Surface and Interfacial Properties

The surface and interfacial tensions were determined according to Hollebone (2011) with modifications. A Du Nouy tensiometer (an accuracy of ± 0.25 mN/m, Fisher Scientific, Model 21) was used at room temperature (22 ± 1ºC). Solutions of the artificial seawater with salinities

of 0, 10, 20 and 30 ppt and pHs of 4-10 and diluted sophorolipid solutions in deionized water were used in this study. First, the surface tension of tap water was measured as the control, then nearly 20 mL of seawater was added to the clean Pyrex Petri dishes (d: 22 mm) and the surface tensions of the seawater solutions were measured. The sophorolipid solutions (0.1 ml) were added to the seawater solutions, then gently mixed and the apparent surface tensions of the mixtures of seawater-sophorolipid were measured. The true ST values were calculated using the Equation 3.1(Hollebone, 2011). Then, the critical micelle concentration (CMC) was determined by plotting two separate curves of the true surface tension values against the sophorolipid concentrations (in logarithmic scale, base: 10). Applying a linear regression for each curve, the CMC (the point of intersection for two linear regressions) was found from setting equal the two regression equations and solving the equations.

The oil-water interfacial tension (IFT) was measured as follows. First, the tensiometer was calibrated with tap water and artificial seawater (30 ppt, pH 7.2). Then the artificial seawater (16 mL) was added to the Pyrex Petri dish (d: 22 mm) followed by the addition of each of weathered diesel, biodiesel and light crude oil (4 mL). The surface and interfacial tensions of oil reduce as the oil undergoes the weathering process. Thus, it's recommended that the ST and IFT measurements be conducted following the oil-water equilibration (e.g., a contact time of 30 min) (Wang et al., 2014). As in the present study, the diesel, biodiesel, and light crude oil were already weathered, the mixtures were gently mixed and let to stand for only 5 min for the oil-water equilibration before the measurements. A 1 mL of sophorolipid solution was added to the mixtures of seawater-biodiesel, seawater-diesel and seawater-light crude oil and the apparent IFTs of mixtures were measured. The true surface and interfacial tensions were calculated from the Eq. 3.1(Hollebone, 2011).

$$\delta = \delta app\left(0.7250 + \sqrt{\frac{(1.452 \times \delta app)}{C^2(D-d)} + 0.04534 - \frac{1.679}{R/r}}\right) \qquad \textbf{Eq. 3.1}$$

Where δ, δ*app*, *R, r, C, D* and *d* are the true surface/interfacial tension, the apparent surface/interfacial tension, the radii of the Du Nouy tensiometer and the wire of the ring, the circumference of the ring, and the densities of the lower and the upper phases, respectively (Hollebone, 2011).

3.2.3 Dispersion Effectiveness: Effect of Salinity, Temperature, and pH on Oil Dispersion

The salinities, temperature, and pH ranges were selected as the representative environmental conditions of natural fresh and saline waters. Salinities of 0, 10, 20 and 30 ppt, temperatures of 8 ± 1ºC, 22 ± 1ºC and 35 ± 1ºC and pH of 6.2, 7.2 and 8.2 were evaluated in this study. The swirling flask dispersant effectiveness test (USEPA, 2011) was followed with modifications, e.g., the diesel (D), biodiesel (BD) and light crude oil (L) and sophorolipid solutions were not premixed. In addition, the fresh oils were weathered at room temperature to simulate the natural weathering process.

Two experiments including control and biodispersion (dispersion by sophorolipid solutions) were designed. Each treatment included solution preparation (containing seawater, sophorolipid biosurfactant and oil), the dispersion test, sampling, solvent extraction, and chemical analysis of dispersed oil (total petroleum hydrocarbons, TPHs) in seawater. The control samples contained the artificial seawater and weathered oils, while the sophorolipid treated samples were prepared by the addition of sophorolipid solutions to the D, BD, and L oil contaminated seawater. Three or more identical samples were prepared for each treatment (the same amount of seawater, weathered oil and sophorolipid solutions). Triplicate samples were separately hexane-extracted and separately analyzed using a gas chromatograph (FID) or a UV-VIS spectrometer. The reproducibility of treatments was evaluated by the relative standard deviation value (RSD). Treatments with the RSD ≤ 15% were accepted and the dispersion tests with the higher RSD were repeated until the RSD ≤ 15% was achieved.

Dispersion Experiment: Mixtures of artificial seawater (100 mL), weathered oil (0.1 mL) and sophorolipid solutions (0.1 mL) were prepared in Erlenmeyer flasks (250 mL). Dispersion samples were shaken for exactly 20 min on an orbital shaker at 150 ± 1 rpm and samples were taken from the mixtures through disposable syringes (30 mL) after a settling time of less than 1 min.

Solvent Extraction: The dispersion samples were thoroughly mixed using a vortex (10 sec, 3000 rpm) and transferred to the separatory funnels (60 or 120 mL). Pure n-hexane (5 ml, 95%, Sigma-Aldrich) were added to the dispersed samples in the funnels, then shaken (15 sec) and

settled for at least 3 min or until two layers were separated. The solvent layer was collected in the amber vials (40 mL) and after three subsequent extractions, the solvent layer was increased to 20 mL with the hexane. The remaining traces of water were removed by the addition of sodium sulfate anhydrous (2 g, ≥ 99%, Fisher Scientific). The extracted oil (1 mL) was syringe-filtered (0.45 µm, PTEF, Fisher Scientific) and stored until analysis with a GC.

Chemical Analysis: Analysis of the dispersed oil was performed by a CP-3800 VARIAN gas chromatograph-Flame Ionization Detector (GC/FID). The GC column (DB-5 fused silica column) information according to the manufacturer is as follows; 30 m long, 0.25 mm inner diameter, 0.25 µm film thickness and -60 to 325ºC temperature limits. A method was created based on the literature (Toxics Cleanup Program, 1997) and the manufacturer recommendations at which the carrier gas (helium), make-up, hydrogen, and airflows were adjusted to 5, 28, 30, and 300 mL/min, respectively. The splitless injection mode was selected according to the manufacturer recommendations. The middle injector and detector temperatures were adjusted to 250ºC. The column oven temperature was adjusted to 50°C (held for 2 min), then increased to 250ºC (held for 6 min at 8ºC/min).

The dispersed oil concentration was determined from the total peak area corresponding to the total petroleum hydrocarbons (TPHs, retention times of 3 to 33 min). The dispersion percentage was calculated as $(C_{disersed\ oil}/\ C_{in}) \times 100$, where C_{in} and $C_{dispersed\ oil}$ are the total concentration of oil and the concentration of dispersed-oil in seawater, respectively.

3.3 Results and Discussion

3.3.1 Effect of Salinity

The dispersion of weathered biodiesel (BD), diesel (D), and light crude oil (L) by sophorolipid biosurfactant was studied at salinities of 0, 10, 20 and 30 ppt. The dispersion of weathered BD, D, and L increased as the salinity increased from 0 ppt to 30 ppt, with a rapid increase from 0 to 10 ppt and slower increase from 10 to 20 and 30 ppt. For example, at an 80 mg/L of SL, the dispersion effectiveness of weathered biodiesel (BD), diesel (D) and light crude oil (L) nearly doubled from 13 to 25%, 7 to 14% and 2.5 to 8%, respectively, as the salinity of seawater (pH 7.2) increased from 0 to 10 ppt. Then the dispersion increased gradually from 25% to 26.5%

(BD), 14% to 15% (D) and 8% to 10% (L) as the salinity further increased from 10 to 20 ppt, and reached 27% (BD), 15.5% (D) and 12% (L) at salinity of 30 ppt. Figure 3.1 shows the oil dispersion by sophorolipid biosurfactant at salinities of 0-30 ppt. The increase in the oil solubilization in the presence of salts also obtained from the study conducted by Klevens (1950a). The lower surface activity of sophorolipid biosurfactant and weaker interfacial film (e.g., due to the dissolution of sophorolipid at the salinity of 0 ppt) seem to be the reasons for the lower dispersion effectiveness of sophorolipid at the salinity of 0 ppt. Therefore, further examinations of the surface activity and dissociation of sophorolipid in the salinities of 0-30 were carried out to verify the relationship between the salinity and the sophorolipid surface activity, dissociations, and dispersion effectiveness.

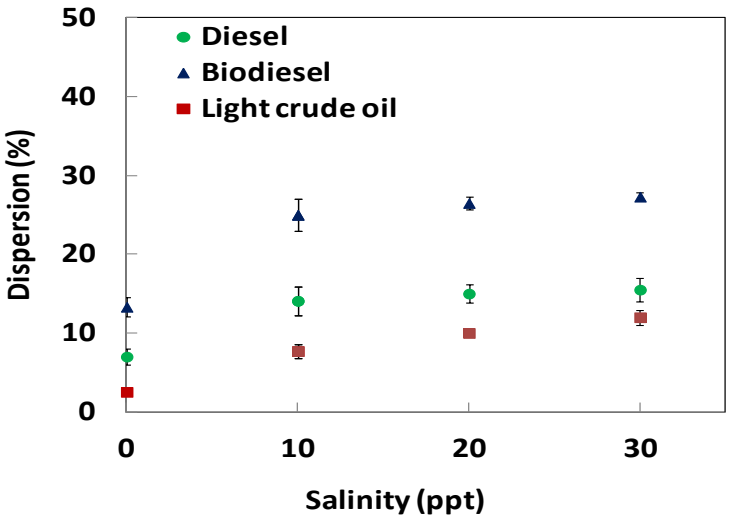

Figure 3.1. Dispersion of weathered diesel, biodiesel and light crude oil by sophorolipid biosurfactant (80 mg/L) at pH 7.2 in the presence of 0-30 g/L NaCl salt at room temperature (the swirling flask dispersant effectiveness test). The error bars show the standard deviation of three identical samples.

Table 3.1 summarizes the results of surface tension measurements with and without sophorolipid biosurfactant (SL) at different salinity (various amounts of NaCl salt (0-30 g/l)) by Du Nouy tensiometer. Results showed an insignificant effect of NaCl salt on the surface activity of SL. For example, the ST of solutions without NaCl salt (0 g/l) dropped from 73 ± 1.5 mN/m to 39 ± 0.5 (n: 3) following the addition of sophorolipid, while the ST of solution with 30 g/L of NaCl salt reduced from 70.25 ± 0.5 mN/m (data from two experiments were combined, n:6) to 33 ± 0.6

mN/m. However, the determination of the critical micelle concentration (CMC) at the salinities of 0 and 30 ppt showed a higher CMC (70 mg/L) value at the salinity of 0 ppt than at the salinity of 30 ppt (38 mg/L, based on the mean of 1-4 ST measurements). The decrease in the CMC of ionic and nonionic surfactants with the increase in the concentrations of electrolytes was previously reported (Attwood and Florence, 2008a; Dutkiewicz and Jakubowska, 2002; Klevens, 1950a; Kumar et al., 2012; Rosen, 2004c).

The potentiometric titration of sophorolipid (pH 5.90 ± 0.2, which is the initial pH of seawater solutions (0-30 ppt, 23.4 ± 0.9°C) when the sophorolipid (41%) was added) was measured at the salinities of 0, 10, 20 and 30 ppt to examine the changes in the sophorolipid structure under different salinities. The pKa (acid dissociation constant) values of 7.04, 6.73, 6.60 and 6.55 at salinities of 0, 10, 20 and 30 ppt were obtained, respectively as the sophorolipid was added to the solutions (Table 3.1). The results showed that the sophorolipid biosurfactant acted as a weak acid in the solutions and was slightly deprotonated in all seawater solutions.

Table 3.1. Surface activity and dissociation of sophorolipid biosurfactant at salinities of 0 to 30 ppt.

Salinity (NaCl, g/l)	**Surface tension (mN/m) of seawater without sophorolipid (n:3)**	**Surface tension (mN/m) of artificial seawater with sophorolipid (n:3)**	**[1]pKa values of Sophorolipid**
0	73 ± 1.47	39 ± 0.5	7.04
10	72 ± 0.0	35 ± 0.4	6.73
20	71 ± 0.4	34 ± 0.5	6.60
30	[2]70.25 ± 0.5	33 ± 0.6	6.55

[1]Titration of a 0.2 g of sophorolipid (41%) in 100 mL of seawater with 0.1 M NaOH (0.5 mL). No pH and temperature adjustments were done before the measurements.

[2]data from two experiments were combined (n:6)

Studies showed that the electrolytes influence the repulsion forces between the hydrophilic parts ("head groups") of the ionic surfactants and the "solubility properties" of the non-ionic surfactants (due to "salting in or out" effects) (Attwood and Florence, 2008a; Biswal and Paria,

2010; Klevens, 1950a; Mukerjee, 1967; Ray, 1971a; Rosen, 2004c). Several reasons were proposed for the influence of electrolytes on surfactants. Study conducted by Shinoda and Takeda (1970) on the effect of salts on the properties (e.g., hydrophilic-lipophilic balance, HLB) of nonionic surfactants in the presence of hydrocarbons through turbidity measurements showed that the types and quantities of salts and the types of hydrocarbons influenced the HLB of nonionic surfactants at the oil-water interface (Shinoda and Takeda, 1970). Moreover, a study conducted by Belk et al. (1989) on the effect of sodium chloride, sodium sulphate, calcium chloride, calcium acetate and magnesium chloride on the dispersion of oils by chemical dispersants showed that the electrolytes differently influence the dispersants. For example, the slight differences in the dispersant effectiveness in the present of sodium chloride and sodium sulphate suggested that the changes of anions are not as important as changes in the cations. A marine formulated dispersant had very high effectiveness in the solution of low concentration calcium ion, but effectiveness significantly decreased as the calcium concentration increased. On the other hand, the dispersant effectiveness only slightly changed in the solutions of low to high concentrations of magnesium chloride (Belk et al., 1989). Klevens' study showed that the viscosity of system increase as the salinity increase possibly due to either formation of unknown micellar structures ("gelation") or formation of the bigger micelles (Klevens, 1950a). The study on the effect of electrolytes on the chemical surfactants also revealed that the "salting out" effect and ionic strength have influenced the dispersion formation (Sterling Jr et al., 2004). However, it was found that the electrolytes had an insignificant effect on preventing the oil coalescent (Sterling Jr et al., 2004). A study conducted by Saberi et al. (2014) showed that the electrolytes even increased the emulsion ("a combination of vitamin E acetate, carrier oil (MCT), and Tween 80 (a nonionic surfactant)") instability by increasing the coalescence process (Saberi et al., 2014). The addition of electrolyte (e.g., KCl, K_2SO_4 and $K_4Fe(CN)_4.3H_2O$) to the aqueous systems containing surfactants (ionic type) influences the solubilization of hydrocarbons such as n-heptane (Klevens, 1950a; Rosen, 2004d) through changes in the surface activity of surfactants (Attwood and Florence, 2008a; Klevens, 1950a; Rosen, 2004d; Schott and Han, 1976; Shinoda and Takeda, 1970), surfactant critical micelle concentration (CMC) and the micellar aggregate formation, size and quantities and the viscosity of system (Attwood and Florence, 2008a; Klevens, 1950a; Rosen, 2004d). For example, lower CMCs were obtained as the result of salting out effect (Schott and Han, 1976). The CMC of surfactants decreases in the presence of

electrolyte due to either decrease in the repulsion forces (e.g., in the case of ionic surfactants) and/or the salting out effect (in the case of nonionic surfactants) (Attwood and Florence, 2008a; Rosen, 2004c, d; Srinivasan and Blankschtein, 2003).

It seems that the level of NaCl salt in solutions influenced the sophorolipid surface-active property and interactions. As the only difference between the solutions in this study was the content of the NaCl salt, the lower dispersion effectiveness in solution without the NaCl salt can be due to the changes in the sophorolipid interactions (Shinoda and Takeda, 1970). That is, at low salinity (0 g of NaCl), the sophorolipid possibly had more interaction with water molecules which increased it solubility in the seawater (salting-in effect). Therefore, the sophorolipid could not effectively interact with the oils. The higher dispersion in the solutions with 10 to 30 g of NaCl seems to be due to the salting out effect, at which the sophorolipid biosurfactant possibly had more interaction with oil than water molecules and effectively adhered to the oils, reduced the oil-seawater IFT and dispersed the oils into the seawater (Klevens, 1950a; Rosen, 2004d). The effect of salting in/out can be detected from the changes in the surfactants critical micelle concentration. The CMCs of 38 and 70 mg/L, respectively, at the salinities of 30 ppt and 0 ppt (30 g and 0 g NaCl salt) can imply the formation of micelles at the lower concentration of sophorolipid biosurfactant at salinity of 30 ppt, which may have resulted in the higher micellar encapsulation of diesel, biodiesel, and light crude oil.

3.3.2 Effect of Temperature

The biodiesel (BD), diesel (D) and light crude oil (L) dispersion by sophorolipid (SL) was studied at temperatures of 8°C, 22°C, and 35°C. Results showed that the dispersion of the diesel, biodiesel, and light crude oil increased as the temperature increased from 8ºC to 22°C. The dispersion of biodiesel reduced slightly at 35ºC, but the dispersion of diesel and light crude oil increased further. For example, at a final concentration of 80 mg/L, the biodiesel dispersion increased from 3.6% at 8ºC to 28% at 22°C and then decreased to 25% at 35ºC. The dispersion of diesel increased from 7% at 8°C to 14% at 22°C and 18% at 35°C and the dispersion of light oil increased from 3% at 8°C to 13% at 22°C and 17% at 35°C. Figure 3.2 shows the variations in the oil dispersion with the temperature at sophorolipid concentrations below and above the CMC. The comparison of dispersion effectiveness at temperatures of 8ºC and 22°C indicates that

the dispersion of diesel and biodiesel increased nearly 50% and the dispersion of light crude oil increased nearly 75% as the temperature increased from 8ºC to 22°C. The highest increase in effectiveness was observed in the biodiesel dispersion followed by the light crude oil and diesel. However, at 8ºC, the dispersion of diesel, biodiesel, and light crude oil was significantly low (less than 5%).

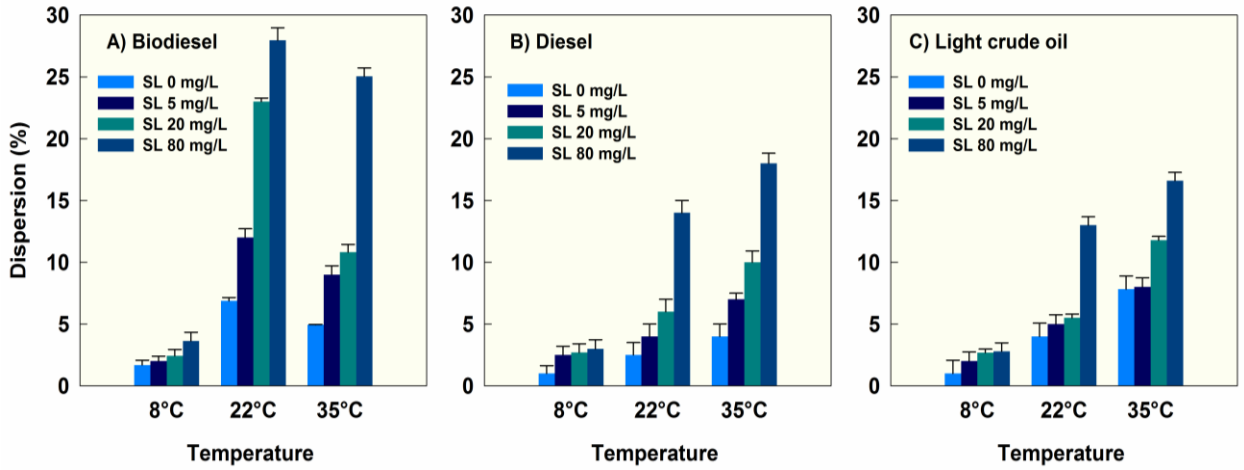

Figure 3.2. Dispersion of weathered A) biodiesel, B) diesel and C) light crude oil at sophorolipid concentrations below (0, 5, and 20 mg/l) and above (80 mg/l) the CMC (38 mg/l) at temperatures of 8ºC, 22ºC, and 35ºC following the swirling flask dispersant effectiveness test. The error bars represent the standard deviation of three identical samples.

Temperature influences the properties of each of dispersant/surfactant, seawater and tested oil (Clayton et al., 1993b). Therefore, it is difficult to clearly state the main reasons for the low sophorolipid dispersion effectiveness at the low temperature. It was found that the low temperature can influence the interaction between the surfactant molecules with each other and with oil (Clayton et al., 1993b; Hollebone, 2011; Attwood and Florence, 2008a) through (i) increase in the solubility of surfactants (e.g., ethoxylated surfactants) (Clayton et al., 1993b), (ii) changes in the hydrophilic-lipophilic balance (HLB) value of surfactants (Attwood and Florence, 2008b; Clayton et al., 1993b), (iii) increase in the viscosities of the surfactant and oil (Clayton et al., 1993b; Fingas et al., 1991). The oil dispersion usually reduces when the viscosities of each of oil and surfactant increase (Clayton et al., 1993b; Trudel et al., 2010). This is because, at low temperature, the surfactants/dispersants cannot reach the oil-water interface, thus fewer oil droplets are formed (Clayton et al., 1993b). Moreover, the low temperature may influence the movement of oil droplet to the aqueous phase (Clayton et al., 1993b). On the other hand, the high

temperature influence (usually increase) the dispersion by (i) reducing the CMC of surfactants (e.g., nonionic), which decreases at high temperatures up to the cloud point (the temperature at which "the nonionic surfactants become turbid") (Attwood and Florence, 2008a) (ii) increasing the size of micellar aggregates (Balmbra et al., 1962), and (iii) influences the viscosities of oils and surfactants (Clayton et al., 1993b).

The changes in the viscosity of oil and sophorolipid (SL) or surface activity of SL at temperatures of 8, 22 and 35°C were not investigated in the present study. However, one factor that seems resulted in the low dispersion of diesel (D), biodiesel (BD) and light crude oil (L) at 8ºC can be the effect of temperature on the oil properties such as viscosity and density. This is because the oil dispersion only slightly improved even when the sophorolipid concentrations increased to 80 mg/L (above the CMC) at 8ºC. Moreover, at 8ºC, the clusters of semi-solid biodiesel were observed on the surface of seawater during the experiment. The changes in the oil properties such as the "cloud point (e.g., 6-13°C in the case of fresh Rothsay biodiesel (Rothsay Biodiesel, 2010)), viscosity and density" with temperature were previously reported (Aworanti et al., 2012; Colcomb et al., 2005; Knothe and Steidley, 2007; Lewis, 2004; Li et al., 2010; Moles et al., 2002; Sjöblom and Simon, 2014; US National Renewable Energy Laboratory, 2009). For example, the viscosity measurements of biodiesel (e.g., B100, NOPEC Corporation, Lakeland, FL) and diesel (e.g., No.1) by Cannon-Fenske viscometer showed that the viscosity of biodiesel and diesel increased from 4.6 cSt to 11cSt and 1.5 cSt to 3 cSt, respectively, with decrease in the temperature from 40 to 10ºC (Tat and Van Gerpen, 1999). The kinematic viscosity measurements of light crude oil at temperatures of 30 and 10ºC also showed that the viscosity of light crude oil increased from 7.14 cSt to 14.22 cSt, respectively (Al-Besharah et al., 1989). Furthermore, the density measurements of biodiesel (B100, synthesized from crude palm oil) and diesel (No.2) at different temperatures showed that the density of biodiesel increased from 850 kg/m^3 to 870 kg/m^3 and the density of diesel increased from 844 kg/m^3 to 858 kg/m^3 as the temperature decreased from 40 to 10ºC, respectively (Benjumea et al., 2008). Variation in the density of biodiesel with temperature was also reported by investigators (Nogueira Jr et al., 2010; Tate et al., 2006a, b). The study suggests that apart from the influence of the low temperature on the properties of SL and seawater, the low dispersion of biodiesel, diesel, and light crude oil at 8ºC possibly can be due to changes in the viscosities and densities of the SL and oils, which

influenced the interaction between the sophorolipid biosurfactant and the denser and more viscous oils.

3.3.3 Effect of pH

Figure 3.3 shows the dispersion effectiveness of weathered diesel, biodiesel, and light crude oil as a function of pH common to the natural seawaters (pH 6-8) at the sophorolipid concentrations below and above the CMC and at salinities of 20 and 30 ppt. As shown, the sophorolipid effectively dispersed diesel, biodiesel, and light crude oil, regardless of the seawater pH. However, it seems that sophorolipid had a slightly better performance at pH 6.2. Slight changes in the oil dispersion at pHs of 6 to 8 indicated the high stability of sophorolipid at the tested pHs. For example, at a given concentration of 80 mg/L, the biodiesel dispersion reached 31%, 30% and 29% at seawater (salinity of 20 ppt) at pH values of 6.2, 7.2 and 8.2, respectively (Figure 3.3.A). To control the effect of salinity, the experiment was repeated at the salinity of 30 ppt. A similar effect was observed at the higher salinity as well. For example, at the same sophorolipid concentration (80 mg/L), dispersions of 35%, 30%, and 29% were acquired at pH values of 6.2, 7.2 and 8.2, respectively (Figure 3.3.B). Similar behavior was also observed in the dispersion of weathered diesel (D) and light crude oil (L). For example, the application of 80 mg/L of sophorolipid to seawater (salinity of 30 ppt) solutions with pH values of 6.2, 7.2 and 8.2 dispersed approximately 12.4%, 11% and 10.7% of weathered diesel, respectively (Figure 3.3.C). This indicated the limited influence of pH on the sophorolipid dispersion effectiveness. The dispersion of weathered light crude oil with the same concentration of sophorolipid also increased from 11.3% to 12.5% and 15% at the pH reduced from 8.2 to 7.2 and finally to 6.2 (Figure 3.3.D).

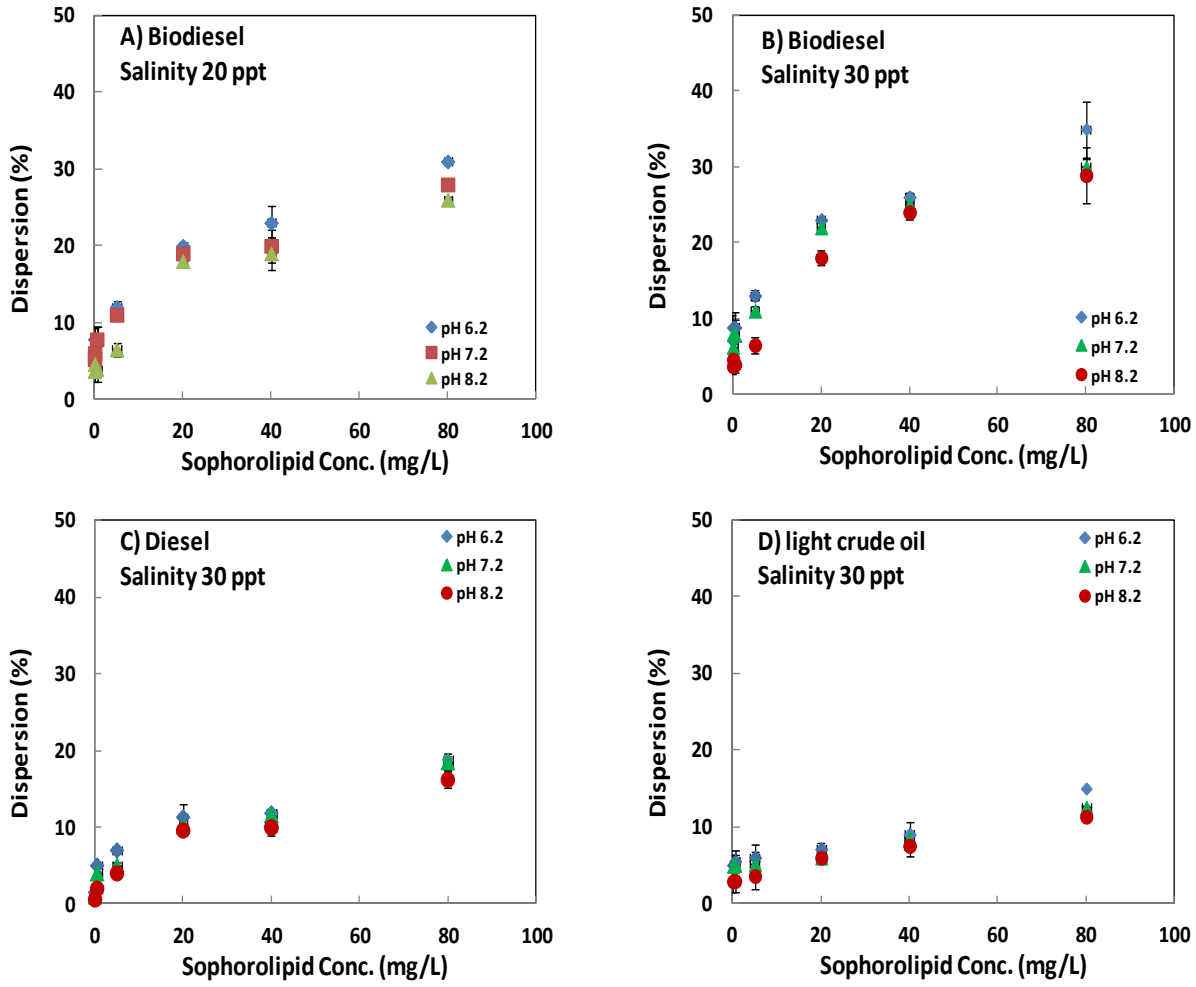

Figure 3.3. Oil dispersion at pHs 6-8; Figures A and B, the dispersion of weathered biodiesel at the salinities of 20 ppt and 30 ppt, Figures C and D, the dispersion of weathered diesel and light crude oil at the salinity of 30 ppt at room temperature.

Oil dispersion was examined by conducting two experiments including surface tension (ST) measurements at pH values of 4 to 10 and interfacial tension (IFT) at pH values of 6-8. The surface tension of artificial seawater (30 ppt, 22°C) solutions at two sophorolipid concentrations of 10 and 100 mg/L, slightly decreased as the pH decreased from pH: 9.89 to pH: 4.45. For example, at a given concentration of 100 mg/L and pH 7.46, the surface tension of 35 mN/m was acquired, while at pH 4.45, the ST was reduced to 32 mN/m. Similarly, at the sophorolipid concentration of 10 mg/L, the surface tension decreased gradually from 70.3 mN/m (data from two experiments were combined (n:6)) to 67 mN/m, at pH 9.98 to pH 5.12. The insignificant changes in the surface activity of sophorolipid at pHs 4 to 10 suggested the stability of sophorolipid at these pHs and pHs 2-10 (Daverey and Pakshirajan, 2010).

The IFT values of the weathered diesel, biodiesel and light crude oil (without sophorolipid) at pHs of 6, 7, and 8 showed that the differences in the IFTs of weathered oils were insignificant at the tested pHs. For example, the IFT of mixtures of biodiesel-seawater decreased from 14.5 mN/m (pH 6) to 14 mN/m (pH 7) and 12 mN/m (pH 8), respectively. The IFT values of 28.5, 28 and 26 mN/m were obtained for the mixtures of weathered diesel oil-seawater and the IFT of 27, 26 and 25 mN/m for the mixtures of weathered light crude oil-seawater at the pH values of 6, 7 and 8, respectively. The interfacial tensions of diesel, biodiesel, and light crude oil dropped rapidly to less than 3 mN/m (diesel and light crude oil) and 1.5 mN/m (biodiesel) as the sophorolipid (80 mg/L) added to the mixtures of oil-seawater at the pHs of 6-8.

The influence of pH on the crude oil-water emulsion stability, interfacial tension, surfactants surface tension, micelles formation, and behavior was previously studies (Albino and Nambi, 2009; Baccile et al., 2012; Shin et al., 2004; Strassner, 1968). For example, a study on the effect of pH (pH ranges from 5 to 8) on the acidic sophorolipid, various micellar behavior ("self-assembly behavior") were specified. At pHs below 5, the effect of pH was on the micellar shape, so that the micelle shape changed from spherical to cylindrical as the concentration of sophorolipid increased. At higher pHs (5-8), the effect of pH was on the "long-range micellar interactions", so that the interactions between the micelles increased. At pHs above 8, the effect of pH was on the intermicellar interaction, so that the micellar structures were broken down due to the effect of pH on the ionization of the carboxyl group in the sophorolipid (Baccile et al., 2012). Results obtained from the ST and IFT, and oil dispersion by sophorolipid suggested the insensitivity of the sophorolipid biosurfactant to pHs 6-8 (Daverey and Pakshirajan, 2010).

3.4 Conclusions

The application of sophorolipid biosurfactant was investigated for the dispersion of weathered biodiesel, diesel, and light crude oil under the salinities of 0, 10, 20 and 30 ppt, temperatures of 8ºC, 22°C and 35ºC and pHs of 6.2, 7.2 and 8.2. The surface and interfacial tension experiments were also carried out under these salinities, pHs, and temperatures to find out the mechanisms involved in the oil dispersion under such environmental conditions. This study highlighted three important points. First, when the temperature and pH were constant, the sophorolipid dispersion effectiveness increased with the increase in the salinity. Secondly, the temperature had

significantly influenced the oil dispersion, regardless of the sophorolipid concentration. Finally, the pH (6-8) was not an influential environmental factor in the oil dispersion by the sophorolipid biosurfactant.

The higher oil dispersion at the higher salinities was due to the influence of NaCl salt on the CMC and the "salting out" effect. The lower oil dispersion at the salinity of 0 ppt can be due to the slightly higher solubilization of sophorolipid (Table 3.1) and the possible formation of oil droplets at high concentration of SL (based on the higher CMC at this salinity). The low effectiveness of dispersants such as Corexit 9500 for oil dispersion in the freshwaters, low salinity waters, and low temperatures was also reported by investigators (Belk et al., 1989; Blondina et al., 1999; Fingas et al., 1991; Lehtinen et al., 1984; Wrenn, 2008). The low dispersion of oils by sophorolipid at temperatures of 8ºC and 35ºC (only biodiesel) may be due to changes in the viscosity and density of diesel, biodiesel and light crude oil in those temperatures (Attwood and Florence, 2008a; Aworanti et al., 2012; Colcomb et al., 2005; Fingas et al., 1991; Lewis, 2004; US National Renewable Energy Laboratory, 2009). Although the insensitivity of SLs to high temperature ("boiling temperature") was reported by investigators (Daverey and Pakshirajan, 2010; Ghojavand et al., 2008), the temperature may also have influenced the sophorolipid properties used in this study. The effect of pH on oil dispersion by sophorolipid can be challenging if the sophorolipid biosurfactant applies at solutions with pHs higher than 8. However, as most natural seawaters have pHs between 6-8, the effect of pH on sophorolipids does not seem to be problematic. The performance of sophorolipid under the studied environmental conditions suggested the applicability of sophorolipid for oil dispersion. However, as one of the reasons that dispersants are used is to increase the natural oil biodegradation, thus, it is important to investigate the effect of SL on oil biodegradation.

Chapter 4: Effect of Sophorolipid Biosurfactant on Oil Biodegradation by the Natural Oil-Degrading Bacteria on the Weathered Biodiesel, Diesel and Light Crude Oil

Connecting text:

In most studies, attention has been paid to the biodegradation by the indigenous microorganisms in the oil-contaminated environments and contributions of natural bacterial populations present in the spilled oil in biodegradation have not been understood. The objective of this study is to investigate the role of natural bacterial populations in the weathered diesel, biodiesel, and light crude oil in oil biodegradation in the presence and absence of sophorolipid biosurfactant.

ABSTRACT

This study investigated the role of natural oil-degrading bacteria in the weathered biodiesel (BD), diesel (D) and light crude oil (L) in oil biodegradation in seawater with and without sophorolipid biosurfactant. Mixtures of artificial seawater and weathered oil with and without sophorolipid dispersant were incubated at 22 ± 1°C and 100 rpm for 28 days. Analysis of the remaining total petroleum hydrocarbons showed the degradation of 43 ± 0.7%, 45 ± 5.7% and 39 ± 4.6% of biodiesel, diesel and light crude oil, respectively, during the natural biodegradation, and 44 ± 5%, 47.5 ± 3.9% and 44 ± 1% of biodiesel, diesel and light crude oil, respectively, with sophorolipid by the existing bacteria after 28 days. Characterization of bacteria isolated from the BD, D and L oil by 16S rRNA pyrosequencing showed that the *Firmicutes* was the dominant phylum in biodiesel (100%) and diesel (53%). The *Actinobacteria* was the dominant in the diesel (47%) and the *Proteobacteria* (97%) and *Actinobacteria* (3%) were the two dominant phyla in the light crude oil. The hydrophobicity results showed that the bacteria consumed the hydrocarbons mainly by changing their cell surface structures in the natural biodegradation treatment and increase in the micellar dispersion and solubilization of hydrocarbons in the biodegradation treatment with the sophorolipid. This study confirmed the significant contribution of natural bacteria in the weathered diesel, biodiesel and light crude oil in the biodegradation and the positive effect of sophorolipid on the biodegradation.

4.1 Introduction

Oil spills are usually referred to as the petroleum based hydrocarbons that enter to the aquatic environments (Bouchez-Naïtali et al., 1999; Liu et al., 2015; McKew et al., 2007). Spilled-oil can last for a long time before it can be cleaned up by the natural removal processes (e.g., evaporation, dispersion and biodegradation) (Fingas, 2011a). Success in the biological treatment of hydrocarbons strongly depends on the presence of active oil-degrading microorganisms in the contaminated site, the bioavailability of hydrocarbons and the environmental conditions (Das and Chandran, 2010; Leahy and Colwell, 1990; Okafor, 2011; Zhang and Miller, 1992). According to Okafor (2011) nearly 0.1-1% and 1-10% of the indigenous "heterotrophic bacterial communities" in the unpolluted and oil-polluted marine environments, respectively, are capable of uptaking the petroleum hydrocarbons (HCs) (Okafor, 2011). It was found that, the microorganisms can consume the HCs through different ways that can occur simultaneously or at different stages of HCs uptake (Bouchez-Naïtali et al., 1999; Ward, 2010). For example, microorganisms may first uptake the HCs that are soluble in seawater and then interact with the hydrophobic hydrocarbons if they can change their cell surface structures to hydrophobic or low hydrophobic states, based on the available HCs (Bouchez-Naïtali et al., 1999; Ward, 2010). Moreover, if they can naturally produce surfactant-like products (known as "biosurfactants", that are composed of two parts, a hydrophilic part and a hydrophobic part), they can also uptake the oil droplets or hydrocarbons that encapsulated within the micelles (structures that form when the biosurfactant molecules interact with water and HCs) (Bouchez-Naïtali et al., 1999; Ward, 2010). Since the main constituents of spilled oil are not readily water-soluble, the uptake of hydrocarbons by the microorganisms is either very limited (Ron and Rosenberg, 2002; Ward, 2010; Zhang and Miller, 1992) or restricted to some microorganisms. For example, *Pseudomonas* species produce particular biosurfactants to uptake the hydrocarbons at different biodegradation periods (Bouchez-Naïtali and Vandecasteele, 2008; Sekelsky and Shreve, 1999; Tzintzun-Camacho et al., 2012). Due to the chemical (e.g., hydrophobic nature of hydrocarbons), microbial (e.g., inability of all oil-degrading microorganisms in biosurfactant production) and environmental limitations (e.g., low temperature), the biological or chemical agents (e.g., chemical dispersants, nutrients) were added to the oil-impacted environments (Klevens, 1950b;

Zhang and Miller, 1992) to accelerate the dispersion and consequently the bioavailability of HCs.

The addition of chemical dispersants to the oil-impacted sites has been extensively practiced over the last few decades (Clayton et al., 1993a; Lessard and DeMarco, 2000). This is because the chemical dispersants have both hydrophilic (water-like) and hydrophobic (water-repellant) parts in their structures (Lessard and DeMarco, 2000). Therefore, they are able to simultaneously interact with oil and water molecules and disperse the spilled oil in the water. When dispersants contact the spilled oil, they influence (usually decrease) the oil-water interfacial tension. This leads to the formation of oil droplets if mixing is provided (Rosen, 2004b). In the presence of sufficient dispersants, the oil droplets are dispersed in the water mainly through encapsulation in the micelles (Rosen, 2004c).

To date, the ultimate goal of application of various additives including chemical dispersants was to increase the bioavailability of spilled oil to the indigenous oil-degrading microorganisms in the oil-contaminated environments (Brakstad and Lødeng, 2005; Lessard and DeMarco, 2000; Lindstrom and Braddock, 2002; McKew et al., 2007; Owsianiak et al., 2009), and the role of oil-degrading microorganisms present in the spilled oil was not understood. The study that investigated the role of natural bacteria on hydrocarbon (e.g., crude oil) biodegradation in seawater showed that nearly 66% of oil was degraded during 56 days of biodegradation by the active bacteria in the spilled-oil (Sheppard et al., 2012). However, the effect of additives (e.g., biosurfactant) on natural bacteria on spilled oil has not been considered in the study.

The main objectives of this study were to determine the identity of bacteria naturally present in the weathered biodiesel, diesel, and light crude oil in the oil biodegradation and to determine the effect of sophorolipid biosurfactant on the biodegradation of diesel, biodiesel, and light crude oil by those bacteria. This study provides information regarding the role of indigenous oil-degrading bacteria in the spilled oil biodegradation in the marine environment and the effect of sophorolipid biosurfactant on their activities.

4.2 Materials and Methods

Chemicals

The sophorolipid biosurfactant was supplied by Ecover Company (Belgium N.V, SL18, 41%). Diesel and light crude oil were purchased from Petro-Canada and biodiesel was purchased from Rothsay Biodiesel Company in Montreal, Canada. Deionized water was used for dilution of the original sophorolipid solution and seawater preparation. The fresh biodiesel, diesel and light crude oil were artificially weathered (following the Wang et al. (1998) method with modifications) under a fume hood for 72 h to simulate the weathering conditions and reduce the effects of volatile hydrocarbons on the biodegradation experiment. The synthetic seawater was a brine solution (30 g NaCl/L) amended with necessary elements for the microbial growth and was prepared following the swirling flask dispersant effectiveness test with slight modifications (USEPA, 2011). The synthetic seawater consisted of NaCl salt (30 g/L), nitrogen and phosphate (N&P) solution, and main & trace element solutions. The N&P stock solution consisted of $Na_2HPO_4.2H_2O$ (18.40 g/L) and KNO_3 (76.30 g/L). The trace element stock solution consisted of $MnSO_4.H_2O$ (30.2 mg/L), H_3BO_3 (57.2 mg/L), $ZnSO_4.7H_2O$ (42.8 mg/L) and $(NH_4)_6Mo_7(O_2)_4$ (34.7 mg/L). The three main element solutions consisted of $MgSO_4.7H_2O$ (22.5 g/L), $CaCl_2.2H_2O$ (27.50 g/L), and $FeCl_2.6H_2O$ (0.25 g/L). All five stock solutions were separately autoclaved (121°C, 20 min) and kept at room temperature (22 ± 1°C). Fresh solutions were prepared as the sign of chemical and biological degradations were observed. Prior to each test, the N&P solution (10 mL), the trace elements (2 mL) and the main element solutions (2 mL of each) and the NaCl salt (30 g/L) were added to the deionized water (1L) (USEPA, 2011). The pH and temperature was recorded and dilute HCl (1%) or NaOH (0.1 N) were used to adjust the pH.

Two sets of hydrocarbons (biodiesel, diesel and light crude oil) were prepared for this study. The first set of hydrocarbons were weathered but not sterilized and specifically used as the source of microbial culture and also the source of hydrocarbon in the biodegradation experiment. The second set of hydrocarbons (already weathered) was used only for the microbiological analysis tests (after the biodegradation experiment). This set was initially sterilized (0.22 µm pore size, d: 25 mm, Fisher Scientific, EMD Millipore MF-Millipore™ Mixed Cellulose Ester Membranes) to remove all present microbial communities in the oils. This oil was only used as the source of hydrocarbons for the microorganisms. The oil was kept in amber vials at room temperature.

4.2.1 Biodegradation Experiment

Biodegradation experiment was carried out according to the modified method adapted from the USEPA-bioremediation agent effectiveness (USEPA, 2011) and the method proposed by McKew et al. (2007). The indigenous microbial communities in the weathered biodiesel, diesel and light crude oil were used without any enrichment. Three treatments including the control (no bacteria), natural (with bacteria) and natural treatment with external sophorolipid addition were designed to investigate the oil biodegradation by the active bacteria present in the tested oils. The control treatment was contained the artificial seawater (20 mL) and sterilized weathered oil (100 µL). The natural treatment was contained the artificial seawater (20 mL) and weathered but not sterilized hydrocarbons (100 µL). The natural treatment with external sophorolipid contained the synthetic seawater (20 mL), weathered but not sterilized hydrocarbons (100 µL) and sophorolipid solution (100 µL, 80 mg/L). Biodegradation vials were incubated on an orbital shaker (Thermolyne AROS) at 100 ± 1 rpm and room temperature (22 ± 1ºC) for 28 days. Samples covering days 0, 7, 14, 21 and 28 (for each oil) were taken to analyze the total petroleum hydrocarbon (for determination of hydrocarbon degradation at different days of biodegradation period), the bacterial enumeration and the bacterial characterization.

4.2.1.1 Determination of Oil Biodegradation

The level of biodegradation at various periods was monitored weekly by analysis of the remaining of total petroleum hydrocarbons (TPHs) at days 0, 7, 14, 21 and 28. When the vials were taken from the shaker, the samples were solvent extraction according to the swirling flask dispersant effectiveness test (USEPA, 2011). First, the samples were centrifuged (10000 rpm, 10 min) to separate the biomass from the aqueous phase. The aqueous phase was extracted (3X) with 5 mL of n-hexane (95%, Sigma-Aldrich Canada Co.) and the extracted hydrocarbons (15 mL) were further diluted by the addition of hexane (5 mL). The analysis of the extracted mixture was conducted by a CP-3800 VARIAN gas chromatograph (GC-FID) at the splitless mode and with the oven and detector temperatures of 250°C and a total run time of 33 min (2 min hold at 40°C and 6 min hold at 250°C). The percentage of biodegraded oil was calculated as $((C_{in}-C_{residual})/ C_{in}) \times 100\%$, where C_{in} is the initial concentration of oil added to the biodegradation samples and the $C_{residual}$ is the concentration of oil remaining at different biodegradation periods.

4.2.1.2 Bacterial Verification

The Bushnell-Hass (B-H) media was used to recover the active indigenous oil-degrading bacteria in the tested oils. The Bushnell-Hass is a specific media that only recovers the oil-degrading bacteria (Pepper and Gerba, 2015). The B-H media was supplemented with the weathered sterilized diesel, biodiesel, and light crude oil as the source of hydrocarbons. The B-H media was prepared by the addition of magnesium sulfate (0.2 g/L), calcium chloride (0.02 g/L), monopotassium phosphate, dipotassium phosphate and ammonium nitrate (1 g/L each), ferric chloride (0.05 g/L) and 10-15 g of solidifier to deionized water (final pH 7.0 ± 0.2 at 22°C, autoclaved at 121ºC, 20 min). The plating was done in two steps including serial dilution and aseptic spreading. The serial dilution of the biodegradation samples was done by diluting the aqueous phase of the biodegradation samples (0.5 mL) of each sampling day with a 4.5 mL of phosphate buffer solution (1M, pH 7.4 at 25ºC, to obtain dilutions of $\geq 10^{-5}$, Sigma-Aldrich). The aseptic spreading of the dilutions (100 µL) was conducted by the spreading of the diluted samples and the sterilized weathered diesel (20 µL), biodiesel (20 µL) and light crude oil (20 µL) on the duplicate Bushnell-Hass plates. The plates were sealed with Parafilm and incubated at room temperature (22 ± 1°C) until the bacterial colonies were observed. The number of observed colonies was reported as the colony-forming units (CFU) per mL of samples.

4.2.1.3 Bacterial Communities Characterization

Characterization of the natural microbial communities was conducted by pyrosequencing of 16S rRNA. Three samples of day-0 of the biodegradation experiment were selected to identify the original bacterial communities in the tested oils. Samples (20 mL) were centrifuged (10000 rpm, 10 min) and the biomass was washed (2X) with n-hexane (95%, Sigma-Aldrich). This step was conducted to remove the oil residue from the biomass. The phenol-chloroform protocol (McKew et al., 2007) was followed for the genomic DNA extraction. Briefly, the biomass was initially transferred to tubes (2 mL) that contained 0.5 g of glass beads and a 1 mL of phenol-chloroform-isoamyl alcohol (25:24:1, Sigma-Aldrich). The mixture was vortexed (5 sec), bead beated (20 sec, Mini Vortex, Fisher Scientific) and centrifuged (12000×g, 5 min, Thermo Scientific). This step was repeated until no layer was observed between two phases. The upper layer was transferred to the clean tubes (2 mL) and the chloroform-isoamyl alcohol (450 µL, 24:1,

Biotechnology grade, Amresco) was added to the tubes, vortexed (10 sec) and centrifuged (12000×g, 5 min). The aqueous phase of this step was transferred to a clean (2 mL) collection tube and 70 µL of sodium acetate (3M) and ice-cold isopropanol (1 mL) were added to the tubes. The mixture was gently shaken and incubated at -20°C overnight. Following the incubation time, the mixture was centrifuged (12000×g, 5 min) and the upper phase was discarded. A 70% ethanol (1mL) was used to wash (2X) the precipitated DNA by centrifuging the mixture at 12000×g for 5 min. The DNA was finally air dried and mixed with 50 µL of ultra-pure distilled water (Invitrogen) and stored at -20°C (McKew et al., 2007).

The purity of the genomic DNA examined by running the genomic DNA on a 1.5% agarose gel electrophoresis using a triethanolamine buffer solution (TEA) (a mixture of Tris base, acetic acid and ethylenediaminetetraacetic acid (EDTA)). The concentration of genomic DNA was quantified using the PicoGreen DNA assay kit (Quant-iT) by spectrophotometer. This step was followed by the amplification of the quantified genomic DNA using the polymerase chain reaction, 16S rRNA. The 16S rRNA contains three forward primers and one reverse primer. The sequences of forward primers are 5′-CCTACGGGRGGCAGCAG-3′, 5′-ACWYCTACGGRWGGCTGC-3′ and 5′-CACCTACGGGTGGCAGC-3′ and the reverse primer sequences are 5′-TACNVGGGTHTCTAATCC-3′. The PCR master mixture (Bioline Co.) for each reaction contained the following components: forward and reverse primers (2.5 µL each), 2.5 µmole $MgCl_2$ (2.5 µL), Taq polymerase enzyme (0.5 µL), 5X reaction buffer (10 µL), dNTP-deoxynucleoside triphosphate (0.5 µL), genomic DNA template (2 µL), ultra-pure distilled water (29.5 µL, Invitrogen). The genomic DNA from *E.coli* and deionized water were used as the positive and negative controls. The hot start PCR cycling conditions were as follows. One cycle of hot start at 94ºC for 5 min was followed by 30 cycles at the same temperature each for 1 min, 30 cycles at 55ºC for 30 sec, 30 cycles at 72ºC for 1.5 min and finally one cycle of extended elongation at 72ºC for 8.5 min. The PCR products were then cleaned with the UltraClean PCR Clean-Up Kit (MO BIO Co.). The final concentration of the products was determined by Bioanalyzer 1000 (Agilent Technologies) and samples were submitted to the McGill Genome Center.

The obtained sequences were submitted to the Ribosomal Database Project (RDP, available at http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp) with the Pyrosequencing Pipeline Initial

Process described by Cole et al., (2009). The trimming process (e.g., mismatch adapters, barcodes and primers) was conducted by the default parameters and the sequences ≤ 150 were not included in the analyzes (Claesson et al., 2009). The average length of the analyzed sequences was around 450 bps. The RDP classifier was used to conduct the taxonomic classification and the bacterial sequences of each sample (e.g., biodiesel, diesel and light crude oil samples) carried out individually. The similarities of ≥ 97% were reported as dominant bacteria in each sample.

4.2.1.4 Determination of Cell Surface Hydrophobicity of Microbial Communities

The modified microbial adhesion to the hydrocarbon protocol (Rosenberg et al., 1980; Zhang and Miller, 1994) was followed to determine the hydrocarbon uptake by the indigenous bacterial communities in the weathered oils. Before the test, the biodegradation samples (20 mL, day-7 of the biodegradation) were enriched by transferring the entire samples to the fresh Luria Broth (Sigma-Aldrich) supplemented with 0.1 mL of sterilized and weathered biodiesel (BD), diesel (D), and light crude oil (L) and incubating for 18-24 h at 100 rpm and room temperature (Orbital shaker, Thermolyne AROS). The enriched samples were then centrifuged (10000 rpm, 10 min) and the biomass was washed (2X) with a buffer solution (pH 7.4, $MnSO_4.7H_2O$ (0.2 g/L), urea (CH_4N_2O; 1.8 g/L), KH_2PO_4 (7.26 g/L) and $K_2HPO_4.7H_2O$ (22.2 g/L)) and then with sterilized synthetic seawater (salinity of 30 ppt, pH 7.2) to remove the impurities. Bacterial cells were diluted in the artificial seawater (30 ppt, pH 7.2) until an absorbance of 1.0 A (A_0) at 600 nm was obtained by a UV-visible spectrophotometer (Lambda 20). The mixture was dispensed in separatory funnels (~25 mL in each) followed by the addition of (i) a 1 mL of weathered sterilized hydrocarbons (biodiesel and diesel were syringe filtered (0.2 µm), and light crude oil was autoclaved at 121ºC for 20 min and cooled to room temperature), (ii) mixture of weathered sterilized hydrocarbons and sophorolipid solution. The mixtures were thoroughly mixed (3000 rpm, 2 min) and the optical density (OD: 600 nm) of biomass-aqueous phase (1.5 mL) of each treatment was measured after 1 h.

The bacterial cell surface hydrophobicity (CSH) was calculated from the decrease in the turbidity of biomass in the aqueous phase (which shows the adherence of biomass to hydrocarbons or sophorolipid) following each treatment (A_1) to the initial absorbance of suspended biomass (A_0).

The CSH of cells-diesel and cells-light crude oil and cells-sophorolipid were calculated from (1 - (A_1/A_0) × 100). The CSH of cells-biodiesel and cells-hydrocarbons (D, BD, and L)-sophorolipid was calculated from (A_1- A_0/A_0) × 100). A positive CSH was reported as the hydrophobicity (tendency to interact with the hydrophobic compounds).

4.3 Results and Discussion

4.3.1 Presence of Indigenous Biodegrading Bacteria in the Weathered Diesel, Biodiesel and Light Crude Oil

Few studies have focused on the role of indigenous bacteria in the spilled oil biodegradation. In this study, the bacteria with a high capability of consuming the weathered biodiesel, diesel and light crude oil as the hydrocarbon source was confirmed through (1) chemical analysis of hydrocarbon (TPHs) concentrations at different days of the biodegradation experiment (2) visual observation of the microbial colonies on the Bushnell-Hass plates and (3) microbial verification by the pyrosequencing technique.

Figures 4.1 and 4.2 show the biodegradation of weathered diesel, biodiesel and light crude oil (total petroleum hydrocarbons) and the corresponding bacterial growth in the natural and sophorolipid treatments. The chemical analysis of total hydrocarbons in the control treatment (artificial seawater only) at different sampling days showed no oil biodegradation, while high levels of biodegradation were observed in the natural treatment at different sampling days. For example, the initial concentration (5000 mg of oil/L of artificial seawater) of weathered biodiesel, diesel, and light crude oil by bacteria was reduced to less than 3000 mg/L during the 28 days of the biodegradation process. The high level of biodegradation of diesel (27 ± 2.2%), biodiesel (28 ± 4%), and light crude oil (30 ± 1%), during the natural treatment and 28 ± 2.64% (diesel), 28 ± 3.3% (biodiesel), and 30 ± 1% (light crude oil) during the biosurfactant treatment occurred during the first 7 days of biodegradation period. The biodegradation continued in the following days in both natural and sophorolipid treatments and slowed down from day 14 to day 28, at which the highest biodegradation reached 43 ± 1% (biodiesel), 45 ± 6% (diesel) and 39 ± 5% (light crude oil) in the natural treatment and 45 ± 5% (biodiesel), 48 ± 4% (diesel) and 44 ± 1% (light crude oil) in the sophorolipid treatment, respectively. Although the statistical analysis

showed no significant difference in the biodegradability of weathered biodiesel, diesel and light crude oil in the presence and absence of sophorolipid biosurfactant (SL), the SL had no inhibitory effect on the tested oil biodegradation.

Analysis of microbial population on the plates with the sterilized biodiesel, diesel and light crude oil (control, only sterilized seawater) showed no microbial growth, while nearly 40000, 19000, 24000 CFU/mL were grown on the Bushnell-Hass plates cultured with the aqueous phase of the day-7 of biodegradation samples (control or natural biodegradation, contained the sterilized seawater and the unsterilized weathered biodiesel, diesel and light crude oil). Same trend was also observed at the sophorolipid treated samples. Nearly, 45000, 21000, 30000 CFU/mL of bacteria was grown on the Bushnell-Hass plates cultured with the aqueous phase of the day-7 of the sophorolipid (80 mg/L) treated biodegradation samples. The bacterial population significantly increased from day-0 to day 7 and continued in the following days in both natural and sophorolipid treatments and slowed down from day 14 to day 28.

The presence of oil-degrading bacteria in oil-contaminated marine environments was previously reported in the literature (Head et al., 2006; Yakimov et al., 2007). Previous studies showed the presence and role of the oil-degrading bacteria in the contaminated environments, where the oil spill occurred, instead of in the spilled oil. This study showed that the oil-degrading bacteria are also present in the spilled oil. The results of this study are in agreement with a previous study (Sheppard et al., 2012) that investigated the capability of the oil-degrading microorganisms present on the weathered crude oil on biodegradation of oil in seawater. Although different culture media (minimal salt media) were used for the microbial growth in their study, a significantly higher bacterial population was recovered from the plates cultured with the weathered crude oil (e.g., 41200 ± 511 CFU/mL) than the plates cultured with the bacterial communities isolated from the seawater (Gulf St. Vincent, SA, Australia) where the levels of 66 ± 3 CFU/mL were determined (Sheppard et al., 2012).

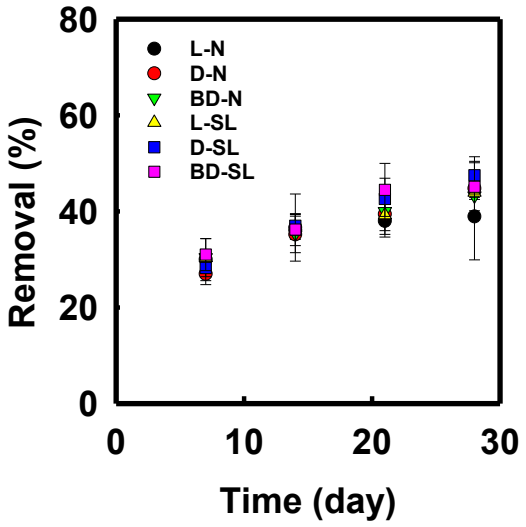

Figure 4.1. Biodegradation of weathered diesel (D), biodiesel (BD) and light crude (L) oil with and without (control or natural biodegradation treatment) sophorolipid (SL) biosurfactant during 28 days of incubation at 100 rpm and 22 ± 1°C.

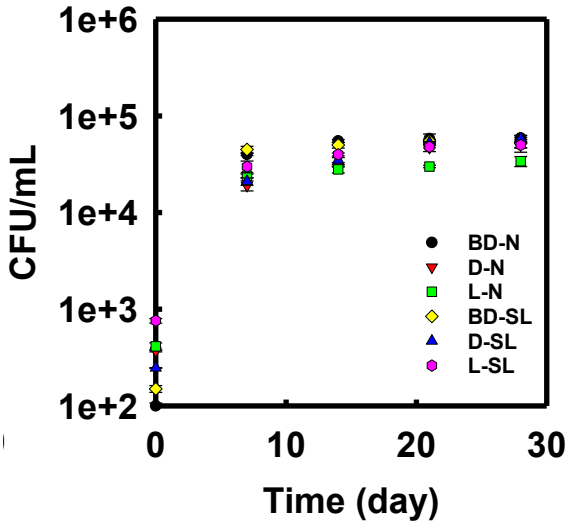

Figure 4.2. Bacterial populations recovered from the weathered biodiesel (BD), diesel (D) and light crude (L) oil with and without sophorolipid (SL) grown on the Bushnell-Hass plates.

The pyrosequencing technique was used to identify the dominant bacteria in the diesel, biodiesel, and light crude oil. Analysis of pyrosequencing results revealed four major phyla including *Firmicutes*, *Actinobacteria*, *Actinobacteria* and *Proteobacteria* in the tested oils. The *Firmicutes* was the dominant phylum in biodiesel (100%) and diesel (53%). The *Actinobacteria* was also dominant in the diesel (47%) oil and the *Proteobacteria* (97%) and *Actinobacteria* (3%) were the dominant phyla in the light crude oil. The majority of the isolated bacteria identified as orders of *Bacillales*, *Actinomycetales* and *Sphingomonadales*. For example, *Bacillales* was the dominant order isolated from the biodiesel oil. Two dominant orders including *Acinetobacter* and *Bacillales* were isolated from the diesel and *Sphingomonadales* was the dominant order in the light crude oil. Table 4.1 summarizes the characteristics of bacteria recovered from the biodiesel, diesel and light crude oil by the pyrosequencing technique. Assessment of the characteristics of the dominant bacteria isolated from the biodiesel, diesel and light crude oil revealed that they either have a high potential for biosurfactant production or are a known oil-degrading bacteria (Bødtker et al., 2009; Ganesh and Lin, 2009; Ron and Rosenberg, 2001). For example, the

biosurfactant production of *Firmicutes* phylum was reported in several studies (Bodour et al., 2003; Kumari et al., 2012; Menezes Bento et al., 2005; Płaza et al., 2008). Similarly, the *Paenibacillus* genus and its members were found to effectively degrade diesel through biosurfactant production (Banat et al., 2010; Ganesh and Lin, 2009).

Table 4.1. Classification of natural bacterial communities present in biodiesel, diesel and light crude oil by pyrosequencing.

Classifications	Biodiesel	Diesel		Light crude oil	
Phylum	*Firmicutes*	*Actinobacteria*	*Firmicutes*	*Proteobacteria*	*Actinobacteria*
Class	*Bacilli*	*Actinobacteria*	*Bacilli*	*Alphaproteobacteria*	*Actinobacteria*
Order	*Bacillales*	*Actinomycetales*	*Bacillales*	*Sphingomonadales*	*Actinomycetales*
Family	*Bacillaceae*	*Dietziaceae*	*Paenibacillaceae*	*Sphingomonadaceae*	*Mycobacteriaceae*
Genus	*Bacillus*	*Dietzia*	*Paenibacillus*	*Sphingomonas*	*Mycobacterium*
Dominancy (%)	100	47	53	97	3

4.3.2 Oil Uptake by Bacteria

The microbial adhesion to hydrocarbon test was used to verify the mechanisms of oil uptake by the natural bacteria in the weathered oils. Figure 4.3 shows the variation in the cell surface hydrophobicity of isolated bacteria under the natural (control) and sophorolipid treatments. In the natural (control) treatment the bacteria were incubated on the diesel, biodiesel and light crude oil. In the sophorolipid treatments, the bacteria were incubated on (i) only the sophorolipid biosurfactant and (ii) the combinations of sophorolipid and diesel, biodiesel and light crude oil.

The results of the cell surface hydrophobicity (CSH) in the control showed that the isolated bacteria recovered from the biodiesel and light crude oil were less hydrophobic (tendency to interact with the hydrophobic compounds) than the isolated bacteria recovered from the diesel. For example, the hydrophobicity values of 2 %, 4.5% and 16% were obtained following 1 h of incubation of bacterial cells on the light crude oil, biodiesel, and diesel, respectively.

The examination of biodiesel-isolated bacteria (e.g., *Bacillus*) incubated on the weathered biodiesel and diesel showed that the bacteria changed the cell surface hydrophobicity (CSH) from 4.5% to 16.6%, as they were exposed to the biodiesel and diesel. This test clearly showed the capability of the bacteria in cell surface modification as exposed to the different types of hydrocarbons. The results of this study are in agreement with the studies on the effect of hydrocarbons on the bacterial cells surface properties (Bouchez-Naïtali et al., 1999; Kaczorek, 2012; Prabhu and Phale, 2003). For example, Bouchez Naïtali et al. (1999) showed that cell surface hydrophobicities of 69% and 21%, respectively, were obtained as *Rhodococcus equi* was cultured on compounds with different properties (e.g., hexadecane, which is insoluble in water and glycerol, which is soluble in water).

The bacteria showed completely different surface properties when exposed to mixtures of hydrocarbons and the sophorolipid biosurfactant. The hydrophobicity of bacteria, which was already incubated on the weathered biodiesel, diesel and light crude oil, decreased as the mixtures of bacterial cells-hydrocarbons were exposed to an 80 mg/L of sophorolipid solution (Figure 4.3). The hydrophobicity of diesel bacteria changed from 16% to 20%. The hydrophobicity of mixtures of the bacterial cell-biodiesel system changed from 4.5% to 3% and the hydrophobicity of the bacterial cell-light crude oil system changed from 2% to 9%.

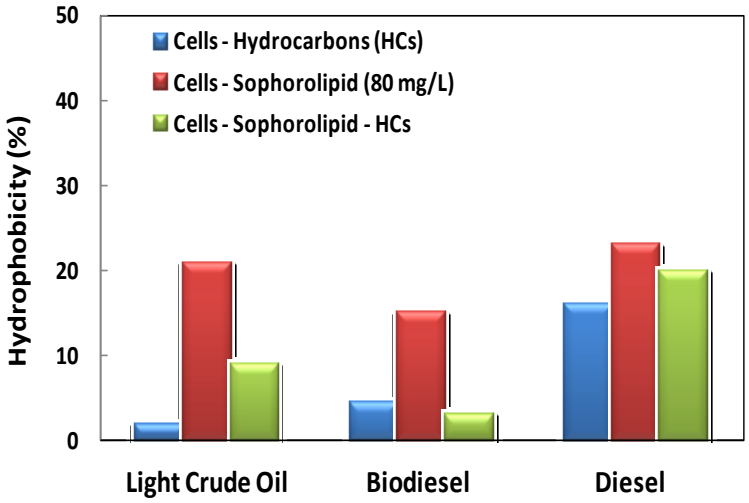

Figure 4.3. Bacterial cell modification following the exposure of bacterial communities in the weathered biodiesel, diesel and light crude oil to the diesel, biodiesel and light crude oil, the sophorolipid biosurfactant individually and in combination. The SD values are ≥ 0.5%.

The bacteria with the high cell surface hydrophobicity are able to directly interact with the hydrophobic compounds such as hydrocarbons, and the bacteria with the low cell surface hydrophobicity can interact with the hydrophilic compounds or adhere to the hydrocarbons that are encapsulated in the micelles (because the outer layer of micelle is hydrophilic) (Bouchez-Naïtali et al., 1999; Franzetti et al., 2010; Giaouris et al., 2009; Kaczorek et al., 2008; Kochkodan et al., 2008; Krasowska and Sigler, 2014; Van Hamme et al., 2003). The ability of microorganisms for cell surface modifications during incubation at different conditions (Kaczorek et al., 2010; Rosenberg and Kjelleberg, 1986; Zhang and Miller, 1994; Zhong et al., 2007) and the microbial cell modifications following the application of biosurfactants were previously reported (Al-Tahhan et al., 2000; Beal and Betts, 2000; Kaczorek, 2012; Kaczorek et al., 2012; Kaczorek et al., 2010; Noordman and Janssen, 2002; Zhang and Miller, 1995; Zhong et al., 2007). The modification of the cell surface hydrophobicity is one of the strategies that microorganisms use to avoid contact with toxic compounds (Bouchez-Naïtali et al., 1999; Krasowska and Sigler, 2014; Torres et al., 2011) or to uptake food (e.g., hydrocarbons) (Kaczorek et al., 2008; Krasowska and Sigler, 2014). For example, some bacteria release vesicles (which is an intercellular structure and its outer membrane is a lipid bilayer) from the outer membrane under unfavorable environmental conditions (Baumgarten et al., 2012), while others

release lipopolysaccharides (LPS) to change the cell surface hydrophobicity (Al-Tahhan et al., 2000).

Production of biosurfactant or emulsifying compounds is another way that bacteria change their cell surface structures. Beal and Betts (2000) showed that the biosurfactant production in the biosurfactant producing bacteria (e.g., PG201, a rhamnolipid-producing bacteria) reduced the level of cell surface hydrophobicity as exposed to phenanthrene. Moreover, a study on the phenanthrene biodegradation by *Pseudomonas* sp. Strain PP2 showed that the biosurfactant production by *Pseudomonas* during biodegradation and modifications of the cell surface hydrophobicity were the two adapted mechanisms by *Pseudomonas* for the phenanthrene uptake (Prabhu and Phale, 2003). Similarly, *Pseudomonas* strains consumed hydrocarbons by the production of rhamnolipid biosurfactants, which accelerated the solubility of hydrophobic substrates. Production of the rhamnolipids made the *Pseudomonas* cell surface less hydrophobic and thus cells were able to uptake the encapsulated hydrocarbons in the micelles through contact with the outer layer of micelles, which is hydrophilic (Franzetti et al., 2010; Van Hamme and Ward, 2001).

One way to increase the hydrocarbon uptake by the microorganism is to add surfactants to the hydrocarbon contaminated systems (e.g., in the case of an oil spill). Although, some studies showed the insignificant effect of the addition of surfactant (e.g., rhamnolipid) on the cell structures (Kaczorek, 2012), the changes in the cell surface hydrophobicity following the addition of biological surfactants was reported by investigators (Beal and Betts, 2000; Zhang and Miller, 1994). Increase in the biodegradation of hexadecane (Beal and Betts, 2000) and octadecane by *Pseudomonas* species (Zhang and Miller, 1994) have highlighted the positive effects of external addition of biosurfactants on the hydrocarbon biodegradation. Similarly, the cell surface properties of gram negative bacteria such as *P. fluorescens* SM, *A. hydrophila* SM, *P. alcaligenes* SM, *A. denitrificans* SM, *P. stutzeri* KS and *F. oryzihabitans* P1 significantly increased in the presence of rhamnolipid (Kaczorek, 2012). A study conducted by Al-Tahhan et al. (2000) showed that the increase in the cell surface hydrophobicity of *Pseudomonas* spp. in the presence of rhamnolipid was because the rhamnolipid biosurfactant changed the outer membrane of the bacteria so that the interaction between the rhamnolipid and the outer membrane of cells

resulted in the loss of fatty acid content due to the release of lipopolysaccharide (LPS) (Al-Tahhan et al., 2000).

Results of the hydrophobicity test showed that the bacterial communities in the diesel, biodiesel and light crude oil modified their cell surface structures based on the availability and the compositions of hydrocarbons. Moreover, the cell surface hydrophobicity was significantly influenced by the types of hydrocarbons and the presence of sophorolipid biosurfactant. For example, the initial less hydrophobic nature of bacteria in the cell-biodiesel system suggests that the bacteria should be able to directly contact the hydrophilic compounds of the biodiesel. On the other hand, the hydrophobic nature of the bacteria in the cell-diesel systems suggest that the direct contact with the hydrophobic compounds of diesel may be the primary way of hydrocarbon uptake by the bacteria.

In the system with the bacterial cells and only sophorolipid (80 mg/L), the exposed bacteria to the sophorolipid biosurfactant also changed the bacterial cell surface hydrophobicity. This behavior seems appropriate, because the hydrophobic structure of the sophorolipid biosurfactant may limited its availability to the bacteria. Therefore, the modification enabled the bacteria to interact directly with the hydrophobic sophorolipid biosurfactant. The decreases in the hydrophobicities of the cell-sophorolipid-diesel, biodiesel and light crude oil mixtures can be because the sophorolipid biosurfactant increased the bioavailability of hydrocarbons (HCs) through the HC encapsulation in the micelles and the bacteria directly contacted with the hydrophilic micelles.

Several reasons led to the high levels of weathered diesel, biodiesel and light crude oil biodegradation in the natural and sophorolipid treatments. The presence of natural oil-degrading bacteria in the weathered biodiesel, diesel and light crude oil (Table 4.1) was the main reason for the oil degradation. The ability of bacteria in cell surface modifications enable the bacteria to uptake the hydrophobic compounds (Figure 4.3). Moreover, the presence of readily consumable hydrocarbons slightly influenced the biodegradation. However, the biodegradation due to the contact with the readily consumable hydrocarbons seems to be limited. This is suggested based on the insignificant natural solubility of biodiesel (BD), light crude oil (L) and diesel (D) in the artificial seawater (e.g., less than 5% for BD and less than 3% for D and L). The ability of

bacteria in the production of HC degrading enzymes and biosurfactant compounds was previously reported by investigators (Banat et al., 2010; Bodour et al., 2003; Bouchez-Naïtali et al., 1999; Das and Chandran, 2010; Ganesh and Lin, 2009; Kumari et al., 2012; Menezes Bento et al., 2005; Płaza et al., 2008). Given the type of isolated bacteria in the biodiesel, diesel and light crude oil, production of biosurfactant by such bacteria during the biodegradation process seemed possible (Table 4.1). However, the surface tension measurements of the culture media (supernatant) of the bacteria recovered from the biodiesel, diesel and light crude oil showed an insignificant decrease in the surface tension during the bioremediation process. The surface tension of supernatant reduced slightly. The surface tension measurements results did not support the significant biosurfactant production by the indigenous bacteria in this study.

Investigations showed that chemical dispersants (e.g., Corexit 9500) and even biological dispersants (e.g., rhamnolipid) did not always lead to enhanced oil mineralization and biodegradation (Bruheim et al., 1997; Lindstrom and Braddock, 2002). However, studies conducted by Koch et al. (1991) suggested that the exposures of non-producing bacteria (e.g., a mutant strain of PG201) to pure rhamnolipid increased the uptakes of the hydrophobic compounds. The biodegradation of diesel, biodiesel and light crude oil in the samples treated with sophorolipid biosurfactant suggested the high availability of hydrocarbons to the bacteria as the results of increase in the solubilization and dispersion of hydrocarbons by sophorolipid biosurfactant and the hydrocarbon encapsulation in the micelles (Figure 4.1), the ability of bacteria in the cell surface modifications based on the available HCs (Figure 4.3) and direct contact with the water-soluble hydrocarbons (e.g., biodiesel), respectively.

4.4 Conclusions

Several studies have assessed the role of oil-degrading bacteria isolated from seawater in the biodegradation of petroleum hydrocarbons (Das and Chandran, 2010; Harayama et al., 2004; Sheppard et al., 2012). Although those studies provided valuable information on the influence of such bacteria in the oil biodegradation in aqueous environments, they did not consider the importance of the microbial communities in the spilled oils in the oil biodegradation (Das and Chandran, 2010; Harayama et al., 2004; Sheppard et al., 2012). This study determined the presence of natural oil-degrading bacteria in the weathered diesel, biodiesel and light crude oil

and the role of the bacteria in oil biodegradation in the presence and absence of the sophorolipid biosurfactant.

The findings from this study revealed the significant biodegradation of tested oils especially light crude oil in both natural and sophorolipid treatments. The biodegradation results and bacterial growth on the Bushnell-Hass media confirmed the presence of active oil-degrading bacteria in the tested oils. A comparison of the results obtained from the biodegradation study and the cell surface hydrophobicity tests suggested that the biodiesel, diesel, and light crude oil biodegradation (in the natural treatment) by the bacteria was because the bacteria were able to uptake the oil droplets or the hydrophilic compounds by the direct interactions as the result of the cell surface modifications. The mentioned mechanism for the natural (control) treatment could have also simultaneously occurred in the treatment with the sophorolipid biosurfactant. However, the hydrocarbon uptake by the bacteria in the sophorolipid treated samples can also be due to the encapsulation of biodiesel, diesel, and light crude oil in the sophorolipid micellar aggregates. This study confirmed that the indigenous oil-degrading bacteria in the weathered diesel, biodiesel and light crude oil had an important role in the oil biodegradation in the seawater and also the positive effect of the external addition of the sophorolipid biosurfactant on the biodegradation.

Chapter 5: Summary and Conclusions

5.1. SUMMARY

This study addressed three objectives. The first objective was to study the applicability of a microbially produced surfactant (sophorolipid) for dispersion of spilled oil in seawater under the environmental conditions affecting oil dispersion and sophorolipid biosurfactant effectiveness. The second objective was to determine the involvement of indigenous bacteria in the weathered diesel, biodiesel and light crude oil in the aquatic oil biodegradation. The third objective was to determine the effect of sophorolipid biosurfactant on oil biodegradation by indigenous bacteria.

Two experimental phases were designed for this study. In the first part of the project, the sophorolipid biosurfactant was used as a dispersant to determine its effectiveness on the weathered biodiesel, diesel, and light crude oil dispersion according to the swirling flask dispersion effectiveness test with modifications (USEPA, 2011). This method was modified to simulate an actual oil spill situation. For example, the oil was artificially weathered (72 h under a fume hood) and the tested oils and sophorolipid biosurfactant were not initially premixed. The experiments were done in two steps. In the first step, the effectiveness of sophorolipid biosurfactant on the dispersion of diesel, biodiesel, and light crude oil was determined. As the effectiveness of sophorolipid for the diesel, biodiesel and light crude oil dispersion was confirmed, the sophorolipid effectiveness was evaluated under different salinities, temperatures, and pHs. Knowing the effect of the environmental factors on the oil dispersion, further experiments were conducted to determine the influencing mechanisms on the oil dispersion. In the second part of the project, biodegradation experiment was conducted to determine the involvement of indigenous bacteria in the weathered diesel, biodiesel and light crude oil in the aquatic oil biodegradation. The effect of sophorolipid biosurfactant on the oil degradation by such bacteria was also studied.

The results of the applicability of sophorolipid biosurfactant for dispersion of weathered biodiesel, diesel and light crude oil as the functions of sophorolipid concentration and quantity, mixing or settling time, salinity, temperature, and pH are summarized as follows.

Objective I: Sophorolipid Biosurfactant Dispersion Effectiveness

In general, in order to bring an insoluble compound (e.g., oil or hydrocarbons) into the water (through dispersion process) the detergent-like products (e.g., dispersants) should be added to the system. Figure 5.1 shows the schematic interaction of sophorolipid biosurfactant with the water and oil molecules in seawater.

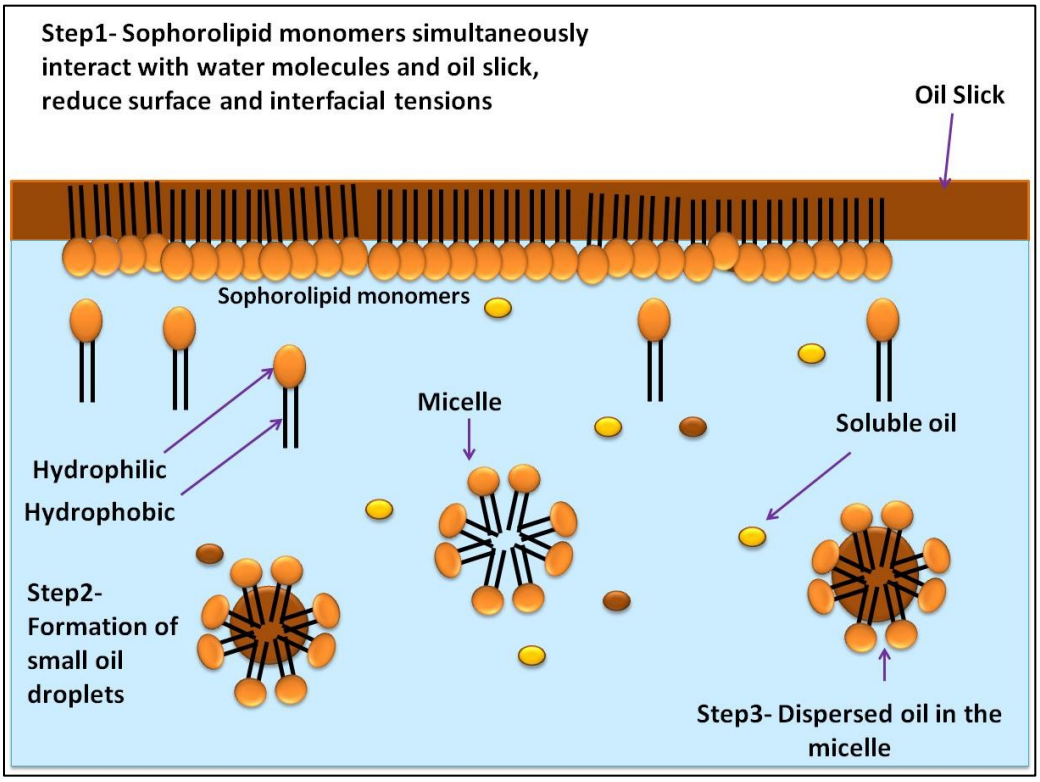

Figure 5.1. Dispersion of oil droplets in seawater as the result of sophorolipid biosurfactant interactions with water and oil molecules. The yellow color represents the water soluble compounds of oil that naturally dissolve/disperse in the water. The brown color shows the oil droplets that form as long as the mixing is provided.

The high surface activity of the sophorolipid used in this study was determined by surface tension analysis. The surface tension of artificial seawater was significantly reduced from nearly 70.25 mN/m (data from two experiments were combined) to 34 mN/m with sophorolipid biosurfactant. Determination of the total petroleum hydrocarbons (TPHs) concentration in the systems treated with various concentrations of sophorolipid solutions showed that the dispersion of biodiesel, diesel, and light crude oil increased as the sophorolipid concentrations and

quantities, mixing, salinity and temperature increased. However, the pH had an insignificant effect on the oil dispersion by sophorolipid. Moreover, the extent of dispersion varied with the oil type and composition.

No oil dispersion was formed at the minimum level of agitation (0 rpm), regardless of the sophorolipid concentrations, and the dispersion occurred only when the mixing increased to maximum (e.g., 150 rpm) (Figure A.3). The stability of dispersed oil was also influenced by the settling time. Oil dispersion was considerably reduced as the settling times (e.g., 10 min) were applied following the shaking (e.g., 20 min, 150 rpm) mainly due to the resurfacing process (Figure A.2). Moreover, the dispersion of oils increased as the salinity increased from 0 ppt to 10 ppt. However, the oil dispersion was not significantly different at the salinities of 10 to 30 ppt. Results showed that the dispersion of oils increased as the temperature increased from 8ºC to 35°C (Figure A.5), with the exception of biodiesel, which showed reduced dispersion at 35ºC.

One of the reasons that the oil dispersion increased at the higher concentration and quantity of sophorolipid seems to be the oil solubilization in the micellar aggregates (Figure 5.2). Moreover, higher quantities of oil droplets were formed when the concentrations of sophorolipid increased above the CMC. The effectiveness of sophorolipid at higher salinities seems to be due to the effect of “salting out” which seems that influenced the CMC of sophorolipid at high NaCl salt concentration. In addition, the low effectiveness of sophorolipid in cold temperature (8ºC) can be due to the changes in the viscosity and density of diesel, biodiesel and light crude oil.

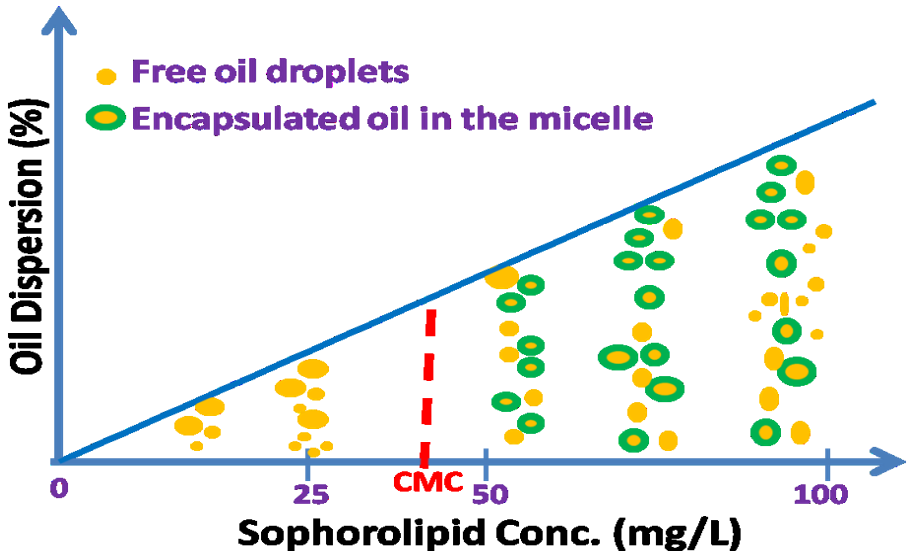

Figure 5.2. Oil droplet formation and encapsulation in the micelles (aggregates of the sophorolipid monomers above a certain concentration).

Objective II: Biodegradation by the Indigenous Bacteria in the Weathered Diesel, Biodiesel, and Light Crude Oil

The characterisation the indigenous bacteria in the weathered diesel, biodiesel, and light crude oil and the determination of the bacterial contribution in the oil biodegradation with and without sophorolipid biosurfactant were another main objective of this research.

The simultaneous removal of weathered biodiesel, diesel, and light crude oil and growth of the bacteria during the biodegradation suggested the presence and contribution of bacteria capable of consuming the weathered biodiesel, diesel, and light crude oil. For example, determination of total petroleum hydrocarbons concentrations at the days of 0 to 28 of biodegradation period showed that the concentration of weathered biodiesel, diesel, and light crude oil reduced nearly 46% and 42%, as the result of biodegradation with and without the sophorolipid biosurfactant. Subsequently, the bacterial population in the biodiesel, diesel, and light crude oil on Bushnell-Hass plates increased from 150, 247, and 760 CFU/ml at day 0 to 52000, 59000, and 5000 CFU/ml, respectively, at day 28. The bacterial communities in the diesel, biodiesel, and light crude oil were verified by the pyrosequencing technique. The phyla including *Firmicutes*, *Actinobacteria*, *Actinobacteria* and *Proteobacteria* in the tested oils were identified. Nearly

100% and 53% of the dominant phylum in the biodiesel and diesel found to be the *Firmicutes*. In addition to the *Firmicutes* phylum, the *Actinobacteria* phylum was also dominant (47%) in the diesel. Nearly 97% and 3% of the bacterial in the light crude oil belonged to the *Proteobacteria* and *Actinobacteria* phyla. Further literature assessment of the dominant bacteria showed that the dominant bacteria are known for their hydrocarbon consuming properties (Bødtker et al., 2009; Ganesh and Lin, 2009; Ron and Rosenberg, 2001). Moreover, the bacteria are known to degrade hydrocarbons through biosurfactant production (Bødtker et al., 2009; Ganesh and Lin, 2009; Ron and Rosenberg, 2001). For example, the biodegradation of diesel was found to be due to the biosurfactant production by *Paenibacillus* genus (Banat et al., 2010; Ganesh and Lin, 2009).

Apart from the influence of environmental conditions, three main reasons were proposed for the variations in the hydrocarbon biodegradation. These include (i) the chemical structure of oils or hydrocarbons (Pitter and Chudoba, 1990; Von Wedel, 1999), (ii) the existence of oil-degrading enzymes such as Acetyl-CoA in the oil-degrading microorganisms (which reduce the lag phase or adaptation time) (Das and Chandran, 2010), and (iii) the presence or absence of toxic or refractory compounds in the oils (Pitter and Chudoba, 1990; Zhang et al., 1998; Zubay, 1983). For example, the high rate of biodegradation of biodiesels is usually attributed to their less complex chemical structures (DeMello et al., 2007; Von Wedel, 1999). Biodiesels are composed of fatty acids ("straight carbon chain with two oxygen"(Von Wedel, 1999)) and also have less toxic or refractory compounds (e.g., benzene, toluene, xylene) in their structures (Von Wedel, 1999). According to Zhang et al., the fatty acids are " biologically active" (due to the presence of oxygen in the biodiesels structures) (Zhang et al., 1998). This may also be due to the fact that the bacteria already have enzymes (e.g., "Acetyl-CoA dehydrogenase") that target the fatty acids (Pitter and Chudoba, 1990; Zhang et al., 1998; Zubay, 1983). On the other hand, the chemical structure of diesel is different than the biodiesels. For example, there are "double bonds and branched and or cyclic compounds" in the diesel (Von Wedel, 1999)". This reduces the uptake ("metabolization") of diesel by the bacteria (Von Wedel, 1999). Moreover, crude oils contain a large amount of unsaturated compounds such as resins, asphaltenes which their degradations by bacteria become difficult (Pitter and Chudoba, 1990; Zhang et al., 1998; Zubay, 1983). They also have toxic compounds such as alkylbenzenes and refractory compounds such as benzenes in their structures. For example, aquatic toxicity studies of plant driven biodiesels ("vegetable

methyl esters") and petroleum products and crude oil showed that the biodiesels had less toxic effect (e.g., lethal concentration (LC50) of 578 ppm) on the tested plants and animals than those of petroleum products and crude oil (e.g., LC50 of 27 ppm) (Von Wedel, 1999). Moreover, as diesel has low biological activities, the oil-degrading microorganisms have to either produce an enzyme or change the existing enzymes to be able to uptake the diesel (Pitter and Chudoba, 1990; Zhang et al., 1998; Zubay, 1983).

The similar rate of the diesel, biodiesel and light crude oil biodegradation can be explained by the fact that the dominant bacteria in the biodegradation samples already produced the required enzymes for the breakdown of the oils (since the recovered bacteria used in this study were already lived in the oils). Therefore, they have spent a minimal time to adapt to the new conditions (e.g., from the oil to the artificial seawater). A study conducted by Siddiqui and Adams (2002) showed a lag phase of only 2 days before the biodegradation of diesel be initiated in the soil samples with the microbial communities that were already exposed to diesel, while it took 6 days for the bacteria to initiate the diesel biodegradation in the sample that previously was not contaminated with the diesel (Siddiqui and Adams, 2002).

Some of the isolated bacteria in the tested oil were reported to produce biosurfactant, thus, it was assumed that the biodegradation of biodiesel, diesel, and light crude oil bacteria may have been through biosurfactant production. However, the measurements of the surface tension of biodegradation samples during the biodegradation period did not support the production of biosurfactant compounds by the bacteria in this study (Javan Roshtkhari, 2014). Therefore, the biosurfactant production by the bacteria was not considered as the adaptive method for oil uptake.

The removal of diesel, biodiesel and light crude oil during the biodegradation experiment seems to be due to three reasons including (i) the presence of bacteria that were able to degrade hydrocarbons in the tested diesel, biodiesel and light crude oil (ii) the ability of such bacteria to change their cell surface hydrophobicity based on the type of hydrocarbons and (iii) the presence of more accessible compounds in the tested oils. The presence of the oil-degrading bacteria in this research was confirmed by (i) the degradation of biodiesel, diesel, and light crude oil during the biodegradation experiment (ii) the growth of bacteria on the Bushnell-Hass media, and (iii)

the microbial verifications by the pyrosequencing technique. The cell surface hydrophobicity tests also showed that the bacteria altered their cell surface structures based on the availability of hydrocarbons. Therefore, it seems that one of the ways that bacteria used to uptake the hydrocarbons was to contact with the available hydrocarbons through modification in the cell surface hydrophobicity (Figure 5.3). Moreover, the degradations of hydrocarbons by bacteria can be due to the presence of less complex (e.g., fatty acid methyl ester, saturated HCs) (Von Wedel, 1999).

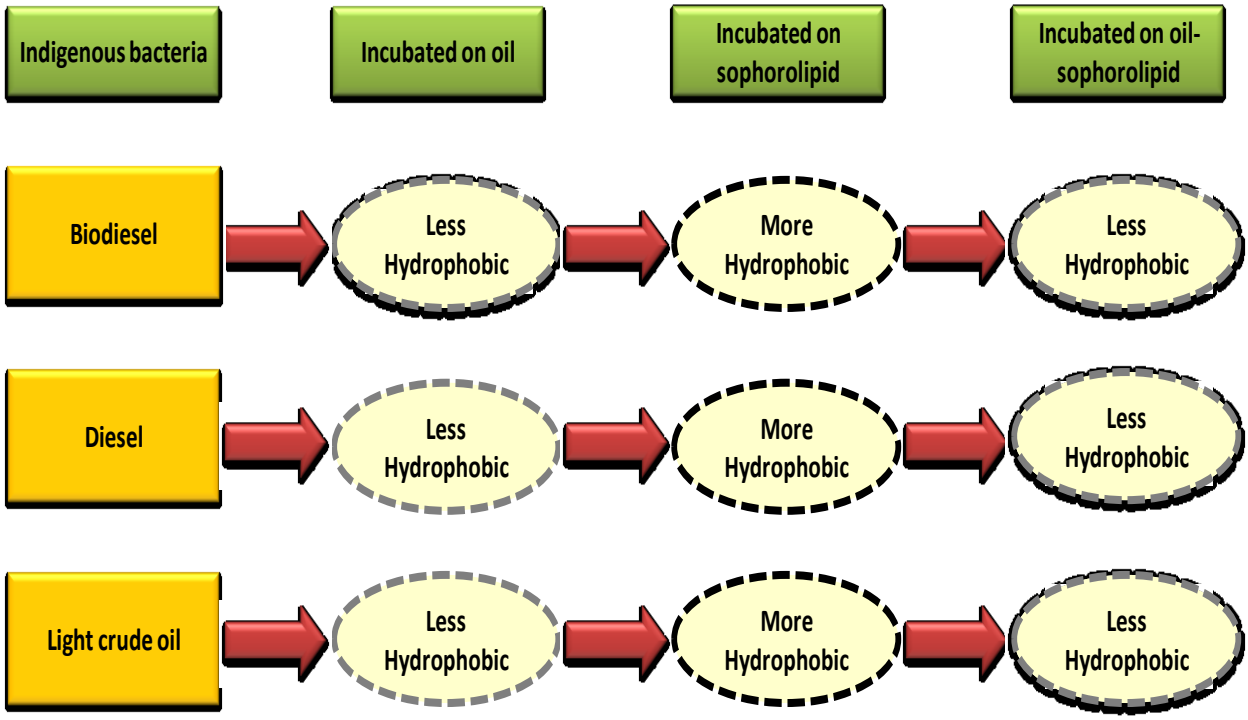

Figure 5.3. Variations in the bacterial cell surface structures as grown on the oil, sophorolipid and oil-sophorolipid during the biodegradation period. Colors represent the state of cell surface modifications (gray: slightly hydrophobic; black: more hydrophobic).

Objective III: Effect of Sophorolipid Biosurfactant on Biodegradation

The removal of diesel, biodiesel, and light crude oil during the biodegradation period especially light crude oil in the sophorolipid treatment implies the existence of bacteria that were able to uptake the hydrocarbons in the tested oils. Mechanisms of diesel, biodiesel and light crude oil degradation in the sophorolipid treatment were also examined by the cell surface hydrophobicity experiment. The results of hydrophobicity tests revealed that the bacteria showed a hydrophobic

effect when exposed to the sophorolipid biosurfactant (Figure 5.3). However, in the presence of both oil and sophorolipid, the cell surface hydrophobicities were slightly lower. This means that the hydrocarbons removal in the sophorolipid treated samples during the biodegradation period can be due in part to increase in the "micellar solubilization" by sophorolipid biosurfactant. However, the mechanisms such as direct contact with the oil and uptakes of the hydrophilic (water-like) parts of diesel, biodiesel and light crude oil by the indigenous bacteria can also influence the oil biodegradation. This research showed that despite the high oil dispersion by the sophorolipid (SL) in the dispersion experiment, the SL only slightly increased the availability of hydrocarbons to the bacteria during the biodegradation process.

5.2. Future Work and Recommendations

Based on the results obtained from the present research the following recommendations need to be considered for further research.

- Optimization of the sophorolipid performance through application of mixtures of sophorolipid and biosurfactants such as rhamnolipid and surfactin for improving the weathered oil dispersion in the seawater.
- Analysis of the sophorolipid dispersion effectiveness through a pilot study to provide reliable baseline information for application of sophorolipid biosurfactant in actual oil spill dispersion.
- Determination of the effect of sophorolipid on oil biodegradation by non-biosurfactant producing bacteria and the influential mechanisms (e.g., stimulation of biosurfactant production).

REFERENCES

Abouseoud, M., Maachi, R., Amrane, A., Boudergua, S., Nabi, A. (2008), Evaluation of different carbon and nitrogen sources in production of biosurfactant by *Pseudomonas fluorescens*. Desalination, 223 (1–3), 143-151.

Al-Besharah, J.M., Akashah, S.A., Mumford, C.J. (1989), The effect of temperature and pressure on the viscosities of crude oils and their mixtures. Industrial & Engineering Chemistry Research, 28 (2), 213-221.

Al-Tahhan, R.A., Sandrin, T.R., Bodour, A.A., Maier, R.M. (2000), Rhamnolipid-Induced Removal of Lipopolysaccharide from *Pseudomonas aeruginosa*: Effect on Cell Surface Properties and Interaction with Hydrophobic Substrates. Applied and Environmental Microbiology, 66 (8), 3262-3268.

Albers, P.H. (1979), Effects of Corexit 9527 on the hatchability of mallard eggs. Bulletin of Environmental Contamination and Toxicology, 23 (1), 661-668.

Albino, J.D., Nambi, I.M. (2009), Effect of biosurfactants on the aqueous solubility of PCE and TCE. Journal of Environmental Science and Health, Part A, 44 (14), 1565-1573.

Allen, C.A., Watts, K.C., Ackman, R.G. (1999), Predicting the surface tension of biodiesel fuels from their fatty acid composition. Journal of the American Oil Chemists' Society, 76 (3), 317-323.

Anton, N., Saulnier, P., Béduneau, A., Benoit, J.-P. (2007), Salting-Out Effect Induced by Temperature Cycling on a Water/Nonionic Surfactant/Oil System. The Journal of Physical Chemistry B, 111 (14), 3651-3657.

Ashby, R.D., Solaiman, D.K.Y., Foglia, T.A. (2008), Property control of sophorolipids: Influence of fatty acid substrate and blending. Biotechnology Letters, 30 (6), 1093-1100.

Attwood, D., Florence, A.T., (2008a), Chapter 4: Surfactants, Physical Pharmacy. Pharmaceutical Press, pp. 43-62.

Attwood, D., Florence, A.T., (2008b), Chapter 5: Emulsions, suspensions and other dispersed systems, Physical Pharmacy. Pharmaceutical Press, pp. 63-81.

Aworanti, O., Agarry, S., Ajani, A. (2012), A Laboratory Study of the Effect of Temperature on Densities and Viscosities of Binary and Ternary Blends of Soybean Oil, Soy Biodiesel and Petroleum Diesel Oil. Advances in Chemical Engineering and Science, 2 (4), 444-452.

Baca, B., Ward, G.A., Lane, C.H., Schuler, P.A. (2005). Net environmental benefit analysis (NEBA) of dispersed oil on nearshore tropical ecosystems derived from the 20 year "Tropics" field study 1. Paper presented at the International Oil Spill Conference. Retrieved from http://ioscproceedings.org/doi/abs/10.7901/2169-3358-2005-1-453

Baccile, N., Babonneau, F., Jestin, J., Pehau-Arnaudet, G., Van Bogaert, I. (2012), Unusual, pH-induced, self-assembly of sophorolipid biosurfactants. ACS Nano, 6 (6), 4763-4776.

Balmbra, R., Clunie, J., Corkill, J., Goodman, J. (1962), Effect of temperature on the micelle size of a homogeneous non-ionic detergent. Transactions of the Faraday Society, 58, 1661-1667.

Banat, I.M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, M.G., Fracchia, L., Smyth, T.J., Marchant, R. (2010), Microbial biosurfactants production, applications and future potential. Applied Microbiology and Biotechnology, 87 (2), 427-444.

Baumgarten, T., Sperling, S., Seifert, J., von Bergen, M., Steiniger, F., Wick, L.Y., Heipieper, H.J. (2012), Membrane vesicle formation as a multiple-stress response mechanism enhances *Pseudomonas putida* DOT-T1E cell surface hydrophobicity and biofilm formation. Applied and Environmental Microbiology, 78 (17), 6217-6224.

Beal, R., Betts, W. (2000), Role of rhamnolipid biosurfactants in the uptake and mineralization of hexadecane in *Pseudomonas aeruginosa*. Journal of Applied Microbiology, 89 (1), 158-168.

Belk, J.L., Elliott, D.J., Flaherty, L.M. (1989). The comparative effectiveness of dispersants in fresh and low salinity waters. Paper presented at the International Oil Spill Conference. Retrieved from http://dx.doi.org/10.7901/2169-3358-1989-1-333

Belore, R.C., Trudel, K., Mullin, J.V., Guarino, A. (2009), Large-scale cold water dispersant effectiveness experiments with Alaskan crude oils and Corexit 9500 and 9527 dispersants. Marine Pollution Bulletin, 58 (1), 118-128.

Benjumea, P., Agudelo, J., Agudelo, A. (2008), Basic properties of palm oil biodiesel–diesel blends. Fuel, 87 (10–11), 2069-2075.

Biswal, N.R., Paria, S. (2010), Effect of Electrolyte Solutions on the Adsorption of Surfactants at PTFE−Water Interface. Industrial & Engineering Chemistry Research, 49 (15), 7060-7067.

Blondina, G., Singer, M., Lee, I., Ouano, M., Hodgins, M., Tjeerdema, R., Sowby, M. (1999), Influence of salinity on petroleum accommodation by dispersants. Spill Science & Technology Bulletin, 5 (2), 127-134.

Blondina, G.J., Sowby, M.L., Ouano, M.T., Singer, M.M., Tjeerdema, R.S. (1997), A modified swirling flask efficacy test for oil spill dispersants. Spill Science and Technology Bulletin, 4 (3), 177-185.

Bodour, A.A., Drees, K.P., Maier, R.M. (2003), Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid southwestern soils. Applied and Environmental Microbiology, 69 (6), 3280-3287.

Bødtker, G., Hvidsten, I.V., Barth, T., Torsvik, T. (2009), Hydrocarbon degradation by *Dietzia* sp. A14101 isolated from an oil reservoir model column. Antonie Van Leeuwenhoek, 96 (4), 459-469.

Bouchez-Naïtali, M., Rakatozafy, H., Marchal, R., Leveau, J., Vandecasteele, J. (1999), Diversity of bacterial strains degrading hexadecane in relation to the mode of substrate uptake. Journal of Applied Microbiology, 86 (3), 421-428.

Bouchez-Naïtali, M., Vandecasteele, J.-P. (2008), Biosurfactants, an help in the biodegradation of hexadecane? The case of *Rhodococcus* and *Pseudomonas* strains. World Journal of Microbiology and Biotechnology, 24 (9), 1901-1907.

Brakstad, O., Lødeng, A. (2005), Microbial diversity during biodegradation of crude oil in seawater from the North Sea. Microbial Ecology, 49 (1), 94-103.

Bruheim, P., Bredholt, H., Eimhjellen, K. (1997), Bacterial degradation of emulsified crude oil and the effect of various surfactants. Canadian Journal of Microbiology, 43 (1), 17-22.

Campo, P., Venosa, A.D., Suidan, M.T. (2013), Biodegradability of Corexit 9500 and dispersed South Louisiana crude oil at 5 and 25^0C. Environmental Science & Technology, 47 (4), 1960-1967.

Canevari, G.P. (1985). The effect of crude oil composition on dispersant performance. Paper presented at the International Oil Spill Conference. Retrieved from http://dx.doi.org/10.7901/2169-3358-1985-1-441

Chandran, P., Das, N. (2012), Role of Sophorolipid Biosurfactant in Degradation of Diesel Oil by *Candida tropicalis*. Bioremediation Journal, 16 (1), 19-30.

Chandrasekar, S., Sorial, G.A., Weaver, J.W. (2006), Dispersant effectiveness on oil spills-impact of salinity. ICES Journal of Marine Science: Journal du Conseil, 63 (8), 1418-1430.

Chapman, H., Purnell, K., Law, R.J., Kirby, M.F. (2007), The use of chemical dispersants to combat oil spills at sea: A review of practice and research needs in Europe. Marine Pollution Bulletin, 54 (7), 827-838.

Chen, Q., Bao, M., Fan, X., Liang, S., Sun, P. (2013), Rhamnolipids enhance marine oil spill bioremediation in laboratory system. Marine Pollution Bulletin, 71 (1-2), 269-275.

Claesson, M.J., O'Sullivan, O., Wang, Q., Nikkilä, J., Marchesi, J.R., Smidt, H., de Vos, W.M., Ross, R.P., O'Toole, P.W. (2009), Comparative analysis of pyrosequencing and a phylogenetic microarray for exploring microbial community structures in the human distal intestine. PLOS ONE, 4 (8), e6669.

Clayton, J.R., Payne, J.R., Farlow, J.S., (1993a), Section 1: Introduction, Oil Spill Dispersants: Mechanisms of Action and Laboratory Tests. CRC Press, pp. 1-4.

Clayton, J.R., Payne, J.R., Farlow, J.S., (1993b), Section 3: Factors Affecting Chemical Dispersion of Oil and Its Measurement, Oil Spill Dispersants: Mechanisms of Action and Laboratory Tests. CRC Press, pp. 14-53.

Clifford, J.S., Ioannidis, M.A., Legge, R.L. (2007), Enhanced aqueous solubilization of tetrachloroethylene by a rhamnolipid biosurfactant. Journal of Colloid and Interface Science, 305 (2), 361-365.

Colcomb, K., Salt, D., Peddar, M., Lewis, A. (2005). Determination of the limiting oil viscosity for chemical dispersion at sea. Paper presented at the International Oil Spill Conference. Retrieved from http://ioscproceedings.org/doi/abs/10.7901/2169-3358-2005-1-53

Cole, J.R., Wang, Q., Cardenas, E., Fish, J., Chai, B., Farris, R.J., Kulam-Syed-Mohideen, A., McGarrell, D.M., Marsh, T., Garrity, G.M. (2009), The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Research, 37 (suppl 1), D141-D145.

Dagnew, M. (2004). Rhamnolipid assisted dispersion and biodegradation of crude oil spilled on water. (Master Thesis), Concordia University, Montreal, Canada. Retrieved from http://spectrum.library.concordia.ca/8348/

Daniel, H.-J., Reuss, M., Syldatk, C. (1998), Production of sophorolipids in high concentration from deproteinized whey and rapeseed oil in a two stage fed batch process using *Candida bombicola* ATCC 22214 and *Cryptococcus curvatus* ATCC 20509. Biotechnology Letters, 20 (12), 1153-1156.

Das, N., Chandran, P. (2010), Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology Research International, 2011, 13.

Daverey, A., Pakshirajan, K. (2009), Production, characterization, and properties of sophorolipids from the yeast *Candida bombicola* using a low-cost fermentative medium. Applied Biochemistry and Biotechnology, 158 (3), 663-674.

Daverey, A., Pakshirajan, K. (2010), Sophorolipids from *Candida bombicola* using mixed hydrophilic substrates: Production, purification and characterization. Colloids and Surfaces B: Biointerfaces, 79 (1), 246-253.

Daverey, A., Pakshirajan, K. (2011), Pretreatment of synthetic dairy wastewater using the sophorolipid-producing yeast *Candida bombicola*. Applied Biochemistry and Biotechnology, 163 (6), 720-728.

Delvigne, G.A. (1985). Experiments on natural and chemical dispersion of oil in laboratory and field circumstances. Paper presented at the International Oil Spill Conference. Retrieved from http://ioscproceedings.org/doi/abs/10.7901/2169-3358-1985-1-507

DeMello, J.A., Carmichael, C.A., Peacock, E.E., Nelson, R.K., Arey, J.S., Reddy, C.M. (2007), Biodegradation and environmental behavior of biodiesel mixtures in the sea: An initial study. Marine Pollution Bulletin, 54 (7), 894-904.

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

Develter, D.W.G., Lauryssen, L.M.L. (2010), Properties and industrial applications of sophorolipids. European Journal of Lipid Science and Technology, 112 (6), 628-638.

Dutkiewicz, E., Jakubowska, A. (2002), Effect of electrolytes on the physicochemical behaviour of sodium dodecyl sulphate micelles. Colloid and Polymer Science, 280 (11), 1009-1014.

Effects, C.U.O.S.D.E., Board, O.S., Studies, D.E.L., Council, N.R. (2005). Oil Spill Dispersants:: Efficacy and Effects, National Academies Press.

Epstein, N., Bak, R., Rinkevich, B. (2000), Toxicity of third generation dispersants and dispersed Egyptian crude oil on Red Sea coral larvae. Marine Pollution Bulletin, 40 (6), 497-503.

Eswari, J.S., Anand, M., Venkateswarlu, C. (2013), Optimum culture medium composition for rhamnolipid production by *pseudomonas aeruginosa* AT10 using a novel multi-objective optimization method. Journal of Chemical Technology and Biotechnology, 88 (2), 271-279.

Fingas, M., (2011a), Chapter 1: Introduction, in: Fingas, M. (Ed.), Oil Spill Science and Technology. Gulf Professional Publishing, USA, pp. 3-5.

Fingas, M., (2011b), Chapter 3: Introduction to Oil Chemistry and Properties, in: Fingas, M. (Ed.), Oil Spill Science and Technology. Gulf Professional Publishing, USA, pp. 51-59.

Fingas, M., (2011c), Chapter 8: Introduction to Spill Modeling, in: Fingas, M. (Ed.), Oil Spill Science and Technology. Gulf Professional Publishing, USA, pp. 187-200.

Fingas, M., (2011d), Chapter 15: Oil Spill Dispersants: A Technical Summary, in: Fingas, M. (Ed.), Oil Spill Science and Technology. Gulf Professional Publishing, USA, pp. 435-582.

Fingas, M., (2011e), Chapter 16: A Practical Guide to Chemical Dispersion for Oil Spills, in: Fingas, M. (Ed.), Oil Spill Science and Technology. Gulf Professional Publishing, USA, pp. 583-610.

Fingas, M., Bier, I., Bobra, M., Callaghan, S. (1991). Studies on the physical and chemical behavior of oil and dispersant mixtures. Paper presented at the International Oil Spill Conference. Retrieved from http://www.ioscproceedings.org/doi/abs/10.7901/2169-3358-1991-1-419

Fingas, M., Huang, E., Fieldhouse, B., Wang, L., Mullin, J.V. (1996), The effect of energy, settling time and shaking time on the swirling flask dispersant apparatus. Spill Science & Technology Bulletin, 3 (4), 193-194.

Fingas, M.F., Kyle, D., Tennyson, E., (1995), Effectiveness: Studies into the Causes of Effectiveness Variations, in: Lane, P. (Ed.), The Use of Chemicals in Oil Spill Response. ASTM International, Philadelphia, p. 92.

Fingas, M.F., Stoodley, R., Laroche, N. (1990), Effectiveness testing of spill-treating agents. Oil and Chemical Pollution, 7 (4), 337-348.

Franzetti, A., Tamburini, E., Banat, I.M., (2010), Chapter 9: Applications of Biological Surface Active Compounds in Remediation Technologies, in: Sen, R. (Ed.), Biosurfactants. Springer New York, pp. 121-134.

Ganesh, A., Lin, J. (2009), Diesel degradation and biosurfactant production by Gram-positive isolates. African Journal of Biotechnology, 8 (21), 5847-5854.

Ghojavand, H., Vahabzadeh, F., Roayaei, E., Shahraki, A.K. (2008), Production and properties of a biosurfactant obtained from a member of the *Bacillus subtilis* group (PTCC 1696). Journal of Colloid and Interface Science, 324 (1–2), 172-176.

Ghurye, G.L., Nedwed, T.J., Tidwell, A.C., Canevari, G.P. (May 2014), Dispersion of High Viscosity Oils. International Oil Spill Conference Proceedings, 2014 (1), 776-782.

Giaouris, E., Chapot-Chartier, M.P., Briandet, R. (2009), Surface physicochemical analysis of natural *Lactococcus lactis* strains reveals the existence of hydrophobic and low charged strains with altered adhesive properties. International Journal of Food Microbiology, 131 (1), 2-9.

Goodbody-Gringley, G., Wetzel, D.L., Gillon, D., Pulster, E., Miller, A., Ritchie, K.B. (2013), Toxicity of Deepwater Horizon source oil and the chemical dispersant, Corexit 9500, to coral larvae. PLOS ONE, 8 (1), e45574-e45574.

Gorin, P.A.J., Spencer, J.F.T., Tulloch, A.P. (1961), Hydroxy fatty acid glycosides of sophorose from Torulopsis magnoliae. Canadian Journal of Chemistry, 39 (4), 846-855.

Gray, J.L., Kanagy, L.K., Furlong, E.T., Kanagy, C.J., McCoy, J.W., Mason, A., Lauenstein, G. (2014), Presence of the Corexit component dioctyl sodium sulfosuccinate in Gulf of Mexico waters after the 2010 Deepwater Horizon oil spill. Chemosphere, 95, 124-130.

Gross, S.A., Avens, H.J., Banducci, A.M., Sahmel, J., Panko, J.M., Tvermoes, B.E. (2013), Analysis of BTEX groundwater concentrations from surface spills associated with hydraulic fracturing operations. Journal of the Air & Waste Management Association, 63 (4), 424-432.

Gudiña, E.J., Rangarajan, V., Sen, R., Rodrigues, L.R. (2013), Potential therapeutic applications of biosurfactants. Trends in Pharmacological Sciences, 34 (12), 667-675.

Harayama, S., Kasai, Y., Hara, A. (2004), Microbial communities in oil-contaminated seawater. Current Opinion in Biotechnology, 15 (3), 205-214.

Head, I.M., Jones, D.M., Röling, W.F. (2006), Marine microorganisms make a meal of oil. Nature Reviews Microbiology, 4 (3), 173-182.

Hirata, Y., Ryu, M., Igarashi, K., Nagatsuka, A., Furuta, T., Kanaya, S., Sugiura, M. (2009a), Natural synergism of acid and lactone type mixed sophorolipids in interfacial activities and cytotoxicities. Journal of oleo science, 58 (11), 565-572.

Hirata, Y., Ryu, M., Oda, Y., Igarashi, K., Nagatsuka, A., Furuta, T., Sugiura, M. (2009b), Novel characteristics of sophorolipids, yeast glycolipid biosurfactants, as biodegradable low-foaming surfactants. Journal of Bioscience and Bioengineering, 108 (2), 142-146.

Holakoo, L. (2001). On the capability of rhamnolipids for oil spill control of surface water. (Master Thesis), Concordia University, Montreal, Canada. Retrieved from http://spectrum.library.concordia.ca/1745/1/MQ68436.pdf

Hollebone, B., (2011), Chapter 4: Measurement of Oil Physical Properties, in: Fingas, M. (Ed.), Oil Spill Science and Technology. Gulf Professional Publishing, USA, pp. 63-86.

Inoue, S., Kimura, Y., Kinta, M., (1980), Dehydrating purification process for a fermentation product, C12P19/44, C12R1/88, C07H3/04, C12P19/46, C07H15/10, B01F17/56 ed. Google Patents.

Javan Roshtkhari, S., (2014), Application of Rhamnolipid and Microbial Activities for Improving the Sedimentation of Oil Sand Tailings. Doctoral Research Proposal, Concordia University, Canada, p. 83.

Jones, T.J., Neustadter, E.L., Whittingham, K.P. (1978), Water-in-crude oil emulsion stability and emulsion destabilization by chemical demulsifiers. Journal of Canadian Petroleum Technology, 17 (2), 100-108.

Joshi-Navare, K., Khanvilkar, P., Prabhune, A. (2013), Jatropha oil derived sophorolipids: production and characterization as laundry detergent additive. Biochemistry research international, 2013.

Kaczorek, E. (2012), Effect of External Addition of Rhamnolipids Biosurfactant on the Modification of Gram Positive and Gram Negative Bacteria Cell Surfaces during Biodegradation of Hydrocarbon Fuel Contamination. Polish Journal of Environmental Studies, 21 (4), 901-909.

Kaczorek, E., Chrzanowski, L., Pijanowska, A., Olszanowski, A. (2008), Yeast and bacteria cell hydrophobicity and hydrocarbon biodegradation in the presence of natural surfactants: rhamnolipides and saponins. Bioresource Technology, 99 (10), 4285-4291.

Kaczorek, E., Jesionowski, T., Giec, A., Olszanowski, A. (2012), Cell surface properties of *Pseudomonas stutzeri* in the process of diesel oil biodegradation. Biotechnology Letters, 34 (5), 857-862.

Kaczorek, E., Urbanowicz, M., Olszanowski, A. (2010), The influence of surfactants on cell surface properties of *Aeromonas hydrophila* during diesel oil biodegradation. Colloids and Surfaces B: Biointerfaces, 81 (1), 363-368.

Kang, S.-W., Kim, Y.-B., Shin, J.-D., Kim, E.-K. (2010), Enhanced biodegradation of hydrocarbons in soil by microbial biosurfactant, sophorolipid. Applied Biochemistry and Biotechnology, 160 (3), 780-790.

Khelifa, A., Fingas, M., Hollebone, B.P., Brown, C.E. (2007, June 5-7). Effects of chemical dispersants on oil physical properties and dispersion. Paper presented at the 30th Arctic and Marine Oil Spill Program, AMOP Technical Seminar, Edmonton, AB, Canada. Retrieved from http://crrc.unh.edu/sites/crrc.unh.edu/files/media/docs/Publications/khelifaal2007_amop_paper-1.pdf

Khelifa, A., So, L. (2009, June 9-11). Effects of chemical dispersants on oil-brine interfacial tension and droplet formation. Paper presented at the 32nd AMOP Technical Seminar on Environmental Contamination and Response, Vancouver, BC, Canada. Retrieved from https://inis.iaea.org/search/search.aspx?orig_q=RN:40100746

Kim, L.H., Jung, Y., Yu, H.-W., Chae, K.-J., Kim, I.S. (2015), Physicochemical Interactions between Rhamnolipids and *Pseudomonas aeruginosa* Biofilm Layers. Environmental Science & Technology, 49 (6), 3718-3726.

Kirby, S.M., Anna, S.L., Walker, L.M. (2015), Sequential Adsorption of an Irreversibly Adsorbed Nonionic Surfactant and an Anionic Surfactant at an Oil/Aqueous Interface. Langmuir, 31 (14), 4063-4071.

Kirkeleit, J., Riise, T., Bråtveit, M., Moen, B.E. (2008), Increased risk of acute myelogenous leukemia and multiple myeloma in a historical cohort of upstream petroleum workers exposed to crude oil. Cancer Causes & Control, 19 (1), 13-23.

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

Klevens, H.B. (1950a), Effect of Electrolytes Upon the Solubilization of Hydrocarbons and Polar Compounds. Journal of the American Chemical Society, 72 (8), 3780-3785.

Klevens, H.B. (1950b), The Solubilization of Polycyclic Hydrocarbons. The Journal of Physical and Colloid Chemistry, 54 (2), 283-298.

Knothe, G., Steidley, K.R. (2007), Kinematic viscosity of biodiesel components (fatty acid alkyl esters) and related compounds at low temperatures. Fuel, 86 (16), 2560-2567.

Koch, A.K., Käppeli, O., Fiechter, A., Reiser, J. (1991), Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. Journal of Bacteriology, 173 (13), 4212-4219.

Kochkodan, V., Tsarenko, S., Potapchenko, N., Kosinova, V., Goncharuk, V. (2008), Adhesion of microorganisms to polymer membranes: a photobactericidal effect of surface treatment with TiO2. Desalination, 220 (1–3), 380-385.

Kosaric, N., Sukan, F.V. (1993). Biosurfactants: Production: Properties: Applications, CRC PressVol. 159, pp. 504.

Kostka, J.E., Prakash, O., Overholt, W.A., Green, S.J., Freyer, G., Canion, A., Delgardio, J., Norton, N., Hazen, T.C., Huettel, M. (2011), Hydrocarbon-degrading bacteria and the bacterial community response in Gulf of Mexico beach sands impacted by the Deepwater Horizon oil spill. Applied and Environmental Microbiology, 77 (22), 7962-7974.

Krasowska, A., Sigler, K. (2014), How microorganisms use hydrophobicity and what does this mean for human needs? Frontiers in Cellular and Infection Microbiology, 4, 7.

Kumar, B., Tikariha, D., Ghosh, K.K. (2012), Effects of electrolytes on micellar and surface properties of some monomeric surfactants. Journal of Dispersion Science and Technology, 33 (2), 265-271.

Kumari, B., Singh, S., Singh, D. (2012), Characterization of two biosurfactant producing strains in crude oil degradation. Process Biochemistry, 47 (12), 2463-2471.

Leahy, J.G., Colwell, R.R. (1990), Microbial degradation of hydrocarbons in the environment. Microbiological Reviews, 54 (3), 305-315.

Lehtinen, C.M., Vesala, A.-M., Allen, T. (1984), Effectiveness of oil spill dispersants at low salinities and low water temperatures. Oil Spill Chemical Dispersants: Research, Experience, and Recommendations, 108-121.

Lessard, R.R., DeMarco, G. (2000), The Significance of Oil Spill Dispersants. Spill Science & Technology Bulletin, 6 (1), 59-68.

Lewis, A. (2004). Determination of the Limiting Oil Viscosity for Chemical Dispersion at Sea. (MCA Project MSA 10/9/180). Alun Lewis Oil Spill Consultant, 10 (10/9/180), 86. Retrieved from http://ukerc.rl.ac.uk/pdf/AE0817_report.pdf

Li, Z., Lee, K., King, T., Boufadel, M.C., Venosa, A.D. (2010), Effects of temperature and wave conditions on chemical dispersion efficacy of heavy fuel oil in an experimental flow-through wave tank. Marine Pollution Bulletin, 60 (9), 1550-1559.

Lin, S.-C. (1996), Biosurfactants: Recent Advances. Journal of Chemical Technology & Biotechnology, 66 (2), 109-120.

Lin, T.-C., Shen, F.-T., Chang, J.-S., Young, C.-C., Arun, A.B., Lin, S.-Y., Chen, T.-L. (2009), Hydrocarbon degrading potential of bacteria isolated from oil-contaminated soil. Journal of the Taiwan Institute of Chemical Engineers, 40 (5), 580-582.

Lindstrom, J.E., Braddock, J.F. (2002), Biodegradation of petroleum hydrocarbons at low temperature in the presence of the dispersant Corexit 9500. Marine Pollution Bulletin, 44 (8), 739-747.

Liu, J.-F., Mbadinga, S.M., Yang, S.-Z., Gu, J.-D., Mu, B.-Z. (2015), Chemical Structure, Property and Potential Applications of Biosurfactants Produced by *Bacillus subtilis* in Petroleum Recovery and Spill Mitigation. International Journal of Molecular Sciences, 16 (3), 4814-4837.

Lourith, N., Kanlayavattanakul, M. (2009), Natural surfactants used in cosmetics: glycolipids. International Journal of Cosmetic Science, 31 (4), 255-261.

Mackay, D., Chau, A., Hossain, K., Bobra, M. (1984), Measurement and prediction of the effectiveness of oil spill chemical dispersants. Oil Spill Chemical Dispersants, Research, Experience and Recommendations, ASTM STP, 840, 38-54.

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

Menezes Bento, F., de Oliveira Camargo, F.A., Okeke, B.C., Frankenberger Jr, W.T. (2005), Diversity of biosurfactant producing microorganisms isolated from soils contaminated with diesel oil. Microbiological Research, 160 (3), 249-255.

Moles, A., Holland, L., Short, J. (2002), Effectiveness in the laboratory of Corexit 9527 and 9500 in dispersing fresh, weathered, and emulsion of Alaska North Slope crude oil under subarctic conditions. Spill Science & Technology Bulletin, 7 (5), 241-247.

Morrison, I.D., Ross, S., (2002), Capter 13 Aqueous Solutions of Surface-Active Solutes, Colloidal Dispersions: Suspensions, Emulsions, and Foams. Wiley, p. 656.

Mukerjee, P. (1967), No title available,. Advances in Colloid and Interface Science, 1, 241.

Mulligan, C.N. (2005), Environmental applications for biosurfactants. Environmental Pollution, 133, 183-198.

Mulligan, C.N. (2009), Recent advances in the environmental applications of biosurfactants. Current Opinion in Colloid & Interface Science, 14 (5), 372-378.

Mulligan, C.N., Yong, R.N., Gibbs, B.F. (2001), Heavy metal removal from sediments by biosurfactants. Journal of Hazardous Materials, 85 (1-2), 111-125.

Muthusamy, K., Gopalakrishnan, S., Ravi, T.K., Sivachidambaram, P. (2008), Biosurfactants: Properties, commercial production and application. Current Science (00113891), 94 (6).

National Research Council, (1989), Chapter 2 Chemistry and Physics of Dispersants and Dispersed Oil, Using Oil Spill Dispersants on the Sea. National Academy Press, Washington, DC, , pp. 45-97.

Neumann, H.-J., Paczynska-Lahme, B., Severin, D. (1981). Composition and properties of petroleum, United States, Halsted Press,New York, NY, pp. 137.

Nguyen, T.T., Edelen, A., Neighbors, B., Sabatini, D.A. (2010), Biocompatible lecithin-based microemulsions with rhamnolipid and sophorolipid biosurfactants: Formulation and potential applications. Journal of Colloid and Interface Science, 348 (2), 498-504.

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

Nitschke, M., Costa, S. (2007), Biosurfactants in food industry. Trends in Food Science & Technology, 18 (5), 252-259.

Nogueira Jr, C.A., Feitosa, F.X., Fernandes, F.A., Santiago, R.l.S., de Sant'Ana, H.B. (2010), Densities and viscosities of binary mixtures of babassu biodiesel + cotton seed or soybean biodiesel at different temperatures. Journal of Chemical & Engineering Data, 55 (11), 5305-5310.

Noordman, W.H., Janssen, D.B. (2002), Rhamnolipid Stimulates Uptake of Hydrophobic Compounds by *Pseudomonas aeruginosa*. Applied and Environmental Microbiology, 68 (9), 4502-4508.

Okafor, N., (2011), Chapter 7: Pollution by Petroleum in Oceans and Seas: Role of Microorganisms in Oil Degradation and Remediation, Environmental Microbiology of Aquatic and Waste Systems, 1 ed. Springer Netherlands, p. 307.

Owsianiak, M., Chrzanowski, Ł., Szulc, A., Staniewski, J., Olszanowski, A., Olejnik-Schmidt, A.K., Heipieper, H.J. (2009), Biodegradation of diesel/biodiesel blends by a consortium of hydrocarbon degraders: Effect of the type of blend and the addition of biosurfactants. Bioresource Technology, 100 (3), 1497-1500.

Pekin, G., Vardar-Sukan, F., Kosaric, N. (2005), Production of Sophorolipids from *Candida bombicola* ATCC 22214 Using Turkish Corn Oil and Honey. Engineering in Life Sciences, 5 (4), 357-362.

Pepper, I.L., Gerba, C.P., (2015), Chapter 10 - Cultural Methods, in: Pepper, I.L., Gerba, C.P., Gentry, T.J. (Eds.), Environmental Microbiology Third ed. Academic Press, San Diego, pp. 195-212.

Perfumo, A., Rancich, I., Banat, I.M., (2010), Chapter 10: Possibilities and Challenges for Biosurfactants Use in Petroleum Industry, in: Sen, R. (Ed.), Biosurfactants. Springer New York, pp. 135-145.

Pitter, P., Chudoba, J. (1990). Biodegradability of organic substance in the aquatic environment, CRC press, pp. 306.

Płaza, G., Łukasik, K., Wypych, J., Nałęcz-Jawecki, G., Berry, C., Brigmon, R. (2008), Biodegradation of crude oil and distillation products by biosurfactant-producing bacteria. Polish Journal of Environmental Studies, 17, 87-94.

Poremba, K., Gunkel, W., Lang, S., Wagner, F. (1991), Marine biosurfactants, III. Toxicity testing with marine microorganisms and comparison with synthetic surfactants. Zeitschrift für Naturforschung C, 46 (3-4), 210-216.

Prabhu, Y., Phale, P. (2003), Biodegradation of phenanthrene by *Pseudomonas* sp. strain PP2: novel metabolic pathway, role of biosurfactant and cell surface hydrophobicity in hydrocarbon assimilation. Applied Microbiology and Biotechnology, 61 (4), 342-351.

Ramachandran, S.D., Hodson, P.V., Khan, C.W., Lee, K. (2004), Oil dispersant increases PAH uptake by fish exposed to crude oil. Ecotoxicology and Environmental Safety, 59 (3), 300-308.

Ray, A. (1971a), No title available,. Nature (London), 231, 313.

Reichert, M.D., Walker, L.M. (2013), Interfacial Tension Dynamics, Interfacial Mechanics, and Response to Rapid Dilution of Bulk Surfactant of a Model Oil-Water-Dispersant System. Langmuir, 29 (6), 1857-1867.

Renkin, M. (2003), Environmental profile of sophorolipid and rhamnolipid biosurfactants. Rivista Italiana delle Sostanze Grasse, 80 (4), 249-252.

Riehm, D.A., McCormick, A.V. (2014), The role of dispersants' dynamic interfacial tension in effective crude oil spill dispersion. Marine Pollution Bulletin, 84 (1–2), 155-163.

Riser-Roberts. (1992). Bioremediation of Petroleum Contaminated Sites, CRC Press.

Ron, E.Z., Rosenberg, E. (2001), Natural roles of biosurfactants. Environmental Microbiology, 3 (4), 229-236.

Ron, E.Z., Rosenberg, E. (2002), Biosurfactants and oil bioremediation. Current Opinion in Biotechnology, 13 (3), 249-252.

Rosen, M.J., (2004a), Chapter 1: Characteristic Features of Surfactants, Surfactants and Interfacial Phenomena, 3 ed. John Wiley & Sons, Inc., pp. 1-33.

Rosen, M.J., (2004b), Chapter 2: Adsorption of Surface-Active Agents at Interfaces: The Electrical Double Layer, Surfactants and Interfacial Phenomena, Third ed. John Wiley & Sons, Inc., pp. 34-104.

Rosen, M.J., (2004c), Chapter 3: Micelle Formation by Surfactants, Surfactants and Interfacial Phenomena, Third ed. John Wiley & Sons, Inc., pp. 105-177.

Rosen, M.J., (2004d), Chapter 4: Solubilization by Solutions of Surfactants: Micellar Catalysis, Surfactants and Interfacial Phenomena, 3 ed. John Wiley & Sons, Inc., pp. 178-207.

Rosen, M.J., (2004e), Chapter 8: Emulsification by Surfactants, Surfactants and Interfacial Phenomena, 3 ed. John Wiley & Sons, Inc., pp. 303-331.

Rosen, M.J., Kunjappu, J.T. (2012). Surfactants and interfacial phenomena, John Wiley & Sons.

Rosenberg, E. (1993), Exploiting microbial growth on hydrocarbons-new markets. Trends in Biotechnology, 11 (10), 419-424.

Rosenberg, M., Gutnick, D., Rosenberg, E. (1980), Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS Microbiology Letters, 9 (1), 29-33.

Rosenberg, M., Kjelleberg, S., (1986), Hydrophobic interactions: role in bacterial adhesion, Advances in Microbial Ecology. Springer, pp. 353-393.

Rothsay Biodiesel (2010), Our Biodiesel Quality. 2012. Retrieved from http://www.rothsaybiodiesel.ca/quality.html

Saberi, A.H., Fang, Y., McClements, D.J. (2014), Effect of Salts on Formation and Stability of Vitamin E-Enriched Mini-emulsions Produced by Spontaneous Emulsification. Journal of Agricultural and Food Chemistry, 62 (46), 11246-11253.

Saeki, H., Sasaki, M., Komatsu, K., Miura, A., Matsuda, H. (2009), Oil spill remediation by using the remediation agent JE1058BS that contains a biosurfactant produced by *Gordonia* sp. strain JE-1058. Bioresource Technology, 100 (2), 572-577.

Sajna, K.V., Sukumaran, R.K., Jayamurthy, H., Reddy, K.K., Kanjilal, S., Prasad, R.B.N., Pandey, A. (2013), Studies on biosurfactants from *Pseudozyma* sp. NII 08165 and their potential application as laundry detergent additives. Biochemical Engineering Journal, 78 (0), 85-92.

Schott, H. (1997), Effect of inorganic additives on solutions of nonionic surfactants. Journal of Colloid and Interface Science, 189 (1), 117-122.

Schott, H., Han, S.K. (1976), Effect of inorganic additives on solutions of nonionic surfactants III: CMC's and surface properties. Journal of Pharmaceutical Sciences, 65 (7), 975-978.

Sekelsky, A.M., Shreve, G.S. (1999), Kinetic model of biosurfactant-enhanced hexadecane biodegradation by *Pseudomonas aeruginosa*. Biotechnology and Bioengineering, 63 (4), 401-409.

Seo, Y., Bishop, P.L. (2007), Influence of nonionic surfactant on attached biofilm formation and phenanthrene bioavailability during simulated surfactant enhanced bioremediation. Environmental Science and Technology, 41 (20), 7107-7113.

Shafir, S., Rijn, J.V., Rinkevich, B. (2007), Short and Long Term Toxicity of Crude Oil and Oil Dispersants to Two Representative Coral Species. Environmental Science and Technology, 41 (15), 5571-5574.

Shah, V., Jurjevic, M., Badia, D. (2007), Utilization of restaurant waste oil as a precursor for sophorolipid production. Biotechnology Progress, 23 (2), 512-515.

Sheppard, P.J., Keryn L, S., Krishna K, K., Sayali S, P., Andrew S, B. (2012), The Importance of Weathered Crude Oil as a Source of Hydrocarbonoclastic Microorganisms in Contaminated Seawater. Microbial Biotechnology, 22 (9), 1185-1192.

Shin, K.-H., Kim, K.-W., Seagren, E. (2004), Combined effects of pH and biosurfactant addition on solubilization and biodegradation of phenanthrene. Applied Microbiology and Biotechnology, 65 (3), 336-343.

Shinoda, K., Takeda, H. (1970), The effect of added salts in water on the hydrophile-lipophile balance of nonionic surfactants: the effect of added salts on the phase inversion temperature of emulsions. Journal of Colloid and Interface Science, 32 (4), 642-646.

Shreve, G., Inguva, S., Gunnam, S. (1995), Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by Pseudomonas aeruginosa. Molecular Marine Biology and Biotechnology, 4 (4), 331-337.

Shu, Q., Wang, J., Peng, B., Wang, D., Wang, G. (2008), Predicting the surface tension of biodiesel fuels by a mixture topological index method, at 313 K. Fuel, 87 (17–18), 3586-3590.

Siddiqui, S., Adams, W.A. (2002), The fate of diesel hydrocarbons in soils and their effect on the germination of perennial ryegrass. Environmental Toxicology, 17 (1), 49-62.

Singer, M., George, S., Tjeerdema, R. (1995), Relationship of some physical properties of oil dispersants and their toxicity to marine organisms. Archives of Environmental Contamination and Toxicology, 29 (1), 33-38.

Sjöblom, J., Simon, S., (2014), Chapter 5: Oil Films: Some Basic Concepts, in: Somasundaran, P., Patra, P., Farinato, R.S., Papadopoulos, K. (Eds.), Oil Spill Remediation: Colloid Chemistry-Based Principles and Solutions. John Wiley & Sons, pp. 127-159.

Smith, M.T. (2010), Advances in understanding benzene health effects and susceptibility. Annual Review of Public Health, 31, 133.

Solomon, G.M., Janssen, S. (2010), Health effects of the Gulf oil spill. Journal of the American Medical Association, 304 (10), 1118-1119.

Srinivasan, V., Blankschtein, D. (2003), Effect of Counterion Binding on Micellar Solution Behavior: 2. Prediction of Micellar Solution Properties of Ionic Surfactant−Electrolyte Systems. Langmuir, 19 (23), 9946-9961.

Sterling Jr, M.C., Bonner, J.S., Ernest, A.N.S., Page, C.A., Autenrieth, R.L. (2004), Chemical dispersant effectiveness testing: influence of droplet coalescence. Marine Pollution Bulletin, 48 (9–10), 969-977.

Strassner, J. (1968), Effect of pH on interfacial films and stability of crude oil-water emulsions. Journal of Petroleum Technology, 20 (03), 303-312.

Swedmark, M., Granmo, Å., Kollberg, S. (1973), Effects of oil dispersants and oil emulsions on marine animals. Water Research, 7 (11), 1649-1672.

Tat, M.E., Van Gerpen, J.H. (1999), The kinematic viscosity of biodiesel and its blends with diesel fuel. Journal of the American Oil Chemists' Society, 76 (12), 1511-1513.

Tate, R.E., Watts, K.C., Allen, C.A.W., Wilkie, K.I. (2006a), The densities of three biodiesel fuels at temperatures up to 300 °C. Fuel, 85 (7-8), 1004-1009.

Tate, R.E., Watts, K.C., Allen, C.A.W., Wilkie, K.I. (2006b), The viscosities of three biodiesel fuels at temperatures up to 300 °C. Fuel, 85 (7–8), 1010-1015.

Torres, S., Pandey, A., Castro, G.R. (2011), Organic solvent adaptation of Gram positive bacteria: applications and biotechnological potentials. Biotechnology Advances, 29 (4), 442-452.

Toxics Cleanup Program. (1997). Analytical Methods for Petroleum Hydrocarbons. (ECY 97-602). Washington State Department of Ecology, 137. Retrieved from https://fortress.wa.gov/ecy/publications/summarypages/97602.html

Trudel, B., Belore, R., Guarino, A., Lewis, A., Mullin, J. (2005). Determining the viscosity limits for effective chemical dispersion: relating Ohmsett results to those from tests at-sea. Paper presented at the International Oil Spill Conference. Retrieved from http://dx.doi.org/10.7901/2169-3358-2005-1-71

Trudel, K., Belore, R.C., Mullin, J.V., Guarino, A. (2010), Oil viscosity limitation on dispersibility of crude oil under simulated at-sea conditions in a large wave tank. Marine Pollution Bulletin, 60 (9), 1606-1614.

Tzintzun-Camacho, O., Loera, O., Ramírez-Saad, H.C., Gutiérrez-Rojas, M. (2012), Comparison of mechanisms of hexadecane uptake among pure and mixed cultures derived from a bacterial consortium. International Biodeterioration & Biodegradation, 70, 1-7.

US National Renewable Energy Laboratory, G. (2009). Biodiesel Handling and Use Guide. United States National Renewable Energy Laboratory (NREL), Golden, CO., 56. Retrieved from http://www.osti.gov/scitech/biblio/938562

USEPA. (1993). Understanding oil spills and oil spill response. USEPA Office of Emergency and Remedial Response, 93 (3), 47. Retrieved from http://www.epa.gov/emergencies/content/learning/pdfbook.htm

USEPA. (2011). Appendix C to Part 300 - Swirling Flask Dispersant Effectiveness Test, Revised Standard Dispersant Toxicity Test, and Bioremediation Agent Effectiveness Test. Environmental Protection Agency 28, 225-247. Retrieved from http://www.gpo.gov/fdsys/granule/CFR-2011-title40-vol28/CFR-2011-title40-vol28-part300-appC

Van Bogaert, I.N.A., Saerens, K., De Muynck, C., Develter, D., Soetaert, W., Vandamme, E.J. (2007), Microbial production and application of sophorolipids. Applied Microbiology and Biotechnology, 76 (1), 23-34.

Van Bogaert, I.N.A., Soetaert, W., (2010), Sophorolipids, in: Gloria, S.-C. (Ed.), Biosurfactants From Genes to Applications. Springer Science & Business Media, Berlin, pp. 178-210.

Van Hamme, J.D., Singh, A., Ward, O.P. (2003), Recent advances in petroleum microbiology. Microbiology and Molecular Biology Reviews, 67 (4), 503-549.

Van Hamme, J.D., Ward, O.P. (2001), Physical and metabolic interactions of *Pseudomonas* sp. strain JA5-B45 and *Rhodococcus* sp. strain F9-D79 during growth on crude oil and effect of a chemical surfactant on them. Applied and Environmental Microbiology, 67 (10), 4874-4879.

Vaughn, S.F., Behle, R.W., Skory, C.D., Kurtzman, C.P., Price, N.P.J. (2014), Utilization of sophorolipids as biosurfactants for postemergence herbicides. Crop Protection, 59 (0), 29-34.

Vipulanandan, C., Ren, X. (2000), Enhanced solubility and biodegradation of naphthalene with biosurfactant. Journal of Environmental Engineering, 126 (7), 629-634.

Von Wedel, R., (1999), Technical handbook for marine biodiesel in recreational boats, Second ed. USEPA, Point Richmond, CA, p. 29.

Wadekar, S., Kale, S., Lali, A., Bhowmick, D., Pratap, A. (2012), Sophorolipid production by *Starmerella bombicola* (ATCC 22214) from virgin and waste frying oils, and the effects of activated earth treatment of the waste oils. Journal of the American Oil Chemists' Society, 89 (6), 1029-1039.

Wang, L., Curran, M., Deng, M., Lui, Q., Xu, Z., Masliyah, J., (2014), Chapter 13: Physicochemical Properties of Heavy Oil-Water Interface in the Context of Oil Removal from Seawater by Froth Flotation, in: Somasundaran, P., Patra, P., Farinato, R.S., Papadopoulos, K. (Eds.), Oil Spill Remediation: Colloid Chemistry-Based Principles and Solutions. John Wiley & Sons, pp. 279-294.

Wang, X.-B., Chi, C.-Q., Nie, Y., Tang, Y.-Q., Tan, Y., Wu, G., Wu, X.-L. (2011), Degradation of petroleum hydrocarbons (C6–C40) and crude oil by a novel *Dietzia* strain. Bioresource Technology, 102 (17), 7755-7761.

Wang, Z., Fingas, M., Blenkinsopp, S., Sergy, G., Landriault, M., Sigouin, L., Foght, J., Semple, K., Westlake, D. (1998), Comparison of oil composition changes due to biodegradation and physical weathering in different oils. Journal of Chromatography A, 809 (1), 89-107.

Ward, O.P., (2010), Chapter 5: Microbial Biosurfactants and Biodegradation, in: Sen, R. (Ed.), Biosurfactants Springer New York, pp. 65-74.

Wells, P., Harris, G. (1979), Dispersing Effectiveness of Some Oil Spill Dispersants: Testing with the 'Mackay Apparatus'. Spill Technology Newsletter, 4, 232-241.

Whang, L.-M., Liu, P.-W.G., Ma, C.-C., Cheng, S.-S. (2008), Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. Journal of Hazardous Materials, 151 (1), 155-163.

Wise, C.F., Wise, J.T., Wise, S.S., Thompson, W.D., Wise, J.P. (2014), Chemical dispersants used in the Gulf of Mexico oil crisis are cytotoxic and genotoxic to sperm whale skin cells. Aquatic Toxicology, 152, 335-340.

Wrenn, B.A. (2008). Dispersion Of Crude Oil And Petroleum Products In Freshwater. (EPA/600/R-08/037). U.S. Environmental Protection Agency, Office of Research and Development, National Risk Management Research Laboratory, Washington, DC: Citeseer, 29. Retrieved from http://nepis.epa.gov/Adobe/PDF/P10016VR.pdf

Yakimov, M.M., Timmis, K.N., Golyshin, P.N. (2007), Obligate oil-degrading marine bacteria. Current Opinion in Biotechnology, 18 (3), 257-266.

Yu, H., Huang, G., Wei, J., An, C. (2011), Solubilization of Mixed Polycyclic Aromatic Hydrocarbons through a Rhamnolipid Biosurfactant. Journal of Environment Quality, 40 (2), 477-477.

Zhang, L., Somasundaran, P., Singh, S.K., Felse, A.P., Gross, R. (2004), Synthesis and interfacial properties of sophorolipid derivatives. Colloids and Surfaces A: Physicochemical and Engineering Aspects, 240 (1), 75-82.

Zhang, X., Peterson, C., Reece, D., Haws, R., Moller, G. (1998), Biodegradability of biodiesel in the aquatic environment. Transactions of the ASAE, 41 (5), 1423-1430.

Zhang, Y., Chen, D., Ennis, A.C., Polli, J.R., Xiao, P., Zhang, B., Stellwag, E.J., Overton, A., Pan, X. (2013), Chemical dispersant potentiates crude oil impacts on growth, reproduction, and gene expression in *Caenorhabditis elegans*. Archives of Toxicology, 87 (2), 371-382.

Zhang, Y., Miller, R.M. (1992), Enhanced Octadecane Dispersion and Biodegradation by a *Pseudomonas* Rhamnolipid Surfactant (Biosurfactant). Applied and Environmental Microbiology, 58 (10), 3276-3282.

Zhang, Y., Miller, R.M. (1994), Effect of a *Pseudomonas* Rhamnolipid Biosurfactant in Cell Hydrophobicity and Biodegradation of Octadecane. Applied and Environmental Microbiology, 60 (6), 2101-2106.

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

Zhong, H., ming Zeng, G., Yuan, X.Z., yan Fu, H., Huang, G.H., Ren, F.Y. (2007), Adsorption of dirhamnolipid on four microorganisms and the effect on cell surface hydrophobicity. Applied Microbiology and Biotechnology, 77 (2), 447-455.

Zubay, G. (1983). Biochemistry (third edition), Columbia University, Wm. C. Brown Publishers, pp. 445.

APPENDICES

Table A.1. Fresh biodiesel diesel and light crude oil characteristics (adapted from the literature data).

Parameter	**Fresh Biodiesel (B100) (US National Renewable Energy Laboratory, G. (2009)**		**Fresh Diesel (Fingas, 2011b)**	**Fresh light crude oil (Fingas, 2011b)**
Physical state	Liquid	Liquid	Liquid	Liquid
Formula	$C_{18}H_{34}O_2$	$C_{19}H_{36}O_2$	$C_{10}H_{20}$ to $C_{15}H_{28}$ (Date, 2011)	-
Molecular weight	282.5	296.5	-	-
Cetane number	-	47.2-55	-	-
Melting point	16	-20	-	-
Density (kg/m^3)	860 – 900		840 at 15°C	780 to 880 at 15°C
Kinematic viscosity (mm^2/s)	1-9 to 6 at 40°C		2 mPa.s at 15°C 1.3-4.1 at 40°C	5 to 50 mPa.s at 15°C
Saturates	-		65-95	55-90
Alkanes	-		35 to 45	-
Cyclo-alkanes	-		30 to 50	-
Waxes	-		0 to 1	0 to 20

Olefins	-	0 to 10	-
Aromatics	-	5 to 25	10 to 35
[1]BTEX	-	0.5 to 2	0.1 to 2.5
[2]PAHs	-	0 to 5	10 to 35
Polar compounds	-	0 to 2	1 to 15
Resins	-	0 to 2	0 to 10
Asphaltenes	-	-	0 to 10
Solubility in water (ppm)	-	40	10-50
[3]IFT (mN/m) at 15°C	-	27	10 to 30

[1]Benzene, Toluene, Ethylbenzene, and Xylenes

[2]Polycyclic aromatic hydrocarbons

[3]Interfacial tension

Table A.2. Variations in the dispersant effectiveness (adapted from the literature data).

Type of oil	Weathering	Dispersant	Salinity (ppt)	Temp (°C)	pH	Mixing	[7]Effectiveness	References
[1]ANS	Fresh	Corexit 9500	22	3	-	150 rpm	< 10%	(Moles et al., 2002)
ANS	Fresh	Corexit 9500	22	10	-	150 rpm	< 10%	
ANS	Fresh	Corexit 9500	22	22	-	150 rpm	15.8%	
ANS	Fresh	Corexit 9500	32	3	-	150 rpm	< 10%	
ANS	Fresh	Corexit 9500	32	10	-	150 rpm	22.3%	
ANS	Fresh	Corexit 9500	32	22	-	150 rpm	18.4%	
ANS	Fresh	Corexit 9527	22	3	-	150 rpm	< 10%	
ANS	Fresh	Corexit 9527	22	10	-	150 rpm	< 10%	
ANS	Fresh	Corexit 9527	22	22	-	150 rpm	35.2%	

Type of oil	Weathering	Dispersant	Salinity (ppt)	Temp (°C)	pH	Mixing	[7]Effectiveness	References
ANS	Fresh	Corexit 9527	32	3	-	150 rpm	< 10%	
ANS	Fresh	Corexit 9527	32	10	-	150 rpm	15.3%	
ANS	Fresh	Corexit 9527	32	22	-	150 rpm	30.5%	
ANS	20%	Corexit 9500	22	3	-	150 rpm	< 10%	
ANS	20%	Corexit 9500	22	10	-	150 rpm	< 10%	
ANS	20%	Corexit 9500	22	22	-	150 rpm	< 10%	
ANS	20%	Corexit 9500	32	3	-	150 rpm	< 10%	
ANS	20%	Corexit 9500	32	10	-	150 rpm	< 10%	
ANS	20%	Corexit 9500	32	22	-	150 rpm	< 10%	
ANS	20%	Corexit 9527	22	3	-	150 rpm	< 10%	
ANS	20%	Corexit 9527	22	10	-	150 rpm	< 10%	
ANS	20%	Corexit 9527	22	22	-	150 rpm	< 10%	
ANS	20%	Corexit 9527	32	3	-	150 rpm	< 10%	

Type of oil	Weathering	Dispersant	Salinity (ppt)	Temp (°C)	pH	Mixing	[7]Effectiveness	References
ANS	20%	Corexit 9527	32	10	-	150 rpm	< 10%	(Li et al., 2010)
ANS	20%	Corexit 9527	32	22	-	150 rpm	< 10%	
[6]IFO180	Fresh	Corexit 9500	[2]SW	16	-	Breaking waves (intensive mixing)	90%	
IFO180	Fresh	SPC 1000	SW	16	-	Breaking waves (intensive mixing)	50%	
IFO180	Fresh	Corexit 9500	SW	10	-	Breaking waves (intensive mixing)	3%	
IFO180	Fresh	SPC 1000	SW	10	-	Breaking waves (intensive mixing)	6%	
IFO180	Fresh	Corexit 9500	SW	10-17	-	Regular wave condition	<15%	
IFO180	Fresh	SPC 1000	SW	10-17	-	Regular wave condition	< 15%	
Phenanthrene	Fresh	Rhamnolipid	[3]DI	25	4	Orbital shaker	[4]4 mg PN/240 mg/l [5]RL	(Shin et al., 2004)
Phenanthrene	Fresh	Rhamnolipid	DI	25	4.5	Orbital shaker	7 mg PN/240 mg/l RL	
Phenanthrene	Fresh	Rhamnolipid	DI	25	5	Orbital shaker	6 mg PN/240 mg/l RL	
Phenanthrene	Fresh	Rhamnolipid	DI	25	5.5	Orbital shaker	5 mg PN/240 mg/l RL	
Phenanthrene	Fresh	Rhamnolipid	DI	25	6	Orbital shaker	3 mg PN/240 mg/l RL	
Phenanthrene	Fresh	Rhamnolipid	DI	25	7	Orbital shaker	1 mg PN/240 mg/l RL	
Phenanthrene	Fresh	Rhamnolipid	DI	25	7.5	Orbital shaker	1 mg PN/240 mg/l RL	

Type of oil	Weathering	Dispersant	Salinity (ppt)	Temp (°C)	pH	Mixing	[7]Effectiveness	References

[1]Alaska North Slope crude oil
[2]SW: seawater
[3]DI: deionized water
[4]PN: Phenanthrene
[5]RL: rhamnolipid
[6]Heavy fuel oil
[7]Effectiveness

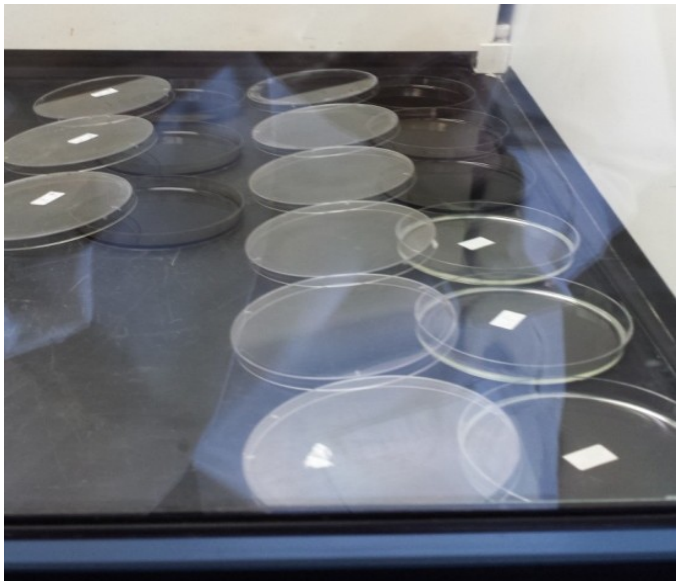

Figure A.1. Weathering of oil in a fume hood for 72 h.

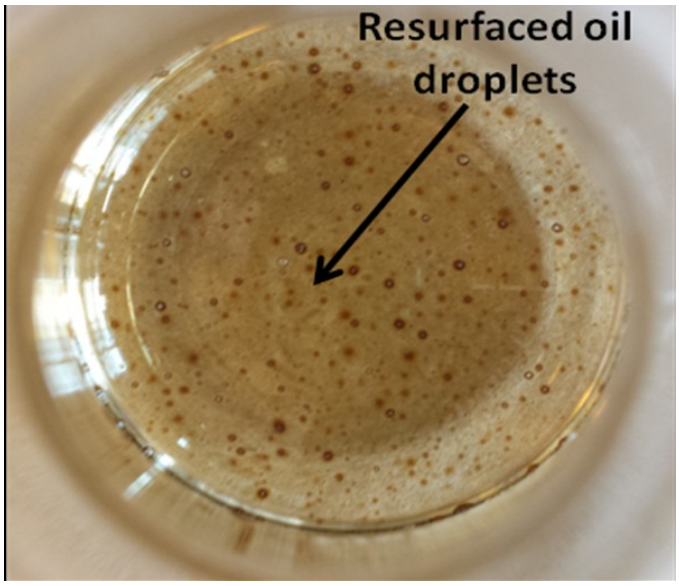

Figure A.2. Resurfaced light crude oil droplets following a 10 min settling time.

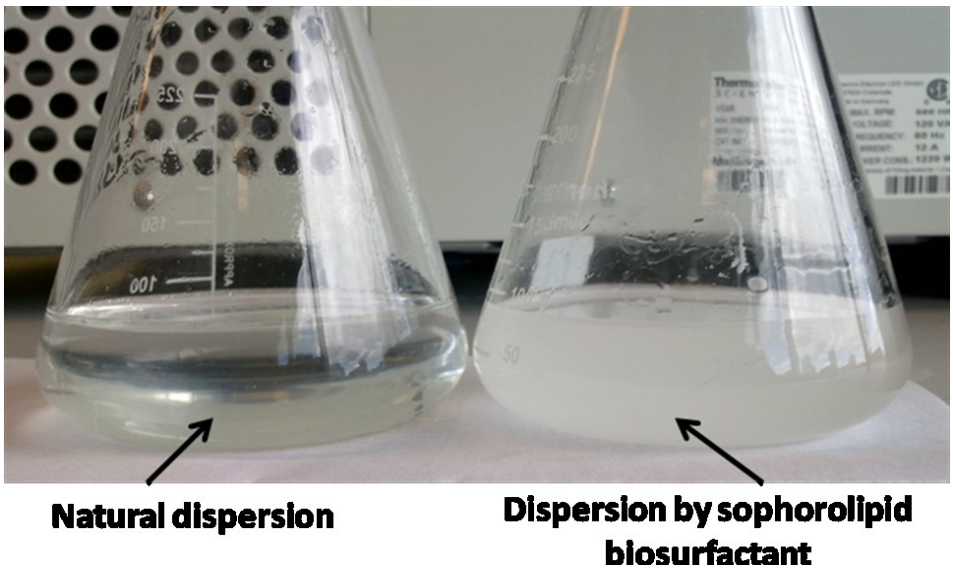

Figure A.3. Natural and biodispersion of the weathered biodiesel in the artificial seawater following a 20 min shaking time and 0 min settling time.

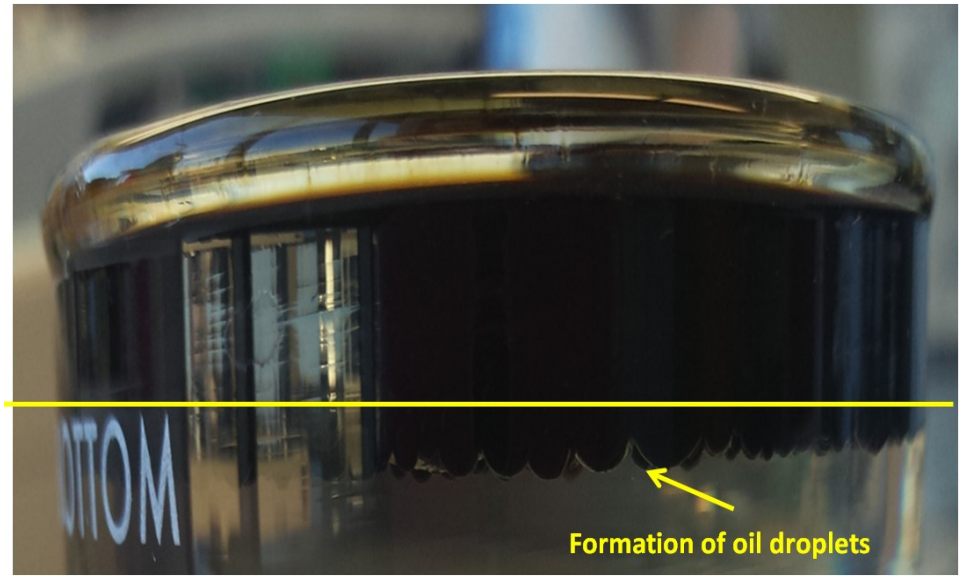

Figure A.4. Formation of oil droplets by the sophorolipid biosurfactant.

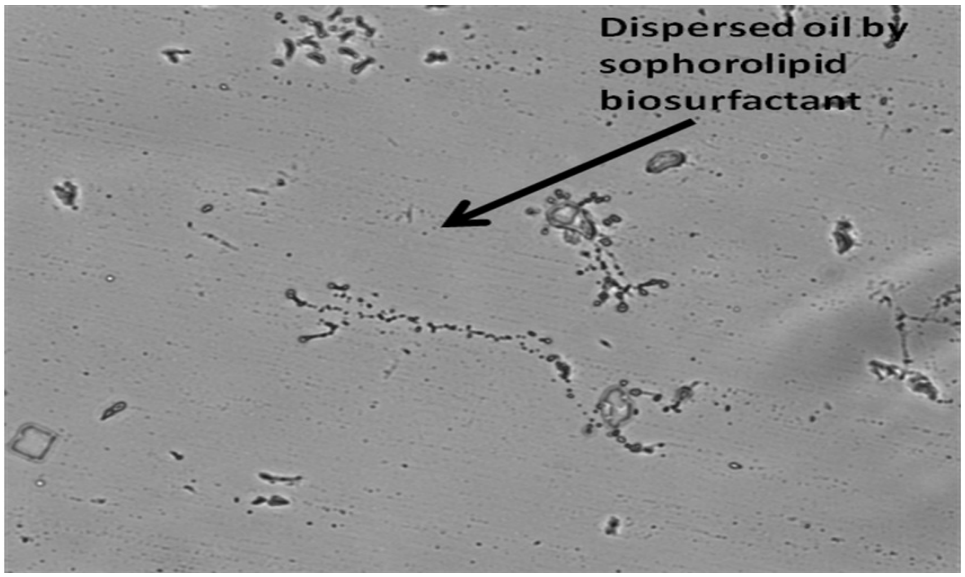

Figure A.5. Microscopic visualization (magnification: 60X) of the weathered diesel dispersion by the sophorolipid biosurfactant (above the CMC, 80 mg/L) at 35°C in the artificial seawater with the salinity of 30 ppt, and pH 7.2 (shaking time: 20 min; settling time: 0 min). Black dots represent the dispersed biodiesel.

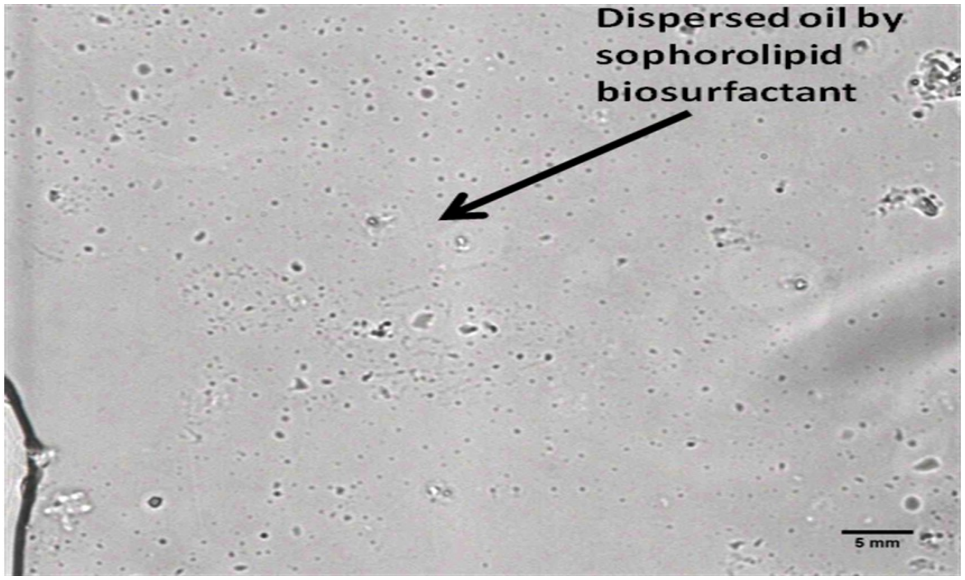

Figure A.6. Microscopic visualization (magnification: 60X) of the weathered biodiesel dispersion by the sophorolipid biosurfactant (below the CMC, 20 mg/L) in the artificial seawater with the salinity of 30 ppt, pH 6.22 and 22 ± 1°C (shaking time: 20 min; settling time: 0 min). Black dots represent the dispersed biodiesel. Scale bar: 5 mm.

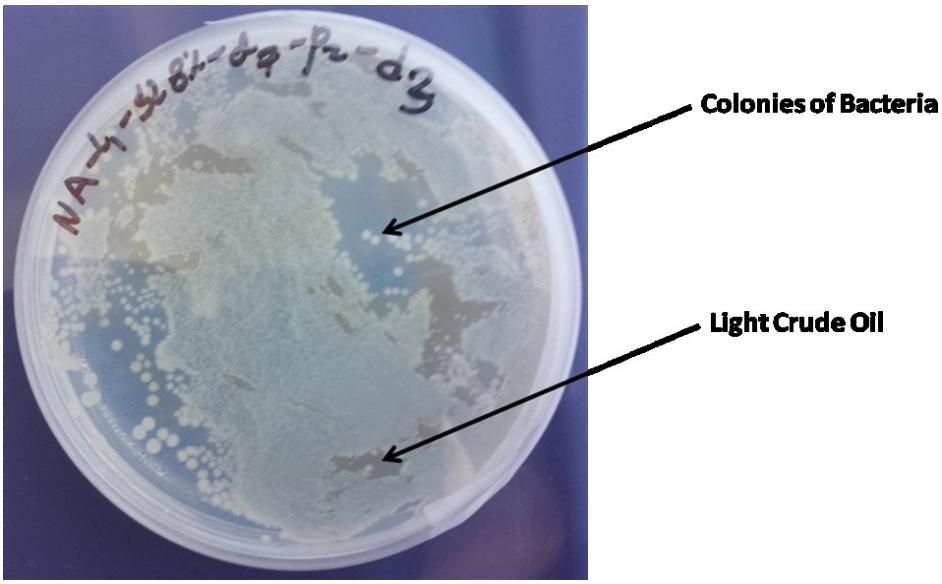

Figure A.7. Growth of bacteria on the weathered light crude oil following seven days of biodegradation period in the presence of the sophorolipid biosurfactant.