

**CAPABILITY OF RHAMNOLIPID AND TWO
BIOLOGICAL PRODUCTS IN BIOREMEDIATION
OF OIL IN MARINE ENVIRONMENT**

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in
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of
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ABSTRACT

Capability of Rhamnolipid and Two Biological Products in Bioremediation of Oil in Marine Environment

Farnaz Vasefy

Each year more than 1.3 million metric tonnes of oil are released into the seas from man-made and natural sources. While mechanical and chemical processes have proven to have limited effectiveness, in many cases biological techniques are promising alternatives because of the lower costs, environmental friendliness associated with them. This study focuses on assessing the effectiveness of a rhamnolipid biosurfactant (JBR 425TM) and two other commercial biological products on biodegradation of weathered light crude oil, heavy crude oil, and diesel fuel spilled on saline water following the USEPA's biological effectiveness test method.

The two products contain bacterial consortia and nutrients and were used as supplementary additives to enhance the biodegradation rate and extent of hydrocarbon compounds. Results show that at 20°C and 35 ‰ salinity, the mixture of rhamnolipid biosurfactant and one of the biological products overall produced the highest percentage of biodegradation at 81% for diesel fuel, 76% for light crude oil, and 64% for heavy crude oil after 28 days of experiment. Degradation rates of oil types for most of the treatments were in the order of diesel fuel > light crude oil > heavy crude oil and generally removal percentage and microbial densities had a direct relationship. The results obtained from this study show the potential for petroleum hydrocarbon removal

enhancement in various oils by the addition of biological agents to the rhamnolipid biosurfactant.

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DEDICATION

This thesis is dedicated to my mother, who taught me patience; diligence, and perseverance and who gave me love, support and spirit. It is also dedicated to my brother, who gave me his continuous support and encouragement.

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LIST OF SYMBOLS

α	Level of significance
‰	Parts per thousand
ANOVA	Analysis of variance
AOR	Additive to oil ratio
API	American Petroleum Institute
ASTM	American society for testing and materials
ATSDR	Agency for toxic substances and disease registry
BTX	Benzene, Toluene, and Xylene
C	Total petroleum hydrocarbon concentration (g/L)
C ₀	Total petroleum hydrocarbon concentration at day 0 (g/L)
CFU	Colony forming units
CMC	Critical micelle concentration
CO ₂	Carbon dioxide
DCM	Dichloromethane
DOR	Dispersant to oil ratio

GC/FID	Gas chromatography/ Flame ionization detector
HCl	Hydrochloric acid
HLB	Hydrophilic-lipophilic balance
k	First order biodegradation rate coefficient (1/day)
K	Second order biodegradation rate coefficient (L/g-day)
NaOH	Sodium hydroxide
NCP	National oil and hazardous substances pollution contingency plan
NRC	National research council
<i>p</i>	Probability
PAH	Polycyclic aromatic hydrocarbons
PCP	Pentachlorophenol
R ²	Determination coefficient
SDS	Sodium dodecyl sulfate
t	Time of incubation (day)
t _{1/2}	Half-life (day)
TPH	Total petroleum hydrocarbon
USEPA	United States Environmental Protection Agency

1 INTRODUCTION

1.1 Background

The use of petroleum hydrocarbons as the main source of energy is widely used around the world. This makes the accidental spill of crude oil and its refined products during extraction, transportation, storage, refining, and distribution operations inevitable. Taking into account the natural seepages of crude oil and deliberate waste disposals, on average, annually more than 1.3 million metric tonnes of oil is released into marine waters worldwide (NRC 2003).

Marine oil spills, in short term, pose great threats and cause extensive damage to the marine and coastal ecosystems because of the toxicity of many hydrocarbon compounds as well as complications aroused by physical coating of flora and fauna by hydrocarbon compounds. In the long run, the recalcitrant fraction of crude oils can remain in a system for many years, resulting in the loss of animals habitat and persistent poisoning of organisms. The type of technology used in the cleanup process of an oil spill depends primarily on the type, characteristics, and amount of the spilled oil. Other important factors include economical and site considerations (Zhu et al. 2001). Meanwhile, the traditional cleanup options including mechanical removal (booms, skimmers, absorbents), washing, in-situ burning, and dispersants are expensive, produce toxic end products, and/or rarely achieve acceptable cleanup levels (OTA 1990).

In the wake of 1989 Exxon Valdez disaster, in which 37,000 metric tons of crude oil spilled into Prince William Sound, Alaska, and bioremediation was successfully applied as an effective secondary treatment process, natural breakdown of organic pollutants by

microorganisms emerged as a viable potential alternative (Bragg et al. 1994; Prince et al. 1994). By definition, bioremediation is the addition of materials to contaminated environments in order to enhance the rate and extent of natural breakdown of organic compounds by microorganisms (USEPA 2004). Bioremediation, being a natural process, is less intrusive and disruptive to the contaminated ecosystem, produces environmental friendly by-products, and is considered less expensive than traditional cleanup methods (Zhu et al., 2001).

The present study evaluates the effectiveness of a rhamnolipid biosurfactant (JBR 425TM) and two other commercial biological products on biodegradation of three different types of oils spilled on saline water following the USEPA's biological effectiveness test method. The hypothesis is that the biosurfactant and the two products, which contain bacterial consortia, dispersants, and/or nutrients, would stimulate natural breakdown of oil hydrocarbon compounds by the indigenous oil microorganisms.

1.2 Objectives

The intention of this research is to evaluate the effectiveness of using the commercial products on biodegradation of spilled oil in saline water. The overall objectives of this research are as below:

- To evaluate the effects of different JBR 425TM concentrations on biodegradation of light crude, heavy crude, and diesel fuel oils.
- To evaluate the effects of different ASAPTM and DegreaserTM ratios on biodegradation of the three oils.

- To determine the effectiveness of different Rhamnolipid to Degreaser™ and/or ASAP ratios on oil biodegradation.

1.3 Thesis Outline

This thesis consists of five chapters, Chapter 1, in a few words, describes the nature of the problem and objectives of this research. Chapter 2 covers the literature review of oil spill response techniques in a marine environment, and mainly discusses the bioremediation process and required information for this research. Chapter 3 discusses the experimental procedures as well as the materials, and methodology used in this research. Chapter 4 explains the results of the experimental studies and the final chapter summarizes the conclusions and shows the recommendations of this study for the future work. Finally, the appendix of this dissertation contains the gas chromatography (GC) calibration curve.

2 LITERATURE REVIEW

2.1 Oil Spill

Despite the great attention given to reducing the magnitude and frequency of oil spills over last decade, the ever-increasing worldwide demand and transport of oil and petroleum products assures their unintentional releases into the marine environment (NRC 2003). According to the U.S. EPA (2006a), from around one billion cubic meters of petroleum hydrocarbon products handled annually, less than 1% is spilled into the environment. The sources that contribute to the release of oil into marine bodies are coastal refinery waste and urban runoff discharges (38%), offshore oil production (3%), and spillage from oil tankers (12%). Crude oil also enters the oceans by natural seepage (47%) from undersea reservoirs (Clark 2001; NRC 2003).

2.2 Crude Oil and Refined Oil Characteristics

Both crude and refined petroleum products contain complex and variable mixtures of thousands of gaseous, liquid, and solid hydrocarbon compounds with different characteristics. These compounds are generally grouped into four broad classes of chemicals. Figure 2.1 shows the structure of some hydrocarbon compounds in crude and refined petroleum (Zhu et al. 2001):

A. Saturates: Alkanes are fully saturated hydrocarbon compounds that normally have a carbon number range from one to around 40, although compounds with carbon numbers of up to 60 have been identified. They are divided into aliphatic and cyclic alkanes (alicyclics), and constitute the most abundant fraction of crude oils. Aliphatic alkanes are

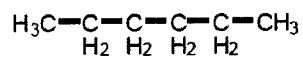
normal, or straight chain, and branched chain hydrocarbons with structures of C_nH_{2n+2} . Alicyclics have saturated ring structures of C_nH_{2n} .

B. Aromatics: Compounds that have at least one benzoid ring in their structure. They are grouped into monocyclic aromatics (benzene, toluene, xylene, etc.) and polycyclic aromatic hydrocarbons or PAHs (anthracene, phenanthrene, etc.). PAHs have at least two fused (naphthalene, etc.) or linked (biphenyl, etc.) aromatic rings. Aromatics are less abundant than saturates and are of particular concern because they are known carcinogens and are recalcitrant to degradation (ATSDR 1999).

C. Resins: Resins are hydrocarbons with polar structures and contain inorganic elements such as nitrogen, sulfur, and oxygen in their structures (pyridines, thiophenes, etc.). They have strong adsorption tendencies and have relatively high molecular weights.

D. Asphaltenes: Consist of high molecular weight compounds that are soluble in aromatic solvents and insoluble in alkane solvents. They generally contain aromatic hydrocarbons with aliphatic and aromatic alkane side chains as well as inorganic elements such as nitrogen, sulphur, oxygen. Metalloids in oils are also associated with asphaltenes.

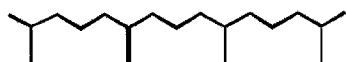
SATURATES



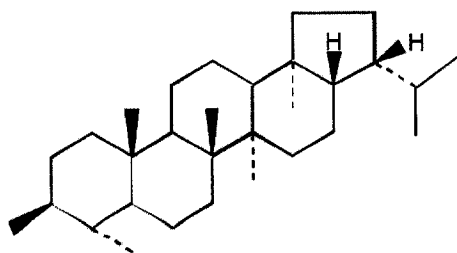
n-hexane



n-heptadecane (n-C₁₇H₃₆)



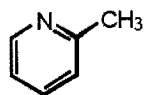
pristane (C₁₉H₄₀)



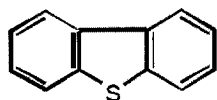
17α(H),21β(H)-hopane

RESINS

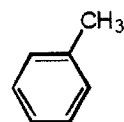
2-methylpyridine



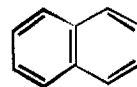
dibenzo-
thiophene



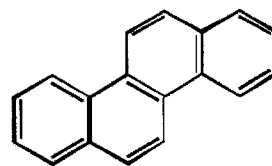
AROMATICS



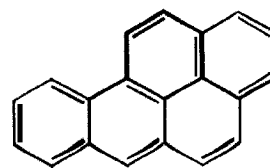
toluene



naphthalene



chrysene



benzo[a]pyrene

ASPHALTENES

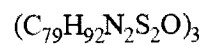


Figure 2.1 Illustration of the structure of some hydrocarbon compounds in crude and refined petroleum (Zhu et al. 2001).

2.2.2 Crude Oil

Crude oil or petroleum is a mixture of a wide range of hydrocarbons carbon numbers from one to over 60. It also contains inorganic elements, such as sulphur, nitrogen, and oxygen, as well as metallic constituents, particularly vanadium, nickel, iron, and copper (Klimisch et al. 2003). According to the American Petroleum Institute, on average, crude oil contains 84% carbon, 14% hydrogen, 1-3% sulfur, 1% nitrogen, 1% oxygen, and 0.1% minerals and salts (Klimisch et al. 2003). Crude oil properties and characteristics differ according to its source (Speight 1991). It could be found in different colors, such as red, green, and brown. Some have a low viscosity with a high volatility, while others have higher densities with a high viscosity and a low volatility.

According to Klimisch et al. (2003), crude oils can be categorized as:

Heavy or Light: The words "light" and "heavy" describe a crude oil's density and its resistance to flow (viscosity). Crude oils are classified as heavy and light according to their API gravity, which is defined as:

$$\text{API gravity} = (141.5/\text{Specific Gravity at } 60^\circ\text{F}) - 131.5$$

Light crude oils have API gravities of more than 33°, light colors, and low metals and sulfur contents. Heavy crude oils have API gravities of less than 28°, dark colors, and high metals and sulfur contents and are rich in asphaltene and aromatic fractions.

Sweet or Sour: The term "sweet" describes crude oils with small amounts of carbon dioxide and sulfur compounds, such as hydrogen sulfide and mercaptans (thiol groups). "Sour" oil is a lower quality crude with higher levels of impurities and toxicity.

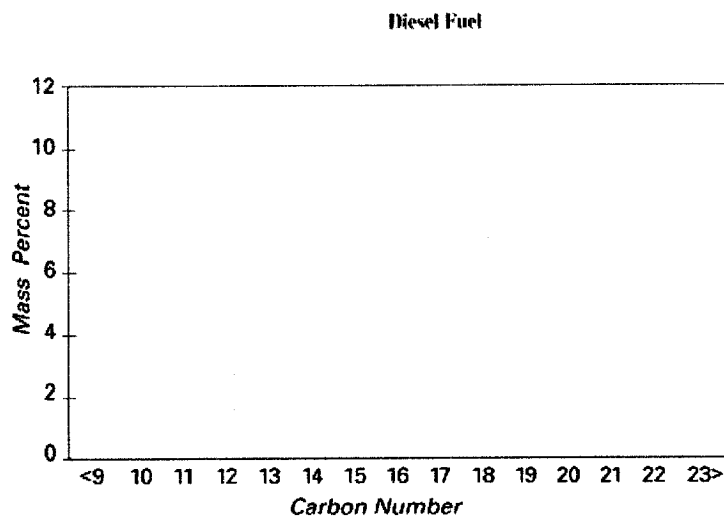
Paraffinic or Naphthenic: Crude oils contain both paraffinic and naphthenic groups. Paraffinic hydrocarbons are the linear molecules with the formula C_nH_{2n+2} , whereas, naphthenic hydrocarbons contain one or more saturated rings in their structure with the general formula C_nH_{2n} . Where paraffinic hydrocarbons are predominant, the oil is referred to as paraffinic crude. A crude oil with a majority of naphthenic hydrocarbons and aromatic hydrocarbons is referred to as naphthenic crude.

2.2.3 Refined Oil Products

Refined petroleum products or petroleum hydrocarbons, such as diesel fuel, gasoline, kerosene, jet fuels, fuel oils, and lubricating oils, are mixtures of organic compounds derived from crude oils through refining processes, such as catalytic cracking and fractional distillation (Zhu et al. 2001). With the exception of gasoline, petroleum hydrocarbons are mostly made by boiling point separation of crude oil fractions from a distillation column (ATSDR 1999). Therefore, these mixtures have a wide variety of hydrocarbons in varying compositions that depend on the origin of oil and the refining processes (Speight 1991; Zhu et al. 2001).

Diesel Fuel

Diesel fuel is obtained in the fractional distillation of crude oil between 200 °C and 350 °C at atmospheric pressure. This middle distillate product is a complex mixture of thousands of individual compounds and particularly contains saturated aliphatics and aromatic hydrocarbons with carbon numbers between 10 and 22 (Song, 2000). It also contains branched chained alkanes as well as straight chain n-alkanes (Christensen and Larsen 1993).



**Figure 2.2 Typical carbon number for diesel fuel
(Chevron Products Company 1998).**

Figure 2.2 illustrates a typical carbon number distribution for diesel fuel. The type and amount of diesel compounds depend on the chemical composition of the petroleum source, refining processes, and the extent of exposure to environmental factors (Block et al. 1991). According to Frankenberger et al. (1987), on average, diesel fuel consists of 35% alkanes, 45% cyclic alkanes, and 24% aromatics.

2.3 Environmental Fate and Weathering of Spilled Oil

In the event of an oil spill into marine waters, a slick of oil initially is formed on the surface of the water. With time the oil slick undergoes a variety of transformations known as weathering and includes: physical changes (spreading, evaporation, and dispersion), chemical changes (emulsification, dissolution, oxidation, and sedimentation) and biological changes (biodegradation) as it is shown in Figure 2.3 (ITOPF 2002).

Over time, the single slick formed will break up, spread out, and cover a vast area of the water surface due to winds, waves, and water turbulences. This process is called spreading and is one of the most important processes during the first few hours after the spill because, by increasing the total surface area of broken oil, it facilitates further processes such as evaporation, dissolution, and biodegradation (Patin 1999; Zhu et al. 2001). Spreading is greatly influenced by the characteristics of the spilled oil such as viscosity, and specific gravity, as well as the temperature and the intensity of water currents and wind speed (Zhu et al. 2001; ITOPF 2002).

Evaporation occurs when the volatile and low molecular weight hydrocarbon fractions, which are among the most toxic constituents of the oil, partition to the gaseous phase. As a result, the heavy and viscous fractions of oil remain in the water (USEPA 1999). Depending on the type of crude oil spilled, evaporation can account for 20-50% of the volume of the spilled oil (NRC 2005). The degree of evaporation also depends on environmental factors, such as the wind velocity, water temperature, and intensity of wave action and water turbulences (Zhu et al. 2001).

The slick and its fragments are dispersed by waves and turbulence at the water surface into droplets of varying size (Patin 1999). Some of the finer droplets will remain suspended in the water column while the larger ones, also called oil-in-water emulsion, will tend to rise and band together at the sea surface (NRC 2003). An emulsion is the dispersion of a liquid in another immiscible liquid in the form of droplets with diameters of at least 0.1 mm (Mulligan et al. 2002). Although oil-in-water emulsions are to a large extent unstable in marine environments, they can be stabilized by continuous agitation, interaction with suspended particulates, and addition of dispersants. Dispersed droplets

can be transported over large distances away from the origin of the spill, thus decreasing the concentration of oil (NRC 2003). Furthermore, by increasing the surface area of the oil compartment, dispersion encourages other natural processes, such as dissolution, biodegradation and sedimentation to occur (IPEICA 2001; Zhu et al. 2001). The extent of dispersion is largely affected by the source and properties of the oil, as well as the environmental conditions of the sea, such as temperature and intensity of the wind. Chemical dispersants have also been applied to oil slicks to enhance the rate and extent of dispersion (ITOPF 2002).

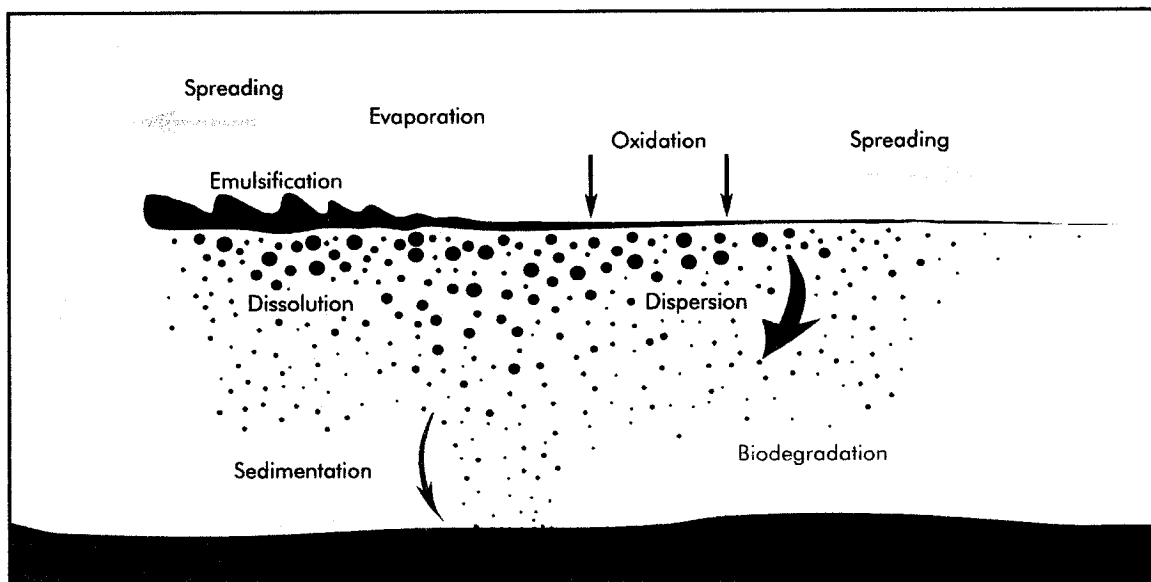


Figure 2.3 Fate of spilled oil in the marine environment (ITOPF 2002).

When sea-water droplets become suspended in the oil by turbulence at the water surface, water-in-oil emulsions are formed. The water-in-oil emulsion is usually very viscous and more persistent than the original oil and is often referred to as ‘chocolate mousse’ because of its appearance (NRC 2003). Oils with over 0.5% asphaltene content readily form stable emulsions, while those containing a lower percentage of asphaltenes are more likely to disperse than form emulsions. Sunlight, heat, and calm conditions may

destabilize and break up previously formed water in oil emulsions (ITOPF 2002). The formation of these emulsions could increase the overall volume of pollutants as well as the amount of floating pollutants (Cormack 1999). In addition, water-in-oil emulsion formation reduces the rate of other weathering processes, and makes the oils resistant to natural degradation (Zhu et al. 2001).

Dissolution is the solubilization of hydrocarbon compounds in water. It depends on the source and composition of the spilled oil, other weathering processes, such as exposure to sunlight, and condition of the spill site (Zhu et al. 2001). Although dissolution is considered to be less important than the evaporation process, and since hydrocarbons are generally not very soluble in water, it is still an important phenomenon, because many of the acutely toxic components of oils, such as benzene, toluene, and xylene (BTX) are the most soluble (NRC 2003). In an average marine oil spill, more than 95 percent of the benzene evaporates while generally less than 5 percent of it dissolves in the water (NOAA 1992). Dissolution is also important because it can affect biodegradation through increasing the availability of hydrocarbons, but at the same time, possibly decreasing biodegradation rates due to greater availability of the more soluble and toxic BTX compounds (ITOPF 2002).

Oxidation or photo-oxidation is the breakdown of hydrocarbon molecules, such as high molecular weight aromatics and polar compounds in the presence of molecular oxygen by natural sunlight into simpler polar or persistent compounds called tars (ITOPF 2002; NRC 2005). The rate and extent of oxidation depends mainly on the intensity and duration of sunlight, although other factors, such as oil viscosity, exposure to oxygen, and the climatic conditions are also involved (Zhu et al. 2001; ITOPF 2002).

The combination of low density of crude oils and high density saline waters cause most types to float in marine environments. However, some dense crude oils and highly weathered products can sink in saline waters (ITOPF 2002; NRC 2002). Sinking is intensified by adhesion of oil to inorganic solid particles, such as sand and sediment as well as organic matter (ITOPF 2002). Sinking and sedimentation are affected by the quantity and properties of suspended material in the sea-water, quantity and characteristics of the spilled oil, the energy of the system, and salinity of the water (NRC 2005).

Biodegradation is one of the principal removal mechanisms for hydrocarbon compounds in the marine environment (Zhu et al. 2001; ITOPF 2002). It is especially important for the non-volatile fraction of oil that is resistant to other processes of weathering. Under favourable conditions, microorganisms metabolize complex hydrocarbon compounds to provide the cell with carbon, energy, and other nutrients required for growth (Atlas and Bartha 1993; ITOPF 2002). These microorganisms, which include bacteria, yeasts, moulds, fungi, unicellular algae and protozoa can partially or completely degrade hydrocarbon compounds. The main factors affecting the efficiency of biodegradation are the quantity, type, and characteristics of the spilled oil, the levels of oxygen and macro-nutrients, such as nitrogen and phosphorus in the water, and environmental factors, such as the temperature, salinity, and pH of the marine water (Zhu et al. 2001).

Different oil fractions have different susceptibilities to degradation by microorganisms based on their molecular structure and size (Leahy and Colwell 1990; Zhu et al. 2001). Simple hydrocarbon fractions, such as aliphatic and aromatic alkanes are degraded to simpler compounds and eventually to carbon dioxide and water. Other more complex

fractions including branched alkanes and polycyclic aromatic hydrocarbons are more recalcitrant and require more time and a consortium of microorganisms to be degraded (NRC 2003). Biodegradation rates are also influenced by concentrations of the oil or oil fractions. Low levels of oil may not be sufficient to stimulate biodegradation; in contrast, high concentrations of hydrocarbons may inhibit biodegradation because of nutrient or oxygen limitations or their toxic effects (Focht and Westlake 1987).

The weathering processes have profound effects on petroleum biodegradation by evaporation of volatile oil components and low-molecular-weight components, such as benzene and smaller *n*-alkanes that are more toxic to aqueous organisms (Atlas 1981; NRC 1985). The oil surface area is important because growth of oil degraders mostly occurs at the oil-water interface (Atlas and Bartha 1992). Therefore, while some weathering processes such as spreading, dispersion, and dissolution stimulate biodegradation by increasing the surface area of the oil, formation of water-in-oil emulsions or mousses that reduce the oil surface area decrease biodegradation (ITOPF 2002). Large aggregates of weathered oil or tar balls are also inaccessible to microorganisms because of their limited surface area (Leahy and Colwell 1990).

2.4 Impacts of Oil Spills in Marine Environment

Petroleum poses a range of environmental risks when released into the environment. The risks from exposure depend on many factors which include the type of hydrocarbon compounds, the route of exposure, the duration of exposure, and the total amount of hydrocarbon compounds (ATSDR 1999).

In marine environments the impacts of oil spills are divided into effects caused by the physical nature and the chemical components of the oil (ITOPF 2006). The first is the physical impacts that continuous large spills have on the marine species, such as birds and air-breathing animals. Oils with low solubility and slow dispersion rates make persistence slicks that may physically coat the fur of birds and marine mammals, shoreline areas, and its living organisms (Cormack 1999; NRC 2003). This can kill marine animals by physical smothering, reduce their fitness through sub-lethal effects, and disrupt the structure and function of marine communities and ecosystems (Cormack 1999; ITOPF 2006).

The second affect is due to the toxicity of many of the individual compounds contained in petroleum. The toxicity of these compounds is governed by the solubility, and consequently their availability to marine organisms. The mobility of these toxicants is greatly enhanced by weathering phenomena, especially dispersion and dissolution, due to waves and water movement at the surface. Once these slicks have dispersed into the water column, toxic sub-lethal or even lethal effects on the lower micro-organisms as well as other vulnerable organisms, such as fish eggs, and larva can occur at even small concentrations of certain compounds or fractions. Higher organisms are also affected by feeding upon those lower on the food chain, which have bio-accumulated toxic material (Cormack 1999; NRC 2003).

The toxic consequences of oil spills can also impact humans through the food chain. Although this risk is minimum, because most petroleum compounds are not persistent and do not accumulate in the tissue, some compounds such as PAHs can accumulate for short periods, when organisms are chronically exposed to them (Cormack 1999).

2.5 Microbial Degradation of Petroleum Hydrocarbons

Over 200 species of microorganisms, including bacteria, fungi, yeast, as well as some algae are able to break down a wide range of petroleum hydrocarbons through metabolism and co-metabolism pathways (Zobell 1973; Cookson 1995). These microorganisms are ubiquitous in pristine and contaminated freshwater, marine, and soil environments. However, their distribution in terms of both species and numbers are dependent on environmental factors as well as previous exposure to hydrocarbon pollutants (Atlas 1981; Leahy and Colwell 1990; Zhu et al. 2001). Metabolism refers to the use of hydrocarbons as a substrate source for growth or energy, whereas, co-metabolism is the transformation of organic compounds without direct benefit to the degrading microorganism (Alexander 1999).

Different species and genera have different biodegradation rates for hydrocarbon compounds and because individual microorganisms have the ability to degrade a certain range of hydrocarbons, for degradation of a mixture of hydrocarbons a consortium of organisms, with a broad catabolic (breakdown) potential is required (Bartha and Atlas 1987; Cookson 1995). In marine environments the major hydrocarbon-degrading microorganisms are bacteria and are usually more abundant in coastlines both in terms of numbers and species (Jordan and Payne 1980; Floodgate 1984; Zhu et al. 2001).

Hydrocarbons that are readily biodegraded are called biodegradable, while compounds that are not biodegradable under natural conditions or degrade extremely slow are considered recalcitrant (Huesemann 1997). The most important factors in determining the biodegradability of a compound are molecular size and structure (Alexander 1999). In

general, the lower the molecular weight of an organic compound, the more biodegradable the compound is (Alexander 1999; Kanaly and Harayama 2000). As stated before, based on their molecular structure, hydrocarbon compounds can be categorized into four main groups of saturates, aromatics, resins and asphaltenes.

Among the saturate fraction of hydrocarbons, n-alkanes are the most susceptible to degradation (Atlas 1981; Zhu et al. 2001). Branched alkanes are less readily biodegradable in comparison with n-alkanes. Cyclo-alkanes are mainly resistant to microbial attack and complex alicyclic compounds such as hopanes are the most persistent components of petroleum (Atlas 1981; Leahy and Colwell 1990).

Among the aromatic hydrocarbons, except for some low molecular weight compounds, such as naphthalene, most are recalcitrant to biodegradation (Hinchee et al. 1994). This is because the mono-aromatics are toxic to microorganisms and the five or more ring polycyclic hydrocarbons can only be degraded through co-metabolism (Zhu et al. 2001).

Resins and asphaltenes are known to be resistant to degradation due to their complex structures (Hinchee et al. 1994). Leahy and Colwell (1990) illustrated that some asphaltenes can be degraded through co-metabolism. Some low molecular weight resin fractions can be biodegraded at very low concentrations as well (NRC 1985).

2.6 Surfactants

Surfactants, or surface-active agents, are amphiphilic molecules that contain a hydrophilic (polar) head and hydrophobic (nonpolar) tail as shown in Figure 2.4. The hydrophilic head groups have a higher attraction to the bulk polar medium (in this case

water) while the hydrophobic tail groups have a lower affinity to the bulk medium (Mulligan, 2005); therefore, these compounds tend to gather at interfaces (liquid/vapour, solid/liquid, and liquid/liquid) (Zajic and Seffens 1984). By accumulating at interfaces, surfactants can replace the bulk solution molecules and reduce the surface and interfacial tensions; thus, facilitating the formation of emulsions between different immiscible liquids (in this case oils), lowering the capillary forces, and increasing the mobility of the contaminant (Mulligan 1998; Mulligan et al. 2001; Urum 2004; Jennings et al. 2006).

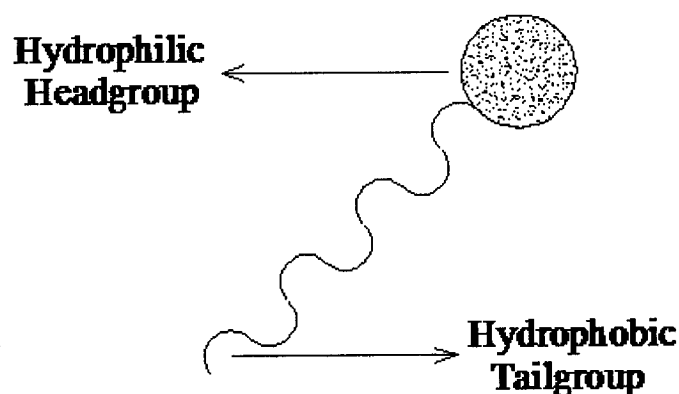


Figure 2.4 Representation of a surfactant monomer.

Each surfactant has a concentration above which the increasing surfactant concentration no longer corresponds with a reduction in the surface and interfacial tension of the solution. This concentration is known as the critical micelle concentration (CMC) and is the maximum concentration of free surfactant monomers and is strongly influenced by pH, temperature, ionic strength and salinity of the medium (Sabatini et al. 1995; Lin 1996; Myers 1999; Mulligan 2005).

The relationship between surfactant concentration, surface tension, solubility and interfacial tension is shown in Figure 2.5. The correlation between surfactant

concentration and the surface tension is used to obtain the critical micelle concentration (Mulligan and Gibbs 2004).

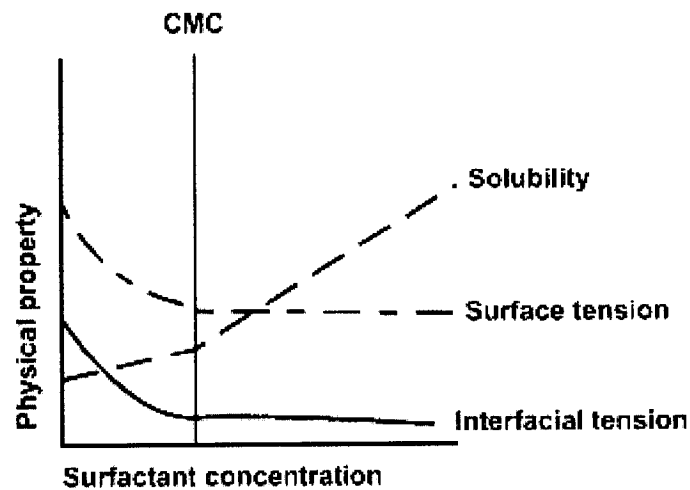


Figure 2.5 The relationship between surfactant concentration, solubility, surface tension and interfacial tension (Mulligan 2005).

Surfactant monomers have lower solubility than when they group to form micelles; however, when the CMC is reached, adding more surfactant will not further decrease the surface tension. To enhance a surfactant's dispersion capabilities however, a concentration higher than the CMC is required, to generate large concentrations of micelles (Holakoo 2002).

Surfactants have the ability to aggregate as micelles once their critical micelle concentration is reached. Through micelle formation hydrophobic compounds are partitioned into the hydrophobic core of the micelle, thus dispersing them beyond their solubility limit in the solution (Sarkar et al. 1989; Falatko 1991; Pennell et al. 1993). Enhanced solubility increases the bioavailability, and subsequently the biodegradation of hydrophobic compounds (Rouse et al. 1994; Miller 1996; Deshpande et al. 1999). At

levels above their CMC surfactants also prevent formation of water-in-oil emulsions. This is achieved by creating a protective film layer around the oil droplet that increases the surface viscosity of the droplets and prevents oil droplets from coalescing together (Schramm 2000).

Surfactants can be judged based on their ability to reduce surface tension (effectiveness) as well as their potential to obtain low critical micelle concentration (efficiency) (Mulligan 2005). Effectiveness or ability to lower surface tension, according to Rosen (1978) is “a measure of the surface free energy per unit area required to bring a molecule of oil phase to the water surface”. For instance, a desirable surfactant can lower the surface tension of water from 72 to 35 mN/m. A surfactant is considered efficient if it has a low CMC level, in other words, less surfactant is needed to decrease the surface tension of a solution (Mulligan 2005).

Surfactants are categorized based on their charge type as well as their hydrophilic-lipophilic balance (HLB). Like many other properties a surfactant's charge types are determined by the hydrophilic head group (Bai et al. 1997) and are grouped as nonionic, anionic, cationic and zwitterionic or dual charged (West and Harwell 1992; Clayton et al. 1993).

Another parameter used to characterize surfactants is hydrophile-lipophile balance (HLB). The HLB is a coding scale of 0 to 20 that indicate the tendency of the surfactant molecule to be soluble in either water or oil (Clayton et al. 1993). Surfactants with a lower HLB value are more lipophilic surfactants (HLB between 3 to 6) and therefore tend to make a water-in-oil emulsion (mousse), while those with a high HLB are more

hydrophilic surfactants (HLB values between 8 to 18) and tend to make an oil-in-water emulsion. Most common surfactant formulations have a HLB range of 9-11, having been shown by experiment to produce more stable oil in water emulsions (Clayton et al. 1993).

Surfactants are extensively used in industries that deal with two or more immiscible phases, such as in petroleum and pharmaceutical industries. In addition, they have been recently used in environmental applications, especially in the decontamination of soils. The desirable properties of surfactants are surface and interfacial tension reduction, solubility enhancement, wettability, toxicity reduction, and foaming capacity (Mulligan and Gibbs 1993; Mulligan 1998; Holakoo 2002).

Examples of their use in environmental application include emulsification of oils in marine waters (Lin and Lin 1995; Becker and Lindblom 2005), enhanced biodegradation of phenanthrene in soil (Yang et al. 2002), and soil washing (Zhang 1998). However, the use of chemical surfactants in environmental applications has been hindered by their persistence to degradation, toxicity, and unavailability of micellized compounds (Mulligan et al. 2001; Cort et al. 2002).

2.7 Biosurfactants

Biosurfactants are surfactants that are naturally produced by microorganisms, such as yeast, bacteria, and fungi from various organic material including oils, sugars, alkanes, and wastes (Mulligan 2005). The biosurfactants molecule contains a hydrophilic portion, which can consist of a mono-, di-, or polysaccharide, amino acid, peptide, carboxylate or phosphate group, and a hydrophobic portion, which is comprised of a saturated or unsaturated hydroxylated fatty acid or fatty alcohol group (Lang and Wullbrandt 1999).

Like their synthetic counterparts, biosurfactants have a tendency to concentrate at interfaces (vapor/liquid, liquid/liquid or solid/liquid), increase the surface areas of insoluble compounds, and consequently increase their mobility, bioavailability, and biodegradation.

Based on their molecular structure biosurfactants can be grouped as glycolipids, lipopeptides, phospholipids, fatty acids, and neutral lipids (Mulligan, 2005). They are also categorized by their electrostatic charge, which is dictated by the hydrophilic head (Urum and Pekdemir 2004). Only a few cationic biosurfactants are identified and the rest are either anionic or neutral (Mulligan 2005).

Biosurfactants have the benefits of surfactants as well as the advantage of being biodegradable and nontoxic (Zhang and Miller 1992). Other advantages include high selectivity and the potential to be used under conditions of extreme pH, temperature, and salinity (Desai and Banat 1997). Biosurfactants can be produced in-situ or ex-situ, and can be recovered and recycled (Moran et al. 2000). Biosurfactants have CMC values which range from 1 to 200 mg/L and are capable of reducing the surface tension of distilled water from 72 to 30 mN/m (Lang and Wagner 1987; Desai and Banat 1997).

They have various uses in the oil, textile, cosmetic, food, and pharmaceutical industries, and in the area of environmental applications as well (Healy et al. 1996). Environmental applications of biosurfactants include ex-situ and in-situ biodegradation of oil contaminated shore lines from the Exxon Valdez oil spill (Bragg et al. 1990; Harvey et al. 1994), mobilization and removal of hydrocarbon (Shabtai et al. 1985; Scheibenbogen et al. 1994), metal (Miller 1995), and PCB (Van Dyke et al. 1993) contaminants from soil,

and dispersion and biodegradation of hydrocarbon mixtures in waters (Atlas and Bartha 1992; Zhang and Miller 1992; Atlas 1993; Dixon 1993; Bertrand 1994).

2.7.1 Rhamnolipid

Rhamnolipids are a class of glycolipid biosurfactants produced from *Pseudomonas aeruginosa* bacteria, which is ubiquitous in many different environments including water, soil, and plants. Rhamnolipids are among the best-studied and most effective biosurfactants, and at the CMC have a characteristic surface tension of 29 mN/m (Itoh and Suzuki 1972; Mulligan 2005).

Although seven homologues of rhamnolipid are known to be produced by *Pseudomonas aeruginosa*, the primary constituents of rhamnolipid mixtures produced in liquid cultures are mono-rhamnolipid and di-rhamnolipid. Mono-rhamnolipid contains one rhamnose connected to a β -hydroxydecanoic acid and di-rhamnolipid contains two rhamnoses attached to the same fatty acid (Figure 2.6).

Various studies have investigated the effect of rhamnolipids on mobilization and biodegradation of organic contaminants and petroleum hydrocarbons. Zhang and Miller (1992) demonstrated that a concentration of 300 mg/L of rhamnolipids increased the mineralization of octadecane from 5% for the control to 20%. Also, Maier and Soberon-Chavez (2000) determined that rhamnolipid addition can enhance biodegradation of hexadecane, octadecane, n-paraffin, and phenanthrene in liquid systems. Beal and Betts (2000) stated that the rhamnolipids increased the solubility of hexadecane from 1.8 to 22.8 mg/L. Other studies indicated that rhamnolipids with the addition of fertilizers

improved biodegradation of aromatic and aliphatic compounds in the aqueous phase and soil reactors (Churchill et al. 1995).

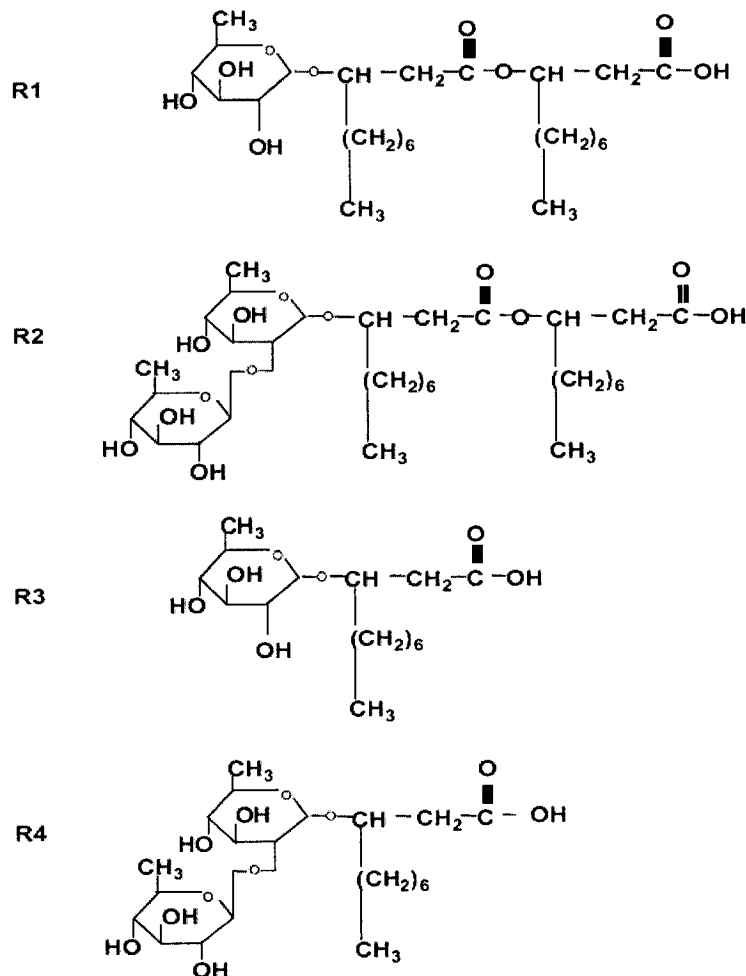


Figure 2.6 Different structures of rhamnolipids produced by *Pseudomonas aeruginosa* (Mulligan 2005)

In more recent studies, rhamnolipid exhibited a promising effect with respect to extracting copper from a low-grade ore (Dahrazma and Mulligan 2004) and pentachlorophenol (PCP) contaminated soil (Mulligan and Eftekhari 2003). Holakoo (2002) examined the potential of rhamnolipid for dispersion and remediation of oil slicks

as well as the effect of different solvents and additives on stabilization of rhamnolipid dispersed oil in saline water. Furthermore, Dagnev (2004) compared the effect of rhamnolipid with a chemical surfactant (Corexit 9500) on crude oil dispersion and biodegradation in saline waters.

2.8 Bioremediation Agents

U.S. EPA has defined bioremediation agents (biological agents) as microbiological cultures, enzyme additives, or nutrients that increase the rate of natural biodegradation in various ecosystems and under a wide range of environmental conditions (Zhu et al. 2004). Depending on the type of application bioremediation agents can also be referred to as bioaugmentation agents and biostimulation agents (Hoff 1993). Currently there are 16 bioremediation agents on the U.S. EPA National Oil and Hazardous Substances Pollution Contingency Plan (NCP) product schedule (Nichols 2001; USEPA 2002). However, appearance on this list does not necessarily mean that these products are approved or certified for use on an oil spill by EPA, and only implies that they are safe and effective under the Bioremediation 28-Day Effectiveness Test (NETAC 1993). This test method compares the extent of biodegradation of artificially weathered crude oil in natural seawater with and without a product under laboratory conditions.

Examples of field application of biological agents include bioaugmentation of contaminated shorelines (Rosenberg et al. 1992) and coastal wetlands (Aldrett et al. 1997a) as well as the biostimulation of coastal shorelines (Swannell et al. 1999), river shorelines (Venosa et al. 2002), coastal water (Sendstad 1980; Venosa et al. 1996; Prince et al. 1999) and soils (Alleman and Foote 1997; Zwick 1997).

2.9 Oil Spill Response in Marine Environment

Strategies for oil spill cleanups vary according to type and amount of spilled oil and characteristics of spill site. Numerous technologies have been developed and assessed for cleanup of oil spills in marine environments, and researchers have described in detail these technologies in a number of reviews, including Doerffer (1992), NOAA and API (1994), U.S. EPA (1999), and Cormack (1999).

Remediation methods are classified as either containment or treatment methods. Containment methods include the isolation, control, and stabilization of the spilled oil to prevent its movement and/or reduce its hazardous effects, whereas treatment methods destroy or remove them. Remediation technologies can also be grouped as ex situ or in situ methods. An ex situ method usually is more expensive and involves excavation and removal of the contaminants and/or contaminated media to a controlled environment for containment or an aggressive treatment. In situ technologies, on the other hand, remove the contaminants in place without pumping, or moving the contaminated media to other locations. In situ methods are less expensive but require longer treatment times (EPA 2004).

Detailed description of all the methods applied to oil spill cleanups beyond the scope of this dissertation, therefore, only a few common methods will be highlighted. These methods are divided into natural, physical, chemical, or biological methods.

2.9.1 Natural Methods

Natural attenuation is a method that uses natural processes, including evaporation, photo-oxidation, biodegradation, and other weathering phenomena (section 3.2) to remove and

degrade oil without any further action. This method is a cost-effective method; however, it is slow and requires a complete and intensive monitoring program to evaluate the extent and effects of removal (Zhu et al. 2001). Furthermore, natural methods are only applied to small spills in remote locations when there is no immediate threat to the ecosystem or humans (OTA 1990).

2.9.2 Physical Methods

According to US EPA (2006b) methods that contain and remove the spilled oil by physical means such as booms, barriers, sorbents, and skimmers are the first response and most widely used treatment methods in the cleanup of marine and freshwater shorelines. The disadvantages of physical methods are that they usually generate contaminated wastes and can only recover 10 to 15% of a major oil spill (Zhu et al. 2001). Commonly used physical methods include:

Booms and skimmers: Booms are floating devices that contain and control the movement of floating oil for later recovery by skimmers (Zhu et al. 2001; USEPA 2006). The major limitation of this method is that the collected oil waste at the end of each cycle has to be disposed of (Clayton et al. 1993).

Sorbents: Sorbents are sponge-like hydrophobic materials that wipe up oil from the contaminated surface. They can be natural organic, natural inorganic, or synthetic. Natural organic sorbents consist of peat moss, straw, hay, sawdust, ground corncobs, feathers, and other carbon-based products; natural inorganic sorbents include materials like clay, perlite, vermiculite, glass wool, sand, or volcanic ash; and synthetic sorbents

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are made of polyurethane, polyethylene, and polypropylene (USEPA 2006b). Again, disposal of the generated waste can be problematic.

2.9.3 Chemical Methods

Chemical methods, involve the use of chemical material, particularly dispersants, to break up and/or stabilize the oil spill in surface waters. These additives also increase the oil-water surface area, thus, enhancing the oil biodegradation rate. This process also reduces the toxicity of oil by diluting it. However, there are doubts regarding the effectiveness and long-term environmental effects of chemical additives.

The effectiveness of chemical treatment methods is influenced by the oil composition, the type, application method and quantity of dispersant, degree of weathering, temperature, water salinity, and thickness of the slick (Clayton et al. 1993; EPA 1999). The application method is by spraying the dispersant onto the slick by airplane or ship. The amount of dispersant used is related to the quantity and quality of the spilled oil; this factor is referred to as the dispersant-oil ratio (DOR), and varies according to the type of oil, type of dispersant, degree of weathering, temperature, and the oil slick thickness, and is one of the most important factors in the successful application of dispersants (EPA 2001).

2.9.4 Bioremediation Method

Bioremediation can be simply defined as the use of any material to enhance the natural biodegradation process (OTA 1990). This can include improving accessibility of materials, such as electron acceptors or nutrients as well as providing specific microorganisms (EPA 1993; Eweis 1998). Bioremediation is based on the fact that a wide range of indigenous microorganisms present in various environments such as soil,

groundwater and marine water can break down and remove a large percentage of oil components (Atlas 1993). These microorganisms catabolize or breakdown hydrocarbons, which serve as substrates, providing the cell with carbon, energy, and other nutrients required for growth (White et al. 2005).

These applications can be grouped into two main approaches as follows (Lee and Merlin 1999):

Bioaugmentation: or seeding is the addition of oil-degrading species or genera to increase the spectrum and population of the existing microbial community. This is when the indigenous soil microorganisms do not have the metabolic capability or necessary population to perform the required remediation (Leahy and Colwell 1990; Roane et al. 2001). The added microorganisms can either be cultured natural microbial strains or genetically engineered variants; however, they should not have pathogenic properties or produce toxic by-products (Maier et al. 2000).

Biostimulation: is the stimulation of the growth of the indigenous oil degrading microorganisms with improving availability of nutrients or co-substrates and/or alternations in the environmental condition to provide favourable conditions, such as an increase in available electron acceptors (Lee and Merlin 1999; Zhu et al. 2001). Spilled oil in general creates an imbalance in the ratio of carbon to nitrogen and phosphorus at the spill site. This is called nutrient limitation and can reduce the rate of natural biodegradation processes but can be eliminated by adding additional sources of nitrogen and phosphorus (Maier et al. 2000; Xia et al. 2006).

Both laboratory studies and field tests have shown that bioremediation, particularly the biostimulation process, can enhance oil biodegradation on contaminated shorelines (Prince 1993; Swannell et al. 1996). Also, in several recent field studies, it was shown that biostimulation is a more effective approach because the addition of hydrocarbon degrading microorganisms did not prove to be more effective than simple nutrient addition (Venosa et al. 1996; Lee and Merlin 1999).

Bioremediation has several advantages and disadvantages over conventional technologies.

Advantages: First and foremost, biodegradation is the only process that can eliminate oil compounds into environmentally friendly by-products, eventually to carbon dioxide and water, using only indigenous microorganisms. Bioremediation is based on natural processes and therefore is less intrusive and disruptive to the ecosystem of the contaminated site. Another advantage is that relative to other applications, bioremediation is cost effective. Finally, bioremediation can be applied in-situ without the need for transporting contaminated media, thus further reducing the cost (Zhu et al. 2001).

Disadvantages: Bioremediation like other technologies also has its limitations. The first disadvantage is that bioremediation can be only effective on the biodegradable compounds, and certain oil fractions are not biodegradable or have extremely low degradation rates (Vidali 2001). Secondly, bioremediation strongly depends on having the appropriate microorganisms in place under suitable environmental conditions, and in marine waters, providing some of optimum conditions, such as temperature and pH, can be problematic if not impossible (Zhu et al. 2001). Finally, compared to other removal

techniques, bioremediation is slower and immediate cleanup may not be feasible (Vidali 2001; Zhu et al. 2001).

2.10 Rationale

As previously mentioned, natural attenuation can be used only under strict conditions, physical methods are proven for the most part ineffective, and there are concerns regarding toxicity of chemical dispersants. Following the successful removal of Exxon Valdez spill with the aid of microbial degradation, bioremediation has been accepted as one of the most promising secondary treatments for oil removal (Bragg et al. 1994; Prince et. al., 1994). However, the rate of natural bioremediation is very slow; therefore, additives are used as supplements to enhance the speed and extent of this process.

In this regard, research was conducted on development of dispersants, nutrient additives, microorganism consortium, etc. to stimulate natural biodegradation (Aldrett et al. 1997; Haines et al. 2005; Xia et al. 2006). More specifically, Holakoo (2002) examined the effect of rhamnolipid on dispersion of crude oil in saline water. Dagnev (2004) continued the research to evaluate enhanced biodegradation of light crude oil supplemented with the rhamnolipid and/or two biological agents and found promising results with all three products. The overall objective of this study was to expand research in this subject and further evaluate the efficiency of two commercial bioremediation products and rhamnolipid biosurfactant on biodegradation of three oil types of diesel fuel, light crude, and heavy crude oil under laboratory conditions.

3 MATERIALS AND METHODS

This chapter describes the materials, methods, and experimental set-up used in this investigation, as well as outlines the analysis and interpretation of data. Lists of chemicals and equipment are given in their appropriate section. If not otherwise stated, chemicals were purchased from Fisher Scientific and distilled water was prepared in the lab. The biodegradation tests follow USEPA's bioremediation agent effectiveness test (USEPA 1996) and it was designed to determine the product's ability to biodegrade oil by quantifying changes in the oil composition resulting from biodegradation. Microbiological analysis was also performed to determine and monitor the viability of the microbial cultures being studied.

3.1 Materials

3.1.1 Test Oils

Three oil samples, light crude oil, heavy crude oil, and diesel fuel were used in this study. All originated from Mexico and were obtained from PETRO-Canada. All of them are low in sulphur content but no further information, such as density or fractional composition was provided.

3.1.2 Saline Water

Seawater salinity is generally between 34 to 36‰ (Stowe 1983). For this study synthetic seawater with salinity of 35‰ (or 35 g/L) was prepared by dissolving commercially available sea salt in distilled water according to the USEPA standard method (1996). The pH of the mixture was then adjusted to 7.7 with 1M solutions of sodium hydroxide

(NaOH) and/or hydrochloric acid (HCl), and allowed to equilibrate at ambient temperature ($22 \pm 2^\circ\text{C}$).

3.1.3 Rhamnolipid Biosurfactants (JBR 425TM)

Rhamnolipid biosurfactant JBR 425TM is an anionic glycolipid biosurfactant in aqueous solution with a rhamnolipids concentration of 25%. It is produced from sterilized and centrifuged fermentation broth of *Pseudomonas aeruginosa* soil-borne bacterium (Jeneil Biosurfactants Co. 2002; USEPA 2006c).

The rhamnolipid molecules are comprised of a fatty acid tail with either one or two rhamnose (sugar) rings at the carboxyl end of the fatty acid. Two major rhamnolipids are mono-rhamnolipid (RLL or R1) with the formula of $\text{C}_{26}\text{H}_{48}\text{O}_9$ and molecular weight of 504 and, di-rhamnolipid (RLLL or R2) with the formula of $\text{C}_{32}\text{H}_{58}\text{O}_{13}$ and molecular weight of 650 (Figure 3.1). The physical properties of JBR 425TM are summarized in Table 3.1.

Table 3.1 Physical properties of JBR 425TM (Jeneil Biosurfactants Co. 2002)

Appearance	Amber solution
Odour	Soapy
Specific Gravity	1.05-1.06
pH	6.5-7.0
Solubility in Water	Soluble at neutral pH
Suitable Diluent	Water and most alcohols

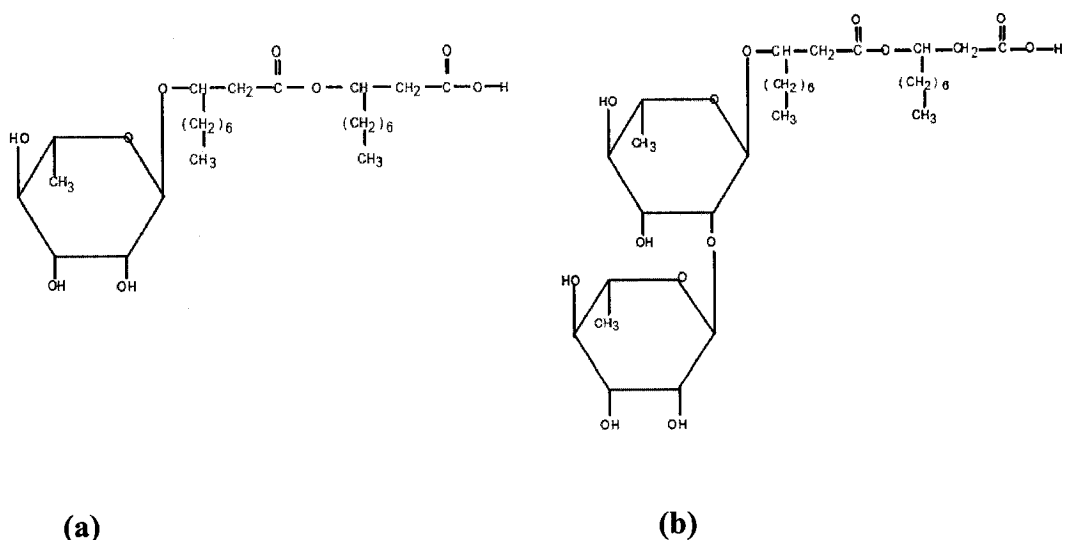


Figure 3.1 Rhamnolipid structure (a) R1 (b) R2 (Jeneil Biosurfactants Co. 2002)

3.1.4 Biomor A.S.A.P Floor Degreaser™

The Biomor ASAP Floor Degreaser™, designated as ASAP™ in this text is a floor degreaser for food service applications. This product is a proprietary blend of surfactants, (tetrasodium ethylenediaminetetraacetate, sodium dodecyl sulfate (SDS)) nutrients, and bio-cleaning agents. ASAP™ can biologically clean grease, fat, oil, and other organic compounds on ceramics, concrete floors as well as resilient tiles. The product is environmentally friendly and biodegradable and does not contain any harsh or harmful chemicals (Avmor 2005a). Table 3.2 presents a summary of the physical and biological properties of ASAP™.

3.1.5 Biomor Industrial Degreaser and Concrete Cleaner™

The Biomor Industrial Degreaser and Concrete Cleaner™, referred to as Degreaser™ in this text, is a biologically formulated product that was originally designed to eliminate petroleum residue or other organic stains from manufacturing equipment, railway, bilges, vehicles, parking lots, soil as well as concrete and asphalt surfaces (Avmor 2005b).

According to Avmor (2005b), DegreaserTM contains a proprietary micro-organism consortium (5 strains) of non-pathogenic bacteria that degrades petroleum products and does not contain any petroleum distillates or solvents. DegreaserTM is environmentally friendly and biodegradable. Some of the physical and biological properties of this product are shown in Table 3.2.

Table 3.2 Properties of DegreaserTM and ASAPTM (Avmor 2005a, b)

Properties	Degreaser ^{TM†}	ASAP ^{TM†}
Physical state & appearance	Opaque white liquid	Opaque blue liquid
pH	10.4-10.7	7.5-8.0
Specific Gravity	1.0-1.01	1.0-1.01
Boiling point	100°C	100°C
Solubility	Easily soluble in cold and hot water	Easily soluble in cold and hot water
Microbial population	7.6 billion microbes per 3.78L, 5 types of strain	200 billion microbes per 3.78L, 5 types of strain

[†] Both products are part of the Domestic Substance List (DSL) with the Canadian government (Avmor 2005a, b).

3.2 Methods

3.2.1 Critical Micelle Concentration (CMC) Measurement

Critical micelle concentration of the rhamnolipid biosurfactant JBR 425TM was determined by measuring the surface tension of biosurfactant at various dilutions. Surface tension was measured with a Semiautomatic FISHER SCIENTIFIC Surface Tensiomat (Model 21). This instrument uses the duNoüy method, which measures the force necessary to separate a platinum-iridium ring from liquid, and has an accuracy of ± 0.25 mN/m. Various rhamnolipid concentrations (0.00001%, 0.0001%, 0.001%,

0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1.0%, 2.0%, 4.0%, 8.0%) were prepared by diluting rhamnolipid biosurfactant JBR 425TM in pH adjusted (pH 7.5) distilled water. The value of the CMC was determined from the inflection point of the graph of surface tension versus the logarithm of the surfactant concentration.

3.2.2 Weathering the Oil

All oils used in this study were artificially weathered by leaving them inside a fume-hood for 72 hours at room temperature ($22 \pm 2^\circ\text{C}$). The main propose of weathering the oil was to stabilize the oils by removing the light hydrocarbons prior to the start of the experiments. Moreover, weathered oil better mimics in situ conditions since in actual oil spills the light fractions are lost due to evaporation.

3.2.3 Experimental Design and Sampling Procedure

The experiment is designed according to the modified USEPA method of the bioremediation agent effectiveness test (USEPA 1996). A 28 day experiment was performed to determine the biodegradation effectiveness of rhamnolipid biosurfactant (JBR 425TM) and two biological agents (ASAPTM, DegreaserTM), individually or in various combinations, for the different oils. Biosurfactant and biological agents were tested under the hypothesis that the addition of these agents will stimulate oil biodegradation.

The experimental setup is comprised of series of 250 mL Erlenmeyer flasks used as reactors. Each batch was prepared by adding 100 mL of synthetic sea-water and 0.2 g of one of the three weathered oil samples. Twelve sets of additives were prepared and added to the flasks according to a designated ratio and/or concentration detailed in Table 3.3.

Flasks were covered with foam stoppers to prevent entry of dust and micro-organisms, while facilitating aeration. Flasks were carried upright, and carefully secured in flask holders on a New Brunswick Scientific INNOVA orbital platform shaker Model 2000 (Figure 3.2) to minimize the amount of oil that might stick to flasks wall prior to shaking. Any flasks with a significant amount of oil splashed on their sides were rejected and prepared anew. To maintain aerobic conditions, as well as simulate sea-water movement, flask contents were swirled at 200 rpm on the orbital shaker throughout the experimental period. Furthermore, to minimize the effect of photodegradation and temperature variations, the shaker was placed inside an incubator (Fisher Scientific Isotemp[®] Incubator model 304) set to $20 \pm 0.5^{\circ}\text{C}$.

Each treatment batch was sampled at four separate intervals of 0 (10 minutes), 3, 7, and 28 days, at which time flasks were completely sacrificed and extracted. Before extraction, 0.5 mL of the aqueous phase were removed from the middle of the flask for microbiological analysis. The remaining contents were moved and transferred into a 250 mL separatory funnel to extract the oil from the oil-water-biomass mixture.

The experiments comprised two distinct stages:

Stage 1: The effect of individual biosurfactant and biological agent additives, using different additive:oil ratios, on oil biodegradation: Based on the recommendations of previous work rhamnolipid concentrations of 2% and three different DORs (1:4, 1:2, 1:1) were selected for investigation (Holakoo, 2002; Dagneu, 2004). Dagneu (2004) also found that the best solvent for diluting the rhamnolipid biosurfactant JBR 425TM was pH adjusted (pH 7.5) distilled water. The outlines of the selected treatments are shown in Table 3.3.



Figure 3.2: Test flasks on INNOVA orbital shaker

Based on the results from that same study, biological agent to oil ratios, which is referred to as the additive to oil ratio (additive:oil) in this research, of 1:4 and 1:8 were selected for the ASAP™ and Degreaser™ products (Table 3.3).

Stage 2: Effect of combination of biosurfactant, and biological agents with different additive to oil ratios on oil biodegradation: After evaluating individual biodegradation efficacy, the effects of adding rhamnolipid to the biological cleaning agents, again using different additive:oil ratios, were assessed. The products, and preferred combined treatments used in this phase, are summarized in Table 3.3.

Table 3.3 Biodegradation experimental procedures.

Treatment additive:oil ratio	Stage 1	Stage 2
	rhamnolipid [†] (1:4)	rhamnolipid (1:4) & ASAP (1:4)
	rhamnolipid (1:2)	
	rhamnolipid (1:1)	rhamnolipid (1:4) & Degreaser (1:4)
	ASAP (1:4)	
	ASAP (1:8)	rhamnolipid (1:4) & Degreaser (1:4) & ASAP (1:4)
	Degreaser (1:4)	
	Degreaser (1:8)	rhamnolipid (1:4) & Degreaser (1:8) & ASAP (1:8)
	Control	

[†] Rhamnolipid concentration used to prepare all ratio treatments was 2%.

3.2.4 Extraction Procedure

Since the extraction method of the USEPA bioremediation effectiveness test (1996) uses dichloromethane (DCM), which dissolves rhamnolipid, a modified version of USEPA standard method 1664A was used for the liquid/liquid extraction of the remaining oil in batch test flasks. This method uses n-hexane to extract organic material from water and wastewaters using a separatory funnel. The summary of the procedure is as follows.

After transferring the remaining flask contents into a 250 mL separatory funnel, the residual hydrocarbon in the Erlenmeyer was washed with 10 mL of Optima[®] n-hexane (Fisher Scientific, meets ACS specifications). The hexane was added to the funnel and the funnel was sealed using glass or Teflon stoppers. The separatory funnel was shaken vigorously for two minutes, with periodic venting to prevent pressure build up due to evaporating solvent. The mixture was then allowed to settle for 10 minutes until the two phases completely separated. The water was then eluted through the stopcock into the

250 mL Erlenmeyer flask and the hexane, with extracted oil, was poured into a designated beaker. This process repeated three times and the combined hexane extract was dried over 10 g of sodium sulfate (granular anhydrous ACS grade) and filtered thorough a Whatman No. 40 filter. The final volume of the hexane was adjusted to 50 mL and was transferred to an amber vial and stored in a refrigerator (maximum of 5 days) at 4°C until analysis.

3.2.5 Gas Chromatography Analysis

Hexane extracts of the samples, blanks, and standards were quantified for total petroleum hydrocarbon concentration by USEPA standard method 8015C using GC/FID. The gas chromatograph was a Varian 3800 GC equipped with a Varian CP-8400 AutoSampler and a flame ionization detector (FID). Separation was achieved using a DB-1 (J & W Scientific) high resolution capillary column coated with 100% dimethylpolysiloxane. The column was 30 meters long with an inner diameter of 0.25 mm and 0.25 µm film thickness capable of withstanding temperatures between -60 to 325°C.

Based on the pervious work by Dagnew (2004) and preliminary trials the following GC configurations were selected: the injection port was split/splitless on a splitless mode using 1 µL injection volume. The carrier gas was helium with a flow rate of 1 mL/min and makeup gas flow rate of 29 mL/min. Hydrogen and air flow rates were set at 35 and 400 mL/min, respectively. Both the injector and detector temperatures were kept constant at 250°C. Initial column oven temperature for each run was set and maintained at 50°C for 2 minutes, then increased to 250°C at a rate of 8°C/min, and finally held at 250°C for 6 minutes, for a total run time of 33 minutes. Hexane-extracted blanks contained seawater and treatment additives but no oil. Blanks were extracted and analysed using the same

procedure as treatment samples. Three point calibration curves were generated by preparing three different concentrations of each oil in saline water which were extracted and analyzed following the procedure delineated above (Appendix). Furthermore, the linearity of the response as well as the method extraction efficiency was determined by directly measuring the GC response to specific amounts of oil dissolved in hexane.

The quantitative analysis of the test samples was done using the Varian Star Chromatography Workstation Version 5.5 software. The software integrates the area under the chromatogram excluding the solvent peak as the total area for that sample. The TPH of each batch was calculated by subtracting the total area of the blank from the total area of each sample. The concentration of the TPH in the sample was determined by using the calibration curve of the known extracted standards.

3.2.6 Microbial Analysis

Variations in the population of heterotrophic bacteria were monitored using the spread plate method (method 9215C) from Standard Methods for the Examination of Water and Wastewater (APHA, AWWA, WPCF 1998). Serial dilutions of each sample were prepared by adding 0.5 mL of the aqueous phase removed from each flask to 4.5 mL of sterilized phosphate buffer adjusted to pH of 7.0, to prepare 1:10 dilutions. Serial dilutions were used to prepare dilutions of up to 10^{-9} .

The Difco™ R2A Agar (Fisher Scientific) media was prepared by dissolving 18.2 g in one litre of distilled water. The solution was sterilized in a Yamato Sterilizer SM300 autoclave at 121°C for 15 minutes and then 20 mL of the sterilized agar was transferred to 100x15 mm sterile disposable plastic petri dishes (Fisher Scientific) with sterile

disposable pipettes. After the agar had solidified the plates were turned upside down and dried by leaving them overnight in the biological hood (Forma Scientific Class II A/B3 Biological Safety Cabinet) at room temperature.

Duplicate plates of appropriate dilutions were prepared and using a sterile pipette to transfer 0.1 mL of each dilution to the plate which was then spread with flame-sterilized bent stainless steel rod. All plate manipulations were performed in the biological hood. The plates were incubated at 28°C, and after seven days colonies were counted and averaged on plates that contained between 30 to 300 colonies. The colony forming units (CFU) per mL of each sample were determined and used to calculate the CFU per mL of original flask liquid based on dilutions (APHA, AWWA, WPCF 1998).

3.2.7 Biodegradation Rate Kinetic Model

In order to evaluate the rate of biodegradation of the total petroleum hydrocarbon in the reactors, the first-order model was used as suggested and developed in the following studies: Simon et al. (2004), Page et al. (2002), Harris et al. (2002), Aldrett et al. (1997), Venosa (1996). For derivation of the model, the reactor mass balance was used in accordance with first-order reaction kinetics. The model was obtained by assuming the biodegradation process as the predominant source of oil loss in the reactors. Other oil loss processes, such as volatilization are assumed to be negligible since the oil was weathered prior to the start of the experiment. The photochemical oxidation process is also believed negligible because the experiments were taking place in an incubator in the dark and the extracted samples were stored in amber vials to reduce any photodegradation of oil.

3.2.8 Statistical Analysis

The results for the additive effectiveness were evaluated using two-way analysis of variance (ANOVA) which can determine data trends and evaluate the magnitude of any differences in microbial density or biodegradation within the various treatments. These analyses were performed using an Excel® environment. Also, the relationship between the microbial densities and percentage loss of the oils in various treatments was studied using a linear regression analysis in an Excel® environment.

4 RESULTS AND DISCUSSION

This chapter presents the oil biodegradation enhancement results, for the three petroleum samples, obtained using the three additives in various combinations: rhamnolipid biosurfactant alone, each commercial additive alone, and rhamnolipid biosurfactant in combination with each commercial additive. All supplements, alone or in combination, were added based on different additive:oil ratios.

4.1 Critical Micelle Concentration (CMC) Measurement

Figure 4.1 shows the semi-log plot of surface tension vs. surfactant concentration obtained for the JBR 425TM rhamnolipid formulation. The surface tension was found to decrease from 72 to a minimum of 29 mN/m, with increasing rhamnolipid concentration. Further increases in biosurfactant concentration did not decrease the surface tension below this minimum value, indicating that the CMC had been reached. Graphical interpolation gave a CMC value of 32 mg/L. Other investigators have reported similar values of 40 mg/L (Zang and Miller 1992) and 35 mg/L (Holkaloo 2002) for this biosurfactant.

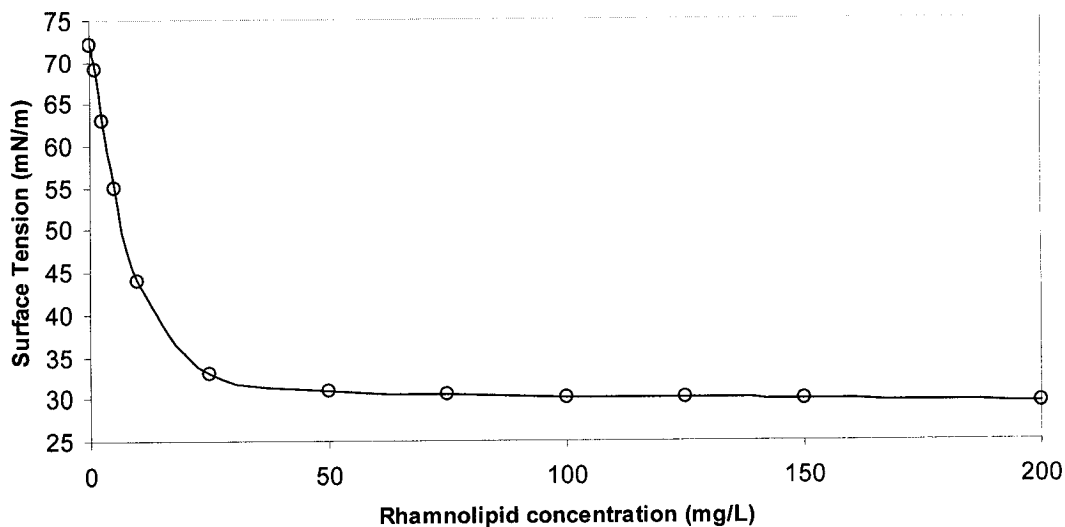


Figure 4.1 Critical Micelle Concentration determination for rhamnolipid JBR 425TM.

4.2 The Weathering of Oils

The three oils were artificially weathered by placing them in a fume hood for 72 hours at room temperature ($22 \pm 2^\circ\text{C}$). The average loss in volume after this weathering period was found to be 4.5, 12.9, and 27.9% for diesel fuel, heavy crude and light crude oils respectively. This decrease in volume is due to evaporation of the more volatile hydrocarbon components.

4.3 Oil Biodegradation Results

4.3.1 Effect of Rhamnolipid JBR 425TM on Oil Biodegradation

A. Total petroleum hydrocarbon reduction:

The effect on petroleum hydrocarbon reduction, as a result of amending oil in reaction flasks with rhamnolipid at dispersant to oil ratio (DOR)* of 1:1, 1:2, and 1:4, on the three

oils were evaluated at 0, 3, 7, and 28 days. The selection of the initial amount of oil in water for the reaction vessel as well as the DOR values was in accordance with a previous study (Dagneu 2004) which demonstrated that biodegradation of light crude oil was effective at a DOR of 1:4. The same study also indicated the possible direct correlation between enhanced oil biodegradation and increased amounts of added rhamnolipid. The results obtained for the three oil samples (diesel fuel, light crude oil, and heavy crude oil) are shown in Figures 4.2, 4.3, and 4.4. It can be seen that the oil-water control (without added rhamnolipid) exhibits the least reduction in petroleum hydrocarbons for all three oil samples when compared to those where biosurfactant was added. Also, a trend is clearly evident whereby increasing the relative level of rhamnolipid resulted in greater oil reduction. The highest percent of petroleum hydrocarbon removals were obtained for rhamnolipid (1:1): about 71% for light, 65% for diesel fuel, and 59% for heavy crude oil. To compare the significance of the treatment effects on oil removal when compared to the control, analysis of variance (ANOVA) was performed. Table 4.1 shows the results of this analysis. The statistical evaluation performed at $\alpha= 0.05$ demonstrated that there are significant differences at the 95% level of confidence between the oil removal efficiencies for all treatments and that for the control. This is inferred by *p*- values of less than 0.05 for each treatment.

*All DOR formulations were prepared using 2% rhamnolipid prepared by diluting JBR 425TM. Since higher concentration formulations are more expensive, it was decided to prepare all DOR formulations with a low concentration of surfactant in keeping with eventual cost benefit considerations.

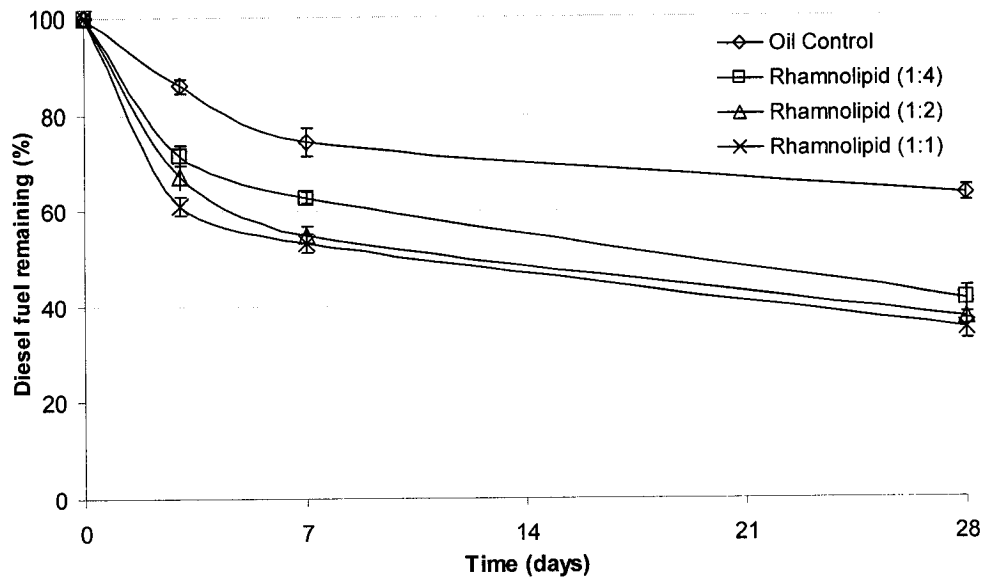


Figure 4.2 Effect of rhamnolipid concentrations on diesel fuel removal over time.

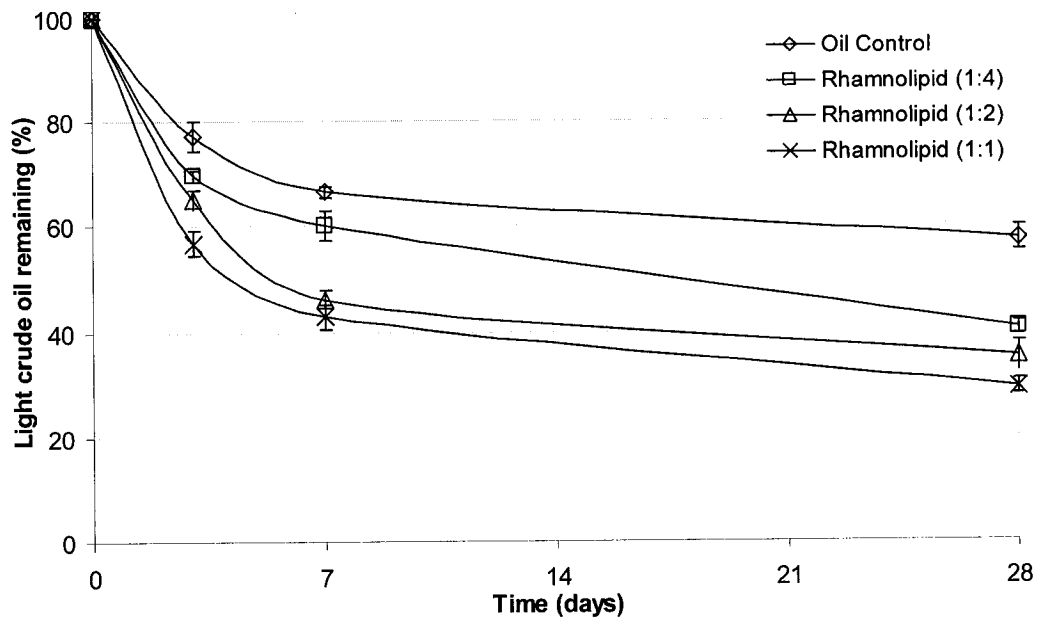


Figure 4.3 Effect of rhamnolipid concentration on *light crude oil* removal over time.

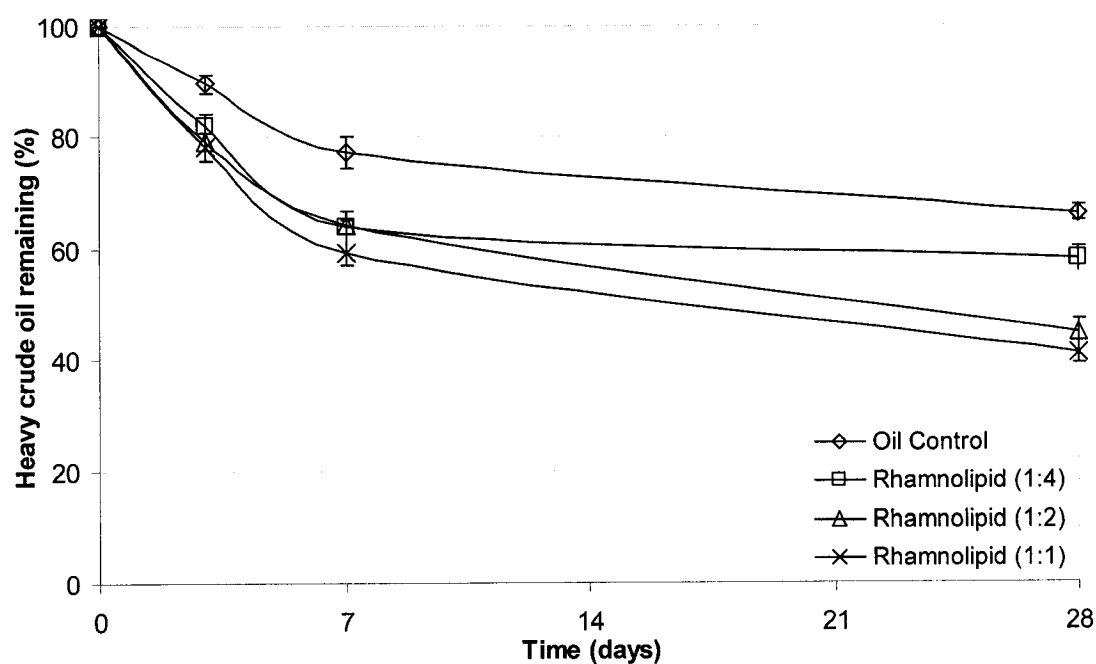


Figure 4.4 Effect of rhamnolipid concentration on *heavy crude oil* removal over time

B. Microbial growth:

Up to this point only, the removal of petroleum hydrocarbons has been discussed but not the means by which that removal occurred. Oil can be dispersed and mobilized into the water compartment through the application of surfactants; thereby, making it more available to microorganisms as a potential source of carbon. Microbiological analysis of the reactor flasks liquid phase, using spread-plate cultures, was performed to determine if increased oil removal was associated with a rise in microbial density. Figures 4.5, 4.6, and 4.7 give the results obtained for the microbiological analysis of control and treatment batches for diesel fuel, light crude, and heavy crude. Results showed that the control reactors had fewer colony forming units per plate than any surfactant treatment DOR for all three oils. Since no bacteria were added to the control batches, the origin of this population derives from an already existing microbial population in the oil sample and/or

that derived from contamination of samples through handling. Microorganisms are ubiquitous in the environment and amending oil with them is unnecessary in order to promote at least some biodegradation of oil, given the right chemico-physical conditions. It is clear from the results that the microbial populations in the control vessels, without added surfactant, were lower throughout the 28 day test period than any of the treatment vessels. Both control and treatment batches exhibited the highest plate counts after 28 days of incubation. The highest rates of microbial density increases were found to occur within the first three days of incubation.

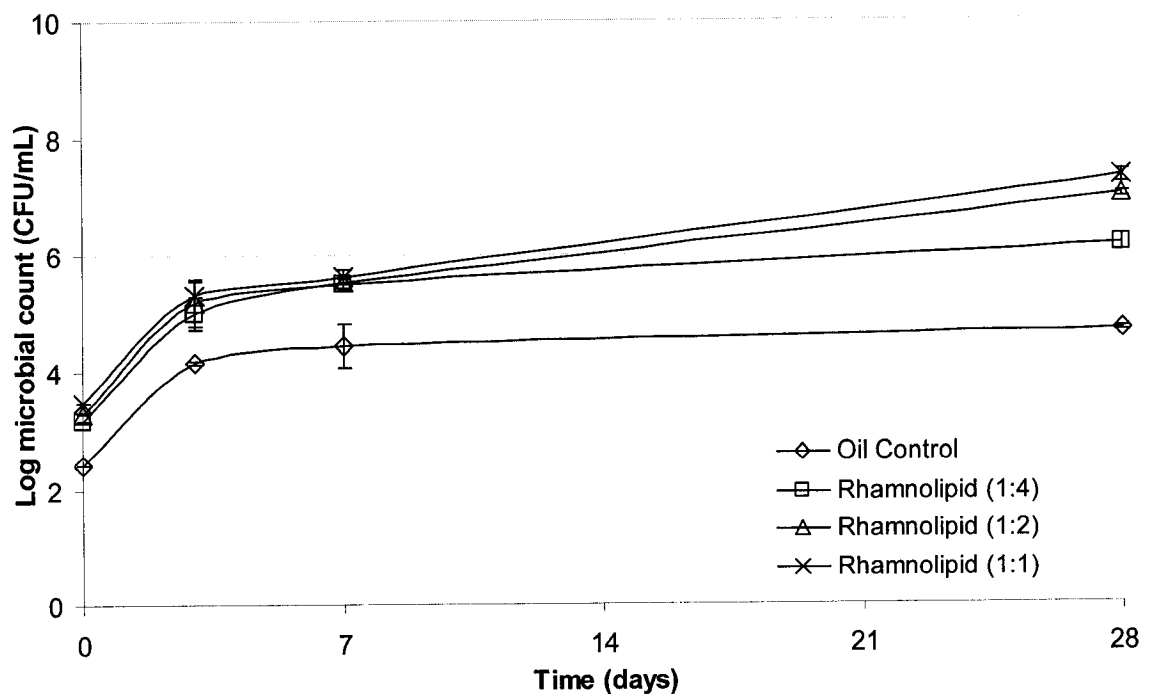


Figure 4.5 Microbial densities for *diesel fuel* over time

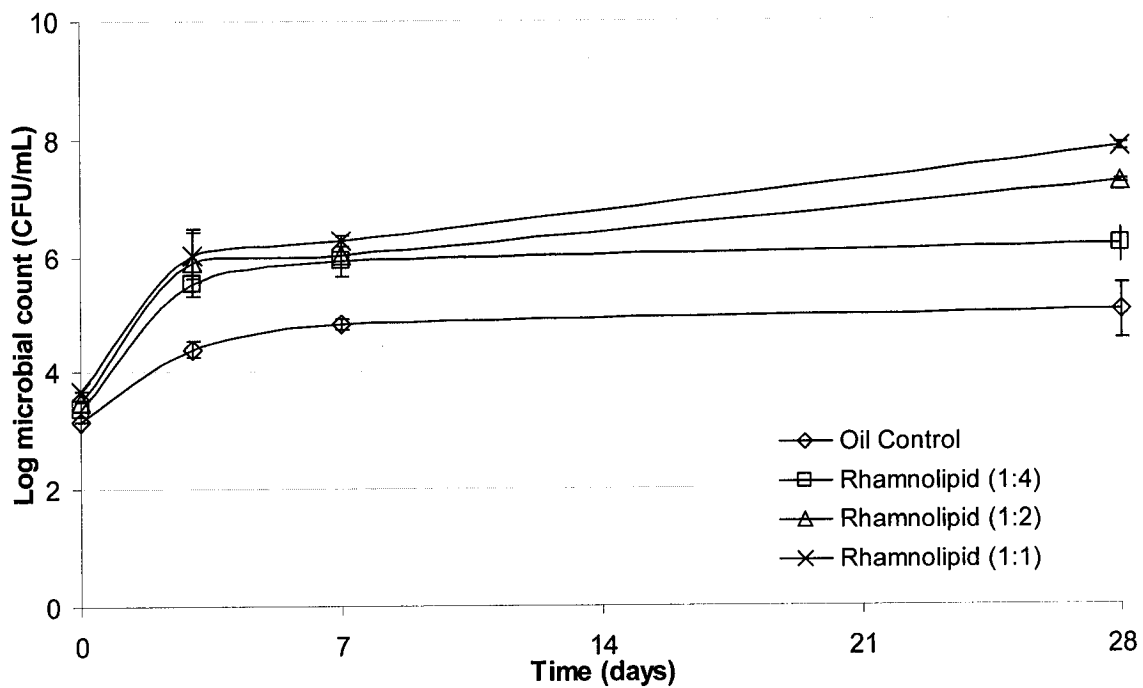


Figure 4.6 Microbial densities for *light crude oil* over time

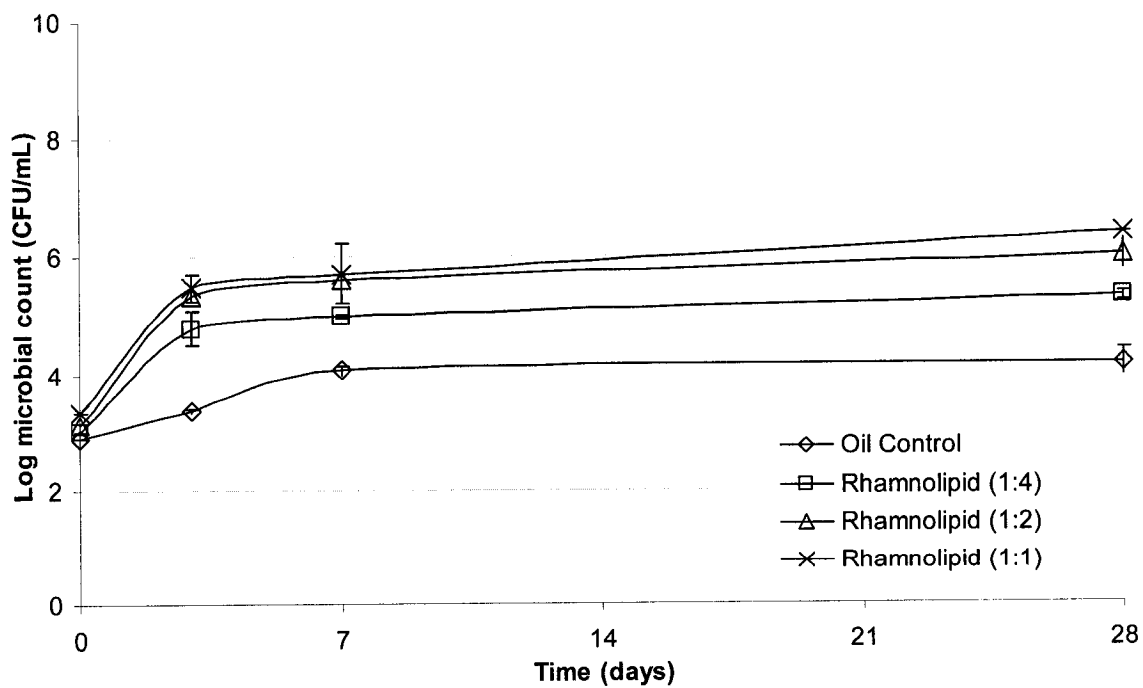


Figure 4.7 Microbial densities for *heavy crude oil* over time

In general, decreases in petroleum hydrocarbons corresponded to increases in microbial densities for both controls and rhamnolipid treatments, which strongly suggest biodegradation. Bacterial growth curves generally display dramatic microbial density increases, after a short lag phase, while substrate is abundant, in what is referred to as an exponential growth phase. As substrate is depleted and becomes limiting, with respect to the now larger microbial population, net microbial growth rate decrease to a point where the population density tends to remain stationary until further substrate depletion results in the beginning of a negative net growth phase. During this phase, endogenous metabolic activity is greater than substrate-based growth and biomass decreases (Maier et al. 2000). Both control and rhamnolipid treatment microbial density plots displayed a pattern similar to this.

The DOR 1:1 treatments, which resulted in the greatest removal in oil, were also consistently associated with highest microbial density estimates. Again, the observed enhanced oil removal efficiency would appear to be a result of increased biodegradation. Although added rhamnolipid is also a potential source of carbon, it accounted for a minute amount of the total available carbon in the batch tests. For example, in the highest rhamnolipid addition treatment, having a DOR of 1:1 prepared from a 2% rhamnolipid solution, the relative contribution of carbon from biosurfactant to that for TPH was at most 0.02:1 or 2%. Also, it should be noted that the CFU data was log transformed, so we are looking at about a 10 to 100 increase in microbial density differences between the control and the 1:4 ratio treatment.

It is impossible here to completely rule out partial loss of oil due to some physical phenomena, such as evaporation, which may have been enhanced as a result of

surfactant addition; however, the fact that weathered oil was used would seem to reduce at least that possibility. In addition, since the contribution of available carbon due to added rhamnolipid was negligible, and since increases in microbial density over control values, paralleled relative increases in TPH removal, biodegradation is more than strongly suggested. Any significant increase in abiotic removal of TPH would have resulted in an increase in TPH removal, but without the corresponding higher microbial densities. The increased loss of TPH is thus believed to be the result of enhanced biodegradation of TPH due to the dispersing action of the added rhamnolipid acting on oil, rendering it more biodegradable.

Also, the growth curves for microbial populations displayed early exponential growth followed by a stationary, or almost stationary growth phase, which is characteristic of bacterial growth on an available substrate, which subsequently becomes limiting. No carbon sources, other than oil, were available to support microbial growth in controls; which exhibited this characteristic growth pattern. It is clear that biodegradation does play a role here in oil removal, and that enhanced microbial growth was associated with an increase in oil removal efficiency. This increase in oil removal efficiency is in the end well related with biosurfactant addition.

One important observation is that although all three DOR treatments did result in significant increases in removal of TPH, when compared to control, the differences within the treatment series were not dramatic. The greatest percent difference in the removal efficiency between the 1:1 and 1:4 DOR treatments was about 10%; which, although significant does not reflect the fact that the amount of added surfactant differs by a factor of four between these treatments. This finding is significant with respect to

cost effectiveness, since rhamnolipid is somewhat more expensive than most commercial chemical surfactants. The lower DOR for effective treatment is the more cost effective.

4.3.2 Effect of Biological Agents on Oil Biodegradation

A. Total petroleum hydrocarbon reduction:

The effect on petroleum hydrocarbon reduction as a result of amending oil in batch tests with either of the two biological agents, ASAPTM and DegreaserTM, on the three oils was evaluated at 0, 3, 7, and 28 days, Figures 4.8, 4.9, and 4.10 shows the total petroleum hydrocarbon analysis for each oil using additive to oil ratios of 1:4 and 1:8. The products were used in their original concentrations without any dilutions to prepare the specified product to oil ratio. Both products showed reductions in TPH greater than that for controls. DegreaserTM added at a ratio of 1:4 had higher percentage removal when compared to its 1:8 addition ratio. It removed about 60% of the diesel and light crude oil but only about 40% of the heavy crude oil, indicating a removal preference for medium over heavier fractions. In general, DegreaserTM slightly outperformed ASAPTM; however, it exhibited a similar pattern in removal efficiency in that it was more effective in reducing diesel and light crude oil than heavy crude oil. ANOVA performed at $\alpha = 0.05$ for these additives (Table 4.1) also shows that oil removals, when compared to control values, were significant.

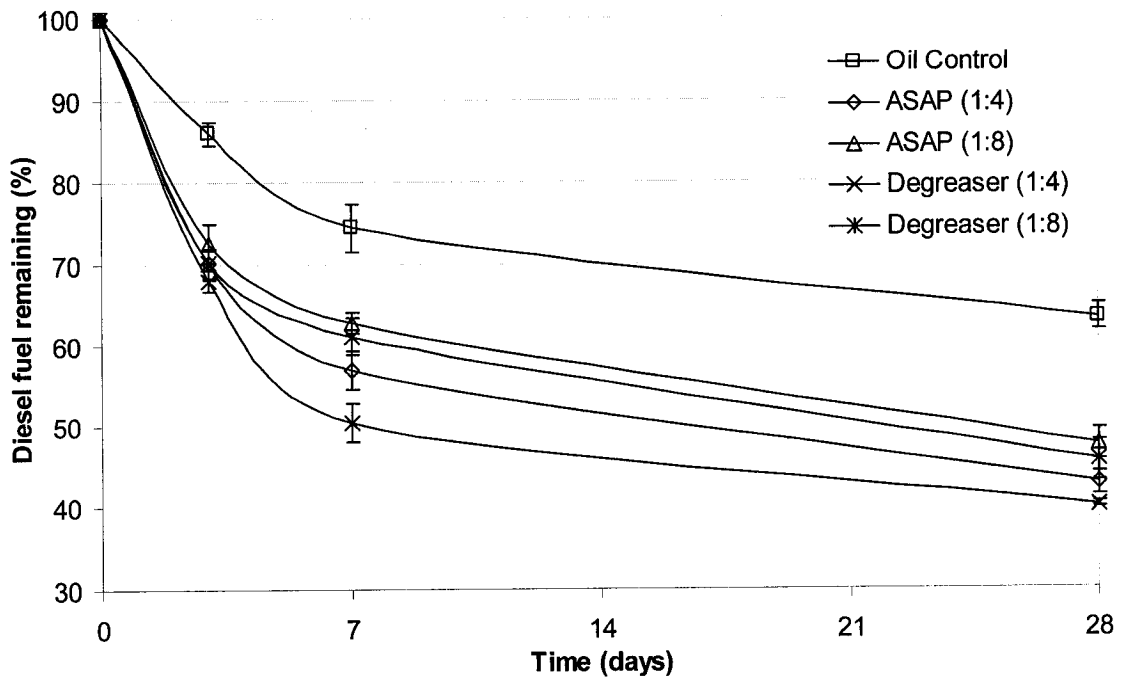


Figure 4.8 Comparisons of the performance of ASAPTM and DegreaserTM with different additive:oil ratios on *diesel fuel* reduction over time.

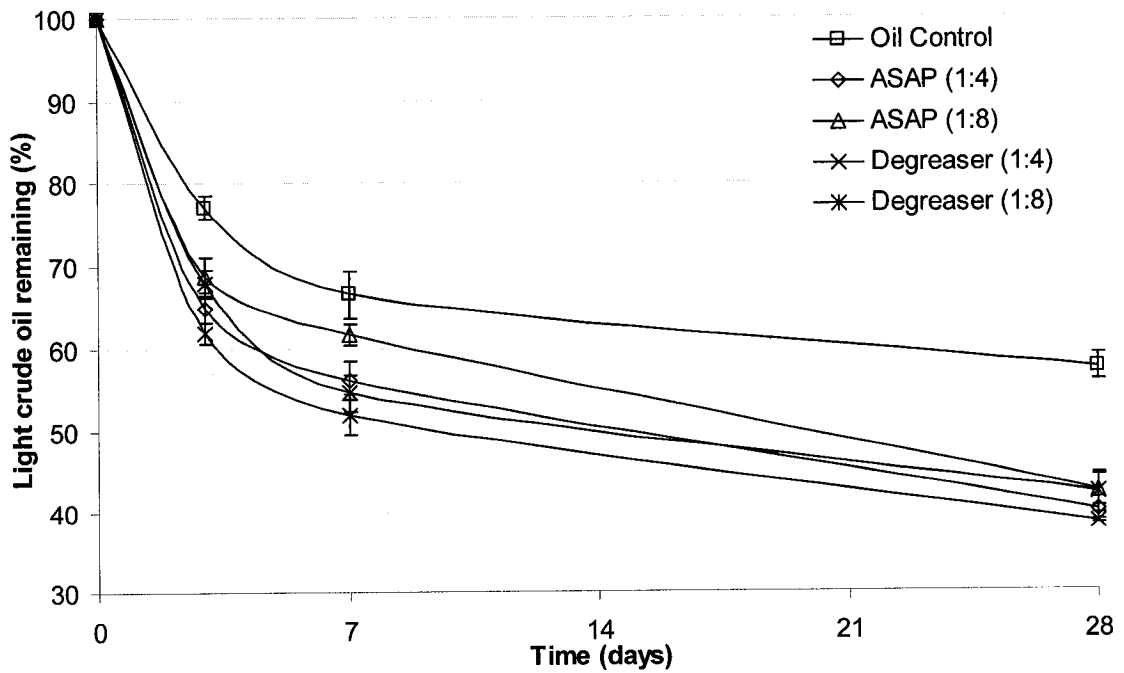


Figure 4.9 Comparisons of the performance of ASAPTM and DegreaserTM with different additive:oil ratios on *light crude oil* reduction over time.

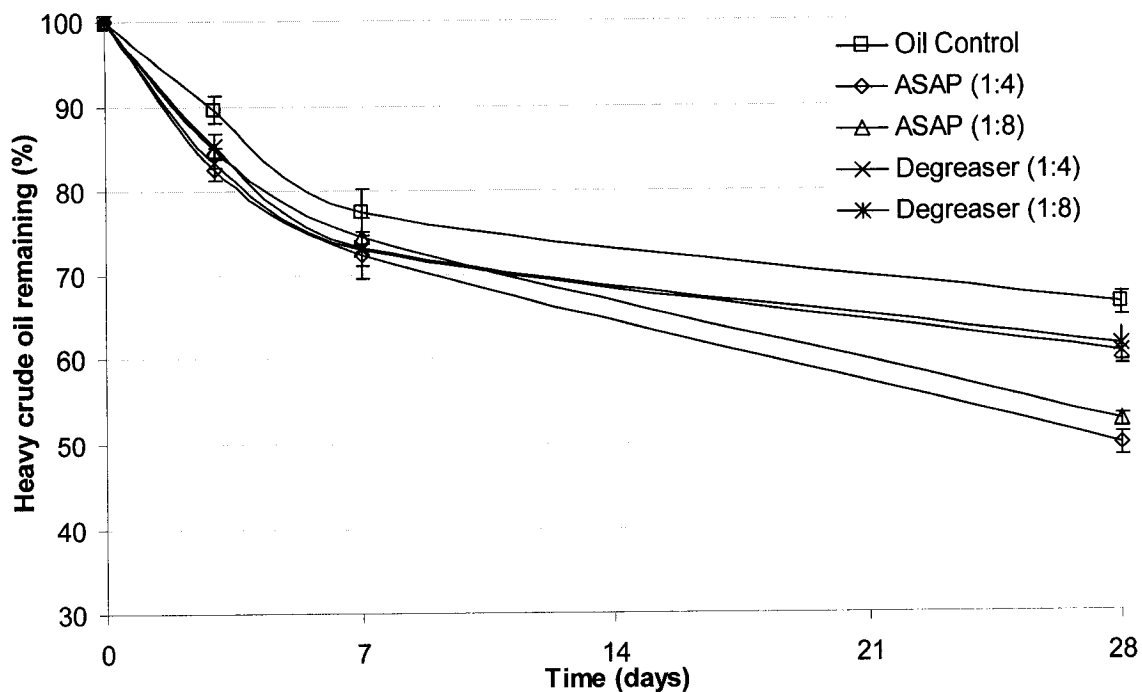


Figure 4.10 Comparisons of the performance of ASAPTM and DegreaserTM with different additive:oil ratios on heavy crude oil reduction over time.

B. Microbial growth:

The results of microbiological analysis of flasks treated with ASAPTM and DegreaserTM are shown in Figures 4.11, 4.12, and 4.13. In general, microbial density was consistently higher in those flasks product was added. However, unlike the rhamnolipid addition tests, the initial plate counts for the biological agent addition tests were much higher than control plate count. This is understandable since both agents contain bacterial populations to aid in the biodegradation of oils.

As with the rhamnolipid-amended flasks, the greatest increases in the microbial populations occurred between days 0 and 3, and then levelled off, almost approaching zero growth by day 28. This zero growth pattern is consistent with microbial growth on a limited substrate supply. From respective TPH removal and microbial growth data, it is

believed that the biological agents also increased TPH removal through enhanced biodegradation. It is possible that some abiotic losses might be masked in the biological agent tests by supplying extra substrate, which might result in an increase in microbial density, not accounted for by TPH biodegradation. However, this seems unlikely. Again, the only logically conceivable possibility for such loss is evaporation, and as mentioned earlier the oils were weathered and stripped of most volatile material. In addition, removal of TPH matches microbial density increases, for with exponential increases in microbial growth during the first 3 days a corresponding sharp decrease in TPH is observed. In addition, as the rate of microbial growth begins to level off the rate of removal of TPH also evens out.

Thus, rhamnolipid as well as the two commercial biological agents seem capable of improving the natural ability of oil to biodegrade in a marine environment. The TPH removal efficiencies for all three were comparable, as an inspection of the total 28 day %TPH removal for each test shows (Table 4.1) with rhamnolipid apparently being slightly more efficient as already mentioned.

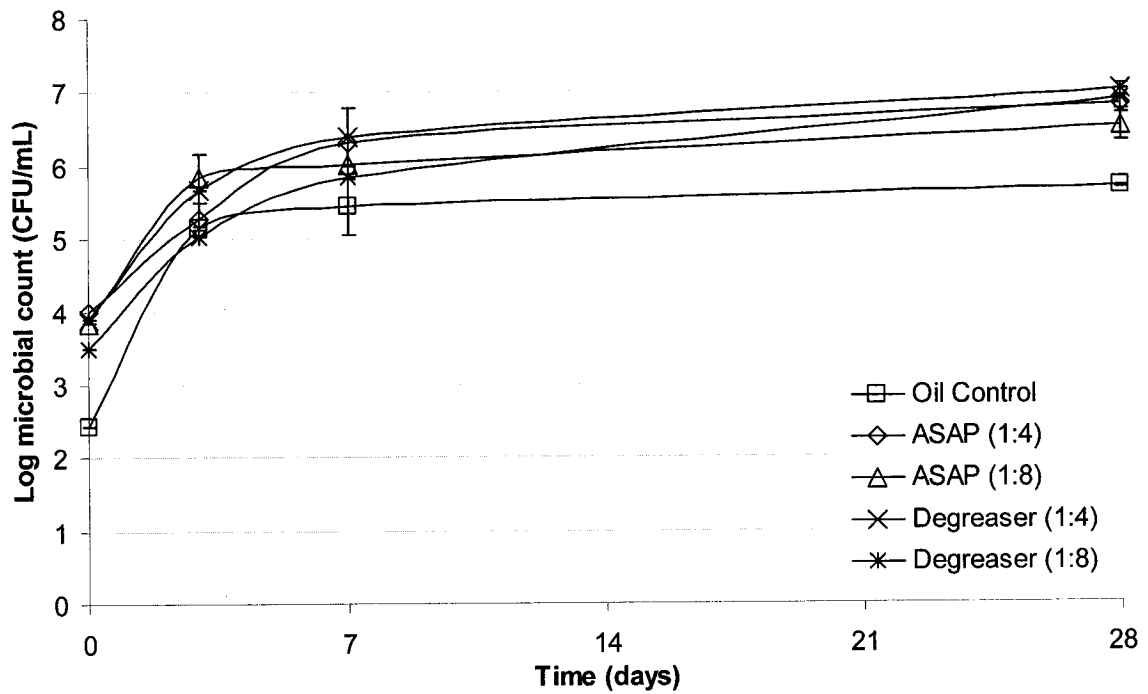


Figure 4.11 Microbial densities for *diesel fuel* over time.

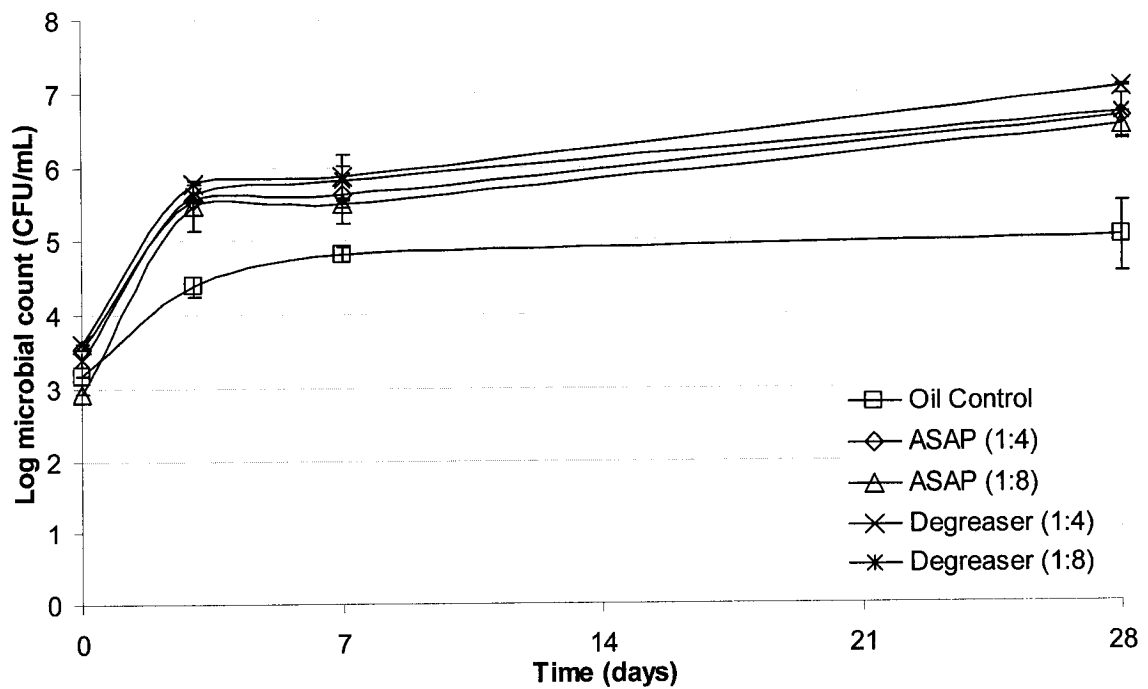


Figure 4.12 Microbial densities for *light crude oil* over time.

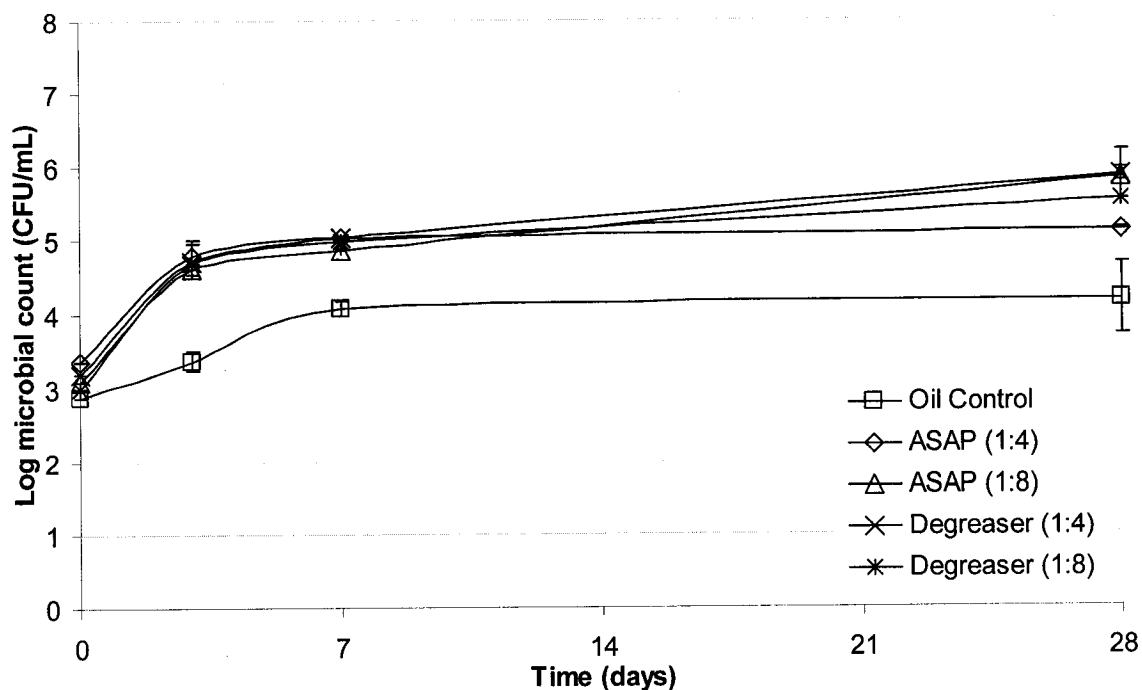


Figure 4.13 Microbial densities for *heavy crude oil* over time.

Table 4.1 Determination of the significant differences between treatments and oil control on diesel, light, and heavy crude oils.

Treatment	Diesel fuel		Light crude oil		Heavy crude oil	
	Diesel removal (%) in 28 days	<i>P</i> value [†]	Light crude removal (%) in 28 days	<i>P</i> value	Heavy crude removal (%) in 28 days	<i>P</i> value
Rhamnolipid (1:4)	58.9	5.05E-03	59.60	4.90E-04	41.9	1.05E-05
Rhamnolipid (1:2)	62.6	2.50E-03	64.72	2.45E-04	55.1	1.35E-05
Rhamnolipid (1:1)	65.0	6.29E-04	70.56	1.07E-05	59.1	6.15E-07
ASAP (1:4)	57.2	4.25E-03	59.85	7.25E-04	50.3	6.42E-06
ASAP (1:8)	52.5	2.50E-02	57.63	1.29E-03	47.5	1.06E-06
Degreaser (1:4)	59.9	1.70E-03	61.43	5.47E-04	39.6	4.65E-04
Degreaser (1:8)	54.4	9.80E-03	57.83	1.60E-03	38.6	8.57E-03

[†] A *p*-value less than 0.05 indicates a significant difference between treatments and the control at the 95% confidence level.

4.3.3 Combined Effect of Rhamnolipid and Biological Agents on Oil Biodegradation

A. Total Petroleum Hydrocarbon reduction:

In evaluating the effect of biological agents combined with rhamnolipid, the concentration of ASAPTM, and DegreaserTM that resulted in the highest TPH removal was selected. Although increasing rhamnolipid concentrations resulted in higher microbial densities as well as better TPH removals, the increase was not proportional to the increase in the concentration of rhamnolipid as discussed earlier. Furthermore, with high concentrations of rhamnolipid (above 4%) complications can arise in handling and application due to its high viscosity. Therefore, in assessing the effect of combined products, rhamnolipid concentration of 2% were chosen again to prepare test mixtures. Figures 4.14, 4.15, and 4.16 show the relative percent of diesel, light crude, and heavy crude oil remaining after treatment by different combinations of rhamnolipid and biological agents.

In general, TPH removal efficiencies in treatments for both biological agents increased by adding rhamnolipid. To illustrate this, the TPH removal efficiency for diesel fuel, for example, using either ASAPTM or DegreaserTM, at an additive to oil ratio of 1:4, were both about 60%, while that for rhamnolipid (1:4) + ASAPTM (1:4) was about 73%, and that for rhamnolipid (1:4) + and DegreaserTM (1:4) was about 81%. No major benefit was obtained by mixing all three additives, which showed a TPH removal efficiency of about 70%. Rhamnolipid plus DegreaserTM also exhibits the best performance when TPH removal efficiency is averaged over the three oils. Again, all treatment removal

efficiencies were found to be significantly different from control values using ANOVA, performed at $\alpha = 0.05$ (Table 4.2).

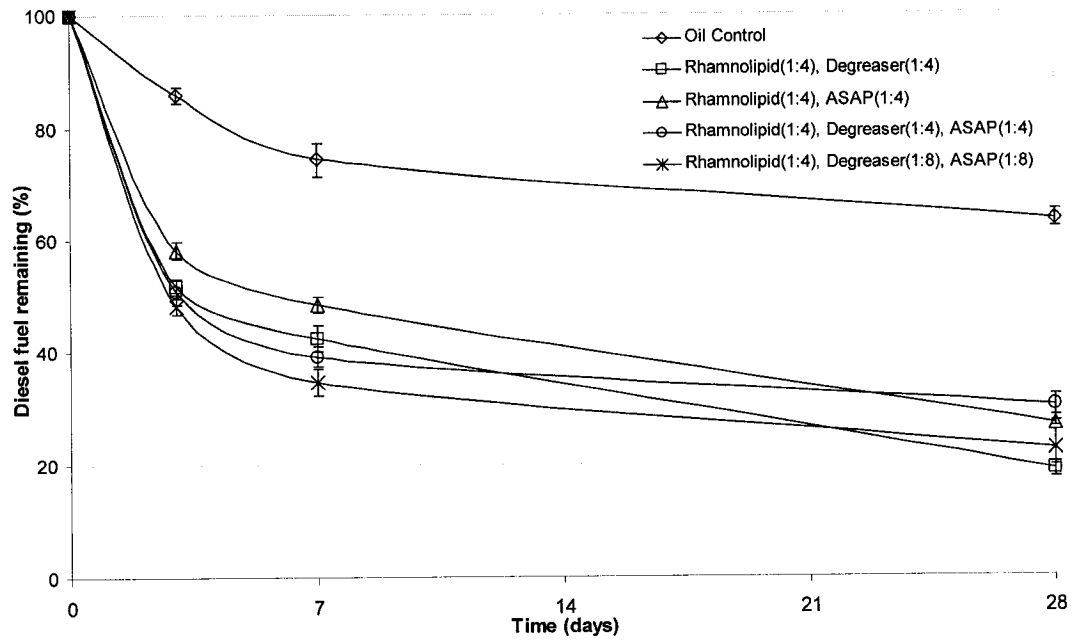


Figure 4.14 Diesel fuel reduction over time using combined treatments.

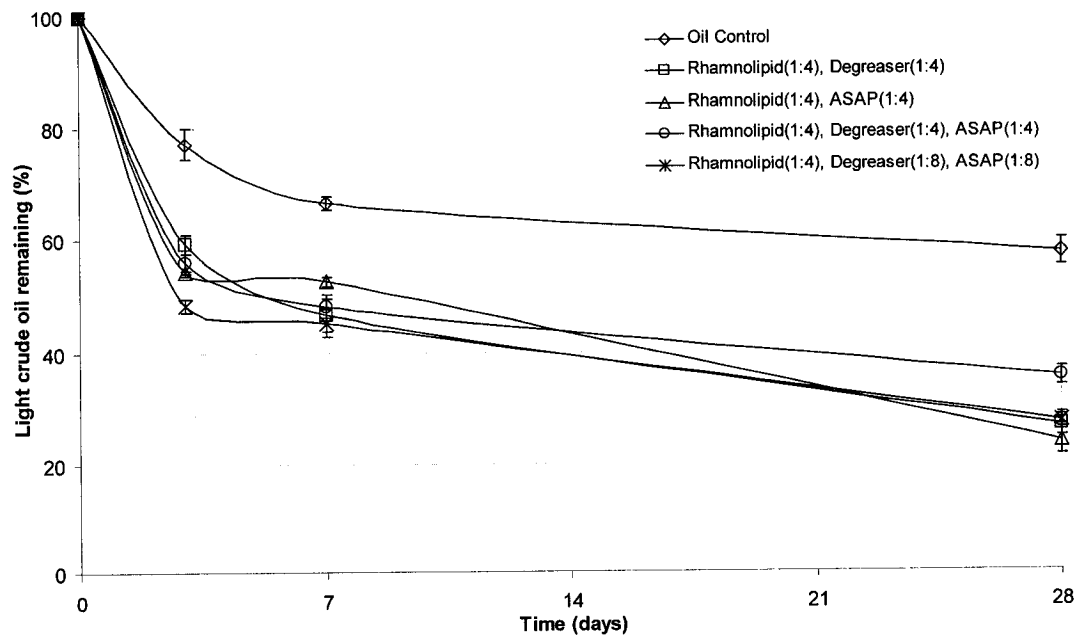


Figure 4.15 Light crude oil reduction over time using combined treatments.

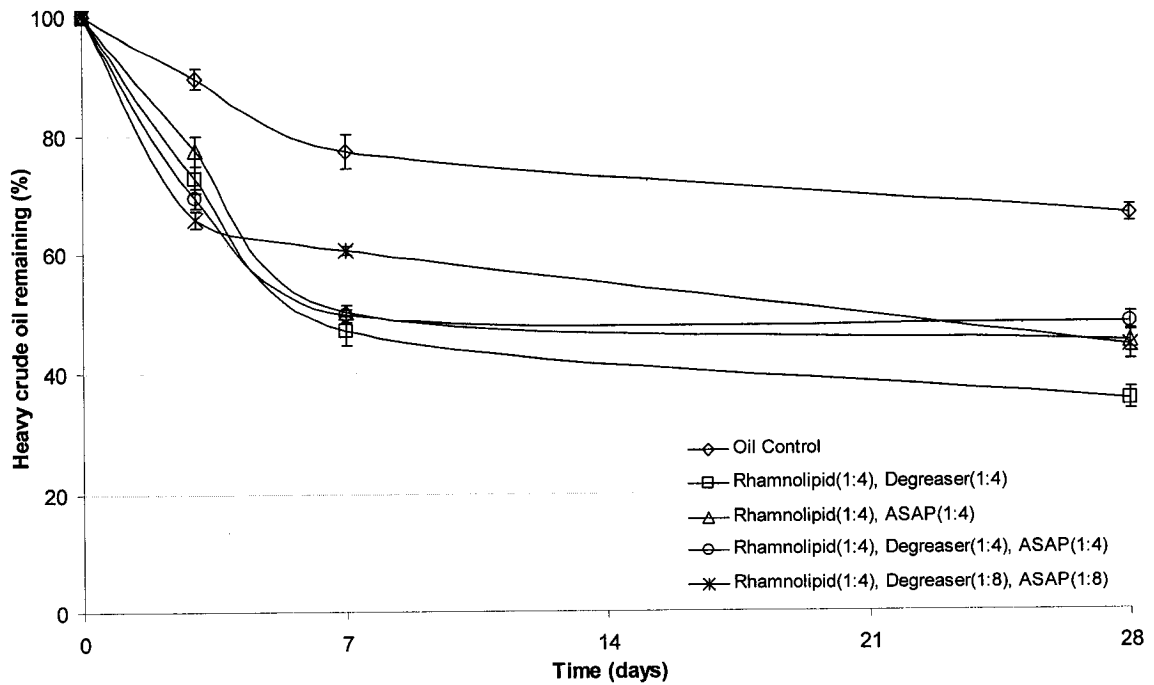


Figure 4.16 Heavy crude oil reduction over time using combined treatments.

B. Microbial growth:

The results of the microbial analysis for combined treatments are given in figures 4.17, 4.18, and 4.19. In general, higher microbial counts were observed for combined treatment batches when compared to those receiving treatments with individual products. Although the same previously observed general trend, where the initial day 0 to day 3 microbial growth rates were high and subsequently tended to level off to a stationary growth phase, was observed in the combined treatment set, the microbial growth rates from day 3 to 28 were greater and did not level out to the same extent. This is most likely related to the enhanced TPH removal observed in the combined agent treatment tests. It would appear that the combination of rhamnolipid and biological agents rendered more TPH available for utilization as the substrate, thus delaying the onset of a stationary growth phase.

In addition, both biological agents contained nutrients, which can increase microbial growth and enhance substrate utilization. Thus the observed increase in TPH removal may be the result of a combination of enhanced TPH bioavailability and added nutrients. However, nutrients in general did not seem to be limiting, since rhamnolipid treatments, which contained no nutrients, showed TPH removals that were greater than those for either biological agent alone. In addition, mineral levels in the liquid phase would be high since it was prepared using sea salt, which can be a source of macro and micronutrients (Zhu et al. 2001). Although any effects due to nutrient addition cannot be strictly assessed, it would seem that enhanced bioavailability is more important here as far as biodegradation is concerned.

In none of the control or treatment tests with individual or combined additive treatments was a drop in microbial density observed; characteristic of an endogenous metabolic stage where the substrate is essentially depleted or unavailable. This observation is in keeping with the TPH removal data, since in no instance was the PH substrate ever completely removed. The relative microbial densities of controls and treatments, as well as the corresponding TPH removals, indicate that although available TPH was limiting in the control group, biodegradation was apparently still proceeding. The natural ability of bacteria to produce biosurfactant in order to enhance exogenous metabolic activity is certainly at work in the control batches; however, augmenting this natural ability through biosurfactant addition, or even the addition of biological agents containing a consortium of bacteria, nutrients, and other chemical additives, which may include surfactants, has been shown here to be a potentially feasible remediation technique with respect to TPH contaminated soil.

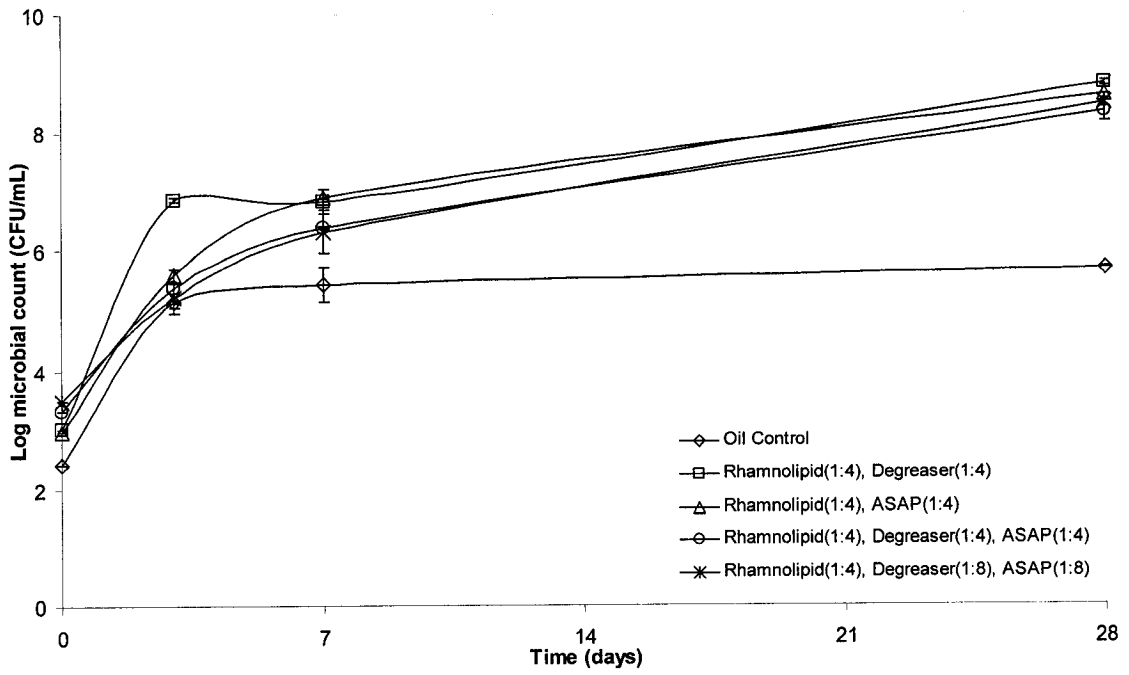


Figure 4.17 Microbial densities of *diesel fuel* over time.

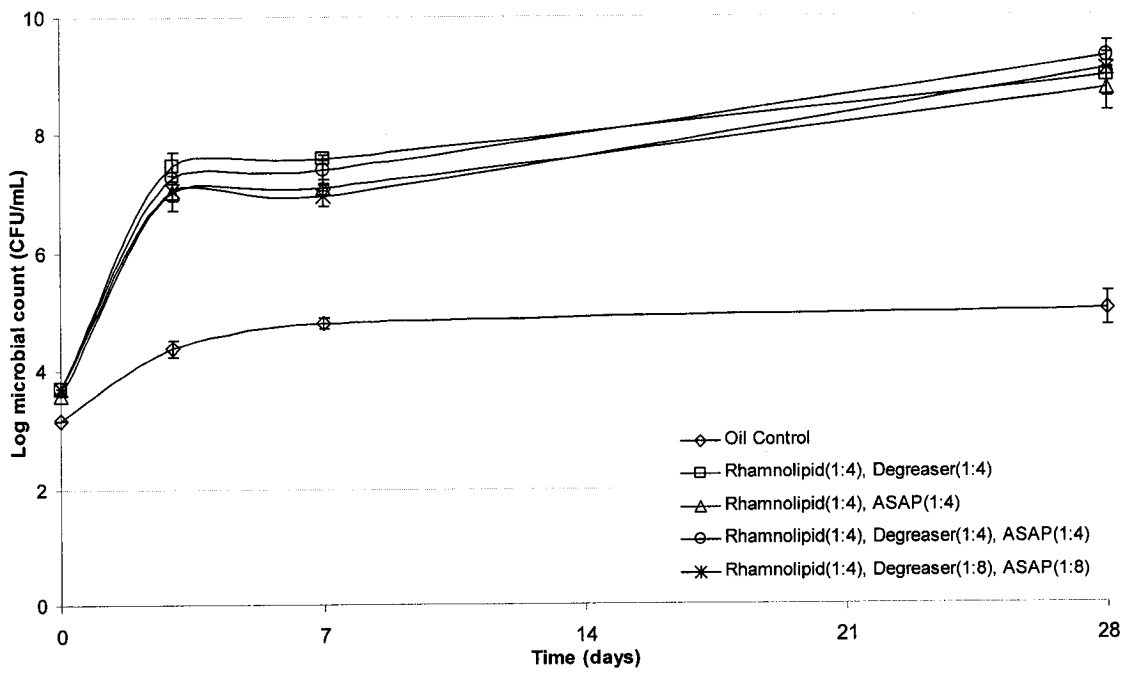


Figure 4.18 Microbial densities of *light crude oil* over time.

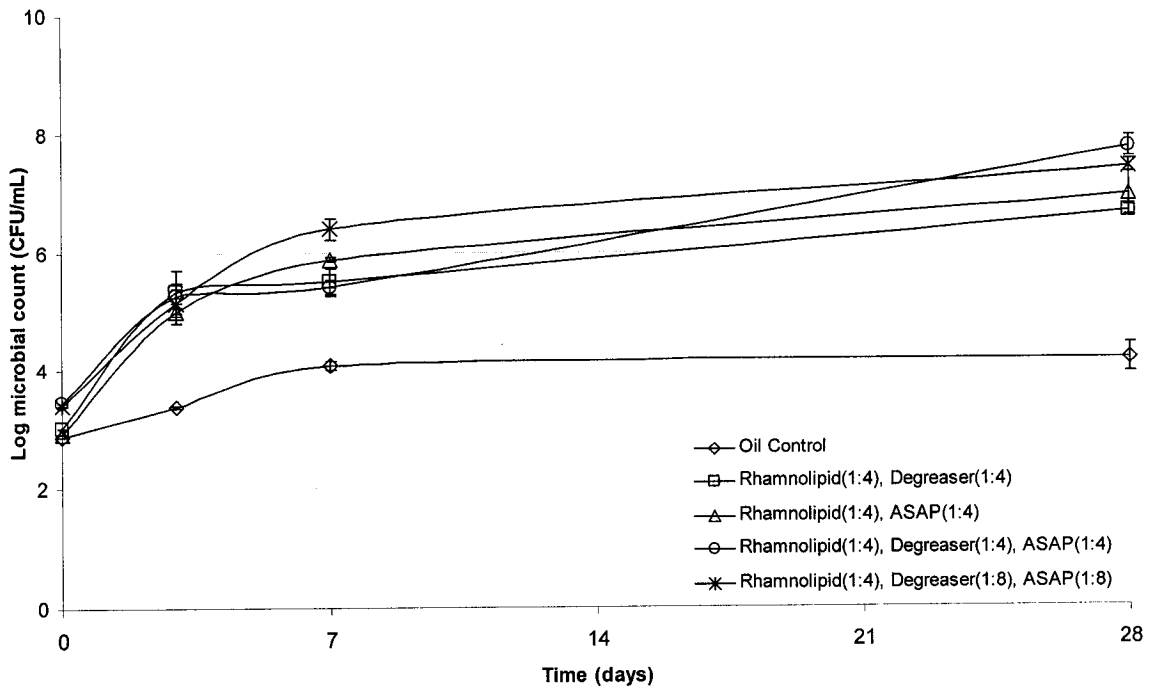


Figure 4.19 Microbial densities of *heavy crude oil* over time.

It should be mentioned at this point that ASAPTM contains chemical surfactants, including SDS, in addition to microorganisms and nutrients. Although SDS has been shown to have an inhibitory effect on the biodegradation of crude oil (Tiehm 1994, Dagneu 2004), very little differences in TPH removal between biosurfactant and ASAPTM treatment was observed. Both seemed to perform at about a similar TPH removal level for individual oils, removing oil within an average range of about 50 to 60% (Table 4.2). To prove any real inhibitory effect due to SDS, or any other chemical surfactant in the formulation, would require two exact formulations, except one would not contain the surfactant under scrutiny; or at least require knowledge of the relative level of the surfactant in the formulation so that it could be tested alone. However, no specifications on its exact contents are available.

Table 4.2 Determination of the significant differences between combined treatments and oil control on diesel, light, and heavy crude oils.

Treatment	Diesel fuel		Light crude oil		Heavy crude oil	
	Diesel fuel removal (%) (28 days)	<i>P</i> value [†]	Light crude removal (%) (28 days)	<i>P</i> value	Heavy crude removal (%) (28 days)	<i>P</i> value
Rhamnolipid (1:4) Degreaser (1:4)	81.3	3.17E-05	73.2	2.90E-06	64.8	1.11E-07
Rhamnolipid (1:4) ASAP (1:4)	73.3	1.15E-04	76.2	1.90E-05	55.2	2.87E-07
Rhamnolipid (1:4) ASAP (1:4) Degreaser (1:4)	70.1	7.56E-05	64.6	3.41E-05	52.0	1.01E-09
Rhamnolipid (1:4) ASAP (1:8) Degreaser (1:8)	77.6	1.93E-05	72.5	2.39E-06	55.8	1.69E-07

[†] A *p*-value less than 0.05 indicates significant differences between treatments and the control at the 95% confidence level.

4.3.4 Oil Biodegradation Kinetics

Kinetics of oil biodegradation is an important consideration when evaluating the efficacy of any oil-bioremediation process. The ability to model and predict oil degradation rates in conjunction with other physical and chemical modelling of oil spills can be of immense help in forecasting potential environmental impacts or choosing between alternative approaches to spill cleanup. Oil biodegradation rates are difficult to predict and are influenced greatly by the type of oil and the presence of other substrates. Environmental factors such as temperature, nutrient concentrations and microbial

population, pH, degree of oil weathering and oxygen tension can also influence the kinetics of oil degradation.

In this study an attempt was made to model TPH removal under the initial assumption that it was mainly achieved through biodegradation. TPH removal rate data for the three different oils were fitted using regression to a simple first-order biodegradation rate model using MS Excel®. The first-order biodegradation rate model is expressed as:

$$\frac{dC}{dt} = -kC \Rightarrow C = C_0 \exp(-kt) \quad \text{(Equation 4.1)}$$

$$\ln \frac{C}{C_0} = -kt \quad \text{(Equation 4.2)}$$

where C is the TPH concentration (g/L of hydrocarbon extracted per litre of hexane), C_0 is the initial concentration of hydrocarbon (g/L of hydrocarbon extracted per litre of hexane), k is the first-order biodegradation rate coefficient (1/day) and t is time (day). Although it is usual to formulate a model based on actual amounts in water, the C/C_0 ratio is the same whether expressed per litre reaction vessel water or per litre hexane, which was used to extract it. It should also be remembered that the TPH is not completely soluble in the liquid phase (especially in the case of the blanks) and that application of this kinetic model based on concentration is an approximation at best.

The biodegradation rate coefficient (k) is the slope of the regression line obtained by plotting $\ln(C/C_0)$ versus time (0, 3, 7, and 28 days) (Equation 4.2). The biodegradation

rate coefficient (k), determination coefficient (R²), and half-lives of the petroleum samples are included in Table 4.3. The half-life of the oils was obtained as follows:

$$t_{1/2} = \frac{0.693}{k} \quad \text{(Equation 4.3)}$$

where t_{1/2} is half-life (day) and k is the first-order biodegradation rate coefficient (1/day).

Table 4.3 First-order biodegradation rate coefficients and half-lives for three oil samples.

Treatment	Diesel fuel			Light crude oil			Heavy crude oil		
	k [†] (1/day)	Half-life (days)	R ²	k (1/day)	Half-life (days)	R ²	k (1/day)	Half-life (days)	R ²
Oil control	0.017	41.0	0.90	0.023	30.5	0.38	0.016	42.8	0.73
Rhamnolipid(1:4)	0.035	20.0	0.73	0.036	19.4	0.67	0.023	30.8	0.40
Rhamnolipid(1:2)	0.039	17.7	0.61	0.043	16.3	0.44	0.031	22.2	0.79
Rhamnolipid(1:1)	0.042	16.5	0.53	0.050	13.9	0.44	0.035	19.9	0.77
ASAP(1:4)	0.034	20.3	0.53	0.037	18.9	0.49	0.027	26.1	0.87
ASAP(1:8)	0.030	23.3	0.56	0.034	20.4	0.62	0.025	28.3	0.89
Degreaser(1:4)	0.037	18.5	0.42	0.039	17.9	0.38	0.020	34.7	0.64
Degreaser(1:8)	0.032	22.0	0.53	0.035	19.7	0.44	0.019	35.7	0.66
Rhamnolipid(1:4) Degreaser(1:4)	0.065	10.6	0.72	0.052	13.3	0.65	0.042	16.5	0.58
Rhamnolipid(1:4) ASAP(1:4)	0.052	13.4	0.66	0.055	12.5	0.73	0.033	20.8	0.41
Rhamnolipid(1:4) ASAP(1:4) Degreaser(1:4)	0.050	13.8	0.18	0.043	16.2	0.27	0.032	22.0	0.08
Rhamnolipid(1:4) ASAP(1:8) Degreaser(1:8)	0.061	11.3	0.37	0.052	13.3	0.37	0.033	21.1	0.45

[†] k represents first-order rate coefficient.

Except for some k values, the determination coefficients were in general low, and in some cases extremely low R² value were obtained. In addition, no really consistent and

meaningful pattern emerged except that determination coefficients were on average lower for mixed addition treatments than for single addition treatments. It can be stated that according to this model biodegradation rate and oil concentration are not linearly correlated. Therefore, an attempt was made to fit TPH removal rates using a second order kinetics model for biodegradation. The second-order biodegradation process was considered according to Suflita et al. (1987) as:

$$\frac{dC}{dt} = -KC^2 \Rightarrow \frac{1}{C} - \frac{1}{C_0} = Kt \quad \text{(Equation 4.4)}$$

$$t_{1/2} = \frac{1}{C_0 K} \quad \text{(Equation 4.5)}$$

where K = second-order biodegradation rate coefficient (L/g-day), C_0 = the initial oil concentration and C = oil concentration at time t (g/L)

According to determination coefficients (R^2) listed in Table 4.4, the second-order model fit the data much better than the first-order model. Table 4.5 lists the average determination coefficients along with the standard deviation for each oil type. The overall average determination coefficient for the second order model was 0.75, while that for the first order model was only 0.55. Half-lives for TPH in the various treatments of course followed the expected trend based on actual removals of: control > biological agent > rhamnolipid > rhamnolipid + biological agent. The obvious importance of having reasonable half-life estimates is the ability to predict the amount of time required to biodegrade or remove the PH contaminant as a function of the treatment.

Table 4.4 Second-order biodegradation rate coefficients and half-lives for three oil samples.

Treatment	Diesel fuel			Light crude oil			Heavy crude oil		
	K [†] (1/day)	Half-life (days)	R ²	K (1/day)	Half-life (days)	R ²	K (1/day)	Half-life (days)	R ²
Oil control	0.010	25.9	0.75	0.012	20.5	0.54	0.011	21.8	0.81
Rhamnolipid(1:4)	0.023	10.8	0.90	0.024	10.5	0.87	0.017	14.1	0.52
Rhamnolipid(1:2)	0.027	9.1	0.84	0.030	8.3	0.69	0.028	8.8	0.91
Rhamnolipid(1:1)	0.031	8.0	0.81	0.039	6.4	0.76	0.034	7.2	0.91
ASAP(1:4)	0.022	11.2	0.75	0.024	10.4	0.74	0.022	11.2	0.95
ASAP(1:8)	0.018	13.5	0.75	0.021	11.8	0.83	0.019	12.7	0.96
Degreaser(1:4)	0.025	9.7	0.65	0.026	9.6	0.66	0.015	16.5	0.76
Degreaser(1:8)	0.020	12.6	0.74	0.022	11.2	0.68	0.014	17.3	0.76
Rhamnolipid(1:4) Degreaser(1:4)	0.068	3.6	0.97	0.042	5.9	0.91	0.042	5.9	0.78
Rhamnolipid(1:4) ASAP(1:4)	0.044	5.6	0.92	0.048	5.2	0.96	0.029	8.4	0.54
Rhamnolipid(1:4) ASAP(1:4) Degreaser(1:4)	0.041	6.0	0.53	0.030	8.2	0.60	0.027	9.0	0.22
Rhamnolipid(1:4) ASAP(1:8) Degreaser(1:8)	0.058	4.3	0.77	0.041	5.9	0.75	0.029	8.5	0.70

[†] K represents the second-order rate coefficient.

Table 4.5 Mean value and standard deviation of determination coefficient for the three oils.

	Diesel fuel		Light crude oil		Heavy crude oil	
	Mean of R ²	SD of R ²	Mean of R ²	SD of R ²	Mean of R ²	SD of R ²
First-order rate	0.56	0.19	0.49	0.14	0.6	0.24
Second-order rate	0.78	0.12	0.75	0.12	0.73	0.22

The rate analyses were performed to investigate the possibility of determining removal rate and half-life for various oil-treatment sets as a means to quantify and even predict removal. As mentioned earlier, complete oil solubility in all tests was most likely not achieved, and some biodegradation may have occurred with oil adhering to the walls of the reaction flasks, and thus applying a kinetic model based on concentration will have drawbacks. However, the removal data did accord fairly well with the second order kinetic model considering that test conditions were not strictly in accordance with the model's basic 'concentration' assumption, when applied to a reactant in water; in this case TPH.

4.4 Discussion

Figures 4.20, 4.21, and 4.22 shows the percent TPH removal for the three oils after 28 days across all treatments, and includes the control value. It also gives microbial density as CFU/mL for day 28. It is clear from these graphs that, if experimental error is taken into account, there is a fairly good correlation between microbial density and TPH removal; which one would expect if PH compounds were removed through the metabolic activity of the corresponding microbial population.

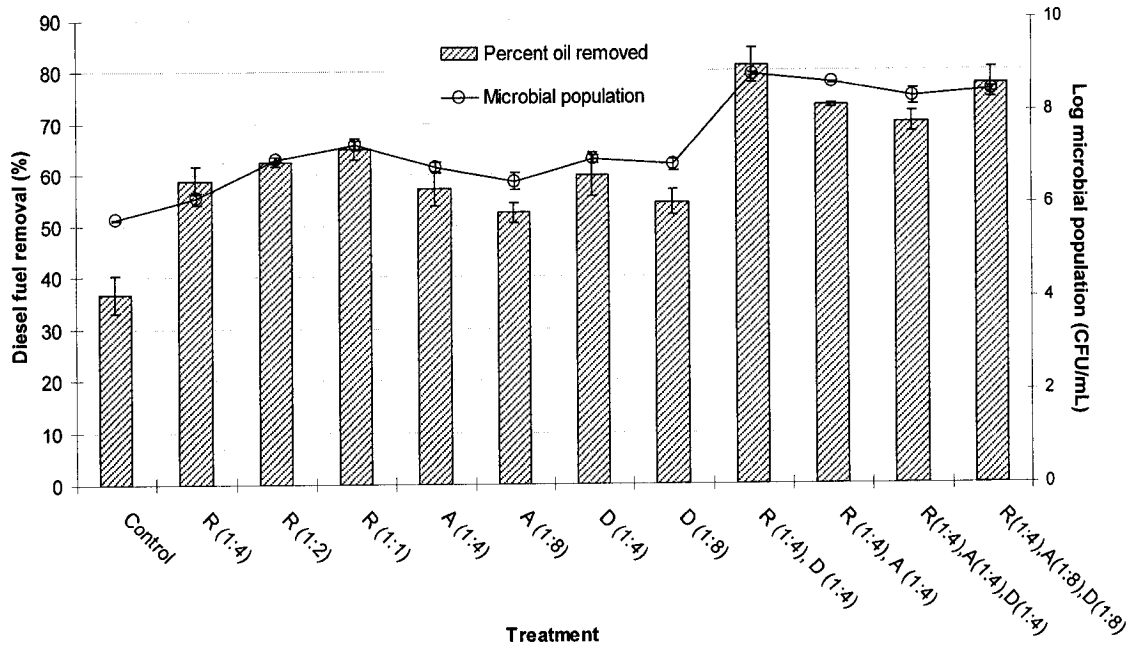


Figure 4.20 Percent TPH removal and microbial population of diesel fuel by various treatments on 28 days of incubation.

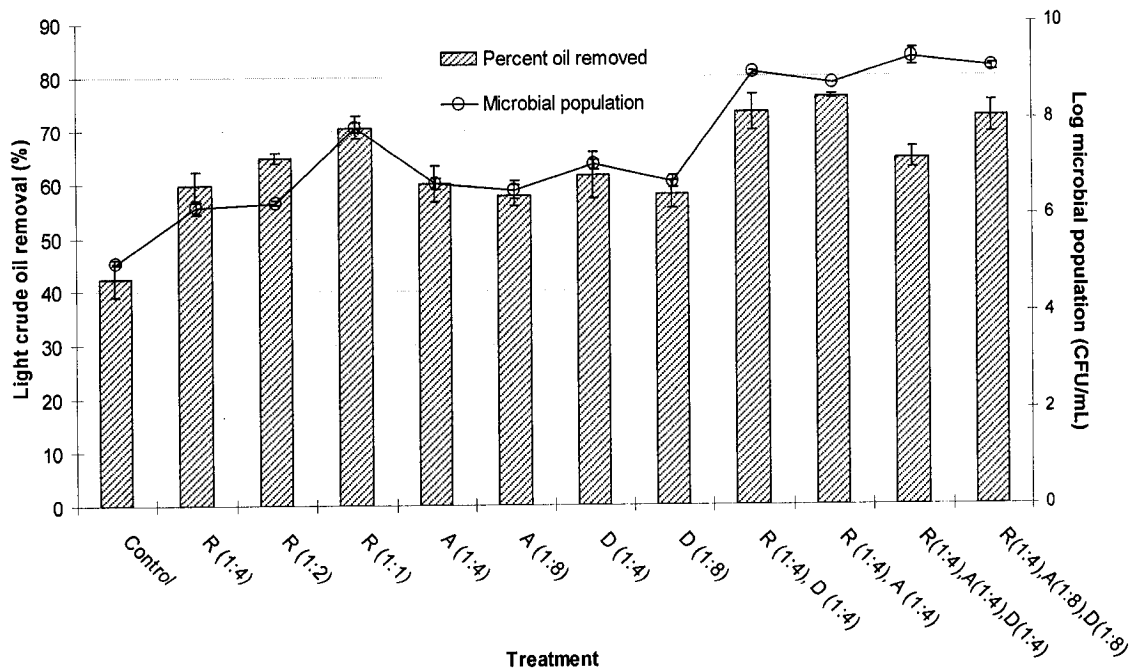


Figure 4.21 Percent TPH removal and microbial population of light crude oil by various treatments on day 28.

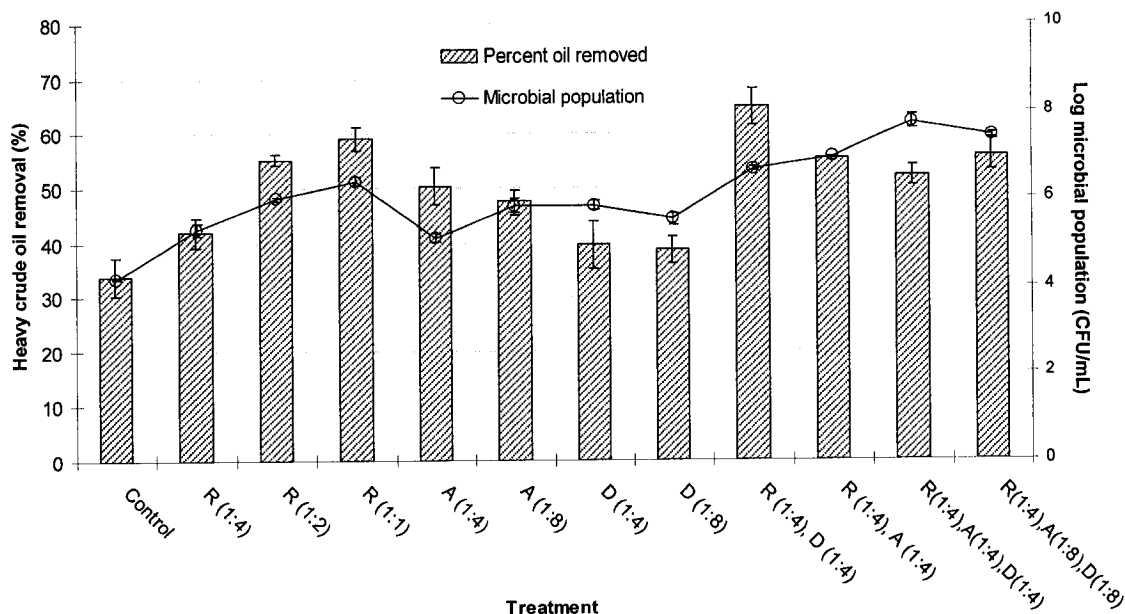


Figure 4.22 Percent TPH removal and microbial population of heavy crude oil by various treatments on day 28.

Figures 4.23, 4.24, and 4.25 give the actual % increase in TPH removal above control values for diesel, light crude, and heavy crude oil. These values are found by subtracting %TPH remaining for treatments from %TPH remaining for controls. The values are indicative of actual TPH removal enhancement for the various treatments. As mentioned before (4.3.3.a), the general trend in TPH removal enhancement with treatment can be seen as: rhamnolipid + biological agent > rhamnolipid > biological agent. Furthermore, for all products tested the %TPH removal was directly related to the quantity of product supplemented.

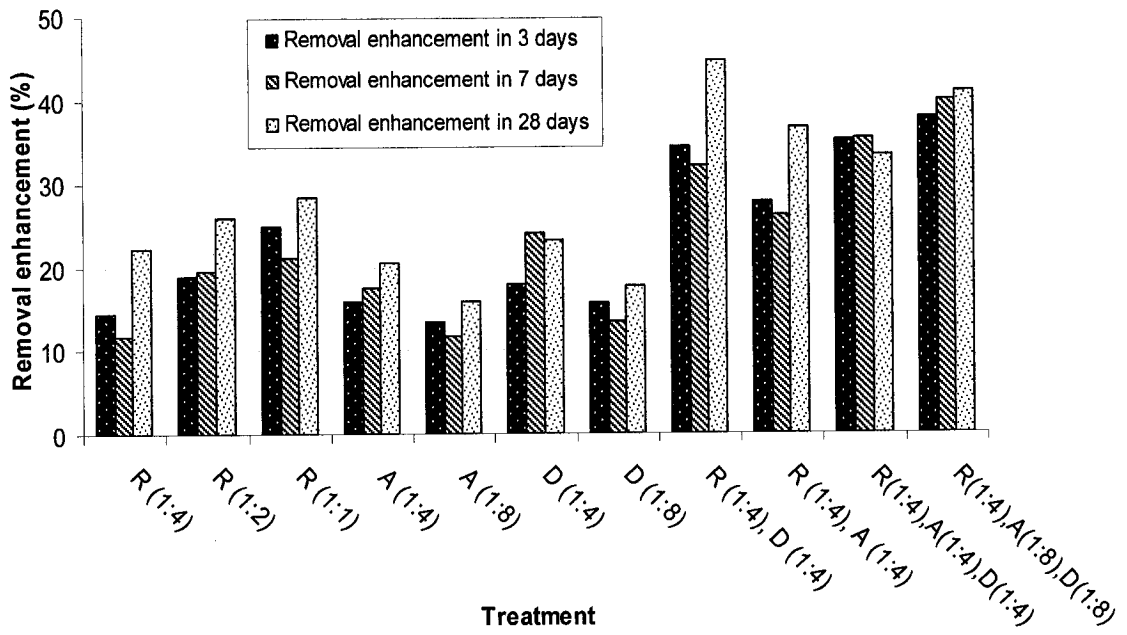


Figure 4.23 Percent TPH removal of treatments relative to oil control over time (diesel fuel).

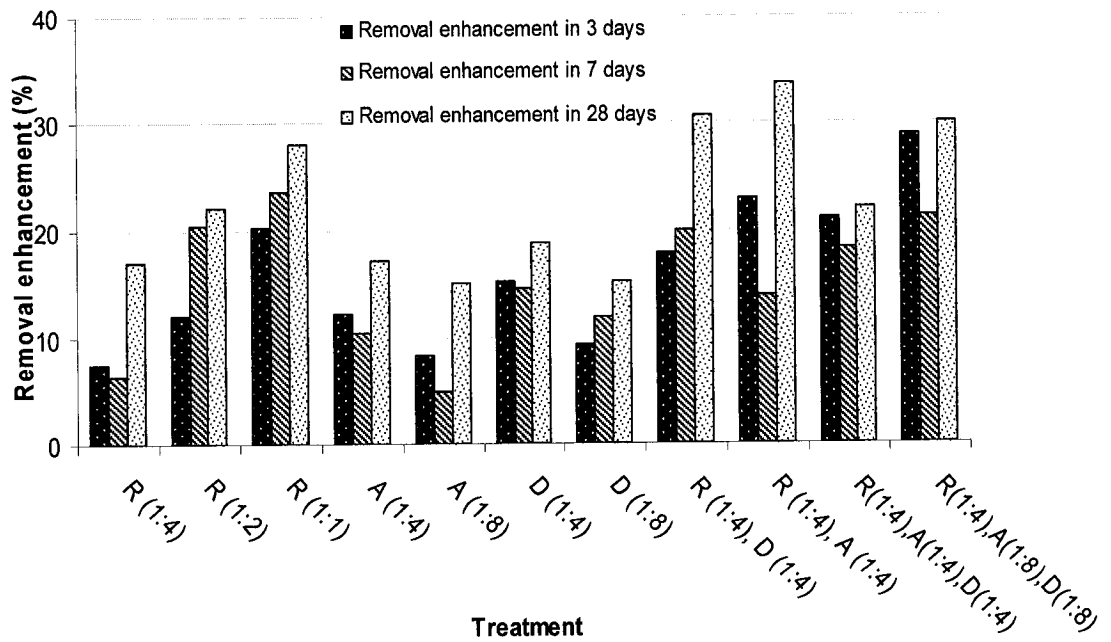


Figure 4.24 Percent TPH removal of treatments relative to oil control over time (light crude oil).

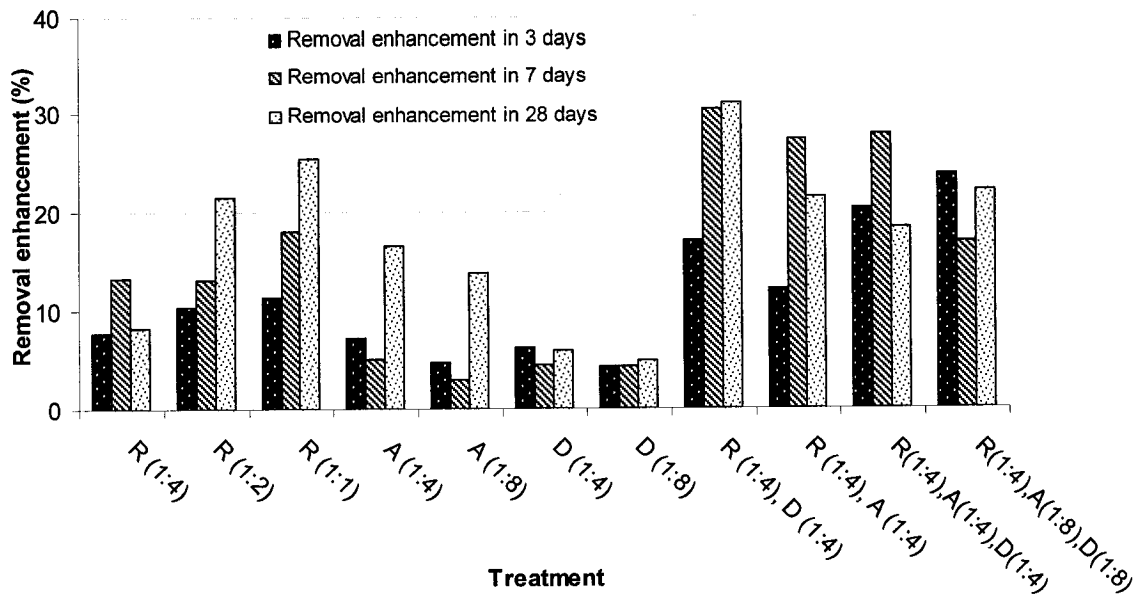


Figure 4.25 Percent TPH removal of treatments relative to oil control over time (heavy crude oil).

Figures 4.26, 4.27, and 4.28 shows the percent TPH removal for the three oils across all treatments, for days 3, 7, and 28, and includes the control value. These graphs are included so that the relative effectiveness of TPH removal across treatments can be more easily compared. Minimum (day 3) and maximum (day 28) percent TPH removal values across all treatments, including control, were 14 and 81% for diesel fuel, 23 and 76% for light crude oil, and 10 and 65% for heavy crude oil, with controls consistently showing the lowest day 3 percent removal. Although TPH removal for heavy crude oil was the lowest (65%), the removal enhancement with respect to the heavy crude oil control (31%) is comparable to maximum removal enhancements for diesel fuel (44%) and light crude oil (34%). More discussion on the performance of difference oil types will follow.

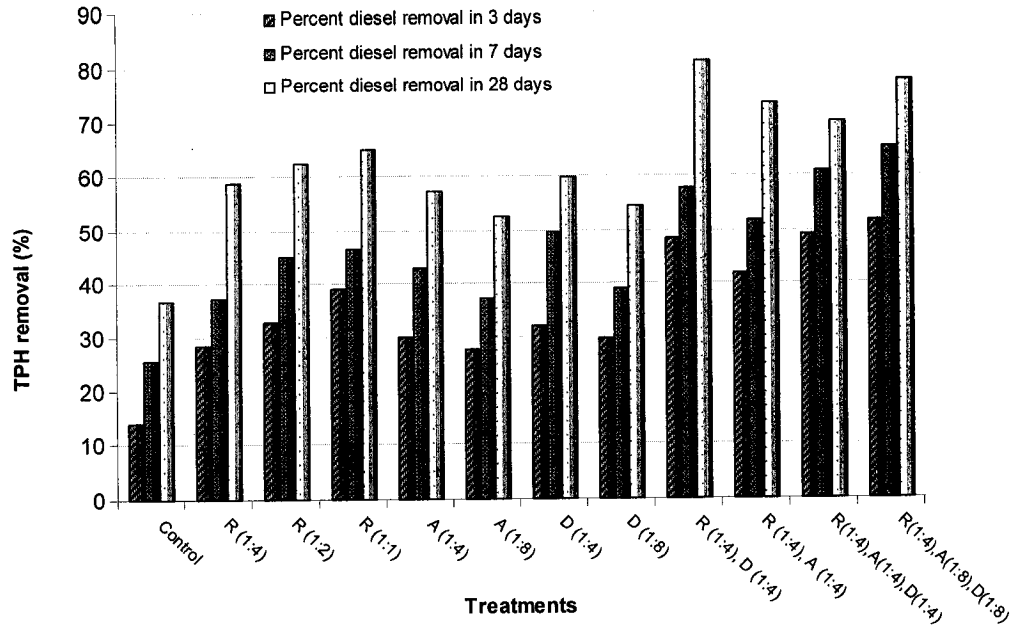


Figure 4.26 Removal comparisons of different treatments on diesel fuel on days 3, 7, and 28.

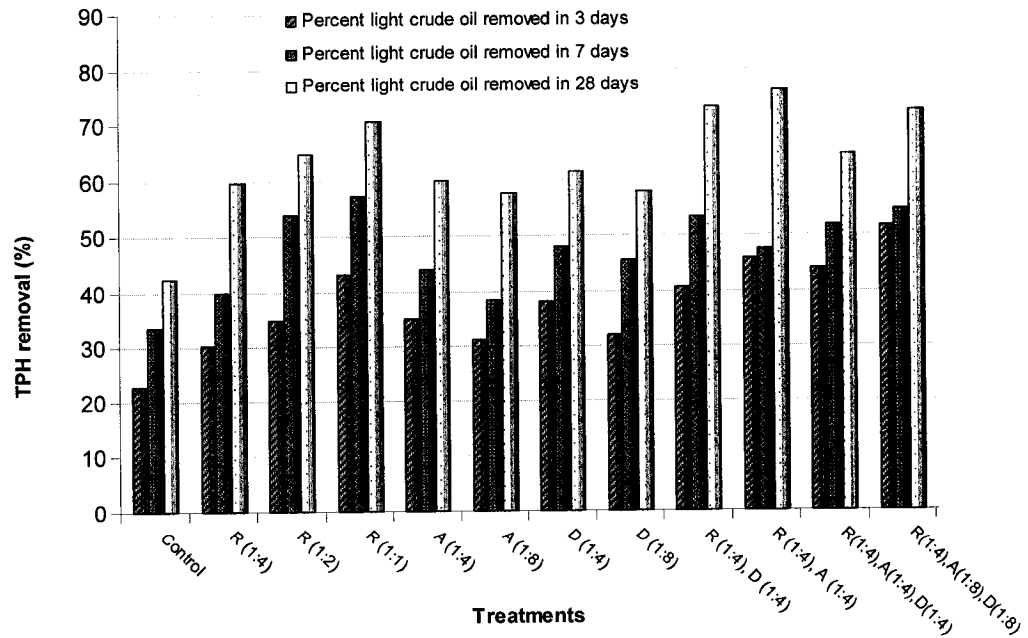


Figure 4.27 Percent TPH removal comparisons of different treatments on light crude oil on days 3, 7, and 28.

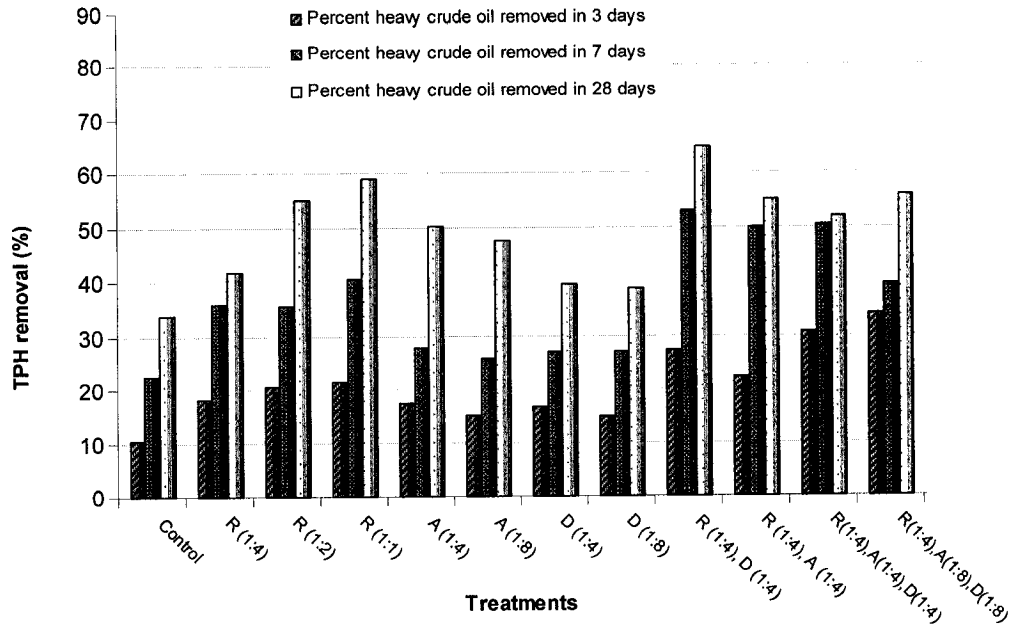


Figure 4.28 Percent TPH removal comparisons of different treatments on heavy crude oil on day 3, 7, and 28.

Chromatograms for weathered oil (day 0 control), weathered oil (day 28 control), treated (day 28) diesel fuel, light crude oil, and heavy crude oil are presented in Figures 4.29, 4.30, and 4.31, respectively. In general, GC separates petroleum hydrocarbon mixtures based on their molecular weights, where the lower the carbon number of the PH, the lower the column retention time. If we compare chromatograms for day 28 control and treatments to day 0 controls a consistent pattern is observed. Although, some removal of hydrocarbons was observed after retention times of about 12 minutes, the majority of hydrocarbons removed are in the lower retention time range (less than 12 minutes) for controls and treatments. In another words, it is the lower carbon number compounds which are more readily biodegraded. The addition of rhamnolipid and biological cleansing agents, alone or in combination, seem to enhance control PH biodegradation patterns as interpreted from the chromatograms, but do not seem to selectively enhance

biodegradation of hydrocarbons not degraded in the controls. It should be stated that no hydrocarbon standards or rigorous peak identification was used in this study. The GC analyses employed were used to determine overall degradation; however, some valuable information regarding the selectivity of hydrocarbon biodegradation was obtained.

Although TPH removals were similar across all three oils, the lowest removals were consistently reported for heavy crude oil. This is understandable in light of GC analyses since the heavy crude oil fraction had less petroleum hydrocarbons than the light crude oil and diesel fuel in the lower retention time range. The highest TPH removal was observed for diesel fuel which although had very little hydrocarbons eluted in the lower retention time range (less than 7 minutes) when compared to the crude oils, had relatively more hydrocarbons eluted at higher retention times (7-12 minutes). Thus it would appear that increased PH biodegradation may have involved more biodegradation of medium range petroleum hydrocarbon which are abundant in diesel fuel.

For a first-order reaction, the rate of reaction is directly proportional to the concentration. As the reactant is consumed its concentration drops, and so does the rate of reaction. In first order reactions the reaction rate drops linearly with decreasing concentration. For a second-order reaction, the reaction rate increases with the square of the concentration, producing an upward curving line in the rate-concentration plot. For this type of reaction, the rate of reaction decreases rapidly (faster than linearly) as the concentration of the reactant decreases.

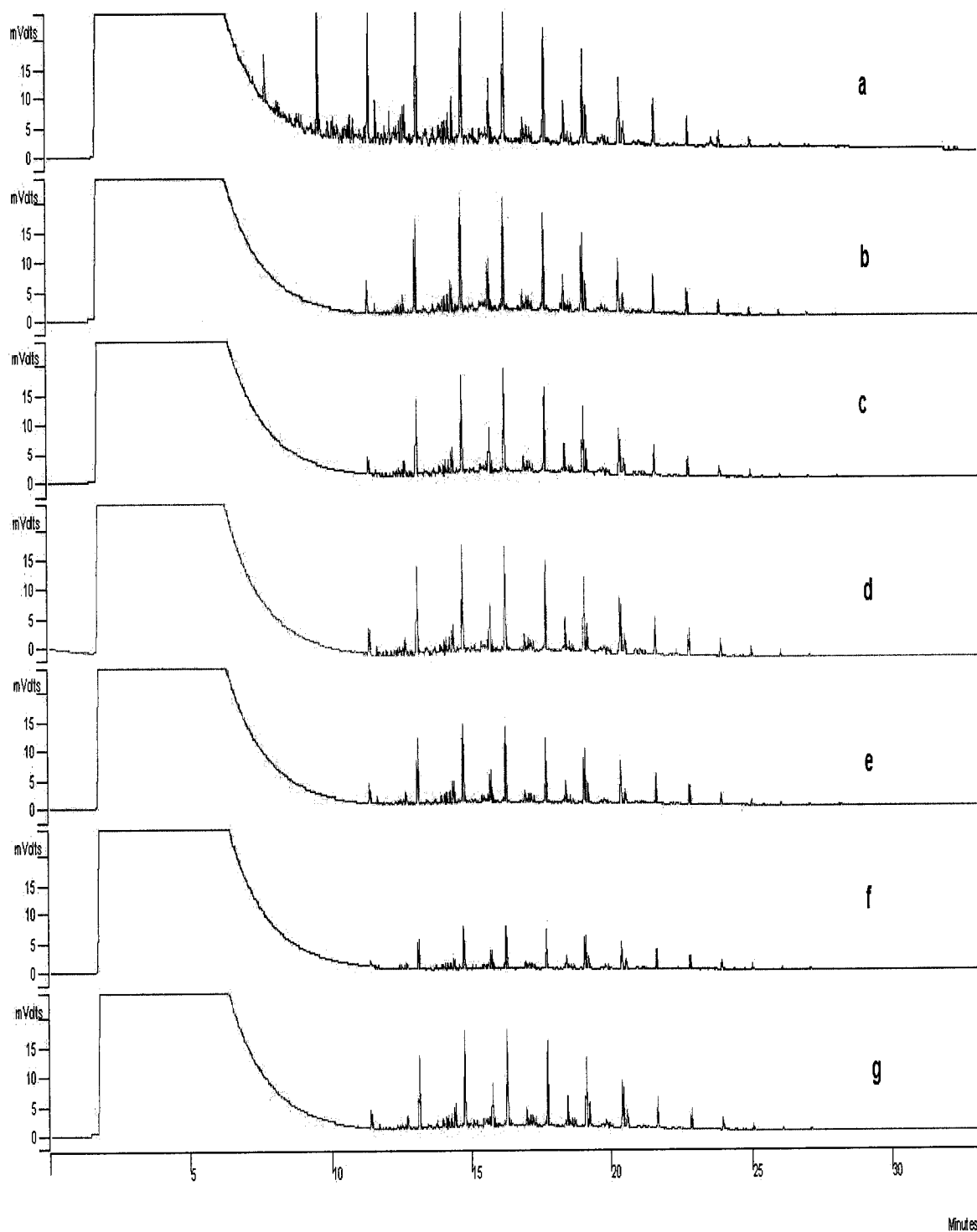


Figure 4.29 Chromatograms of weathered diesel sample and some selected treatments in hexane from 28 days flask batch reactors. (a) diesel fuel control (0 day), (b) diesel fuel control (28 days), (c) rhamnolipid (1:4), (d) Degreaser (1:4), (e) rhamnolipid (1:4) with Degreaser (1:4), (f) rhamnolipid (1:4) with ASAP (1:4), (g) rhamnolipid (1:4) with Degreaser (1:8) and ASAP (1:8).

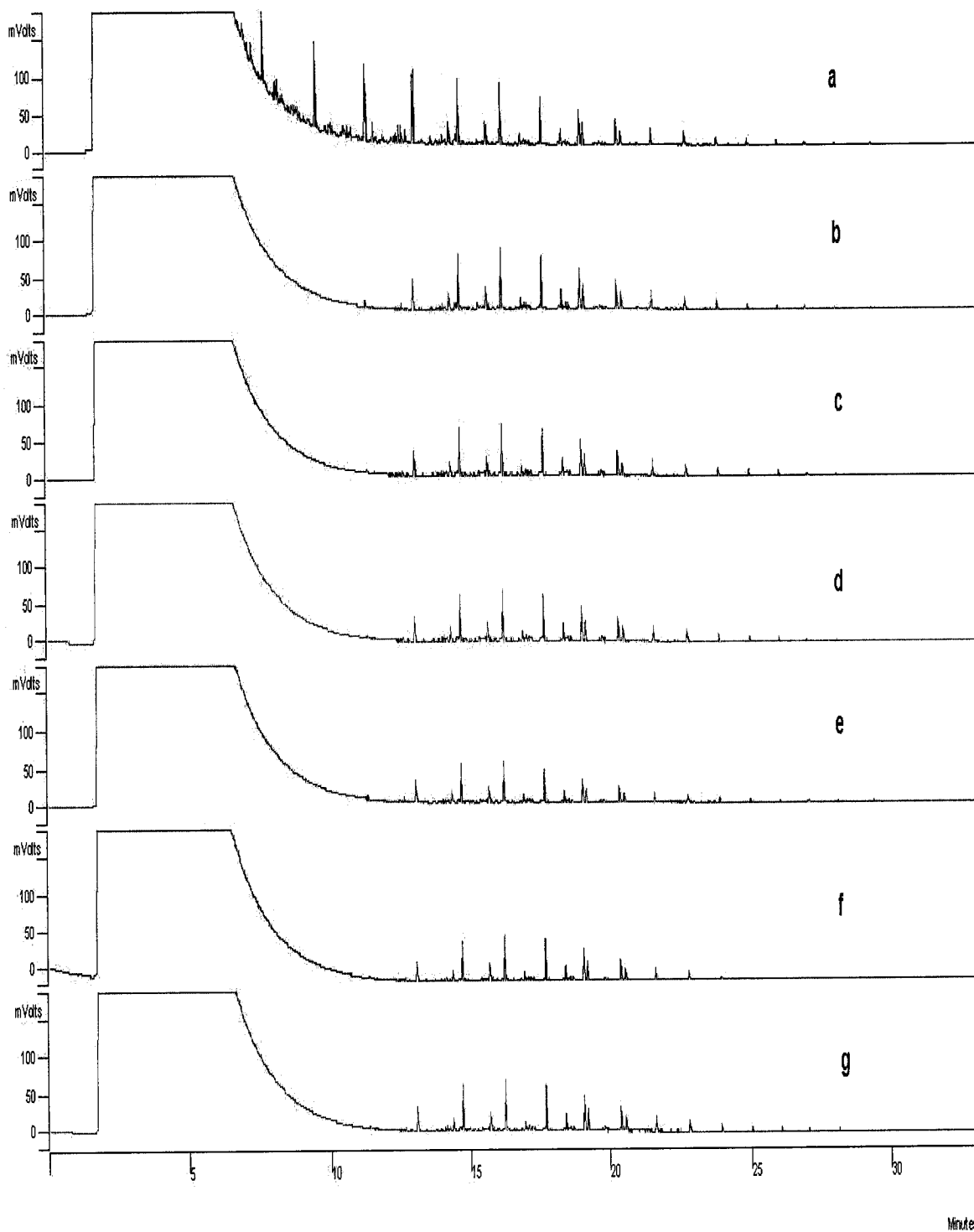


Figure 4.30 Chromatograms of weathered light crude oil and some selected treatments in hexane from 28 day flask batch reactors. (a) light crude oil control (0 day), (b) light crude oil control (28 days), (c) rhamnolipid (1:4), (d) Degreaser (1:4), (e) rhamnolipid (1:4) with Degreaser (1:4), (f) rhamnolipid (1:4) with ASAP (1:4), (g) rhamnolipid (1:4) with Degreaser (1:8) and ASAP (1:8).

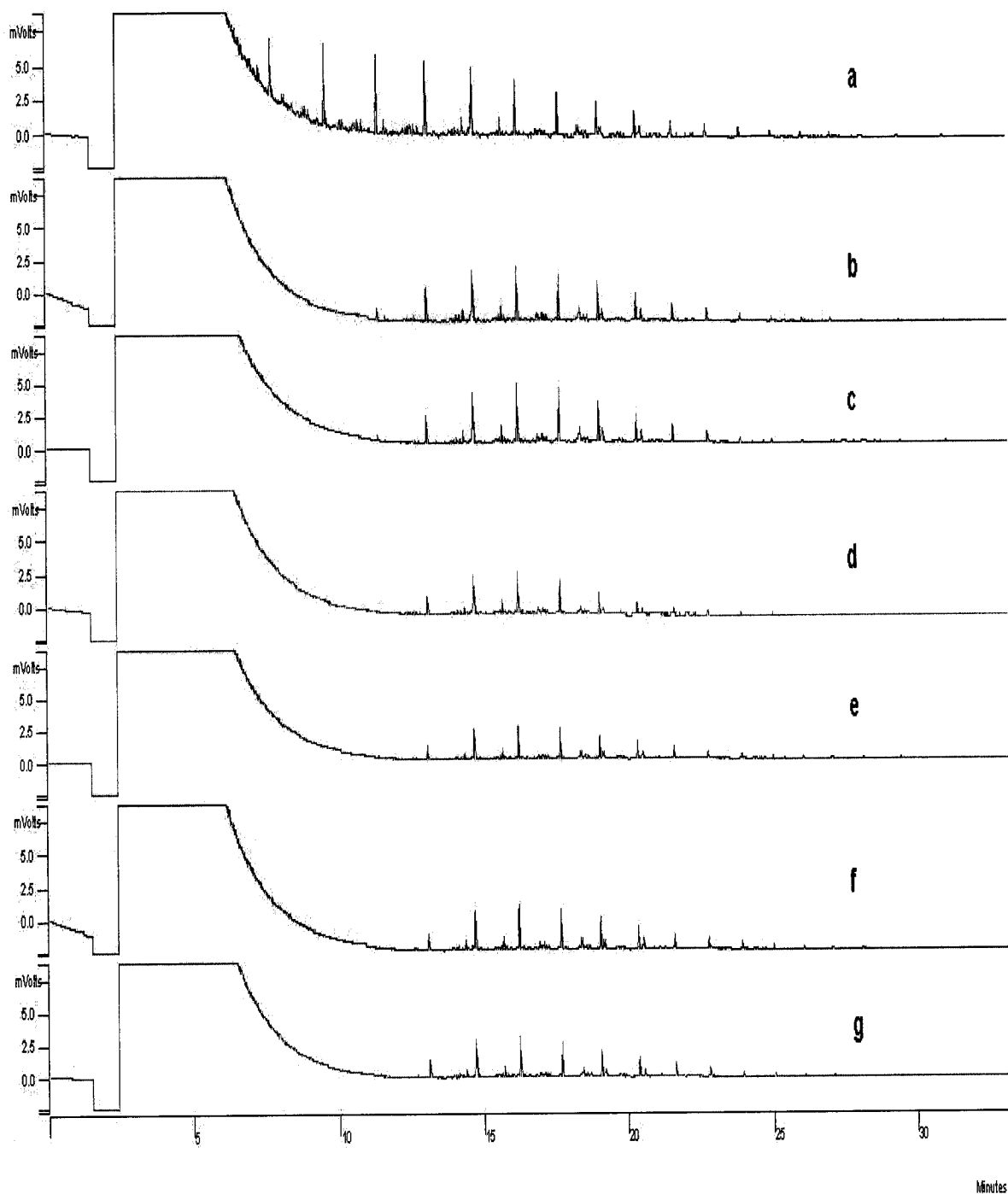


Figure 4.31 Chromatograms of weathered heavy crude oil and some selected treatments in hexane from 28 day flask batch reactors. (a) heavy crude oil control (0 day), (b) heavy crude oil control (28 days), (c) rhamnolipid (1:4), (d) Degreaser (1:4), (e) rhamnolipid (1:4) with Degreaser (1:4), (f) rhamnolipid (1:4) with ASAP (1:4), (g) rhamnolipid (1:4) with Degreaser (1:8) and ASAP (1:8).

Since TPH removal fits the second order model more closely, the implication is that as initial dispersed PH (which represented a part of the initial oil) as well as dispersant, was utilized as substrate by an exponentially growing microbial population, TPH was removed exponentially in second order fashion. As the microbial population increased, the biosurfactant produced was also increasingly subject to endogenous metabolism causing a reduction in further TPH availability. In addition, the amount of more readily dispersed petroleum hydrocarbon compounds had by this time been reduced. The combination of these events could result in a dramatic fall in TPH utilization, again characteristic of second order kinetics (Suflita et al. 1987; Dagnew 2004)

Although control batches also displayed this pattern, without added surfactant, a similar interpretation is valid since as TPH is depleted, albeit more slowly than in amended batches, the naturally produced biosurfactants have less oil to disperse and that oil remaining fraction is now more difficult to disperse. Surfactant must be produced in ever-increasing amounts if the former TPH removal rates are to be maintained. However, this biosurfactant is also biodegraded by the large microbial population, and the relative TPH removal rate may thus fall dramatically. With respect to added biological agents it could be said that they are merely enhancing the TPH degradation characteristics of the control batch through increasing original microbial density and supplying nutrients. Natural biosurfactant production would also take place here as well. Any organic chemical additive or surfactant could also presumably be biodegraded once its initial solubilization task was performed.

However interpreted, it would seem that the combination of TPH first requiring dispersion by a surfactant (alone or perhaps in combination with other biodegradable

additives), that is itself is subject to biodegradation, results in TPH biodegradation rates that follow second order kinetics better than first order. It should be added in conclusion that this kinetic model and its interpretation are not in any way considered to be a scientific elucidation of the fate of TPH, but are given in order to present some possible interpretations of the results obtained in this study, and perhaps stimulate future considerations for work in the complex area of oil biodegradation kinetics. Similar results were obtained by Dagneu (2004) using both first and second-order biodegradation rate model for light crude oil.

5 CONCLUSIONS AND FUTURE WORK

5.1 Conclusions

The results of this study have demonstrated that supplementary biosurfactant addition was able to significantly increase the extent and rate of oil removal in simulated seawater, compared to oil in water alone. The various DORs employed (1:1, 1:2 and 1:4) were able to facilitate TPH removals of about 59 to 65% TPH from diesel fuel, 60 to 71% TPH from light crude, and 42 to 59% TPH from heavy crude. Although higher DOR ratios were correlated with higher TPH rates of removal, with subsequent doublings of biosurfactant levels, increased removals were not spectacular, as is evident from the relatively narrow percent removal ranges cited above.

The finding that the lowest level of biosurfactant application (DOR 1:4) was able to remove TPH at levels comparable to the highest level of application (DOR 1:1) is important. Even though dispersant to oil ratios were prepared using 2% biosurfactant (in other words the ratios of actual surfactant to oil are relatively low: 1:200 for the lowest DOR and 1:50 for the highest), the higher cost of biosurfactant compared to chemical surfactants, when a large oil slick is involved, makes the difference between a DOR of 1:1 and one of 1:4 significant.

Both biological agents tested also significantly enhanced TPH removal with respect to control levels. Removals were comparable to those achieved by biosurfactant addition. ASAPTM, applied at additive to oil ratios (AOR) of 1:4 and 1:8 was able to facilitate the removals of between 52 and 57% TPH from diesel fuel, 58 to 60% TPH from light crude oil, and 47 to 50% TPH from heavy crude. DegreaserTM tested at the same AOR levels

was able to facilitate the removal of between 54 and 60% TPH from diesel fuel, 58 to 60% TPH from light crude oil, and 47 to 50% TPH from heavy crude.

Treatments involving a combination of rhamnolipid and biological agent displayed the best overall TPH removal efficiencies. A combination of rhamnolipid and Degreaser™ resulted in %TPH removals of about 81% for diesel fuel, 73% for light crude oil and 65% for heavy crude oil. Combinations of rhamnolipid and ASAP™ resulted in %TPH removals of about 73% for diesel fuel, 76% for light crude oil and 55% for heavy crude oil. The mixture of rhamnolipid with both biological agents did not improve TPH removal compared to the mixture of rhamnolipid and a single biological agent.

The findings of this study show that removal of PH from weathered oil salt-water systems was reliably related with increases in microbial density, following a pattern consistent with biodegradation of a limited substrate supply. Abiotic loss of TPH was essentially ruled out. Rhamnolipid was believed to enhance biodegradation through dispersion of oil into the aqueous phase; thereby, rendering it more bioavailable. The exact process by which the biological agents enhanced TPH biodegradation is not known due to the complex nature of these additives, but may be due in part to their proprietary oil-degrading bacterial population. In addition, chemical surfactants as well as added nutrients may have helped to disperse oil and enhance biodegradation rates for TPH, respectively.

Biodegradation rates seemed to follow second order kinetics as opposed to first order. The overall average determination coefficient for the second order model was 0.75, while that for the first order model was only 0.55. Fitting batch test TPH removal data to a

kinetic model was seen as a way to develop half-life estimates for oil biodegradation in order to rank or predict treatment efficacy.

The enhancement of TPH removal with the addition of biosurfactant to biological agents, also point to the possibility of enhancing biological cleaning agents with naturally occurring, readily biodegradable alternative surfactants; thus, avoiding the use of potentially toxic chemical surfactants that may have an inhibitory effect on biodegradation of the very material they are dispersing.

5.2 Future Work

Although lab scale testing cannot reproduce the dynamic environmental conditions involved in actual oil spills, it is still possible to shed light on the process of oil dispersion and biodegradation using laboratory experiments. Some possible avenues for future study are listed below.

- To determine the effect of various parameters, such as dissolved oxygen level, pH, water turbulence, and temperature on oil dispersion and biodegradation.
- To assess biodegradation of oil under conditions where photodegradation can occur. Metabolic inhibition of bacterial populations can be used to assess TPH loss through photodegradation. Metabolically active microbial populations could assess the combined effects of biodegradation and photodegradation. Comparing these results with results obtained for identical tests, but without light, could help determine how sunlight affects oil dispersion and biodegradation.

- To evaluate the potential effect of other biosurfactants, such as sophorolipids, surfactin, etc. on oil dispersion and biodegradation.
- To evaluate the effect of oil properties, such as composition, sulfur content, metal content, etc. on oil dispersion and biodegradation.
- To study TPH biodegradation using more rigorous analyses techniques such as GC-MS so as to better characterize and compare differences in hydrocarbon biodegradation selectivity.

6 REFERENCES

- Aldrett, S., Bonner, J.S., McDonalds, T.J., Mills, M.A., Autenrieth, R.L. (1997a). "Degradation of crude oil enhanced by commercial microbial cultures." Proceedings of 1997 International Oil Spill Conference, American Petroleum Institute, Washington DC, pp995-996.
- Aldrett, S., Bonner, J.S., Mills, M.A., Autenrieth, R.L. and Stephens, F.L. (1997b). "Microbial degradation of crude oil in marine environments tested in a flask experiment." *Water Research*, Vol.31, pp.2840-2848.
- Alexander, M. (1999). "Biodegradation and bioremediation" Academic Press, 2nd Ed., San Diego, CA, pp.190-202, 271-279, 282-283, 287.
- Alleman, B.C., and Foote, E.A. (1997). "Evaluation of amendments for enhancing microbial activity in soils from site 18 at MCAGCC Twentynine Palms California." Battelle, Columbus, OH. Performing Organization Report Number D.O. 1795. Sponsoring Agency Report Number TCN 96-026.
- American Public Health Association (APHA), American Water Works Association (AWWA), Water Pollution Control Federation (WPCF). (1998). "Standard methods for the Examination of Water and Wastewater." 20th Ed., *American Public Health Association*, pp. 9-34 to 9-40, New York.
- Atlas, R. M. (1993). "Bacteria and bioremediation of marine oil-spills." *Oceanus*, Vol., 36, pp. 71-81.

- Atlas, R. M., and Bartha, R. (1992). "Hydrocarbon biodegradation and oil spill bioremediation." *Adv. Microb. Ecol.*, Vol. 12, pp. 287–338.
- Atlas, R.M. (1981). "Microbial degradation of petroleum hydrocarbons: an environmental perspective." *Microbiological Reviews*, Vol.45 No. 1, pp.180-209.
- Atlas, R.M. (1995). "Petroleum biodegradation and oil spill bioremediation." *Marine Pollution Bulletin*, Vol.31 No. 4-12, pp.178-182.
- Atlas, R.M., and Bartha, R. (1993). "Microbial ecology: fundamentals and applications." Addison-Wesley publishing Company, 3rd Ed.
- ATSDR (Agency for Toxic Substances and Disease Registry). (1999). "Toxicological profile for total petroleum hydrocarbons (TPH)." U.S. Department of Health and Human Services, Public Health Service, Atlanta, GA.
- Avmor Ltd. Biomor ASAPTM floor degreaser technical data sheet (2005a). 950 Michelin, Laval, Quebec, Canada. www.avmor.com
- Avmor Ltd. Biomor Industrial degreaser and concrete cleanerTM technical data sheet (2005b). 950 Michelin, Laval, Quebec, Canada. www.avmor.com
- Bai, G., Brusseau, M. L., Miller, R. M. (1998) "Influence of cation type, ionic strength, and pH on solubilization and mobilization of residual hydrocarbons by a biosurfactant." *Journal of Contaminant Hydrology*, Vol. 30, No. 3, pp. 265-279.
- Banat, I.M., Makkar, R.S. and Cameotra, S.S. (2000). "Potential commercial applications of microbial surfactants." *Appl. Microbiol. Biotechnol.*, Vol.53, pp. 495-508.

- Becker, K. W., Lindblom, G. P. (2005). "Performance evaluation of a new versatile oil spill dispersant." International Oil Spill Conference, IOSC 2005, pp. 6147.
- Bertrand, J. C., Bonin, P., Goutx, M., Gauthier, M., and Mille, G. (1994). "The potential application of biosurfactants in combating hydrocarbon pollution in marine environment." *Res. Microbiol.* Vol. 145, pp. 53–56.
- Bielefeldt, A. R., Cort, T. (2005). "Dual substrate biodegradation of a nonionic surfactant and pentachlorophenol by *Sphingomonas chlorophenolica* RA2." *Biotechnology and Bioengineering*, Vol. 89, No. 6, pp. 680-689.
- Blenkinsopp, S., Sergy, G., Wang, Z., Fingas, M. F., Foght, J., and Westlake, D. W. S. (1995). "Oil spill bioremediation agents: Canadian efficiency test protocols." *Proceedings of 1995 International Oil Spill Conference*, American Petroleum Institute, Washington, DC, pp91–96.
- Block, R.N., Allworth, N., and Bishop, M. (1991). "Assessment of diesel contamination in soil." *Hydrocarbon Contaminated soils*, Vol. 1, pp. 35-148.
- Bragg, J. R., Prince, R. C., Harner, E. J., and Atlas, R. M. (1994). "Effectiveness of bioremediation for the Exxon Valdez oil spill." *Nature*, Vol. 368, pp. 413–418.
- Chevron Products Company Diesel Fuel Technical Review (1998). , Chevron U.S.A. Inc., www.chevron.com
- Christensen, L.B., Larsen, T.A., (1993). "Method for determining the age of diesel oil spills in the soil." *Groundwater Monitoring Review*, pp. 142-149.

- Clark, R.B., (2001). "Marine pollution" 5th Ed. Oxford University Press, Oxford, New York.
- Clayton, J.R., Payne, J.R., Farlow, J.S. (1993). Oil spill dispersants and mechanisms of action and laboratory tests, CRC Press, Boca Raton, Fl.
- Cookson, J.T. (1995). "Bioremediation engineering: design and application" New York, McGraw-Hill.
- Cormack D., (1999). "Response to marine oil pollution- review and assessment" Kluwer Academic Publishers, U.K.
- Cort, T.L., Song, M.S., and Bielefeldt, A.R. (2002) "Non ionic surfactant effects on pentachlorophenol degradation." *Water Research* Vol. 36, pp. 1253–1261.
- Dagneu, M. (2004). "Rhamnolipid Assisted Dispersion and Biodegradation of Crude oil Spilled on Water" MAsc. Thesis, Concordia University, Montreal.
- Desai J, Banat I. (1997). "Microbial production of surfactants and their commercial potential." *Microbiol Mol. Rev.*, Vol. 61, pp. 47–64.
- Deshpande, S., Shiau, B.J., Wade, D., Sabatini, D.A., Harwell, J.H. (1999). "Surfactant selection for the enhancing ex-situ soil washing." *Water Res.*, Vol. 33, pp. 351-360.
- Dixon, B. (1993). "Oil eaters in nature." *Bio/Technology*, Vol. 11, pp. 525.
- Doerffer, J.W., (1992). "Oil spill response in the marine environment" Pergamon Press, Oxford, U.K.

- Eweis, J. B. (1998). "Bioremediation Principals", McGraw-Hill, Boston.
- Falatko D.M., Novak J.T. (1992). "Effects of biologically produced surfactants on the mobility and biodegradation of petroleum hydrocarbons." *Water Environment Res.* Vol. 64, pp. 163-169.
- Floodgate, G., (1984). "The fate of petroleum in marine ecosystems." *Petroleum Microbiology*, In Atlas Ed., Macmillan Publishing Company, New York, pp. 355-398.
- Foght, J.M. and Westlake, D.W.S. (1987). "Biodegradation of hydrocarbons in freshwater". *Vandermeulen and Hrudey Ed.*, Oil in Freshwater: Chemistry, Biology, Countermeasure Technology. Pergamon Press, New York, pp. 217-230.
- Frankenberger, W.T.Jr., Emerson, K.D. and Turner, D.W. (1989). "In situ bioremediation of an underground diesel fuel spill: a case history." *Environmental Management*, Vol. 13, No. 3, pp.325-332.
- Geerdink, M.J., Van Loosdrecht, M.C.M., and Luyben, K.Ch.A.M., (1996). "Biodegradability of diesel oil." *Biodegradation*, Vol. 7, pp. 73-81.
- Haines, J.R., Kleiner, E.J., McClellan, K.A., Koran, K.M., Holder, E.L., King, D.W., Venosa, A.D. (2005). "Laboratory evaluation of oil spill bioremediation products in salt and freshwater systems" *J. Ind. Microbiol. Biotechnol.*, Vol. 32, pp. 171-185.

- Harris, B.C., Bonner, J., Dimitriou-Christidis, P., McDonald, T., Sterling M., Fuller, C., and Autenrieth, R. (2002). "Nutrient Effects on the Biodegradation Rates of Chemically-Dispersed Crude Oil" *Proceedings of the Twenty-fifth Arctic and Marine Oil spill Program (AMOP) Technical Seminar*, Environment Canada, Ottawa, ON, Canada.
- Harvey, S., Elashvili, I., Valdes, J.J., Kamely, D., and Chakrabarty, A.M. (1990). "Enhanced removal of Exxon Valdez spilled oil from Alaskan gravel by a microbial surfactant." *Bio/Technology*, Vol. 8, pp. 228–230.
- Healy, M.G., Devine, C.M. and Murphy, R. (1996). "Microbial production of biosurfactants." *Resources, Conservation and Recycling*, Vol. 18, pp. 41-57.
- Hinchee R.E., Leeson, A., Semprini, L., Kee Ong, Say. (1994). "Bioremediation of chlorinated and polycyclic aromatic hydrocarbon compounds." *Relative Rates of Biodegradation of Substituted Polycyclic Aromatic Hydrocarbons* Ed., CRC Press, Inc, Lewis Publishers, pp.189-200.
- Hoff, R. (1993). "Bioremediation: An overview of its development and use for oil spill cleanup." *Marine Pollution Bulletin*, Vol. 26, No. 9, pp. 476-481.
- Holakoo, L. (2002). "On the capability of rhamnolipids for oil spill control of surface water." MAsc. Thesis, Concordia University, Montreal.
- Huesemann, M.H. (1997). "Incomplete hydrocarbon biodegradation in contaminated soils: limitations in bioavailability or inherent recalcitrance." *Bioremed. J.* Vol.1, No.1, pp. 27-39.

- IPIECA (International Petroleum Industry Environmental Conservation Association) (2001). "Dispersants and their role in oil spill response." *IPIECA Report Series*, 2nd Ed., Vol. 5, UK. www.ipieca.org
- Itoh, S., Suzuki, T. (1972). "Effect of rhamnolipids on growth of *Pseudomonas aeruginosa* mutant deficient in *n*-paraffin-utilizing ability." *Agric. Biol. Chem.*, Vol. 36, pp. 2233-2235.
- ITOPF (International Tanker Owners Pollution Federation Limited) (2006). "Effects of marine oil spills." <http://www.itopf.com/effects.html>
- ITOPF (The International Tanker Owners Pollution Federation Limited) (2002). "Fate of marine oil spills." *Technical information paper., London, UK.* www.itopf.com
- Jeneil Biosurfactants Co. (2002). JBR 425 product data sheet, Revision4, Saukville, WI. <http://www.biosurfactant.com/>
- Jordan, R.E. and Payne, J.R., (1980). "Fate and weathering of petroleum spills in the marine environment." *Ann Arbor Science Publishers, Inc.* Ann Arbor, MI.
- Kanaly, R.A., and Harayama, S. (2000). "Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria" *J. Bacteriol.*, Vol. 182, No. 8, pp. 2059–2067.
- Klimisch, H.J., Andreae, M., Tillman, U., (2003). "Robust Summary of information on crude oil" *American Petroleum Institute.*

- Lang, S. and Wagner, F. (1987). "Structure and properties of biosurfactants; in biosurfactants and biotechnology." pp. 21-45, New York, Marcel Dekker.
- Lang, S. and Wullbrandt, D. (1999). "Rhamnose lipids-biosynthesis, microbial production and application potential." *Appl. Microbiol. Biotechnol.*, Vol. 51, pp.22-32.
- Le Floch, S., Merlin, F.X., Guillerme, M., Dalmazzone, C., and Le Corre, P. (1999). "A field experimentation on bioremediation: Bioren." *Environmental Technology*, Vol. 20, pp. 897-907.
- Leahy, J.G., Colwell, R.R. (1990). "Microbial Degradation of hydrocarbons in the environment." *Microbial Reviews*, Vol. 53, No.3, pp. 305-315.
- Lee, K., Merlin, F.X., (1999). "Bioremediation of oil on shoreline environments: development of techniques and guidelines" *Pure and Applied Chemistry*, Vol. 71, No. 1, pp161-171.
- Lin, C. Y., Lin, C. M. (1995). "Emulsification characteristics of marine fuel oils with water" *Journal of Ship Research*, Vol. 39, No. 1, pp. 86-94.
- Lin, S. (1996). "Biosurfactants: recent reviews." *J. Chem. Tech. Biotechnol.*, Vol. 66, pp. 109-120
- Maier, R.M., Pepper, I.L. Gerba C.P. (2000). "Environmental Microbiology." Academic Press, San Diego, California, USA.

- Maier, R.M., Soberon-Chavez, G. (2000). "Pseudomonas aeruginosa rhamnolipids: biosynthesis and potential applications." *Applied Microbiology and Biotechnology*, Vol. 54, pp. 625–633.
- Makkar R, Cameotra S. (2002). "An update on the unconventional substrates for the biosurfactant production and their (new) applications." *Appl. Microbiol. Biotechnol.*, Vol. 58, pp. 428–434.
- Miller, R. M. (1995). "Biosurfactant-facilitated remediation of metal-contaminated soils." *Environ. Health Perspect.*, Vol. 103, pp. 59-62.
- Miller, R.M. (1996). "Biological processes affecting contaminant fate and transport." I. L. Pepper, C. P. Gerba and M. L. Brusseau (eds.), *Pollution Science Academic Press Inc.*, U.S.A., pp. 78–91.
- Mulligan, C.N. (1998). "On the capability of biosurfactants for the removal of heavy metals from soil and sediments" PhD. Thesis, McGill University, Montreal.
- Mulligan, C.N. (2005). "Environmental applications for biosurfactants" *Environmental Pollution*. Vol.133, pp. 183-198.
- Mulligan, C.N. and Gibbs, B.F. (2004). "Types, production, applications of biosurfactants" *Proc.Indian natn. Acad.*, B70, No.1, pp. 31-55.
- Mulligan, C.N., Yong, R.N. and Gibbs, B.F. (2001). "Surfactant-enhanced remediation of contaminated soil: a review." *Engineering Geology*, Vol. 60, pp. 371–380.

Myers, D. (1999). "Surfactants, interfaces and colloids." *Principles and Applications*, 2nd Ed. Wiley, New York.

National Environmental Technology Application Center (1993). "Evaluation methods manual: Oil spill response bioremediation agents." University of Pittsburgh Applied Research Center, Pittsburgh, PA.

Nichols, W.J. (2001) The U.S. Environmental Protection Agency: National Oil and Hazardous Substances Pollution Contingency Plan, Subpart J Product Schedule (40 CFR 300.900). *Proceedings of 2001 International Oil Spill Conference*. American Petroleum Institute, Washington DC, pp1479-1483.

NOAA (National Oceanic and Atmospheric Administration). (1992). "An introduction to oil spill physical and chemical processes and information management." *NOAA Hazardous Materials Response and Assessment*, Division Report, Seattle, WA.

NOAA and API (1994). "Options for minimizing environmental impacts of freshwater spill response," *National Oceanic & Atmospheric Administration and American Petroleum Institute*.

NRC (National Research Council) (1985). "Oil in the Sea: Inputs, Fates, and Effects" Committee on Oil in the Sea: Inputs, Fates, and Effects, the National Academies Press, Washington, D.C.

- NRC (National Research Council) (2003). "Oil in the Sea III: Inputs, Fates and Effects." Committee on Oil in the Sea: Inputs, Fates, and Effects, National Academies Press, Washington, D.C.
- NRC (National Research Council) (2005). "Oil Spill Dispersants: Efficacy and Effects" Committee on Understanding Oil Spill Dispersants: Efficacy and Effects, National Research Council, The National Academies Press.
- Office of Technology Assessment (1990). "Coping with an oiled sea: an analysis of oil spill response technologies." OTA-BP-O-63, Washington, DC.
- Page, C.A., Bonner J.S., McDonald, T.J., and Autenrieth, R.L. (2002). "Behavior of a Chemically-Dispersed Oil in a Wetland Environment", *Water Research*, Vol. 36, pp. 3821-3833.
- Patin, S., Translator: E. Cascio (1999). "Environmental impact of the offshore oil and gas industry" EcoMonitor Publishing.
- Pennell, K.D., Abriola, L.M., Weber, W.J. Jr. (1993). "Surfactant enhanced solubilization of residual dodecane in soil columns." *Environ. Sci. Technol.* Vol. 27, pp. 2332-2340.
- Prince, R. C., Bare, R. E., Garrett, R. M., Grossmann, M. J., Haith, C. E., Keim, L. G., Lee, K., Holtom, G. J., Lambert, P., Sergy, G. A., Owens, E. H., and Guenette, C. C. (1999). "Bioremediation of a marine oil spill in the Arctic." Alleman & A. Leeson (eds), *In-Situ Bioremediation of Petroleum Hydrocarbon and Other Organic Compounds*, Battelle Press, Columbus, OH, pp. 227-232.

Prince, R. C., Clark, J.R., Lindstrom, J.E., Butler, E.L., Brown, E.J., Winter, G., Grossman, M.J., Parrish, P.R., Bare, R.E., Braddock, J.F., Steinhauer, W.G., Douglas, G.S., Kennedy, J.M., Barter, P.J., Bragg, J.R., Harner, E.J., and Atlas, R. M. (1994). "Bioremediation of the Exxon Valdez oil spill: monitoring safety and efficacy." *Hydrocarbon Bioremediation*. R.E. Hinchee et al. Eds., Lewis Publishers, Boca Raton, Florida, pp107-124.

Prince, R.C. (1993). "Petroleum spill bioremediation in marine environments." *Critical Rev. Microbiol.*, Vol. 19, pp. 217-242.

Pritchard, P.H, Mueller, J.G, Rogers, J.C., Kremer, F.V. and Glaser, J.A (1992). "Oil spill bioremediation: experiences, lessons and results from the Exxon Valdez oil spill Alaska." *Biodegradation* Dixon, B. (1993). "Oil eaters in nature." *Bio/Technology* Vol. 3, pp. 109-132.

Rahman, K.S.M., Banat, I.M., Rahman,T.J., Thayumanavan, T., and Lakshmanaperumalsamy, P. (2002b). "Bioremediation of gasoline contaminated soil by bacterial consortium amended with poultry litter, coir-pith and rhamnolipid biosurfactants". *Bioresource Technol.* Vol. 81, pp. 25-32.

Rahman, K.S.M., Rahman, T.J., Lakshmanaperumalsamy, P. and Banat, I.M. (2002a). "Occurrence of crude oil degrading bacteria in gasoline and diesel station soils." *J. Basic Microbiol.* Vol. 42, pp. 286-293.

- Roane, T. M., Josephson, K. L., and Pepper, I. L. (2001). "Dual-bioaugmentation strategy to enhance remediation of co-contaminated soil." *Appl Environ Microbiol.*, Vol. 67, No. 7, pp. 3208–3215.
- Ron, E.Z. and Rosenberg, E. (2002). "Biosurfactants and oil bioremediation". *Current Opinion in Biotechnology*, Vol.13, pp. 249–252.
- Rosen, M.J. (1978). "Surfactants and interfacial phenomena." John Wiley and Sons, New York.
- Rosenberg, E., Lagmann, R., Kushmaro, A., Taube., R., Adler, R., and Ron, E.Z. (1992). "Petroleum bioremediation: a multiphase problem." *Biodegradation*, Vol. 3, pp. 337-350.
- Rouse, J.D., Sbatini, D.A., Suflita, J.M. and Harwell, J. H. (1994). "Influence of surfactant on the microbial degradation of organic compounds" *Critical Reviews in Environmental Science and Technology*, Vol. 24, pp. 325-370.
- Sabatini D.A., Knox, R.C., and Harwell, J.H. (1995). "Surfactant-enhanced subsurface remediation: emerging technologies" *Oxford University Press*, UK.
- Sabatini, D.A., Knox R.C., and Harwell J.H. (1995). "Emerging technologies in surfactant-enhanced subsurface remediation." D. A. Sabatini, R. C. Knox, and J. H. Harwell (eds.), *Surfactant enhanced subsurface remediation*, ACS Symposium Series 594, American Chemical Society, Washington, D. C. pp. 1-6.

- Scheibenbogen, K., Zytner, R. G., Lee, H., and Trevors, J.T. (1994). "Enhanced removal of selected hydrocarbons from soil by *Pseudomonas aeruginosa* UG2 biosurfactants and some chemical surfactants." *J. Chem. Technol. Biotechnol.* Vol. 59, pp. 53–59.
- Schramm, L.L. (2000). "Surfactants: fundamentals and applications in the petroleum industry." *American Chemical Society*, Washington, DC.
- Sendstad, E. (1980). "Accelerated biodegradation of crude oil on Arctic shorelines." *Proceedings of the Third Arctic and Marine Oil Spill Program Technical Seminar*, Environment Canada, Ottawa, Canada.
- Shabtai, Y., and Gutnick, D. L. (1985). "Tolerance of *Acinetobacter calcoaceticus* RAG-1 to the cationic surfactant cetyltrimethylammonium bromide: role of the bioemulsifier emulsan." *Appl. Environ. Microbiol.* Vol. 49, pp. 192–197.
- Shreve, G.S., Inguva, S., Gunman, S. (1995). "Rhamnolipid biosurfactant enhancement of hexadecane biodegradation by *Pseudomonas aeruginosa*." *Molecular Marine Biology and Biotechnology*, Vol. 4, pp. 331–337.
- Simon, M.A., Bonner, J.S., Page, C.A., Townsends, R. T., Muellera, D. C., Fullera, C. B., and Autenrieth, R. L. (2004). "Evaluation of two commercial bioaugmentation products for enhanced removal of petroleum from a wetland." *Ecol. Eng.* Vol. 7, pp. 263–277.
- Song, C., (2000). "Introduction to chemistry of diesel fuels in chemistry of diesel fuels." Edited by Song, C., Hsu, C.S., Mochida, I., *Taylor & Francis*, New York.

- Speight, J. G. (1991). "The chemistry and technology of petroleum." Marcel Dekker, New York.
- Stowe, K. S. (1983). "Ocean science." *Wiley*, New York.
- Suflita, J.M., Smolenski, W.J., and Robinson, J.A. (1987). "Alternative nonlinear model for estimating second-order rate coefficients for biodegradation." *Appl Environ Microbiol.* Vol. 53, No. 5, pp. 1064–1068.
- Swannell, R.P.J., Lee, K., and McDonagh, M. (1996). "Field evaluations of marine oil spill bioremediation." *Microbiological Reviews*, Vol. 60, No. 2, pp. 342-365.
- Swannell, R.P.J., Mitchell, D., Lethbridge, G., Jones, D., Heath, D., Hagley, M., Jones, M., Petch, S., Milne, R., Croxford, R., and Lee, K. (1999). "The use of bioremediation to treat an oiled shoreline following the Sea Empress Incident." B.C. Alleman & A. Leeson (eds), *In-Situ Bioremediation of Petroleum Hydrocarbon and Other Organic Compounds*, Battelle Press, Columbus, OH, pp. 239-244.
- Urum, K., Pekdemir, T. (2004). "Evaluation of biosurfactants for crude oil contaminated soil washing." *Chemosphere*, Vol. 57, pp. 1139-1150.
- USEPA (U.S. Environment Protection Agency) (1993). "Bioremediation resource guide" Office of Solid Waste and Emergency Planning.

USEPA (U.S. Environment Protection Agency) (2004). "Treatment technologies for site cleanup: Annual status report (eleventh edition)." Office of Solid Waste and Emergency Planning. Cincinnati, OH.

USEPA (U.S. Environment Protection Agency) (2006a). *Office of emergency program*.
<http://www.epa.gov/oilspill/overview.htm>

USEPA (U.S. Environment Protection Agency) (2006b). *Office of emergency program*.
<http://www.epa.gov/oilspill/oiltech.htm>

USEPA (U.S. Environment Protection Agency) (2006c). Rhamnolipid biosurfactant (110029) Fact Sheet. www.epa.gov/pesticides/biopesticides/ingredients/factsheets/factsheet_110029.htm

USEPA (U.S. Environment Protection Agency). (1999). "Understanding Oil Spills and Oil Spill Response" *EPA 540-K-99-007, Office of Emergency and Remedial Response*.

USEPA (US Environment Protection Agency). (1996). "Bioremediation agent effectiveness test" *Title 40 code of federal regulations, Pt. 300, Appendix C, Narragansett, R.I.*

Van Dyke, M. I., Gulley, S. L., Lee, H., and Trevors, J. T. (1993). "Evaluation of microbial surfactants for recovery of hydrophobic pollutants from soil." *J. Ind. Microbiol.*, Vol. 11, pp. 163–170.

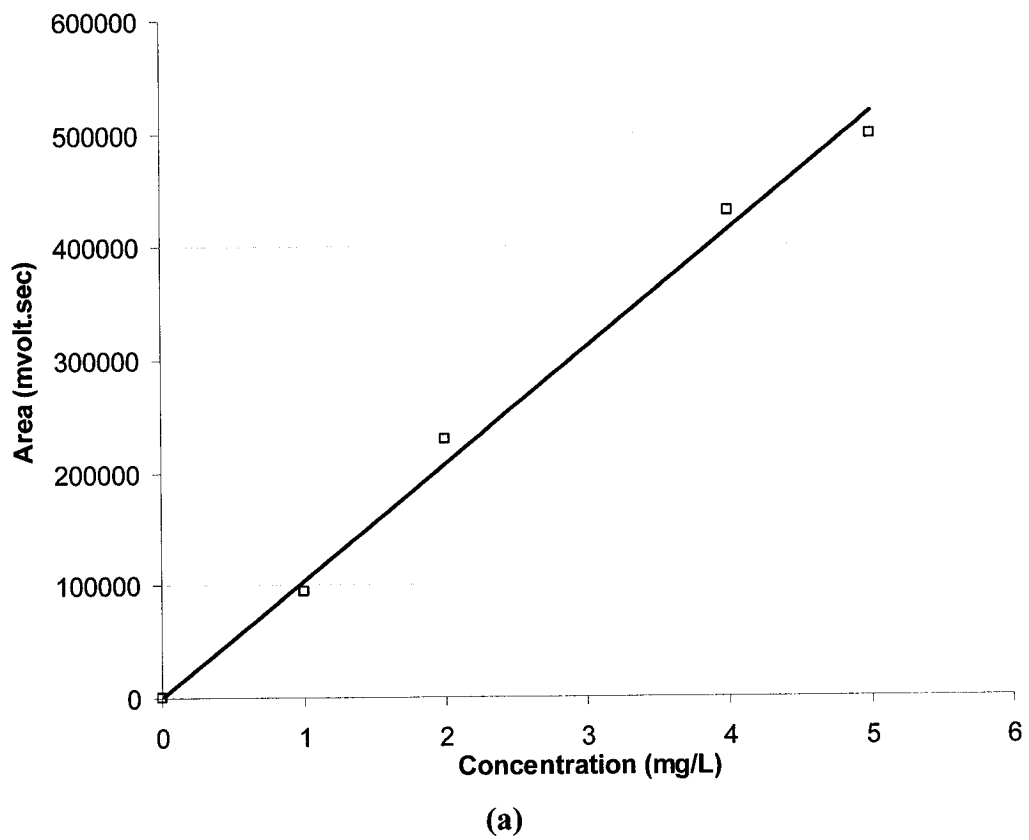
- Venosa, A. D., Lee, K., Suidan, M. T., Garcia-Blanco, S., Cobanli, S., Moteleb, M., Haines, J.R., Tremblay, G., and Hazelwood, M. (2002). "Bioremediation and biorecovery of a crude oil-contaminated freshwater wetland on the St. Lawrence River." *Bioremediation Journal*, Vol. 6, No. 3, pp. 261-281.
- Venosa, A. D., Suidan, M. T., Wrenn, B. A., Strohmeier, K. L., Haines, J. R., Eberhart, B. L, King, D.W., and Holder, E. (1996) "Bioremediation of experimental oil spill on the shoreline of Delaware Bay." *Environmental Science and Technology*, Vol. 30, pp. 1764-1775.
- Venosa, A.D. (1998). "Oil spill bioremediation on coastal shorelines: a critique." S.K. Sikdar & R.I. Irvine(Eds.), *Bioremediation: Principles and Practice*. Vol. III. *Bioremediation Technologies*. Technomic, Lancaster, PA, pp259-301.
- Venosa, A.D., Haines J.R., Nisamaneepong W., Govind R., Pradhan S., and Siddique B. (1992). "Efficacy of commercial products in enhancing oil biodegradation in closed laboratory reactors." *J. Ind. Microbiol*, Vol. 10, pp. 13-23.
- Venosa, A.D., Suidan, M.T., Wrenn, B. A., Strohmeier, K. L., Haines, J. R., Eberhart, B. L, King, D.W., and Holder, E. (1996). "Bioremediation of experimental oil spill on the shoreline of Delaware Bay." *Environmental Science and Technology*, Vol. 30, pp. 1764-1775.
- Vidali, M. (2001). "Bioremediation. An overview." *Pure Appl. Chem*. Vol. 73, No. 7, pp. 1163-1172.

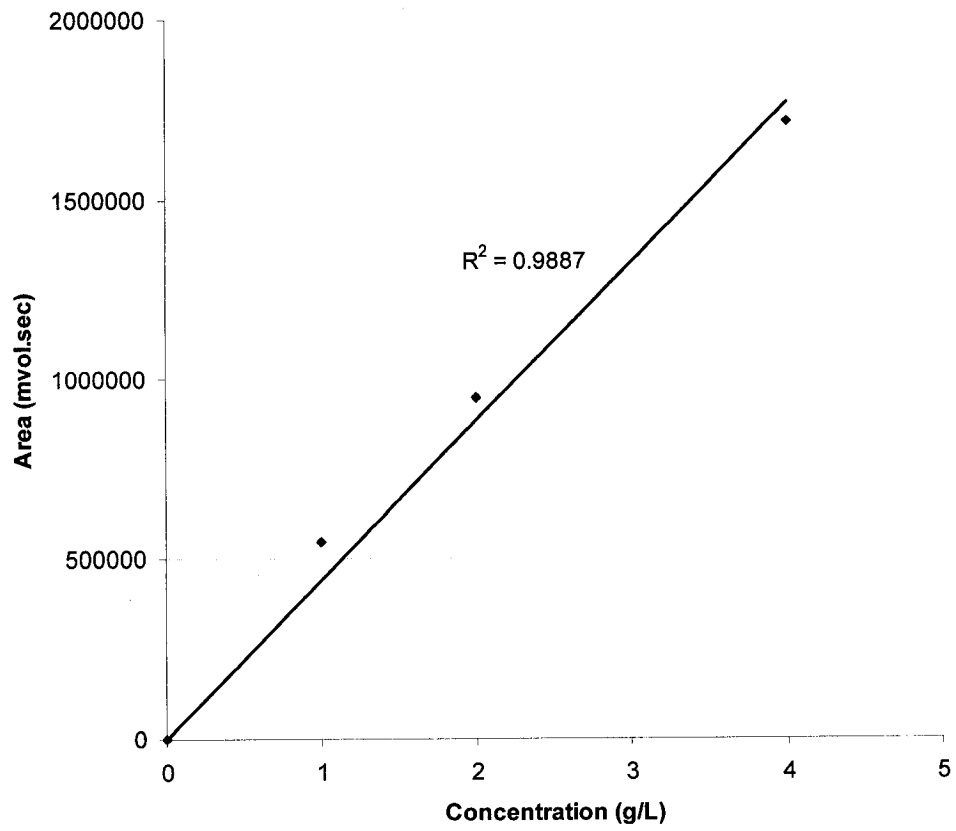
- West, C.C., and Harwell, J. H. (1992). "Surfactants and subsurface remediation." *Environ. Sci. Technol.* Vol. 26, pp. 2324-2330.
- White, R.E., Bertola, G. (2005). "Principles and Practices of Soil Science, the soil as a natural resource." Blackwell Publishing Professional, 4th Ed.
- Xia, W. X., Li, J. C., Zheng, X. L., Bi, X. J., and Shao, J. L. (2006). "Enhanced biodegradation of diesel oil in seawater supplemented with nutrients." *Eng. Life Sci.*, Vol.6, No. 1, pp. 80-85.
- Yang, J., Liu, X., Long, T., Yu, G., She, P., Liu, Z. (2002). "Solubilization and biodegradation of phenanthrene in aqueous nonionic surfactant systems." Proceedings of the Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds, pp. 591-598.
- Zhang, C., Valsaraj, K. T., Constant, W. D., Roy, D. (1998). "Surfactant screening for soil washing: Comparison of foamability and biodegradability of a plant-based surfactant with commercial surfactants." *Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances & Environmental Engineering*, Vol. 33, No. 7, pp. 1249-1273.
- Zhang, Y. and Miller, R.M. (1992). "Enhanced octadecane dispersion and biodegradation by a pseudomonas rhamnolipid surfactant (biosurfactants)." *Applied and Environmental Microbiology*, Vol.58, No.10, pp.3276-3282.

- Zhang, Y. and Miller, R.M. (1995). "Effect of rhamnolipid (biosurfactants) structure on solubilization and biodegradation of n-alkanes." *Applied and Environmental Microbiology*, Vol.61, No.6, pp.2247-2251.
- Zhu, X., Venosa, A.D., Suidan, M.T., and Lee, K. (2001). "Guidelines for the Bioremediation of Marine Shorelines and Freshwater Wetlands" *U.S. Environmental Protection Agency Office of Research*, Cincinnati.
- Zhu, X., Venosa, A.D., and Suidan, M.T. (2004). "Literature review on the use of commercial bioremediation agents for cleanup of oil-contaminated estuarine environments." U.S. Environmental Protection Agency National Risk Management Research Laboratory.
- Zobell, C.E. (1973). "Microbial degradation of oil: Present status, problems, and perspectives." *The Microbial Degradation of Oil Pollutants*, In Ahearn and Meyers Eds., Publication No. LSU-SG-73-01, Louisiana State University, Baton Rouge, LA, pp3-16.
- Zwick, T.C., Foote, E.A., Pollack, A.J., Boone J. L., Alleman, B.C., Hoepfel, R.E., Bowling L. (1997). "Effects of nutrient addition during bioventing of fuel contaminated soils in an arid environment. In: *In-Situ and On-Site Bioremediation: Volume 1*, Battelle Press, Columbus, OH, pp. 403-409.

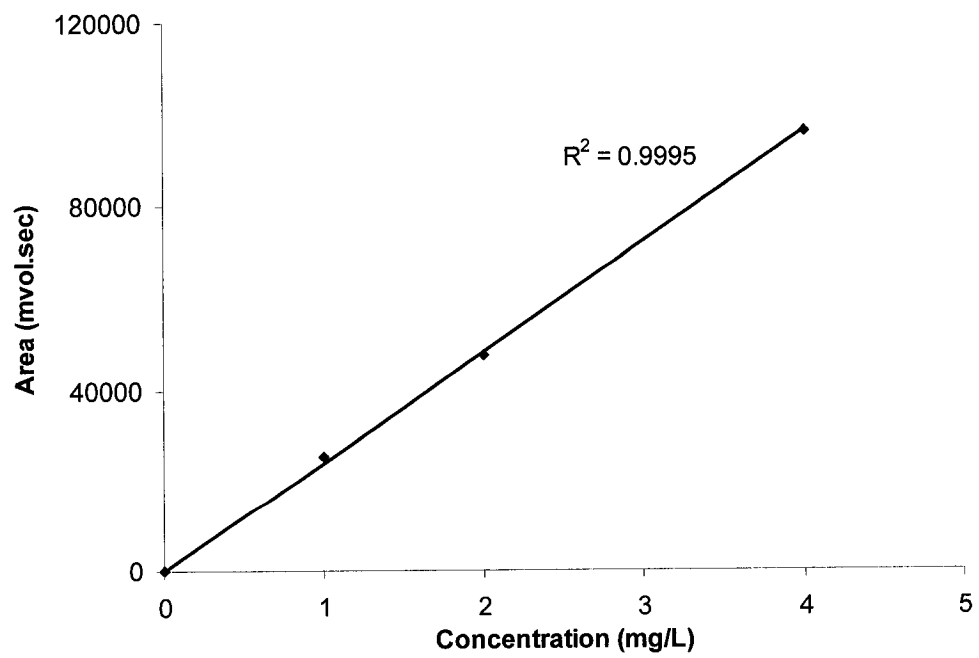
7 APPENDIX

Gas chromatography (GC) calibration curve (a) diesel fuel, (b) light crude oil, (c) heavy crude oil





(b)



(c)