NOTE TO USERS

RHAMNOLIPID ASSISTED DISPERSION AND BIODEGRADATION OF CRUDE OIL SPILLED ON WATER

Martha D. Dagnew

A Thesis

In

Building, Civil and Environmental Engineering

Presented in Partial Fulfillment of the Requirements
for the Degree of Masters of Applied Science at
Concordia University
Montreal, Quebec, Canada

December 2004

Your file Votre référence
ISBN: 0-494-04352-0
Our file Notre référence
ISBN: 0-494-04352-0

ABSTRACT

RHAMNOLIPID ASSISTED DISPERSION AND BIODEGRADATION OF CRUDE OIL SPILLED ON WATER

Martha D. Dagnew

An oil spill caused by ship, pipeline, or oil platform disaster is a significant threat to marine and shoreline ecosystems. Booms and skimmer systems have proven to be ineffective responses. Chemical dispersants have proven effective, but they do not work on weathered oil and may pose health hazards. To reduce the toxicity and enhance biodegradation of the dispersed oil, biosurfactants can be used as opposed to chemical surfactants for open water oil spill response applications. This study examined the effect of rhamnolipid biosurfactant, JBR 425™, on the dispersion and biodegradation of BRENT crude oil spilled on surface water. Crude oil dispersion and biodegradation experiments were conducted according to the methods currently required for listing dispersants on the National Contingency Planning schedule (USEPA 1996).

At 20±2°C and 35‰ salinity, the rhamnolipid biosurfactant dispersed 10.2%, 12%, 22%, 33%, 41%, 50%, 55%, 57%, 68%, 79% and 82% of fresh BRENT crude oil into the water column when applied at 0.125%, 0.25%, 0.5%, 1.0%, 1.5%, 2%, 3%, 4%, 6%, 8% and 12% concentrations, respectively. Additional tests for conditions relevant to cold (10±2°C) temperature regions and weathered spilled BRENT oil showed slightly lower performance. Monitoring biodegradation of crude oil over 3, 7, 14 and 35 days and fitting the results into first and second order kinetics showed higher crude oil biodegradation

rate for reactors treated with bio-dispersant (solution of rhamnolipid biosurfactant in pH 7.5 buffer) and biological agents in comparison to natural biodegradation. On the other hand application of the chemical dispersant (Corexit 9500™) dispersed the crude oil very effectively, however had an inhibitory effect on its biodegradation. The results obtained from this study show the potential of biosurfactants in cleaning oil spills effectively with lower toxicity and higher stability against lower temperature and degree of oil weathering.

ACKNOWLEDGEMENTS

I would like to express my gratitude to all those who gave me the possibility to complete this thesis. I especially would like to thank my supervisor, Dr. Catherine Mulligan, for her guidance, motivation, encouragement, and understanding during the course of this study. I have appreciated her support and also her working style: promoting self-confidence and autonomy. I am also grateful to Mr. R. Parisella for helping me carry out the chemical and microbiological analysis as well for providing constructive comments during my research. I also acknowledge my graduate committee Drs. J. Hadjinicolaou, J. Powlowski and R. Zemeureanu.

I am grateful to the faculty, staff and my friends in the department of Building, Civil and Environmental engineering for their assistance in every aspect of my study. The financial support received from my supervisor and Faculty of Engineering, Concordia University is fully acknowledged. Special thanks to PETRO-Canada for funding the project. Also thanks to Jeneil Biosurfactant and Avmor Company for supplying the biological products.

Finally, I would like to thank my families for their continuous support and encouragement. Especial note of thank also goes to my husband, G. Bitsuamlak, for his love, support and proof reading of the manuscript.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

NOMENCLATURE

α	Level of significance
ANOVA	Analysis of variance
ANS	Alaska North Slope
ASRIT	Activated sludge respiration inhibition
ASTM	American society for testing and materials
BFT	Baffled flask test
C	Concentration of crude oil (gL^{-1})
C_0	Concentration of crude oil at day 0 (gL^{-1})
C_{14}	Concentration of crude oil after 14 days of incubation (gL^{-1})
C_3	Concentration of crude oil after 3 days incubation (gL^{-1})
C_{35}	Concentration of crude oil after 35 days of incubation (gL^{-1})
C_7	Concentration of crude oil after 7 days incubation (gL^{-1})
CDO	Chemically dissolved oil
CFU	Colony forming units
CMC	Critical micelle concentration
CO_2	Carbon dioxide
CV	Coefficient of variation
DCM	Dichloromethane
DOR	Dispersant to oil ratio
DSL	Domestic substance list
EC	Environment Canada
EC_{50}	The concentration of a toxic substance which produces a

	designated effect in 50% of the exposed test organisms tested under specific test conditions and time periods.
FID	Flame ionization detector
GC	Gas chromatography
H_2O	Water
H_2SO4	Sulfuric acid
HCl	Hydrochloric acid
HLB	Hydrophilic-lipophilic balance
HPLC	High performance liquid chromatography
k	First order biodegradation rate coefficient (day^{-1})
k_t	First order biodegradation rate coefficient at time, t (day^{-1})
K	Second order biodegradation rate coefficient ($Lg^{-1}day^{-1}$)
K_t	Second order biodegradation rate coefficient at time, t($Lg^{-1}day^{-1}$)
LC_{50}	The concentration of a toxic substance which is fatal to 50 percent of the organisms tested under specific test conditions and time periods.
M	Mass of crude oil (g)
NCP	National contingency planning
NRC	National Research Council
OECD	Organization for Economic Development
p	Probability
PAH	Polycyclic aromatic hydrocarbon
SDS	Sodium dodecyl sulphate

SFT	Swirling flask test
t	Time of incubation
USEPA	United States Environmental Protection Agency
UV-VIS	Ultraviolet-visible

1 Introduction

1.1 Statement of purpose

Oil spills pose a significant hazard for terrestrial and marine ecosystems. In a report published in 2002 by the National Research Council (NRC), the world production of crude oil by the end of 2000 was about 80 Mbbl (11 Mt/ day) and this is expected to increase by 1.9% per year in the next decade (NRC 2002). Approximately 40% of the world's oil travels by water sometime between its production and final consumption. According to the report, the average total worldwide annual release of crude oil from all known sources to the sea has been estimated as 1.3 Mt/year. Of this, 47% comes from natural seeps, 38% from consumption of oils (operational discharges from ships, pipeline, and coastal facility, and discharges from land-based sources), 12% from accidental spills from ships and 3% from extraction of oils. Although efforts have been expended to minimize anthropogenic releases into the environment, accidents are virtually unavoidable when such large volumes of crude oil are being transported globally, especially since oceanic transport is the only feasible means of intercontinental movement.

Once oil is discharged into the marine environment, it is immediately subjected to a variety of physical, chemical and biological changes. Examples of such processes may include evaporation, dispersion, photochemical oxidation, adsorption onto suspended particulate material, sinking and sedimentation as well as microbial degradation. These processes cause important changes in the physical properties of the original pollutant, which in turn may have a positive effect on its potential impact on the marine

environment. However these natural processes are slow and often the economic and environmental impact of oil spills on coastal areas can be immense.

Oil spill occurrences may result in loss of habitat for economically important species of fish, birds, other marine animals, and damage to sensitive wetlands along the coast. Recovery of the environment from an oil spill could also take many years; hence, there is a considerable incentive to quickly clean up these areas after spill, but the efforts can be expensive and themselves destructive. The choice of spill response techniques is dependent on the spilled oil properties such as its viscosity, composition, pour point temperature, and other environmental and socio economic factors. Thus, for an effective and timely response pre-established contingency plans that consider the above factors are required.

In line with this, several techniques have been used to reduce the environmental and economical impacts. Cleaning up techniques include conventional oil spill responses such as mechanical recovery i.e. collection of oil either with booms, skimmers and absorbents as the first priority choices of the responders, but this rarely is easy, nor very effective (maximum 10% recovery) after a large spill. In addition to this, the associated cost with these response techniques is higher ($12,527 per Mt of spilled oil) (Holakoo 2002). On the contrary, the use of dispersants at lower cost ($2,137) can enable significant fractions of oil to be removed from the sea surface by dispersing the oil into the water column. However, the use of present day (chemical) dispersants raise concerns in regards to the dispersant's toxicity and effectiveness as well the fate and impact of chemically dispersed oil. A research effort is therefore required towards formulation of environmental friendly dispersants to reduce their toxicity and further enhance the biodegradation of the

dispersed oil in the marine environment.

1.2 Objectives and scope of the study

One of the debates in applying chemical dispersants as oil spill response tool is their toxicity to the marine environment. To resolve this issue, environmental friendly alternatives i.e. utilization of biosurfactants (bio-dispersants) as opposed to chemical surfactants (dispersants) for cleaning up oil spills are required. The main objective of this research is, therefore, to determine the efficacy of rhamnolipid biosurfactant (JBR 425™) for oil spill dispersion and further enhancement of bioavailability and hence biodegradation of crude oil spilled on surface water.

The specific objectives of the research are to:

1. Evaluate the effectiveness of rhamnolipid biosurfactant on crude oil dispersion and investigate the effects of solvent type, temperature and degree of oil weathering on its efficacy under laboratory conditions.

2. Determine whether rhamnolipid biosurfactant application not only reduces the risk of the oil polluting the shoreline (by dispersing the oil), but also enhances the rate of removal from the environment by increasing the bioavailability and biodegradation of crude oil spilled on surface water. The study will evaluate the rate of biodegradation for naturally, chemically and biologically dispersed oil under laboratory conditions.

1.3 Plan of thesis

This thesis consists of five chapters, a list of references and appendices. Chapter 2 describes the necessary theoretical background in oil spill dispersants including their formulation and mechanisms of action. It presents reviews of previous research findings in the potential of dispersants for oil spill response, their limitations and recent advances in relation to oil spill dispersant effectiveness testing, as well the potential of biosurfactants for oil spill response. Chapter 3 describes the experimental design as well the methods and materials used. Experimental results obtained in the study are presented and discussed in Chapter 4. Chapter 5 summarizes the findings of this study and presents recommendations for future work.

2 Background knowledge and literature review

2.1 Introduction

This chapter reviews the current literature on the potential of dispersants for oil spill response, their limitations and recent advances in relation to dispersant effectiveness testing methods, as well the potential of biosurfactants (which is the subject of the present research) for oil spill response. To facilitate the comprehension of the literature review and the present study, background knowledge on oil spill dispersants is also presented. This includes dispersant's formulation and mechanisms of action, dispersant effectiveness and their role in the oil biodegradation process.

2.2 Oil spill dispersants

When oil is spilled at sea, some proportion will be naturally dispersed by mixing action caused by waves. This process can be slow and proceed to only a limited extent for most cases (Fiocco and Lewis 1999). Dispersants are used to accelerate the removal of oil from the surface of the sea by improving the rate of natural dispersion of oil and thus prevent it from coming ashore. In addition, dispersants generate droplets that are typically much smaller than would otherwise form by the natural energy of the sea, which often results in recolesence of the dispersed oil to the surface (Fiocco and Lewis 1999). The rationale behind dispersant use is therefore that dispersed oil will have decreased environmental and economical impact compared to oil remaining on the surface of the sea, which can eventually come to the shoreline. Dispersants/ surfactants can be chemically synthesized (chemical surfactant) or microbially produced (biosurfactant). At present, the most common ones used for spill response are chemical dispersants. Details

are discussed below.

2.2.1 The chemistry of dispersants

Dispersants are liquid blends of surfactants, solvents and additives, designed to accelerate break up of oil slicks into fine droplets that disperse naturally in the sea (Fiocco and Lewis 1999). The actual compositions of the specific dispersant products differ significantly in terms of the type of surfactants and solvents used in their formulation. This in turn affects the rate of application, dispersion efficiency, toxicity and choice of application methods.

2.2.1.1 Solvents

The solvent content of a dispersant has functions including: solubilizing the surfactant or blend of surfactants, providing a viscosity appropriate for dispersant application systems and assisting in the penetration of the oil slick and further diffusion of the surfactant to the oil-water interface (Clayton et al. 1993; Myers 1999). Solvents in use for dispersants can generally be categorized into three classes: hydrocarbon, water and water miscible solvents (Clayton et al. 1993).

Hydrocarbon-based: The hydrocarbon-based solvents could be based on aromatic or non-aromatic hydrocarbon solvent based systems. The former were used by earlier generation dispersants on the *Torrey Canyon* spill over 30 years ago on the coast of England. They contain aromatic compounds such as benzene, toluene and xylene (Clayton et al. 1993). Due to their toxicity on marine organisms, they are no longer in use. However, the non-aromatic hydrocarbon solvents have relatively lower toxicity and

are still used in modern day dispersants. These types of solvents have high affinities to oil and promote penetration and mixing of surfactants into high viscosity and weathered oils (Clayton et al. 1993). Examples of such solvents in current use include ethylene glycol monobutyl ether, dipropylene glycol monomethyl ether, de-aromatized kerosene and isoparaffinic solvents (Fiocco and Lewis 1999).

Water-based: Due to its low affinity to the oil slick, the use of a water-based solvent is more effective for fresh oil spills and low viscosity oils (Clayton et al. 1993). Both the water and non-aromatic hydrocarbon solvent-based systems allow only lower contents of surfactants (15 to 25%) thus require high application rates, e.g. Dispersant to Oil Ratio (DOR) of 1:3, and additional mechanical agitation, such as ship propellers (Fiocco and Lewis 1999).

Water miscible hydroxyl compounds: As compared with the hydrocarbon-based solvents, water miscible solvents have relatively lower toxicity and are also used by most of modern day dispersants (Fiocco and Lewis 1999). Examples include oxygenated compounds such as glycols and glycol ethers (Clayton et al. 1993). These types of solvents allow much higher surfactant contents to about 65%, resulting in concentrated dispersants. Thus they are effective at lower application rates, e.g. 1:20 DOR (Fiocco and Lewis 1999; Mackay 1995).

2.2.1.2 Additives

Additives may or may not be present in dispersant formulations. If present their main purpose is to increase the biodegradability of the dispersed oil (for example nutrients, micro organisms), improve the dissolution of the surfactant and/ or increase stability of

the dispersed droplets (Clayton et al. 1993).

2.2.1.3 Surfactants

Surfactants, often called surface-active agents, are the most important components in the dispersant mixture for the actual dispersion process (Clayton 1993). They are compounds containing water seeking hydrophilic (head) and oil seeking lipophilic (tail) groups as shown in Figure 2.1. Because of this distinct nature, when present in relatively low concentrations, they reduce the free energy of the system by replacing the bulk molecules of higher energy at the interface (Clayton 1993, Mulligan and Gibbs 2004).

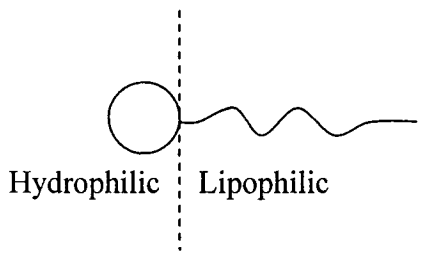


Figure 2.1 Schematic representation of surfactant molecule

Surfactants can either be chemically synthesized (synthetic) or microbially produced (biosurfactant). A property that all surfactants have in common is that they reduce the surface tension of a liquid medium. Synthetic surfactants are of petrochemical origin and are the most used for present day dispersant formulations. Depending on the charge of the hydrophilic group they can be classified as non-ionic, anionic, cationic and amphoteric surfactants. Chemical dispersant formulations contain only non-ionic and anionic surfactants (Clayton et al. 1993).

In order to facilitate the comprehension of the present work, it has been found necessary

to discuss the surface activity and effect of surface-active material addition between two liquids in a more detail. In general the potential energy at the interface between two immiscible (oil and water) molecules is higher than those in the bulk material (Myers 1999). Besides the existing interaction with the bulk phase, the molecules at the interface will experience net force field (increased energy) due to adjacent phase interactions. As a result of this increased energy of surface molecules more work is required to move them from the bulk phase to the surface/adjacent phase. So with the application of surface-active materials, the molecules will be absorbed preferentially at available interfaces, replacing the higher energy bulk phase molecules and resulting in a reduction of the free energy of the system as a whole. Overall, this reduces the interfacial surface tension and promotes the miscibility of the two phases with minimal energy. In essence the lowering of interfacial surface tension will promote formation of a greater interfacial surface area for a constant amount of mixing energy applied to the system.

Due to the above dual characteristic, surfactants are useful in industry applications dealing with multiphase systems and can be used industrially as adhesives, flocculants, emulsifiers for emulsion polymerization, wetting and phase dispersion for cosmetics and textiles, and foaming agents for food processing, and detergents for household and industrial cleaning, or solubilization for agrochemicals. Recently they are used in environmental applications such as bioremediation of contaminated land sites (Mulligan and Gibbs 2004). However selection of specific types of surfactants for a required application will vary depending on the distinct properties of each surfactant. Properties of surfactants that influence their efficacy include: efficiency (low Critical Micelle Concentration (CMC)), effectiveness (surface tension reduction), solubility (Hydrophilic-

Lipophilic Balance (HLB)), biodegradability, toxicity, as well pH, and temperature stability (Myers 1999; Mulligan and Gibbs 2004). For example, in pharmaceuticals the required characteristics of surfactants is biocompatibility and low toxicity (Myers 1999) while in oil spill response low toxicity, biodegradability, HLB as well as pH and temperature stability are the most required characteristics. The most common indices for evaluating surfactant activity for oil dispersion are its CMC, effectiveness and HLB, and are discussed below.

Efficiency: The efficiency of a surfactant indicates the amount of the surfactant required to reach the Critical Micelle Concentration (CMC) (Clayton et al. 1993; Mulligan and Gibbs 2004). Higher efficiency surfactant is thus achieved when the CMC is attained at lower surfactant concentration (Mulligan and Gibbs 2004). The CMC is defined as the minimum concentration necessary to initiate micelle formation (Becher 1965). Micelles are small surfactant aggregates composed of a number of surfactant molecules held by forces such as hydrophobic, Van der Waals', electrostatic, and hydrogen bonding interactions (Lin 1996). Since no molecular bonds are formed, micelle stability could easily be transformed with changes in pH, temperature and salinity conditions of the medium (Lin 1996; Mulligan and Gibbs 2004).

From an oil spill response application point of view, the CMC is the concentration at which surfactant molecules form a uniform monolayer at the oil-water interface. As the oil water interface is already occupied by a complete monolayer of surfactant molecules, a further increase in the concentration of surfactants beyond the CMC results in a slight decrease of the interfacial tension (Figure 2.2, adopted and modified from Mulligan and Gibbs 2004). In oil spill response, to have maximum effect and for the benefit of the

doubt, surfactants should be applied higher than their CMC value (Schramm 2000). The CMC value is unique to the surfactant and is also dependent on the temperature, pH and salinity of the medium; thus for better performance it is required to determine its value for a given condition. The CMC value can be obtained by plotting the different physical properties of surfactant versus its concentration and the break in the curve will indicate the CMC value (Figure 2.2).

Effectiveness: The effectiveness of a surfactant is the magnitude of the decrease in the oil-water interfacial tension to the CMC of the surfactant. Thus a surfactant's effectiveness is considered greater when the magnitude of the reduction in the interfacial tension to the CMC of the surfactant is greater. The relationship between oil-water interfacial tension, surface tension, surfactant concentration, and efficiency as well effectiveness is shown in Figure 2.2. Oil spill dispersant effectiveness can be evaluated indirectly using field and/ or laboratory scale. The different methods used to evaluate oil spill dispersant effectiveness are discussed in section 2.3.

Hydrophilic-Lipophilic Balance (HLB): Another index of surfactants is the HLB. It is a coding scale of 0 to 20 that will characterize the tendency of a specific surfactant to be solubilized in an oil phase (low HLB) or aqueous phase (high HLB) (Clayton et al. 1993; Mulligan and Gibbs 1993). Thus, lower HLB surfactants will favour water in oil emulsions, while higher HLB value surfactants favour oil in water emulsion. Dispersant formulations usually have an overall HLB value of 9 to 11, which derives from the mixture of lipophilic and hydrophilic surfactants with HLB values of about 5 and 15, respectively (Clayton et al. 1993).

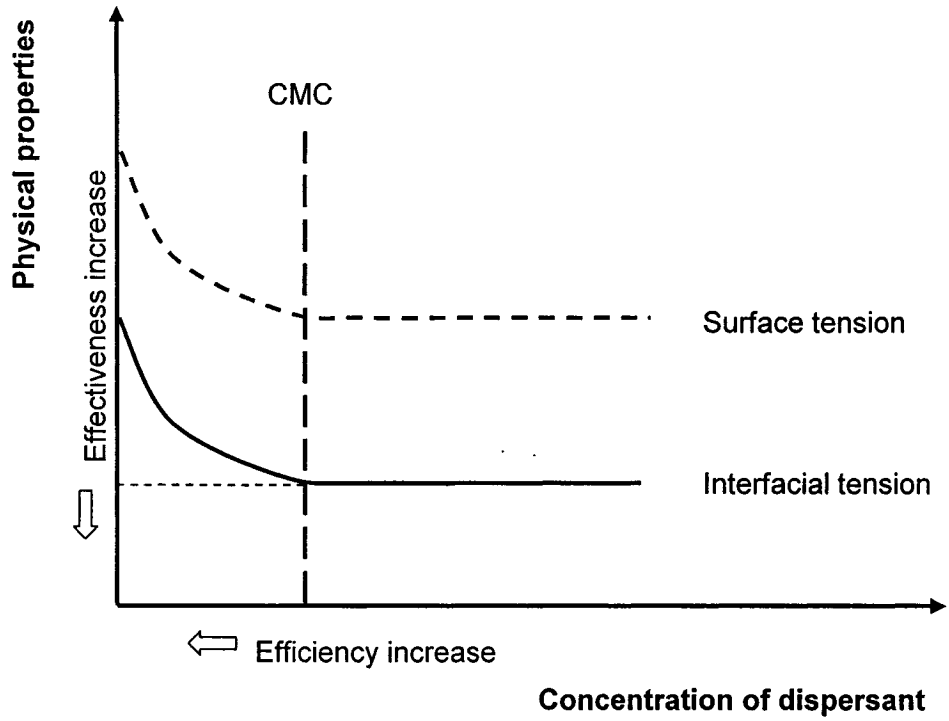


Figure 2.2 The relationship between oil-water interfacial tension, surface tension, surfactant concentration, and efficiency as well as effectiveness (adopted and modified from Mulligan and Gibbs 2004)

2.2.2 The physics of dispersant action

2.2.2.1 Mechanism of dispersion

Enhanced solubility of spilled oil by dispersants is mainly through decreasing interfacial tension, thereby increasing the aqueous dispersion of crude oils at the molecular level (Myers 1999; Fiocco and Lewis 1999; Clayton 1993; and Zhang and Miller 1992). Additional dispersion can be achieved through physical surfactant interaction with slightly water soluble organic compounds; in this case the increase below the CMC is due to hydrophobic interactions between surfactant monomers and slightly soluble organic

compounds and the increase above the CMC due to surfactant encapsulation of slightly soluble organic compounds into micellar aggregates (Zhang and Miller 1992).

Detailed description of the oil dispersion mechanism is given in Figure 2.3. In spill response operation, the dispersant is delivered with a spraying system mounted either from ship or airborne platform. Upon application the dispersant molecules break through the oil slick and reach to the oil water interface with the help of the solvent and the momentum of the droplet spray. As it reaches to the oil-water interface, the dispersant molecules spread and orient at the oil water interface with the hydrophilic groups in the water phase and hydrophobic groups in the oil phase. In doing so, they will reduce the oil-water interfacial surface tension thereby weakening the cohesive strength of the oil film. Thus, with minimum mixing energy (wind/ wave action at the sea) small droplets of oil will break away and enter into the underlying water either individually and/ or by being encapsulated/ pseudosolubilized by the surfactant micelles (Hommel and Ratledge 1993; Fiocco and Lewis 1999; Clayton et al. 1993).

2.2.2.2 Mechanism of dispersion stability

Once dispersed resurfacing and further coalesce of the oil droplets, if they encounter each other, is prevented due to the presence of surfactant molecules surrounding the oil water interfaces of the droplets (Clayton 1993; Fiocco and Lewis 1999; Schuumer 2000). This is accounted for by the presence of the hydrophilic groups of the surfactant molecules at the oil water interfaces of droplets. In addition, Clayton (1993) and Swannell et al. (1999) have described that, if the dispersant action formed small enough droplets, the wave action can keep the small droplets suspended, as opposed to resurfacing or being

deposited on the sediments.

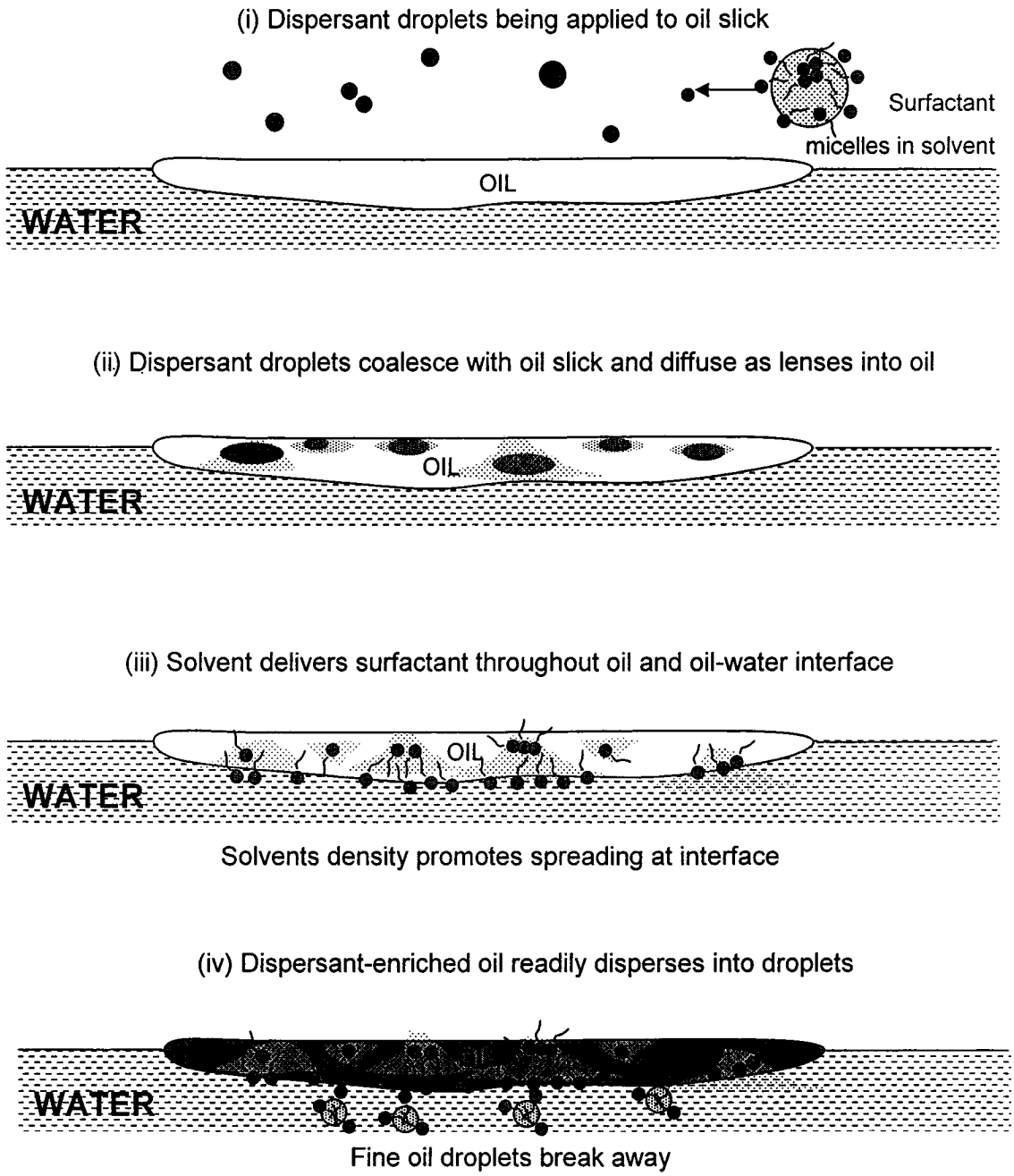


Figure 2.3 Detailed mechanism of chemical dispersion (adopted and modified from Fiocco and Lewis 1999)

2.2.3 Dispersant effectiveness

2.2.3.1 Factors affecting dispersant effectiveness

The major factors affecting the effectiveness of the dispersants in an operational condition are composition of the dispersant product, dispersant application system, composition and state of the oil being dispersed, the dispersant to oil ratio, mixing energy to the system, salinity and temperature (Moles et al. 2002). Prior to their use in field application, a specific oil dispersant product is required to be registered under the National Contingency Plan (NCP) product schedule. Listing of a dispersant product on the NCP requires evaluation and passing of one of the laboratory dispersant effectiveness tests according to the regulations in different regions of the world.

2.2.3.2 Laboratory dispersant effectiveness testing

Despite the continuous effort to develop laboratory and field techniques for measuring the dispersant effectiveness, it is still a difficult task due to the different factors affecting its performance. In the laboratory, factors such as degree of oil weathering, dispersant to oil ratio, salinity and temperature can easily be simulated. However, it is difficult to simulate: (1) the method of application of the dispersant to the oil; (2) the method of mixing of the dispersant into the oil; and (3) the subsequent dispersion of the dispersant/ oil mixture into the water column (Clayton et al. 1993).

To improve the accuracy of laboratory dispersant effectiveness results, over the years, a large number of laboratory tests have been developed by oil spill researchers. So far about 35 methods of testing dispersant effectiveness have been developed (Clayton et al. 1993; Fingas et al. 1999; Sorial et al. 2004a). These tests can generally be classified in

four categories (Clayton et al. 1993): (1) tank tests with water volumes ranging from 1L to 150 L; (2) shake flask tests that are conducted on a relatively smaller scale; (3) interfacial tension tests that measure properties of the treated oil instead of dispersant effectiveness strictly; and (4) flume tests using flowing water systems for simulating real-world conditions of oil spills. In the above tests the dispersant effectiveness is determined by measuring the percent dispersed oil concentration in the water column, percent oil remaining in the surface slick, measuring the dispersed oil droplet size and/ or measuring the interfacial tension (Macnaughton et al. 2003).

In Canada and the United States for a product to be listed on the product schedule, dispersants have been required to pass the shake flask method (Sorial et al. 2004a). In this case, the dispersants are required to have at least a 45% effectiveness value in dispersing two specific oil types i.e. Prudhoe Bay and South Louisiana crude oils under laboratory conditions (Sorial et al. 2004a). As a result, the biosurfactant effectiveness test method used in this research is based on shake flasks and further discussion will be focused on it.

Application of the shake flask method for measuring dispersant effectiveness essentially consists of adding crude oil and dispersant in a flask containing known amounts of sea water and the contents of the flask mixed in an orbital shaker for a specific time, followed by a known settling time. At the end of the settling period, known amounts of sample water are taken from the flask, which is then extracted and measured using either a spectrophotometer or Gas Chromatography (GC) to determine the quantity of the oil that had been dispersed in the water column. There are various shake flask methods that use a variety of test conditions including the dispersant to oil ratio, shaking and settling time,

shaking speed and energy, type of test flasks and method of dispersant addition. Amongst the most commonly used shake flask methods are Environment Canada's Swirling Flask Test, EC SFT (Fingas et al. 1989; 1996; 1999; and ASTM 2002), USEPA's Swirling Flask Test (USEPA SFT) which is the modification of EC SFT (USEPA 1996), Modified Swirling Flask Test (Blondina et al. 1997) and the newly developed Baffled Flask Test, BFT (Sorial et al. 2004a and b). The similarities and differences between different shake flask methods are shown in Table 2.1. More detailed discussion regarding the impact of operational variables such as mixing energy, settling time, shaking time and rotational speed on dispersion effectiveness as well performance of each method can be found in Sorial et al. (2004a and b) and Blondina et al. (1997).

Table 2.1 Differences in operational variables between different shake flask methods

Operational variables	**Method**			
	EC SFT	**USEPA**	**MSFT**	**BFT**
Rotational speed (rpm)	150	150	150	200
Mixing time (minutes)	20	20	20	10
Settling time (minutes)	10	10	10	10
Dispersant addition	Pre-mixed with oil	Pre-mixed with oil	One drop	Oil followed by dispersant
Flask type	Spout	Spout flask	Modified Erlenmeyer	Baffled Trypsinizing
Solvent for extraction	70:30 Dichloromethane: Pentane	Dichloromethane	Dichloromethane	Dichloromethane
Analysis	GC	Spectrophotometer	GC	Spectrophotometer

Although these laboratory methods are simple and straightforward, they are not meant for predicting the actual percent effectiveness to be achieved in the sea by using a specific product. Anticipated results (percent effectiveness values) can be used for: comparison of different dispersant formulations under similar conditions, recommendation of dispersant formulations for oil spill response, to quantify and document the role of different factors affecting dispersants performance, to provide data for use by on-scene coordinators for emergency response and contingency planning and as inputs to mathematical models for predicting dispersant performance (Brandvik and Daling 1998; Fingas et al. 1989).

2.3 Dispersant enhanced biodegradation of spilled oils

It has been reported that many indigenous microorganisms are capable of degrading petroleum hydrocarbons, such as alkanes, paraffins and aromatics (Atlas 1995). Biodegradation of petroleum hydrocarbons involves progressive or sequential reactions, in which certain organisms may carry out the initial attack on the petroleum constituents, producing intermediate compounds. These compounds are subsequently utilized by different groups of organisms in the process that results in further biodegradation and conversion to microbial biomass, carbon dioxide (CO_2) and water (H_2O) over the following days to months (Venosa and Zhu 2003; Harris et al. 2002). In the long run, biodegradation may be the eventual fate of oil spilled at sea that cannot be collected or burnt. Stimulating biodegradation is thus, an important option for maximizing the removal of oil from the environment and minimizing the environmental impact of a spill.

Biodegradation of crude oil is often limited by low substrate solubility, reducing bioavailability to the degrading microorganisms. Bioavailability refers to the partitioning and transport of compounds for uptake by microorganisms for biodegradation. Miller and Bartha (1989) demonstrated that alkane biodegradation is limited partly because of limited transport into the cells. Similarly, Harris et al. (2002) investigated the effect of dispersed oil to water ratio on the oil's rate of biodegradation and concluded that the bulk concentration of the oil in the water column was not a factor controlling the biodegradation rate. Rather it is controlled by partitioning of compounds between the two phases, particle size distribution and the competency of the microbial culture, as well as temperature and nutrients.

It has been suggested that dispersants may enhance oil bioavailability by creating more surface area in terms of multiple small oil droplets, allowing for increased biodegradation of the oil. However, conclusions from laboratory studies regarding enhanced microbial degradation oil using oil dispersants have been contradictory (Prince et al. 2003) as presented in sections 2.3.1-, 3- and 4.

The discrepancies in the dispersant performance are due to the properties of the dispersant (surfactant) used, presence of different hydrocarbon types that interact with the surfactant micelle differently and presence of different micro organisms that use different ways to access the substrate (Van Hamme and Ward 1999). Micro organisms have been shown to access the substrate through: (a) direct interaction with soluble hydrocarbons (b) ingesting surfactant pseudosolubilized substrates and (c) increasing their cell surface hydrophobicity to adhere directly to large oil droplets (Zhang and Miller 1992; Tiehm 1994; Nelson et al. 1996; and Harris et al. 2002). A model of bacteria-oil interaction is

shown in Figure 2.4.

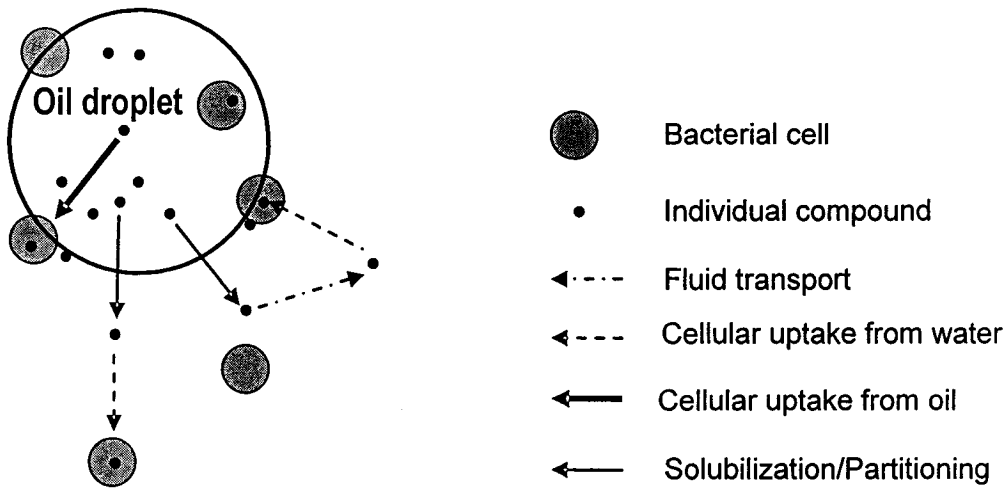


Figure 2.4 Model of bacteria oil interaction (Harris et al. 2002)

2.3.1 Biodegradation in the presence of chemical dispersants

Although significant research has been conducted to study the impact of chemical dispersants on the fate of dispersed crude oil, results are often conflicting. Some researchers have proposed that chemical dispersants/ surfactants have insignificant effect on oil biodegradation (Macnaughton et al. 2003; Page et al. 2002; and Van Hamme and Ward 1999), while others suggest a positive effect (Varadaraj et al. 1995; Mulyono et al. 1994; Van Hamme and Ward 1999; and Vipulanandan and Ren 2000) and others a negative effect (Tiehm 1993; Van Hamme and Ward 1999; Vipulanandan and Ren 2000).

Macnaughton et al. (2003), compared the effect of a chemical dispersant (Corexit 9500™) on the dispersion, biodegradation and microbial colonisation of Forties crude oil at 15°C and Alaska North Slope (ANS) crude oil at 8°C. Results suggest enhanced

biodegradation with the Forties Crude oil (15°C). However the findings with ANS crude oil at 8°C are at odds with the above results i.e. biodegradation was not confirmed. Similarly, Page et al. (2002) evaluated the behaviour and effect of a Chemically Dissolved Oil (CDO) in a wetland environment to determine if the dispersed oil enhanced or inhibited the recovery of the wetland. The results indicated that CDO did not affect the rate of petroleum biodegradation as compared to the rates of the oiled control treatment. However, they stated that the dispersed oil was prone to flushing as compared to the control.

In a further effort to identify the main factors controlling the biodegradation of oil by surfactants, Van Hamme and Ward (1999) studied effects of the physicochemical properties of Igepal CO-630 (nonylphenol ethoxylate) surfactant including HLB, oil to surfactant ratio, fractions and molecular structure of the crude oil on its biodegradation by a mixed bacterial culture. HLB values ranging from 4.6 to 18.2 showed no inhibitory effects but found enhanced biodegradation with an HLB value of 13. Further comparison between seven different surfactants of HLB 13: Alkamuls 600 DO, Antarox P-104, Chemal TDA-9, Chemeen DT-15, Hamposyl L, Sandopan DTC acid and a control showed mixed results. Only one surfactant, Igepal CO-630, increased biodegradation by 57% while surfactants from other chemical classes had no effect or were inhibitory. Inhibitory effects of surfactants were associated with their use in either as potential carbon sources or toxic agents in a microbial system.

Tiehm (1994) investigated the fundamental interaction of polycyclic aromatic hydrocarbons (PAHs) and synthetic surfactants including sodium dodecyl sulphate (SDS) and others (Brij 34, Triton X-102, Genapol X-150, Arkopal N-300, Sapogenat T-300,

Pluronic PE6400, Tegopren 5851m and Marilpal 013/90). Results showed that, while SDS has the potential to solubilize phenanthrene, biodegradation was inhibited by the addition of SDS. This was accounted for by the mineralization of the SDS that resulted in release of sulphuric acid (H_2SO_4), which resulted in acidification of the medium. Also the biodegradation of naphthalene was inhibited in the presence of surfactants, Marlipal 013/90 and Triton X-102, due to their toxic effects associated with their higher lipophilicity while others showed no significant effect. Similar comparisons of two synthetic surfactants by Page et al. (2002), nonionic (Titon X-100) and anionic (SDS), and a *Pseudomonas* sp. biosurfactant for enhanced solubility and biodegradation of naphthalene showed that the biosurfactant effectively solubilized more naphthalene at neutral pH as compared to SDS and Triton X-100. In terms of biodegradation, while SDS inhibits the biodegradation of naphthalene, an increased biodegradation was achieved with the other two.

Overall, the review of the literature showed that, although there is a potential in enhancement of biodegradation by synthetic dispersants, often they resulted in either inhibiting and/ or having no significant effect in biodegradation. The major factors associated with the inhibitory effect of the synthetic surfactants are their toxicity (increased liphophilicity associated with both the solvent as well as the surfactant component of the synthetic dispersants) and substrate competition between the surfactants and the oil droplets (due to their higher CMC values, it requires higher concentration for their effectiveness). More on the controversial issues in the use of synthetic dispersants for oil spill dispersion and biodegradation are discussed below.

2.3.2 Controversial issues in existing oil spill dispersants

Although many reasons support dispersant use, dispersants have continued to be controversial in many parts of the world, with the main issues being effectiveness and environmental acceptability (Lewis and Aurand 1997). The relative toxicity, low biodegradability and limited efficiency at low concentrations reduce the potential for the application of synthetic surfactants in the open water.

Etkin (1999) summarizes a collection of toxicological studies conducted over the last 3 decades involving dispersants and chemically dispersed oil (CDO). Some of the conclusions showed increased toxicity for dispersants or CDO as opposed to whole oil, including studies by Avolizi and Nuwayhid (1974), Ordzie and Garofalo (1981) and Tiehm (1994). Others such as Swannell and Daniel (1999) and Page et al. (2002) suggest mixed results. On the other hand, Prince et al. (2003) pointed out that in practical applications the toxicity of dispersants is less important compared to the effectiveness due to very rapid dilution to extremely low levels in the water column. However, this won't be the reason for cases where the droplets are already encapsulated by the surfactant micelles and where the mechanism of oil and microbe interaction is by digesting surfactant encapsulated oils.

Currently, synthetic dispersant application has not been approved in different regions of the world for waters less than 10m in depth or less than 3 nautical miles from shore (USEPA 1996a), mainly due to their toxicity and the effect of the dispersed oil itself. However, if less toxic and better biodegradable dispersants are introduced, they could possibly be used on shorelines. This purpose could be served by biosurfactants (subject of

this study) whose primary function is to facilitate microbial life in environments dominated by hydrophilic-hydrophobic interfaces.

2.3.3 Biosurfactants as candidates for oil dispersion & biodegradation

Biosurfactants are microbially produced surfactants with clearly defined hydrophilic and hydrophobic groups (Healy et al. 1996). The hydrophilic group can be ionic or non- ionic and consist of mono-, di-, or polysaccharides, carboxylic acids, amino acids, or peptides (Lin 1996). The hydrophobic groups are usually saturated, unsaturated, or hydroxylated fatty acids (Lin 1996). For their production, micro organisms could use either alkanes only, water soluble only or both alkane and water soluble substrates as carbon sources (Matsfuji et al. 1997; Lin 1996).

Depending on their biochemical nature and the microbial species producing them, biosurfactants are classified into five groups: (1) glycolipids (2) phospholipids and fatty acids (3) lipopeptide/ lipoproteins, (4) polymeric surfactants, and (5) particulate surfactants (Edwards et al. 2003; Lin 1996; Healy et al. 1996).

Unlike synthetic surfactants, microbially produced compounds are easily biodegradable and have lower levels of toxicity, lower CMCs (effective at relatively lower concentration), possibility of *in-situ* production and have the ability to be synthesized from renewable feedstock (Lin 1996; Mulligan and Gibbs 2004). These surfactants can reduce surface tension from 72 to less than 30 mN/m and generally have CMC values ranging from 1 to 200 mg/L (Lang and Wagner 1987).

Despite all these advantages, biosurfactants have not yet been employed extensively in

industry because of technical and economic reasons (Lin 1996). With public awareness and as environmentally compatibility is becoming an important factor for selecting industry chemicals, environmental concerns weigh the economic balance in favour of biosurfactants.

Over the years, biosurfactants have found a significant use in industry, such as for oil recovery, textiles, pharmaceuticals and cosmetics (Healy et al. 1996). They can have various uses, particularly for environmental applications such as bioremediation and clean up of beached oils (Lin 1996). Examples of such applications include the acceleration of the degradation of hydrophobic hydrocarbons in an oil-contaminated beach (Bruheim et al. 1997), soils (Bai et al. 1997), and soil slurries in bioreactors (Oberbremer et al. 1990). Most of these studies have used well-characterized biosurfactants such as *Pseudomonas aeruginosa* rhamnolipids (Bai et al. 1997), *Torulopsis bombicola* sophorolipids (Oberbremer et al. 1990), *Rhodococcus erythropolis* trehalose dimycolipids (Bruheim et al. 1997) and *Bacillus sp.* Lichenysins (Yakimov et al. 1995). Some examples of commercial biosurfactant formulations used specifically for oil recovery include the emulsifier Bio-EM™, emulsion stabilizer Emulsan™, and the emulsifier Alasan™. However no major work has been published on the use of biosurfactants for open water spill response.

2.3.4 Rhamnolipids

The type of biosurfactant used in this study is rhamnolipids from the glycolipids group which is produced by *Pseudomonas aeruginosa* (Jeneil Biosurfactant Co. 2002). They are one of the few well-studied and commercially available biosurfactants. There are four

types of rhamnolipids (Dahrazma and Mulligan 2004): Type I, (R1) is L-rhamnosyl-β-hydroxydecanoyl-β-hydroxydecanoate, molecular mass = 504 Da; Type II, (R2) is L–rhamnosyl – β – L– rhamnosyl – β – hydroxydecanoyl – β –hydroxydecanoyl – β–hydroxydecanoate, molecular mass = 660 Da; Type III (R3) is one rhamnose attached to β–hydroxydecanoic acid and Type IV (R4) is two rhamnoses attached to β–hydroxydecanoic acid. Structures of R1 and R2 rhamnolipids are shown on Figure 2.5 .

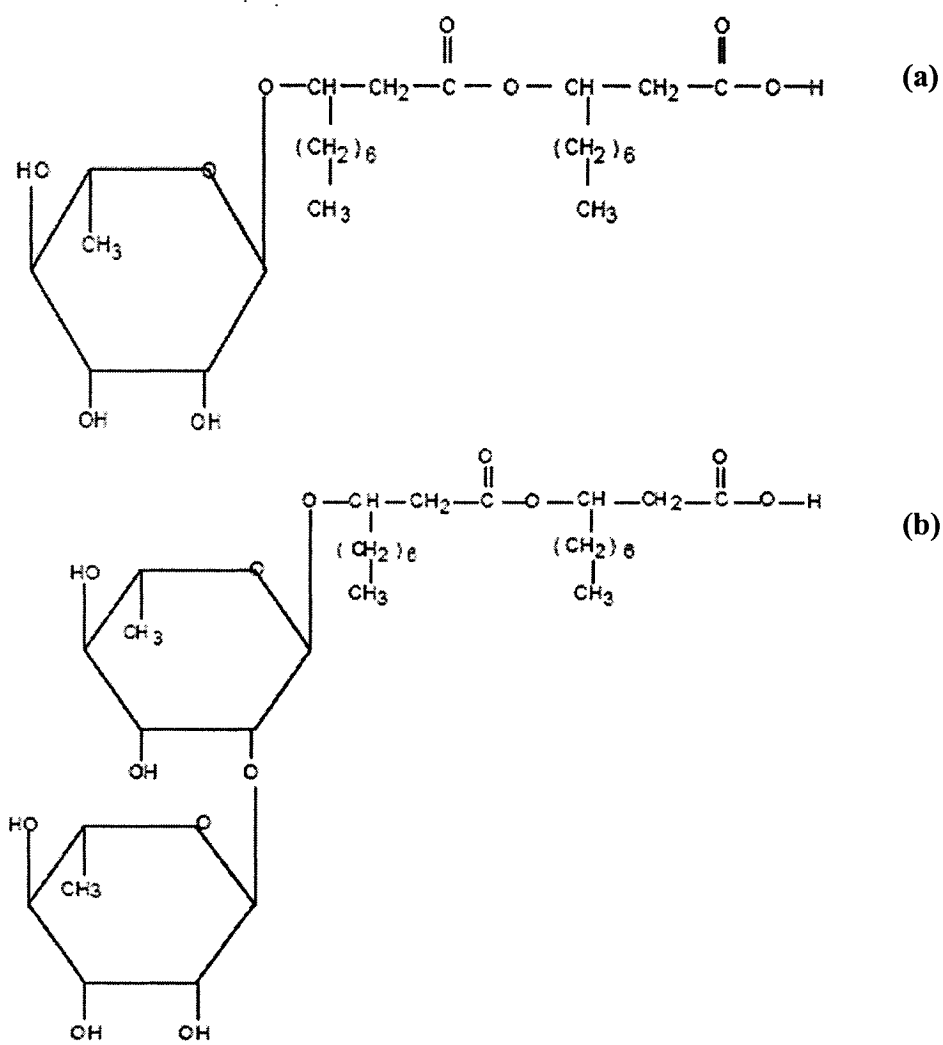


Figure 2.5 Rhamnolipid structure (a) R1 and (b) R2 (Jeneil Biosurfactant Co. 2002)

Rhamnolipids have shown promising results in various environmental applications such as for the extraction of copper from a low-grade ore (Dahrazma and Mulligan 2004), and for remediation of pentachlorophenol (PCP) contaminated soil (Mulligan and Eftekhari 2003). Also, promising results have been shown in the areas of cleaning up beached oils. Harvey et al. (1990) have tested rhamnolipids on the beaches in Alaska after the *Exxon Valdez* tanker spill and found that they release three-times more oil as compared with the water alone. Rahman et al. (2003) tested their effectiveness for enhancing bioremediation of n-alkanes in petroleum sludge using a bacterial consortium and showed pre-treatment of hydrocarbon-contaminated soil with biosurfactants enhanced bioavailability of the hydrocarbons to the microbial population.

Zhang and Miller (1992) have also examined the potential of the rhamnolipids for enhanced octadecane dispersion and biodegradation. Results from their experiments have shown that in the presence of 50 mg/L of rhamnolipid, an increase of more than 4 orders of magnitude over the solubility of octadecane in pure water was obtained. Very recently work has been done by Holakoo (2002) indicating the potential in the application of rhamnolipids to oil slicks. Holakoo (2002) examined the use of different solvents and additives to increase the dispersion and stabilization of the rhamnolipid dispersed oil in the water. A mixture of 8% JBR 425 + 60% ethanol + 32% octanol was recommended as the best performing formulation. When applied to 400 mL of saline water and 0.4 mL of oil at a dispersant to oil ratio of 1:8, the mixture dispersed 82% of the crude oil as measured at 0 minutes settling time. After 2 minutes settling time, the amount of dispersed oil decreased to 21%. Although there was good rationale for using such solvents, introduction of petroleum-based solvents by itself will have an adverse effect on

the marine environment. Thus more research needs to be done in identifying solvents that could dissolve the rhamnolipid and improve the contact with the oil without additional damage to the environment.

3 MATERIALS AND METHODS

The main objective of this research is to determine the efficacy of rhamnolipid biosurfactant (JBR 425™) for oil spill dispersion and further enhancement of bioavailability and hence biodegradation of crude oil spilled on surface waters. This chapter discusses the experimental design as well the methods and materials used. The experiment has two phases: phase-1 focused on evaluation of the efficacy of rhamnolipid for oil spill dispersion and phase-2 focused on the biodegradation of the dispersed crude oil. Oil dispersion and biodegradation studies were conducted in the laboratory according to the methods currently required for listing dispersants on the National Contingency Planning (NCP) product schedule (USEPA 1996).

3.1 Capability of rhamnolipid for dispersing spilled oil

3.1.1 Experimental design

The experiment was designed to evaluate the capability of rhamnolipid biosurfactant (JBR 425™) for dispersing crude oil and its effectiveness was evaluated and formulated with respect to different conditions discussed below.

Experiment-1 Effect of solvent: Solvents are required in the dispersant formulation both to dissolve the biosurfactant and transport the surfactant molecules to the oil water interface (section 2.1.1). The effect of four different types of solvents; laboratory distilled water, pH adjusted distilled water (pH 7.5), ethanol and artificial seawater, were tested in their efficacy for use in the bio-dispersant formulation. While selecting the solvents, emphasis was mainly on water-based solvents for sustaining the biological nature of the

rhamnolipid biosurfactant, thereby limiting the introduction of chemicals to the bio-dispersant formulation.

To compare the effect of solvents, the bio-dispersant was formulated using a 2% rhamnolipid concentration and the bio-dispersant was applied to the oil with a dispersant to oil ratio of 1:4. The dispersant to oil ratio (DOR) and rhamnolipid concentration may not be the optimum values. However they were considered as a starting point since results based on these rates and concentration were effective in previous research (Holakoo 2002). However, the type of solvent used by Holakoo (2002) was hydrocarbon-based, which might be toxic to the marine environment. In the present study it was required to determine the optimum concentration and the DOR of the bio-dispersant formulation using the water-based solvents.

Experiment-2 Effect of Dispersant to oil ratio and rhamnolipid concentration: Once the solvent was identified, the effects of DOR and rhamnolipid concentration on efficacy of the formulated bio-dispersant were evaluated using an incomplete factorial design with two factors. The first factor, dispersant to oil ratio, had 6 levels including DORs such as 1:1, 1:2, 1:4, 1:8, 1:12 and 1:16. The rhamnolipid biosurfactant concentration was the second factor with 5 values: 0.5%, 1%, 2%, 4%, 8% and 12% resulting in a 6×5 factorial experiment. Each group was replicated three times. The experimental setup is outlined in Table 3.1. The experiment was conducted at a temperature equivalent to moderate temperature regions (20±2°C) and using Fresh BRENT crude oil.

Table 3.1 Partial factorial experimental design

Rhamnolipid concentration	Dispersant to oil ratio (DOR)					
	1:1	1:2	1:4	1:8	1:12	1:16
0.5 %	3[†]	3	3	3	0	0
1 %	3	3	3	3	0	0
2 %	3	3	3	3	0	0
4 %	3	3	3	3	0	0
8 %	3	3	3	3	3	3
12 %	3	3	3	3	3	3

[†] Numbers represent the number of replicates

Experiment 3 Effect of temperature and degree of oil weathering: Further to the formulation of the optimal "bio-dispersant" which is a mixture of rhamnolipid biosurfactant and a selected solvent, the stability of dispersion effectiveness was tested under conditions relevant to cold (10±2°C) temperature regions and weathered (72 hours weathered oil) spilled BRENT oil. This will help to simulate the potential applicability of the bio-dispersant mixture in cold regions and/ or on weathered oil, which is the fate of the spilled oil, a day or two after spill occurred, respectively. Temperature and degree of oil weathering were known to affect the effectiveness of the present day synthetic dispersants. This experiment will assist in identifying the impact of these parameters (i.e. temperature and degree of oil weathering) on the bio-dispersant's effectiveness.

In all of the above cases, the effectiveness of the bio-dispersant was tested using the swirling flask method (USEPA 1996a) and the results were compared with chemical dispersant (Corexit 9500™) and natural dispersion. The dispersant effectiveness test method and the composition, as well properties of the materials used in the experiment,

are described below.

3.1.2 Preparation of test substances

3.1.2.1 The oil

A BRENT Blend crude oil (Origin: North Sea, UK) was used for this experiment; it was acquired from PETRO Canada. It is a common crude oil imported into Canada. The original oil has a density of 0.83 g/mL at 15.6°C, API gravity of 38.3 at 15.6°C, kinematic viscosity of 19.12 CST at 20°C, a pour point of -23°C, and a sulphur content of 0.4% by weight. It is a light crude oil with 72% saturates, 23% aromatics, 4% resins and 1% asphaltenes. The technical information was obtained from PETRO Canada and Environment Canada (Environment Technology Centre 2001). For experiments that required weathered oil, the oil was artificially weathered by leaving it in the fume hood for 72 hours (at 20±2°C), thus reducing the oil weight by about 25 %.

3.1.2.2 The synthetic seawater

The synthetic seawater was prepared by dissolving 35 g of sea salt in 1 L of distilled water (i.e., to give a salinity of 35‰) as per the test procedure of USEPA (1996). The pH of the synthetic water was adjusted to 7.7 using 1M sodium hydroxide (NaOH) and/or hydrochloric acid (HCl) solution. Following this preparation, the synthetic seawater was allowed to equilibrate to the ambient temperature of the laboratory (20±2°C).

3.1.2.3 Corexit 9500™

The chemical dispersant (Corexit 9500™) was obtained from OHMSETT Co. It is one of the most commonly used chemical dispersant types for an oil spill response. The actual composition of this dispersant is not clearly known due to commercial confidentiality. However, Edwards et al. (2003) stated that it is blend of fatty esters, glycol ethers and oxyalkylates in a paraffinic solvent. Some of the physical properties are shown in Table 3.2 (USEPA 1995). When using Corexit 9500™ for an oil spill response, a dispersant to oil ratio of 1:50 to 1:10 is recommended (USEPA 1995). This rate varies depending on the type of oil, degree of weathering, temperature, and thickness of the slick. In this study, a DOR of 1:20 was adopted consistently, following the recommendations in various literatures (Moles et al. 2002).

3.1.2.4 Rhamnolipid biosurfactant, JBR 425™

The biosurfactant, rhamnolipid is a metabolic by-product of *Pseudomonas* aeruginosa. JBR 425™ is an aqueous solution of rhamnolipids at 25% concentration and was obtained from Jeneil Biosurfactant Co. It is produced from sterilized and centrifuged fermentation broth, which has had all protein removed (Jeneil Biosurfactant Co. 2002). The part that constitutes the other 75% is not defined by the vendor. Chemically, JBR 425™ contains a mixture of two rhamnolipids: RLL (R1) and RRLL (R2). It is anionic biosurfactant with molecular formulae of $C_{32}H_{58}O_{13}$ and $C_{26}H_{48}O_9$. The properties of JBR 425™ in comparison to Corexit 9500™ are shown in Table 3.2.

Table 3.2 Physical properties of JBR 425™ and Corexit9500™

Typical properties	JBR 425™ (Jeneil Biosurfactant 2001)	Corexit9500™ (USEPA 1995)
Specific gravity	1.05-1.06	0.949
pH	6.5-7.0	6.4
Solubility in water	Soluble in fresh and sea water at neutral pH	Soluble in fresh water but dispersible in sea water
Suitable Diluents	Water, most common alcohols	The solvent used is confidential and in application it is used as concentrate
Volatility	Not volatile	Solvents are volatile
Toxicity	• 68.4% on day 10 of the 28 day test, OECD 301D[†] • EC50 > 1000 mg/L, OECD209[†] • EC50 of 36.1 mg/L, OECD 202[†]	• LC 50 of 25.2 mg/L 96-hr for *Menidia beryllina*

[†]Toxicity tests reported by Jeneil Biosurfactant (Jeneil Biosurfactant Co. 2002) as accomplished in accordance with Organization for Economic Cooperation and Development (OECD): EOCD 301D for ready biodegradability, OECD 209 for activated sludge respiration inhibition (ASRIT), and OECD 202 for aquatic toxicity to daphnia.

3.1.3 CMC measurement of the biosurfactant

Prior to deciding on how much biosurfactant to add, it is vital to identify the CMC of the biosurfactant. CMC refers to the minimum concentration of the biosurfactant required for the formation of micelles. For optimal performance, the concentration of the biosurfactant needs to be of at least equal to its CMC value. In this experiment, the CMC value of the biosurfactant was determined indirectly using surface tension measurements of solutions of various dilutions. First, twelve concentrations of JBR 425™ (0.00001%,

0.0001%, 0.001%, 0.005%, 0.01%, 0.05%, 0.1%, 0.5%, 1.0%, 2.0%, 4.0% and 8.0%) were prepared by diluting the biosurfactant in pH adjusted (pH 7.5) distilled water. Then the surface tension is measured using a FISHER SCIENTIFIC Surface Tensiomat (Model 21). Subsequently the surface tension measurements were plotted against the rhamnolipid concentration using the semi log curve (the rahmnolipid concentration values were in logarithmic). The plot was then fitted to two best-fit linear functions for ≤ 30 and > 50 mg of rhamnolipid per litre concentration, respectively. The CMC of rhamnolipid was then determined at the point where the two best-fit curves intersected.

3.1.4 Laboratory dispersant effectiveness testing

For an oil spill-treating agent to be used either in the United States or Canada, it must be listed on the National Contingency Plan (NCP) product schedule. For example in the United States for a product to be listed on the product schedule, dispersants have been required to pass the USEPA SFT, described in Appendix C, Subpart J of 40 CFR 300.900 (USEPA 1996a) while in Canada it should satisfy the American Society for Testing Material (ASTM) SFT (ASTM 2002). Basically, the two procedures are quite similar, with very small differences (section 2.3.2). In this study, the effectiveness of the bio-dispersant was evaluated using the swirling flask test method (USEPA 1996a) with slight modifications to meet the requirements of the bio-dispersant. The test procedure and modifications made are described below.

The swirling flask method (modified from USEPA 1996a): All the bio-dispersant effectiveness tests were conducted at laboratory temperature (i.e. about 20±2°C) unless otherwise mentioned. 120 mL of synthetic seawater was added to the 250 mL

Erlenmeyer flask with a stopper on the top and a glass pipette insert with an extended tube connected to it (Figure 3.1). The addition of the synthetic water was followed sequentially by addition of oil and finally by the dispersant. This type of application simulates natural conditions better than the USEPA's premixed oil and dispersant application (Sorial et al. 2004 a and b; and Blondina et al. 1997).

A volume of 100 μL of oil was added directly onto the surface of the synthetic water using a microlitre syringe. The dispersant was added in a similar manner at the required dispersant to oil ratio. Once the oil and the dispersant were added, the flask was placed on a gyratory INNOVA New Brunswick Scientific shaker table (Model 2000) for 20 minutes at 150 rpm speed of rotation to induce a swirling motion to the liquid contents. At the end of the shaking period, the flask was allowed to remain stationary for 10 minutes. This allows the oil that reforms a slick to return to the water's surface. At the end of this settling time, 30 mL sample was collected from the bottom of the water column through an extended tube inserted into the flask (Figure 3.1). The use of this sampling procedure limits the sampling bias introduced by the disturbance of the resurfaced oil and prevents resurfaced oil from coming together with the sample. With the use of such arrangement most of the sample is collected only from the water column not the surface. Finally the 30 mL sample is then transferred to 40 ml amber vial and stored at 5^0C until extraction.

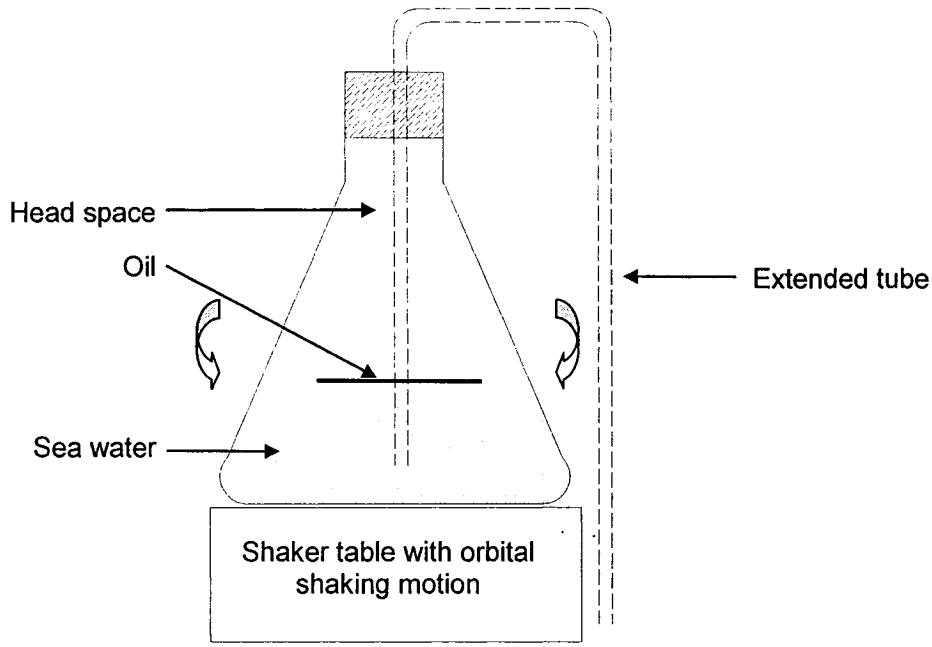


Figure 3.1 Modified Erlenmeyer test flask

Oil extraction: The oil was extracted using slight modification to the liquid/ liquid extraction method as listed in the USEPA test method (USEPA 1996a). For extracting the oil from the sample (oil-synthetic sea water mix), the 30 ml sample was transferred to a 60 ml Erlenmeyer separatory funnel. Then 5 ml of high performance liquid chromatography (HPLC) grade hexane was added to the funnel, followed by vigorous shaking for 15 seconds and the funnel was allowed to remain in a stationary position for 2 minutes to allow phase-separation of the water and hexane. Then the hexane layer was drained from the separatory funnel into a 25 ml graduated cylinder. The hexane extraction process was repeated three times. Finally, the combined three extracts were adjusted to a final volume of 20 mL with additional hexane and transferred to 40 mL amber vial and stored at 5°C until the time of analysis. The solvent, dichloromethane, DCM (used in the standard procedure) was replaced by hexane, as DCM was found to

dissolve the rhamnolipid biosurfactant.

Oil standard preparation: Prior to measurement, the hexane-extracted standards were prepared according to the EPA swirling flask test (USEPA 1996a) for calibrating the UV-visible spectrophotometer. For all experiments, five point standard curves were generated on a daily basis by using five concentrations: 0.125, 0.25, 0.5, 1 and 2 g of oil per litre of hexane. To prepare the standard: 27.5, 55 and 110, 220 and 440 μL of oil was added to 30 ml synthetic seawater. Then the oil-water mixture was extracted in a similar way to that of the samples. Example calibration curves are shown in Appendix A.

Oil analysis: After the oil sample and standards were extracted, the absorbance of the standard and actual oil samples were measured using a PERKIN ELMER Lambada Ultraviolet-Visible (UV-VIS) spectrophotometer (Model 40) at wavelengths of 340, 370 and 400 nm as in the USEPA SFT (US EPA 1996a). A 10 mm cuvette with 1 cm path length was used for measurement and hexane was used as the reference blank. All the samples were either in duplicate or triplicate. Each sample was measured three times to minimize the measurement error.

Blanks: For each sample, the analytical method blanks that involve an analysis of synthetic seawater, synthetic dispersant (Corexit9500™) and/ or bio-dispersant (JBR 425™) were made following the above test procedures and values were deducted according to the requirements.

Percent effectiveness calculation: The percent dispersant effectiveness, which is defined as the concentration of the dispersed oil in the water column was finally determined by dividing the UV-VIS spectrophotometer readings of the hexane extract by the total

concentration of the oil (equation 3.1). The total concentration of the oil refers to the initial amount of the oil added to the test flask.

$$Effectiveness(\%) = 100 * \frac{V_{extract} * V_{water}(C_{340nm} + C_{370nm} + C_{400nm})}{3 * V_{sample} * M_{oil\ added}} \quad \textbf{(Eqn. 3.1)}$$

Where: $V_{extract}$ is the volume of the hexane extract i.e. 20 mL; V_{water} is the volume of the synthetic water used in the experiment: 120 mL and V_{sample} is the volume of the sampled dispersant, synthetic water and oil mixture from the water column (30 mL). The concentration values $C_{340nm,\ 370\ nm\ and\ 400\ nm}$ refer to the concentration of the oil as measured at the respective wavelengths and the $M_{oil\ added}$ refers to the initial amount of crude oil added to the flasks. Figure 3.2 shows a pictorial summary of the test procedure.

3.1.5 Statistical analysis

To detect statistically significant differences between the different treatments/ experimental set-ups, a one-way Analysis of Variance (ANOVA) is used. This test was used to evaluate if there exists any significant difference between the solvents used for the formulation of the bio-dispersant on its efficacy (Experiment-1). As well, the analysis helped to determine if there is a significant effect of temperature and degree of oil weathering on the performance of the formulated bio-dispersant in comparison to the synthetic dispersant and natural conditions (Experiment-3). Finally, a similar statistical test procedure was used to test the experimental repeatability and to identify which of the rhamnolipid concentrations and/ or DOR could significantly affect its effectiveness. All comparisons were conducted at $\alpha = 0.05$. The analysis was done in Matlab® environment. An example of a Matlab® function used is given in Appendix B.

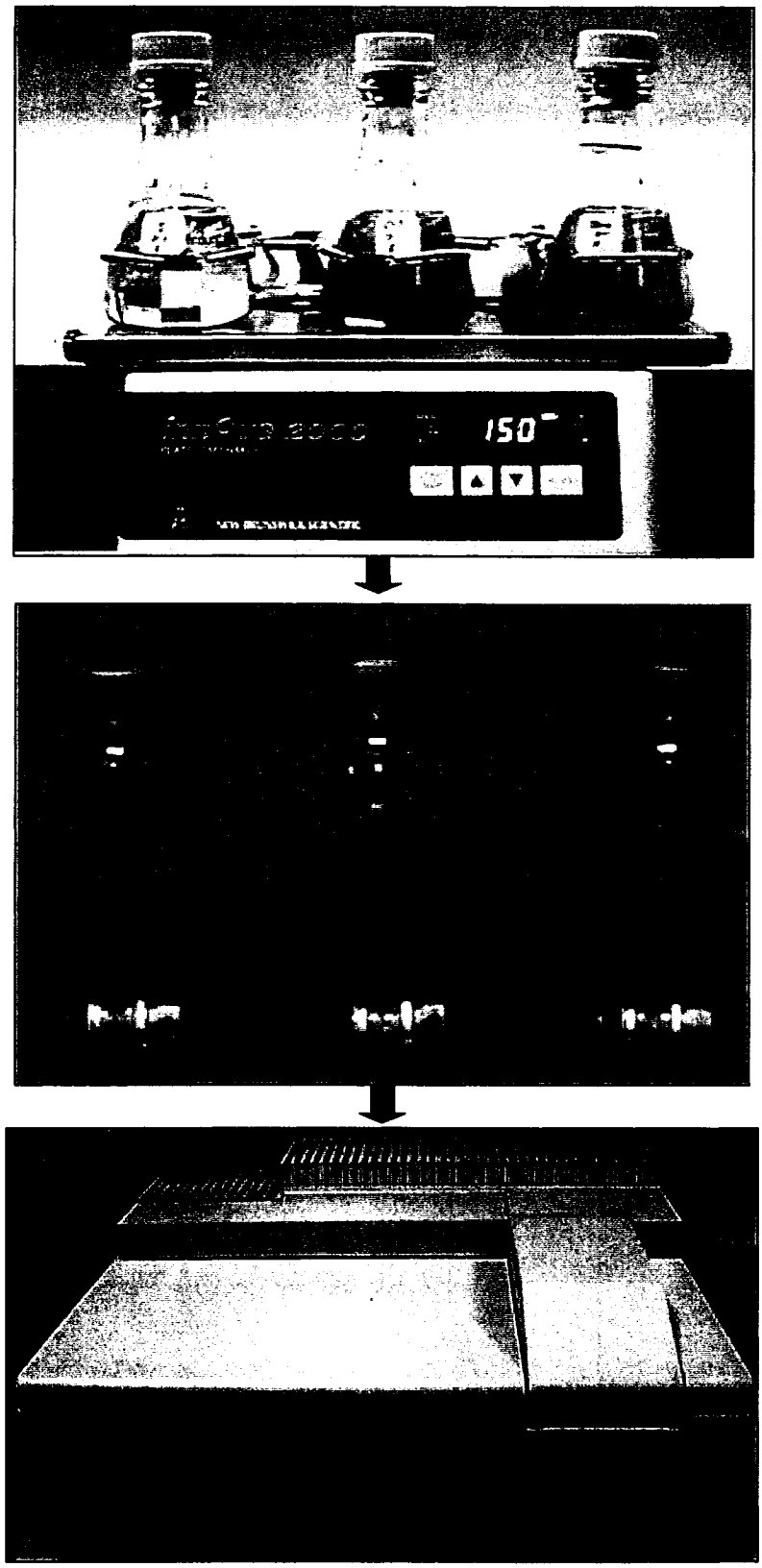


Figure 3.2 Pictorial description of the dispersant effectiveness test

3.2 Rhamnolipid capability for enhancing biodegradation of spilled oil

3.2.1 Experimental design

As discussed in the literature review (section 2.3), application of dispersants may enhance oil bioavailability and increased biodegradation rate of the oil by creating more surface area in terms of multiple small oil droplets. However, conclusions from laboratory studies regarding enhanced microbial degradation of oil using oil dispersants are contradictory. In this study, a biodegradation experiment was carried out to evaluate the hypothesis that the addition of the formulated bio-dispersant (mixture of JBR 425™ and selected solvent) enhances the biodegradability of the oil.

An experiment involving 80 reactors (8 treatments × 5 sampling times × 2 replicates) was conducted to evaluate the biodegradation effect of different treatments over a 35-day incubation period. The experimental setup is shown in Table 3.3. The various treatments tested for their effects in the oil biodegradation were: JBR 425™ bio-dispersant (DOR of 1:4); JBR 425™ bio-dispersant (DOR of 1:8); Corexit 9500™; two biological cleansing agents (ASAP™ and degreaser™) and combined bio-dispersant and biological cleansing agents (JBR 425™ bio-dispersant (DOR of 1:4) with ASAP™, and JBR 425™ bio-dispersant (DOR of 1:4) with degreaser™) as well as an oil only control. For this experiment, a 2% concentration of JBR 425™ was used. The effect of Corexit 9500™ on the biodegradation was tested mainly for comparison of the results with biological treatments. The combined bio-dispersant and biological cleansing agent was used with the hypothesis that addition of the former will stimulate crude oil biodegradation prior to its own biosurfactant production.

Table 3.3 Biodegradation study experimental set up

Treatments	Time (days)				
	0[‡‡]	3	7	14	35
Bio-dispersant (2% applied at DOR of 1:4)[†]	1	2	2	2	2
Bio-dispersant (2%, applied at DOR of 1:8)[‡]	1	2	2	2	2
Corexit9500 (applied at DOR of 1:20)[¶]	1	2	2	2	2
ASAP (applied at DOR of 1:8)[§]	1	2	2	2	2
ASAP (1:8)[§] ×Bio-dispersant (2%, 1:4)[†]	1	2	2	2	2
Degreaser (applied at DOR of 1:4)[#]	1	2	2	2	2
Degreaser (1:4)[#] × Bio-dispersant (2%, 1:4)[†]	1	2	2	2	2
Oil only (control)[††]	1	2	2	2	2

[†]Rhamnolipid concentration delivered to the oil water interface was 0.5%

[‡]Rhamnolipid concentration delivered to the oil water interface was 0.25%

[¶]12.5µL corexit was applied as concentrate

[§]31.25µL of ASAP added as concentrate

[#]62.5µL of Degreaser added as concentrate

[††]0.25mL of oil was added

[‡‡]The amount of oil added to each reactor at day 0 is the same and the control was not killed control rather it is used for representing the natural biodegradation of the oil.

Each reactor was sampled sacrificially at t = 0, 3, 7, 14 and 35 days for monitoring the petroleum composition and bacterial population. Detailed methods of biodegradation study and composition as well as properties of the materials used are described below.

3.2.2 Preparation of test substances

The biodegradation experiment was conducted using BRENT crude oil, synthetic sea water, Corexit9500™, bio-dispersant JBR 425™ and two biological cleansing agents (Avmor ASAP floor degreaser™ and Avmor industrial degreaser and concrete cleaner™). Throughout the biodegradation experiments, an artificially weathered BRENT crude oil was used. The oil was weathered by leaving it in the fume-hood for 72

hours at 20±2°C. The main purpose of weathering the oil was to make it stable which would give consistency to the biodegradation results by alleviating the problem of losses due to volatilization. Also this simulates the practical oil spill scenario better as the biodegradation process often occurred on the heavier fraction of the oil as the light fraction of petroleum is lost faster than it can be biodegraded.

Details on the composition and properties of the first four materials (BRENT crude oil, synthetic seawater, Corexit9500™ and bio-dispersant JBR 425™) are already given in section 3.1.2, the composition and material property of the two cleansing agents is outlined below.

3.2.2.1 Avmor ASAP floor degreaser™

The Avmor ASAP bio-action floor degreaser™, referred in this text as ASAP™, was obtained from Avmor company. The product was originally designed for cleaning fat, oil and grease around food processing floors and equipment with no food contact (Avmor 2003a). It contains a proprietary blend of surfactants (Tetrasodium ethylenediaminetetraacetate, Sodium Dodecylbenzene Sulfonate (SDS) and nutrients) and bio-cleaning agents. A summary of the physical properties of this product is presented in Table 3.4.

To determine the amount of the ASAP™ to be used in the biodegradation test, a preliminary experiment was conducted using the bioremediation effectiveness test procedure (USEPA 1996a) outlined below (section 3.2.3). In this preliminary experiment, the effect of different ASAP™ to oil ratios, 1:50, 1:25, 1:12.5, 1:8.3, 1:6.5, 1:5, 1:2.5 and 1:1.25, on the biodegradation of 0.25 mL BRENT crude oil were evaluated over 14 days.

Relatively better results were obtained for ASAP™ to oil ratios of 1:8.3 to 1:5, which is equivalent to 1.58×10^6 to 2.65×10^6 microbes. Results of this preliminary experiment are shown in Appendix C.

3.2.2.2 Avmor industrial degreaser and concrete cleaner™

The Avmor industrial degreaser and concrete cleaner™, called as Biomor degreaser™ is a biological cleansing agent originally designed for cleaning gas stations, train trucks, runways and processing machines (Avmor 2003b). The product was obtained from the Avmor Company. This product is described as containing a proprietary micro-organism consortium that degrades petroleum products. It doesn't contain petroleum solvents or chemical surfactants, making it environmental friendly (Avmor 2003b). Some of the physical and biological properties are summarized in Table 3.4. In this study, a degreaser to oil ratio of 1:4 (equivalent to 1.21×10^5 microbes) was used.

Table 3.4 Properties of Biomor ASAP™ and Biomor Degreaser™ (Avmor 2003)

Properties	Degreaser™	ASAP™
Specific gravity	1.0-1.01	1
pH	10.4-10.7	7.5 to 8
Microbial population	7.6 billion microbes per 3.78 L	200 billion microbes per 3.78 L
Boiling point	100°C	100 ° C
Solubility	Easily soluble in water	Easily soluble in water

Both products have 5 strains (bacteria) and the bacteria present in the product are part of the Domestic Substance List (DSL) with the Canadian government.

3.2.3 Laboratory biodegradation study

The biodegradation test follows EPA's bioremediation agent effectiveness test (USEPA 1996b) designed to determine a product's ability to biodegrade oil by quantifying changes in the oil composition resulting from biodegradation.

Experimental setup: A 35 day long experiment is performed to assess the degree of oil degradation in the presence of bio-dispersant (JBR 425™), biological cleansing agents (ASAP™ and Degreaser™) as well combined bio-dispersant and biological cleansing agents in comparison to the synthetic dispersant (Corexit9500™) and natural biodegradation. The crude oil biodegradability was evaluated with a series of batch experiments using 250 ml Erlenmeyer flasks as incubation reactors. Each flask was filled with 100 ml of synthetic seawater, 0.22 g of weathered BRENT crude oil and one of the following treatments: JBR 425™ (DOR of 1:4); JBR 425™ (DOR of 1:8); Corexit 9500™ (DOR of 1:20); ASAP™ (Agent to Oil Ratio (AOR) of 1:8); Degreaser™ (AOR of 1:4); combined JBR 425™ (DOR of 1:4) with ASAP™ (DOR of 1:8); combined JBR 425™ (DOR of 1:4) with Degreaser™ (DOR of 1:4) and oil only control. After adding the different treatments, the flasks were covered with cotton plugs and placed on a gyratory INNOVA New Brunswick Scientific shaker table (Model 2000) at 200 rpm for maintaining aerobic conditions and simulating the sea movement. For the experimental period, the flasks were kept in a dark incubator at 20°C to prevent it from being exposed to light and minimize loss of crude oil by photochemical oxidation.

Sampling procedure: After 0, 3, 7, 14 and 35 days of rotary shaking and incubating at 20°C, the reaction vessels were sacrificed. Prior to the total petroleum hydrocarbon

analysis, a 0.5 ml sample of the aqueous phase was removed for the microbiological analysis. The remaining sample was immediately extracted for total petroleum hydrocarbon analysis using the procedure outlined below. At the time of the sampling event, physical observations of each flask were recorded.

Oil extraction: For extracting the oil from the oil-water-biomass mixture, the remaining contents of the flask were placed in a 250 mL separatory funnel and the flask was rinsed into the funnel using 10 mL of hexane. Liquid/ liquid extraction was then repeated three times using 10 mL of hexane for each extraction. After each solvent, addition the funnel was shaken vigorously for 2 minutes and allowed to settle for 10 minutes to facilitate hexane and water layer partitioning (USEPA 1996b). Finally, the combined three extracts were then dried using anhydrous sodium sulphate, adjusted to a final volume of 50 mL with additional hexane, transferred to an amber vial and refrigerated at 5°C until analysis.

Oil standard preparation: Three point standard curves were generated on a daily basis using three concentrations: 1, 2, 4 g of weathered oil per litre of hexane. To prepare the standard, a specific amount of oil 0.05, 0.1 and 0.2 g respectively was added to 100 mL synthetic seawater. Then the oil-water mixture was extracted in a similar manner to the samples. Example calibration curves are shown in Appendix D.

Blanks: For each sample, analytical method blanks that involve an analysis of Corexit 9500™, JBR 425™, ASAP™ and degreaser™ were made following similar test procedures. In this case 12.5 µL, 62 µL, 30 µL and 62 µL of Corexit 9500™, JBR 425™, ASAP™ and degreaser™ were added to 100 mL of synthetic seawater, extracted and analysed using GC. Chromatograms are shown in Appendix E.

Petroleum chemistry analysis: The standard, sample and blank hexane extracts were analyzed quantitatively and qualitatively for total petroleum hydrocarbon concentration using a 3800 VARIAN Gas Chromatography (GC) equipped with a Flame Ionization Detector (FID) and auto sampler. Chromatographic separations were conducted by using a DB-5 fused silica column of 30 m long, 0.25 mm inner diameter, 0.25 μm film thickness and -60 to 325°C temperature limits. Helium was used as carrier gas with a flow rate set at 1 mL/ min and make-up gas at 29 mL/ min. The flame gas, hydrogen and airflow rates were set at 35 and 400 mL/ min, respectively. The injection port temperature and the detector temperature were 250°C. The GC oven temperature is programmed from 50 to 250°C at 8°C/min with a 2 min hold at 50°C and a 6 min hold at 250°C giving a total run time of 33 min. The injection volume was 1 μL in the splitless mode with direct injection. The chromatogram method outlined above was established after experimentation.

After the chromatogram of each sample was obtained, quantitative analysis of the test samples was done with VARIAN Star 5.5 chromatography workstation software. The software uses an automated area and peak height integration method. The reported total petroleum hydrocarbon concentration were obtained by calculating the total area in the chromatograms (excluding those of the solvent peaks) and after deduction of the areas of the blank analyses, and relating these with those of the areas of the known standards.

In addition, the concentration of the oil is measured using PERKIN ELMER Lambada Ultraviolet-Visible (UV-VIS) spectrophotometer (Model 40) following a similar procedure as in section 3.1.4.

Microbiological analysis: For enumeration of heterotrophic bacteria, 0.5 mL of the clear aqueous phase removed from each flask is added to 9.5 mL of sterile phosphate buffer, pH 7.0 (1:10 dilution). Using a sterile technique, the sample was mixed and serial dilutions were performed to 10^{-9} dilutions. Microbial densities were determined using a pour plate method as described in the Standard Methods for Examination of Water and Wastewater test method 9215 B (SMW 2000).

Plate counts for heterotrophic micro-organisms were performed by spreading 0.1 mL samples from each dilution onto Petri dishes containing *R2A* agar (Difco Laboratories, Detroil, IL, USA) in duplicate for each dilution. The agar plates were incubated at 28°C for 7 days. After 7 days all Colony Forming Units (CFU) on selected plates were counted promptly and plates were stored at 5°C. Finally, the number of bacteria colony (CFU) was calculated by multiplying the counted CFUs per plate and the corresponding dilution.

3.2.4 Kinetic model for substrate disappearance

For further comparison of the product efficacy and to evaluate the rate of disappearance of the total petroleum hydrocarbon, a first-order biodegradation model was proposed and used to model the biodegradation of the crude oil from the reactors. This model has been used by previous biodegradation research (Page et al. 2002; Harris et al. 2002; and Aldrett et al. 1997). The model was derived by making a mass balance on the reactor, and assuming a first order rate. For the mass balance, biodegradation was assumed to be the only (dominant) oil loss source. Other oil loss processes such as volatilization are assumed negligible since the oil is weathered before onset of the experiment. Photochemical oxidation was also assumed negligible as the experiments were conducted

in dark environment and the samples after being extracted were kept in amber vials.

3.2.5 Statistical analysis

An analysis of variance (ANOVA) procedure in a Matlab® (as shown in Appendix B) environment was used to assess if significant differences exist between the various treatments in their biodegradation rate, microbial density and percent oil loss in the reactors. In addition a linear regression analysis was also performed in an Excel® environment to evaluate if a relationship existed between the quantified microbial densities in the treatments and the % loss of crude oil concentration.

4 Results and Discussion

4.1 Rhamnolipid capability for oil spill dispersion

An objective of this research has been to determine the effectiveness of a commercial rhamnolipid biosurfactant (JBR 425™) for dispersing BRENT crude oil under different conditions. To achieve this, an oil spill dispersion experiment has been designed and conducted using a modified swirling flask method (USEPA 1996a). The effectiveness of dispersants has been determined by adding 0.1 ml of oil and a dispersant in a flask containing 100 ml of synthetic seawater. The content of the flask was then mixed in a shaker for 20 minutes followed by a 10 minute settling time. At the end of this period, 30 ml of sample water is taken; the oil was extracted using hexane and measured using a UV-VIS Spectrophotometer to determine the quantity of oil dispersed in the water column. The experiment also compared the effectiveness of rhamnolipid with the current commercially available chemical dispersant (Corexit 9500™) and natural dispersion. This section presents and discusses the effectiveness results.

4.1.1 Determination of Critical Micelle Concentration (CMC)

Knowing the CMC value of a surfactant is a prerequisite for selecting the concentration of a biosurfactant to achieve optimal performance. Thus prior to deciding the amount of rhamnolipid biosurfactant to use in the bio-dispersant formulation, its CMC was determined indirectly from the relationship of surface tension and rhamnolipid concentrations by measuring surface tension of various dilution solutions. Results (Figure 4.1) from this analysis showed that, the surface tension decreased, from 72 to 30 mN/m, with an increase in rhamnolipid concentration, from 0 to 50 mg/L, and beyond that an

increase in rhamnolipid concentration had a very small effect on surface tension. This shows that low rhamnolipid concentrations (< 30 mg/L) have a strong effect on surface tension, while high concentrations (> 50mg/L) have a negligible effect. The CMC of rhamnolipid has been determined as 35 mg/L (0.0035%) using the semi-log curve of surface tension versus rhamnolipid concentration as shown in Figure 4.1. This result is close to previously reported rhamnolipid CMC value by Zhang and Miller (1992) and Holakoo (2002), which was equal to 0.004% and 0.006%, respectively.

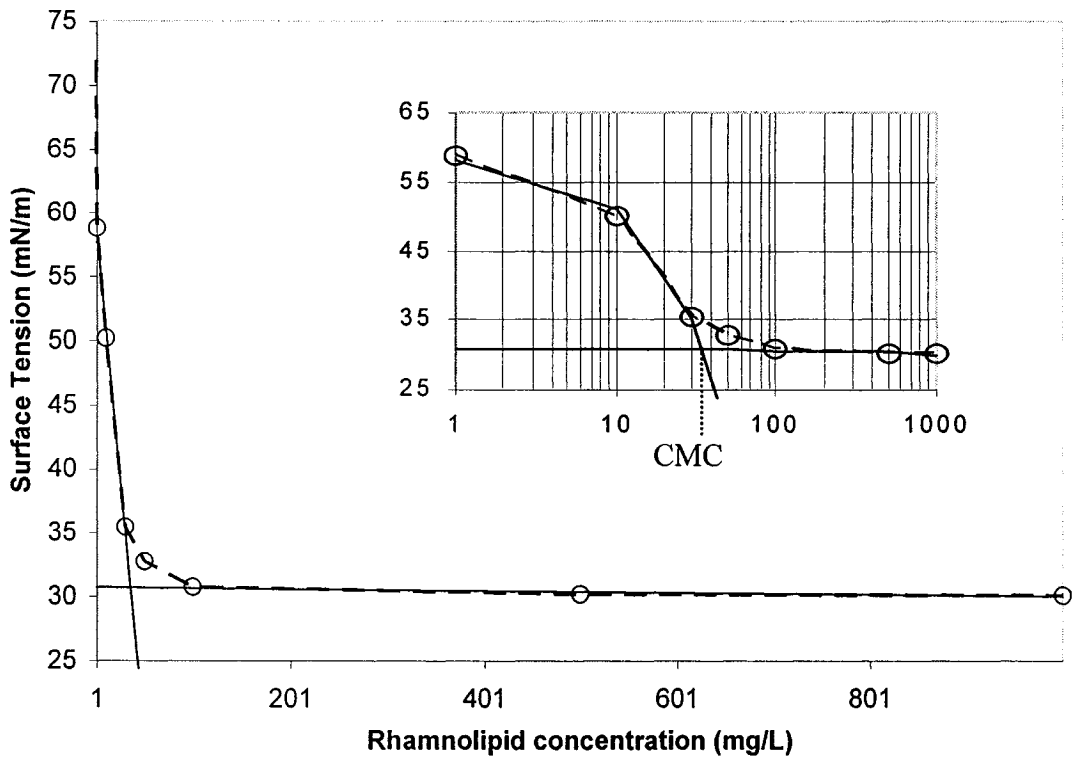


Figure 4.1 Surface tension versus rhamnolipid concentration. Symbols:-Θ- , experimental data; ——, best-fit linear functions at ≤ 30 and > 50 mg of rhamnolipid per litre. Semi log view of the graph indicates the CMC value at the point where the two best-fit curves intersect.

4.1.2 Effect of solvent on efficacy of rhamnolipid

Solvents in a bio-dispersant formulation play an important role by dissolving the biosurfactant and lowering its viscosity thus easing application as well contact between the dispersant and oil interface. Rhamnolipids are soluble in water and most alcohols (Jeneil Biosurfactant Co. 2002). Comparison of the efficacy of the bio-dispersant formulated with 2% rhamnolipid solutions in four different types of solvents such as pH 7.5 buffered distilled water, alcohol, distilled water and artificial seawater is shown in Figure 4.2. For each of the four solvents, three replicates were done and the variation between the samples of a given solvent is shown in the figure by the standard deviation bar. The initial amount of oil added is equal to 0.1 ml (USEPA 1996a) and the bio-dispersant to oil ratio used in all cases is 1:4, resulting in a net rhamnolipid concentration of 0.5% (i.e. 2%/4) which is equal to rhamnolipid to crude oil ratio of 1:200 (i.e. 0.1 ml of oil/0.0005 ml pure rhamnolipid) and/or JBR 425™ to oil ratio of 1:50 (0.1 ml of oil/0.002 ml JBR 425™), respectively.

The results showed better dispersion of crude oil with the use of pH buffered solution (pH=7.5) in comparison to the ethanol, synthetic seawater and distilled water solutions (p= 0.002, at 0.05 α). This is in agreement with the observation by Zhang and Miller (1992) in which the dispersion of octadecane by rhamnolipid solutions was reported as quite sensitive to pH and was highest at a pH between 7.0 and 7.5. Better efficacy of rhamnolipids at this pH is related with surface activity and formation of micelles. It has been reported that the micelle formation process of rhamnolipid starts at a pH greater than 6.8. Also the highest surface tension reduction is achieved with a pH in the range of 7 to 7.5. As dispersion is mainly an interfacial and micelle phenomenon, the relative

higher performance at pH range of 7 to 7.5 can thus be accounted for it. It can be noted that the percent effectiveness (Y-axis in Figure 4.2) displays the concentration of the dispersed oil in the water column using different solvent types.

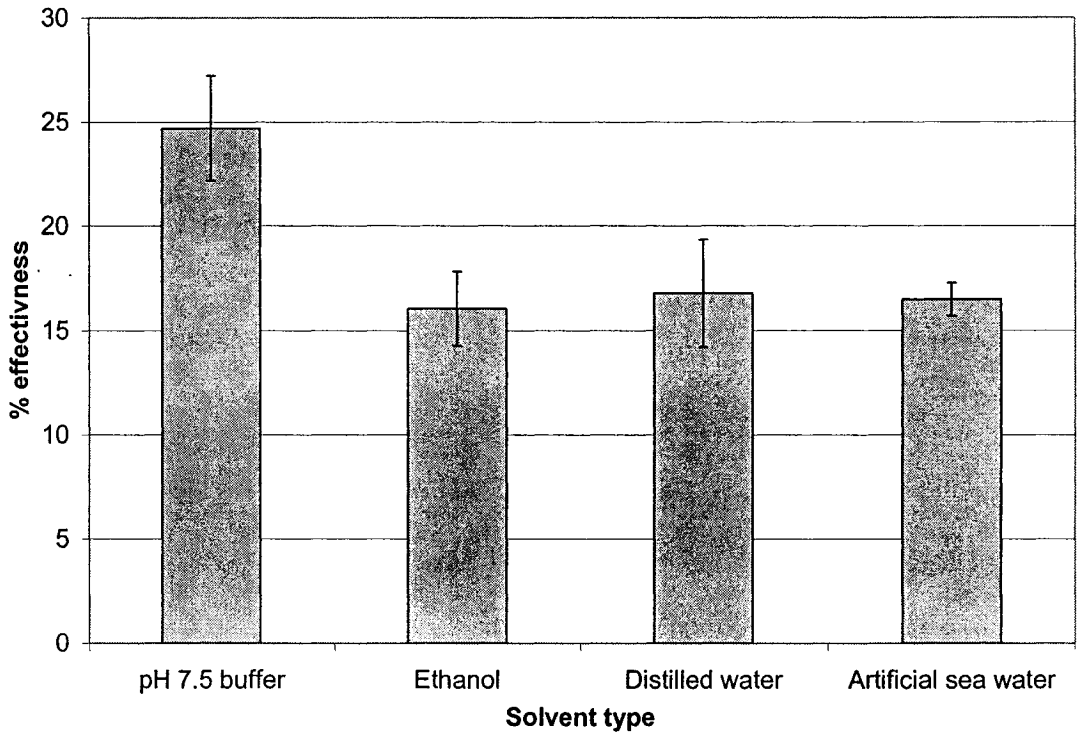


Figure 4.2 Effect of solvent type on rhamnolipid effectiveness

4.1.3 Effect of rhamnolipid concentration and DOR

4.1.3.1 Rhamnolipid concentration

Subsequent to the selection of a suitable solvent, the effect of rhamnolipid concentration on efficacy of the bio-dispersant was evaluated. In real situations, the concentration of rhamnolipid for use in the formulation of bio-dispersant is a balance between effectiveness and cost of the biosurfactant. Figure 4.3 and Table 4.1 show the dispersion of fresh BRENT crude oil at different rhamnolipid concentrations at a pH 7.5-buffered

solvent. The reported effectiveness values are averages of at least three samples. The y-axis (Figure 4.3) displays the concentration of the dispersed oil in the water column.

Figure 4.3 also shows that the rhamnolipid dramatically increased the dispersion of fresh BRENT crude oil with an increase in concentration. In comparison to natural dispersion the rhamnolipid biosurfactant enhanced dispersion of crude oil by more than 50 orders of magnitude, from 1.62 to 82%. At 22± 2°C and 35‰ salinity, the rhamnolipid biosurfactant dispersed 10.2% to 82% of fresh BRENT crude oil into the water column when 0.1 ml rhamnolipid solution is applied with 0.125% to12% pure rhamnolipid concentrations, respectively (Table 4.1). In all cases, the initial amount of oil added is equal to 0.1 ml (USEPA 1996a) resulting in a rhamnolipid to crude oil ratio of 1:800 to 1:8.3, respectively (Table 4.1). From Figure 4.3, it can be observed that at a rhamnolipid concentration < 2% (rhamnolipid to oil ratio of 1:50), there is a sharp increase in crude oil dispersion and after that the crude oil dispersed at a slower rate with increasing rhamnolipid concentration.

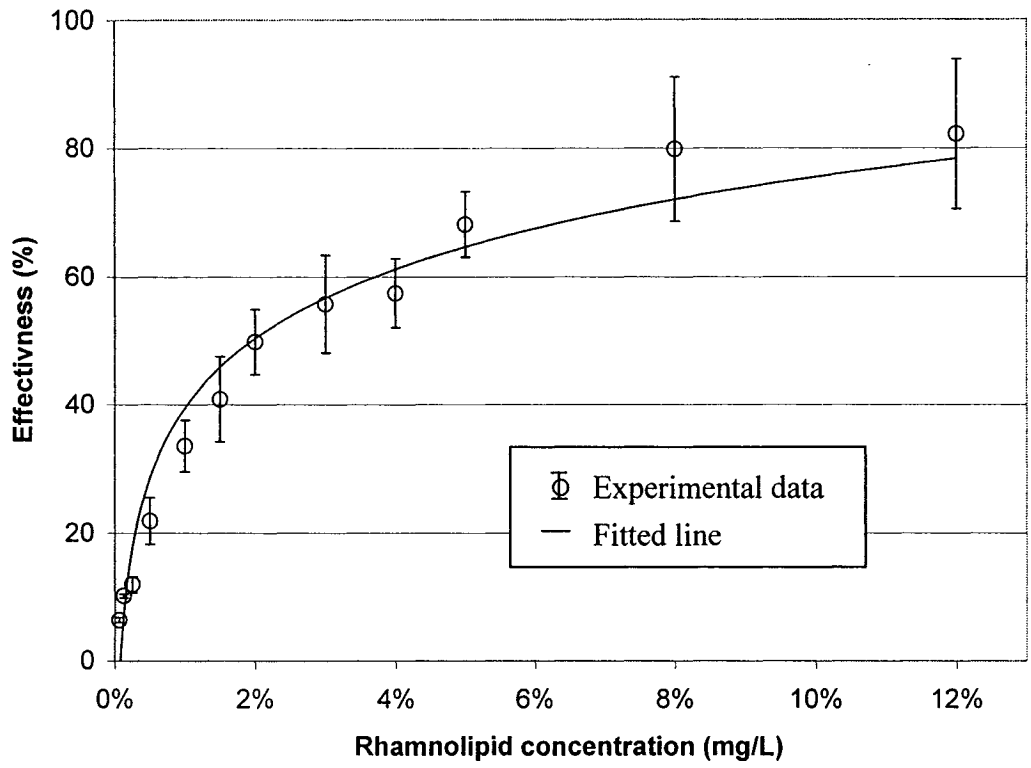


Figure 4.3 Effect of rhamnolipid concentration on its effectiveness

Theoretically the enhancement of dispersion of crude oil using biosurfactants is by lowering the interfacial tension between the oil-water interfaces and/ or by physical interaction of biosurfactants with the hydrocarbon compounds (Zhang and Miller 1992). In the present study, the rhamnolipid has been applied at a concentration greater than its CMC value (0.0035%), showing that the enhancement of crude oil dispersion is more dominated by the physical biosurfactant interaction with hydrocarbon compounds which resulted in dispersion of the crude oil into the water column by encapsulation of hydrocarbon compounds into micellar aggregates.

To finally recommend the optimal concentration of the rhamnolipid for use in an oil spill response, the USEPA guideline has been considered. In accordance with the guideline,

for listing the bio-dispersant in a NCP schedule it is required to have an effectiveness value of at least 45% using the test method followed for this study (USEPA 1996a). It can be clearly observed from Figure 4.3 that, the required dispersion effectiveness value (45%) has been achieved with the use of a 1.5 to 2% rhamnolipid concentration. This concentration has been obtained by diluting 6 to 8% JBR 425™ in a pH 7.5 buffer solution. However it should be noted that this results are specific to the experimental conditions that simulated an oil spill scenario of temperature equal to 22± 2°C, 35‰ salinity of the synthetic seawater and if the dispersant is applied immediately after a spill i.e. prior to oil weathering. A change in any of these conditions could possibly affect the performance of the biosurfactants and hence may require addition of more or less concentration accordingly.

Table 4.1 Effectiveness of JBR 425™

Rhamnolipid concentration (%)	Rhamnolipid to crude oil ratio	JBR 425™ to crude oil ratio	Dispersion effectiveness (%)
0.125	1:800	1:200	10.2
0.25	1:400	1:100	12
0.5	1:200	1:50	22
1.0	1:100	1:25	33
1.5	1:66.7	1:16.7	41
2	1:50	1:12.5	50
3	1:33.3	1:1.33	55
4	1:25	1:1	57
6	1:16.7	1:0.67	68
8	1: 12.5	1:0.50	79
12	1:8.3	1:0.33	82

4.1.3.2 Bio-dispersant to oil ratio

Once the required concentration of rhamnolipid for acceptable performance is known, the decision needs to be made on how to deliver the required rhamnolipid concentration to the oil-water interface. In this regard the bio-dispersant (JBR 425™ and pH buffered distilled water mixture) can either be prepared as a concentrate and applied with a lower DOR or can be formulated at more dilution and applied at a higher DOR. Usually this depends among other factors on the dispersant's effectiveness using either of the two approaches (concentrates at a lower DOR or less concentrates at a higher DOR). The other factor required for the selection of either of the two approaches is the dispersant application system. On one hand less concentration at a higher DOR implies a large volume and may make application of the dispersant difficult in real situations. On the other hand concentrates are usually viscous and it may become difficult to apply using the spray nozzle and/ or to bring sufficient contact between the dispersant and the oil.

Results in Table 4.2 shows the percent dispersed crude oil values obtained with the use of a bio-dispersant formulated with varying rhamnolipid concentration and applied at different dispersant to oil ratios. Overall in terms of effectiveness, delivering the same rhamnolipid concentration either using concentrates at a lower DOR or less concentrates at a higher DOR showed no significance difference. For example along the shaded diagonal (Table 4.2), the net rhamnolipid concentration delivered to the oil water interface for all the combinations is 0.5%. This has been delivered as 0.5%, 1%, 2%, and 4% with a dispersant to oil ratio of 1:1, 1:2, 1:4 and 1:8, respectively. The percent dispersion along this diagonal varied between 18.4 to 27.9% showing no specific trends. The statistical evaluation carried out at α= 0.05 also indicated that there were no

significant differences between the amounts of dispersed oil along the diagonal. Similar observations have been obtained on the other diagonals as well.

The results in this study have useful practical implications for spill responders. For example the fact that there is no significant difference in efficacy by adding concentrated bio-dispersants with lower DOR indicates that one may use the lower ends of the diagonal to decrease the volume of the dispersants. These results have also importance in demonstrating the repeatability of the tests suggesting precision of the evaluation methods used in the study.

Table 4.2 Percent dispersed oil as a function of rhamnolipid concentration and DOR

Rhamnolipid concentration	Bio-dispersant to oil ratio (DOR)				
	1:1 Mean† (SD)	1:2 Mean† (SD)	1:4 Mean† (SD)	1:8 Mean† (SD)	1:12 Mean† (SD)
0.5%	18.4 (0.1)‡	12.6 (4.4)	9.6 (1.0)	6.38 (0.8)	
1%	38.9 (9.6)	22.6 (7.9)‡	10.7 (0.6)	10.7 (0.05)	
2%	44.7 (16.7)	30.6 (3.5)	27.9 (12.3)‡	12.7 (2.5)	
4%	62.7 (7.7)	48.4 (9.4)	31.2 (9.1)	18.6 (6.5)‡	
8%	79.9 (22.4)	52.1 (13.4)	56.2 (3.9)	25.2 (6.1)	
12%	82.2 (23.3)	68.2 (10.2)	55.7 (15.3)	40.8 (13.8)	41.6 (10)

†Percent of dispersed crude oil in the water column; average of 3 replicates

‡The amount of rhamnolipid delivered along the diagonal is equal (0.5%) and the dispersion variability along the diagonal is not significant.

4.1.4 Stability of dispersion

Subsequent to the dispersion of the oil slick, the stability of dispersed oil droplets is of great importance since unstable emulsions coalescence and reform the slick. The stability was evaluated by indirectly measuring the amount of dispersed oil remaining in the water column 2, 5 and 10 minutes after energy in the form of shaking was no longer applied. Figure 4.4 shows the comparison in the stability of droplets formed for a BRENT crude oil dispersed using JBR 425™ (equivalent to 2% rhamnolipid concentration and DOR of 1:4), Corexit 9500™ (DOR of 1:20) and control.

In general it was observed that the percent-dispersed crude oil dropped very rapidly with settling time. Large droplets can be seen resurfacing in the first few minutes after energy in the form of shaking is no longer applied. As shown by the percent decrease, the rapid change at the 10-minute mark is significant and after about 10 minutes, although not shown in this figure, there is little change of effectiveness with time. This observation is also in agreement with Fingas et al. (1999). To account for this factor, the reported laboratory effectiveness results were measured after 10 minutes settling time. At this time, 30% and 50% of the chemically and biologically dispersed oil droplets respectively reformed into the slick (Figure 4.4). The effectiveness of the bio-dispersant containing 0.5% rhamnolipid decreased from 48% at 2 minutes settling time to 23% after 10 minutes settling time. Practically this indicates that future addition of emulsion stabilizers to the formulation of bio-dispersants may improve the stability of biologically dispersed oil droplets, thereby increasing its performance at lower concentration.

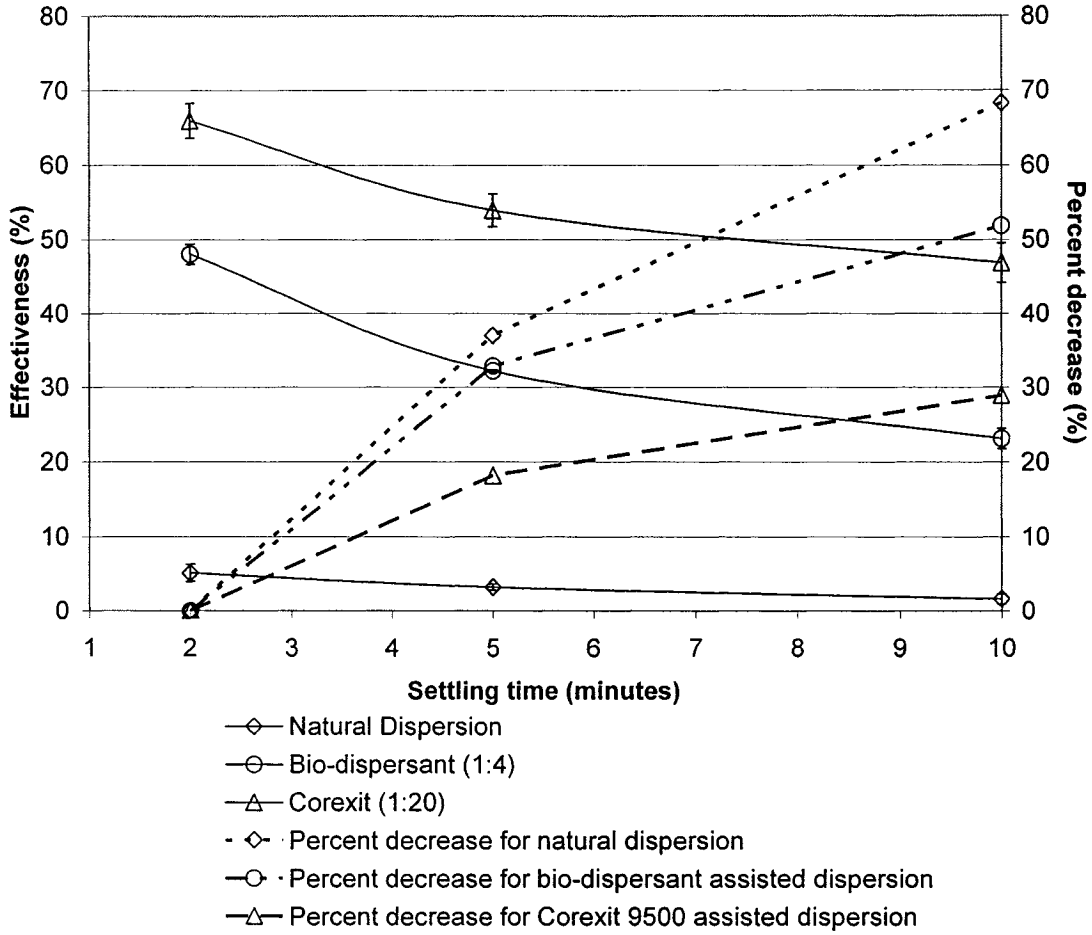


Figure 4.4 Stability of dispersed crude oil

4.1.5 Effect of temperature and oil weathering

Once the formulation of the optimal "bio-dispersant" which is a mixture of the rhamnolipid biosurfactant and a selected solvent as well as its associated optimum concentration, DOR, and settling time was identified, its dispersion effectiveness was compared for conditions relevant to cold (10±2°C) and moderate temperature regions (22±2°C) using fresh and weathered (25% weight loss by evaporation) spilled BRENT oil. In all cases, the effectiveness of the bio-dispersant (solutions of JBR 425™) has been compared with chemical dispersant (Corexit 9500™) and with natural dispersion. The

bio-dispersant used had a 2% rhamnolipid concentration and applied with a DOR of 1:4 resulting in a final 0.5% rhamnolipid concentration at the oil-water interface. All the experiments were conducted with at least three replicates.

Effect of temperature: Figure 4.5 shows the dispersion of fresh crude oil at 22±2°C and 10°C under natural conditions and in the presence of JBR 425™ or Corexit 9500™. In all cases, the dispersion of fresh oil decreased slightly with a decrease on temperature. The average proportion of dispersed oil by Corexit 9500™ was 46.9% and 39.8% at 22±2°C and 10°C, respectively and this difference was not significant (at α=0.05, p=0.18). Similarly the bio-dispersant assisted dispersion of Fresh BRENT crude decreased from 23.1% to 18.8% at 22±2°C and 10±2°C, respectively. However the decrease was not significant (at α=0.05, p=0.07). It can be seen that the natural dispersion of crude oil was negligible both at 22 and 10±2°C as shown in Figure 4.5.

The above results showing no significant reduction in dispersant effectiveness on fresh BRENT crude oil at lower temperature is in agreement with previous tests suggesting that temperature has little effect on dispersant effectiveness (Ross 1997). However research results by Moles et al. (2002), showed a significant decrease in Corexit 9500™ and 9527™ dispersant effectiveness on fresh Alaska North Slope (ANS) crude oil at sub-arctic temperature (5°C). In general lower temperatures increase the viscosity of the dispersant and the oil itself, while in turn affects the dispersant's performance as well as the dispersability of the oil itself. In the case of JBR 425™, the product has been demonstrated to be stable at different temperatures. As well most chemical dispersants are formulated to have low viscosities at low temperatures, offsetting any increase in viscosity at lower temperatures (Ross 1997). Thus low temperature (10°C) may not

affect both the chemical and biological dispersants used in this study. Regarding its effect on the crude oil, the increase in viscosity of light crude oils like BRENT crude oil is not profound (in comparison to heavy and medium crude oil) resulting in an overall low impact of temperature on the amount of dispersed crude oil.

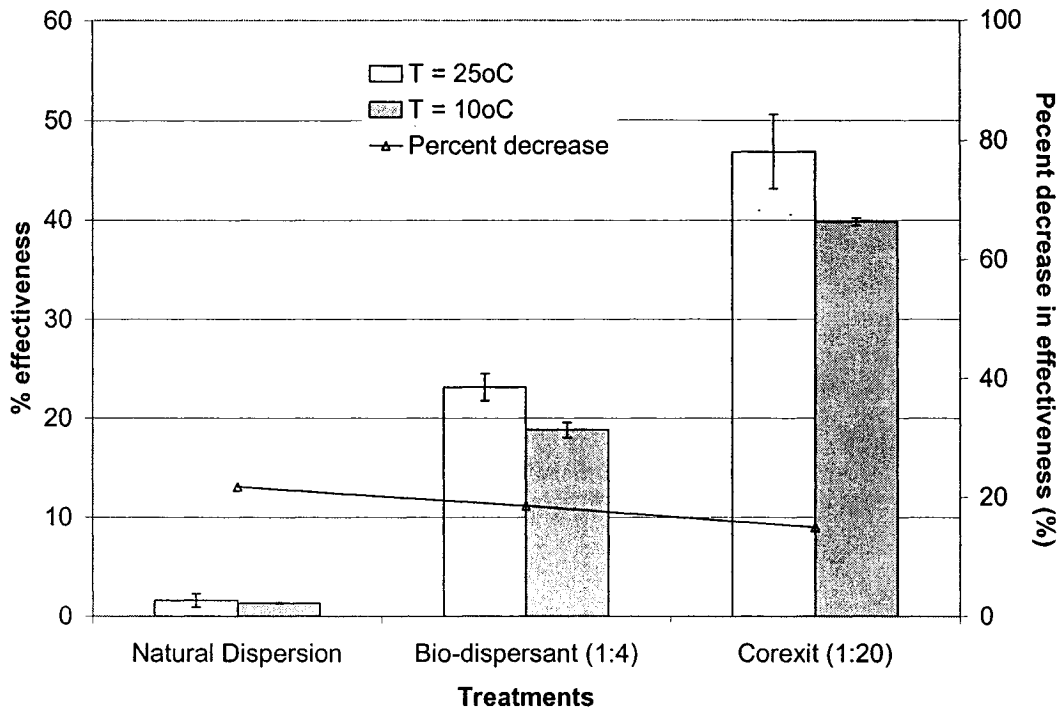


Figure 4.5 Effect of temperature on the natural, bio-dispersant and Corexit 9500™ assisted dispersion of fresh BRENT crude oil

Effect of weathering: Figure 4.6 shows the dispersion of fresh and weathered crude oil at 22±2°C under natural conditions and in the presence of JBR 425™ or Corexit 9500™. In all cases, the dispersion of oil decreased with an increase in oil weathering but with different levels of significance. The dispersion of crude oil in the presence of Corexit 9500™ depended strongly on the degree of oil weathering. The proportion of dispersed fresh and weathered BRENT oil was 46.9% and 32.4 %, respectively and this difference was significant (at α=0.05, p=0.04). However the JBR 425™ assisted dispersion of fresh and weathered BRENT crude showed no significant difference (at α=0.05, p=0.28) with

the percent effectiveness value of 23.1% and 20.3%, respectively.

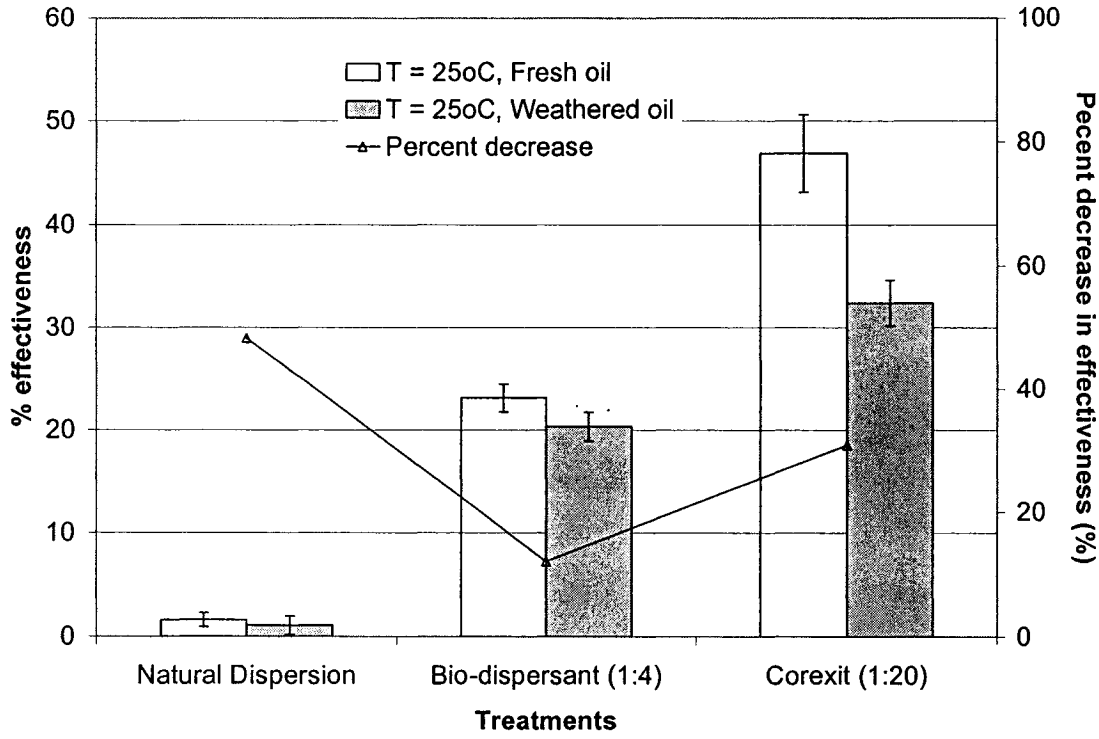


Figure 4.6.Comparison of fresh and weathered crude oil dispersion between the bio-dispersant, Corexit 9500 and natural conditions

4.1.6 Summary of bio-dispersant effectiveness results

This study evaluated the capability of the rhamnolipid biosurfactant (JBR 425™) for dispersing crude oil spilled on surface water. Accordingly the effect of four different types of solvents, concentration of rhamnolipids, dispersant to oil ratio, temperature and degree of oil weathering on the efficacy of JBR 425™ for dispersing BRENT crude oil has been quantified. The study also investigated the stability of the dispersed oil droplets formed in comparison to Corexit 9500™ assisted and natural dispersion. Application of the rhamnolipid biosurfactant enhanced natural dispersion of crude oil by more than 50 orders of magnitude. For listing this product into the NCP schedule, a formulation that

uses a 1.5 to 2% rhamnolipid concentration in pH 7.5-buffered distilled water is recommended. This can be delivered as a concentrate, for example 8% with a lower DOR (1:4), or more dilute (0.5%) with a higher DOR (1:0.125) without affecting its performance. The rhamnolipid if applied at this level can disperse the same amount of oil compared with Corexit 9500™. Economically one litre of JBR 425 costs $29.50 US and Corexit costs $2.30 US (Holakoo 2002). Therefore, the cost of delivering 2% rhamnolipid solution (which is equal to 8% of JBR 425 in pH 7.5 buffered distilled water) per mega tonne of spilled oil is $698,784 US while Corexit at DOR of 1:20 is $138,552 US. To reduce the cost of JBR 425, an equivalent amount of JBR 215 (which contains 15% rhamnolipid and costs $4.84 US per litre) can be used at a cost of $114,648 US. Using rhamnolipid biosurfactant is also advantageous considering the fact that it has shown consistent performance irrespective of temperature as well as oil property changes due to weathering. This result agrees well with the already documented biosurfactant advantages for use in soils (Mulligan and Gibbs 1993).

4.2 Capability of rhamnolipid on enhancing crude oil biodegradability

Subsequent to testing the potential of bio-dispersant (rhamnolipid biosurfactant solution in a pH 7.5 buffered distilled water) for crude oil dispersion, an experiment was conducted to assess its capability in enhancing the biodegradation of crude oil. In addition to the bio-dispersant, the effect of the introduction of two biological agents: ASAP™ (commercial biological product containing bacterial consortium, surfactant and nutrients) and Degreaser™ (product containing bacterial consortium and nutrients), on crude oil biodegradation have also been evaluated. The crude oil biodegradability has been studied with a series of batch experiments using 250 ml Erlenmeyer flasks as

incubation reactors. As described in Chapter 3, each flask is filled with 100 ml of seawater, 0.22 gm (0.25 ml) of weathered BRENT crude oil and one of the above treatments. The flasks have been plugged with cotton and shaken on a shaker table at 200 rpm and 20°C. After 0, 3, 7, 14, and 35 days each reactor was sacrificed and the flasks were analyzed quantitatively and qualitatively for total petroleum hydrocarbon concentration (using GC/FID). Also the reactors were analyzed for microbial densities using a pour plate method. Like the dispersion efficiency test the effectiveness of the bio-dispersant was compared with the chemical dispersant (Corexit 9500™) and natural biodegradation. All the biodegradation experiment was conducted in duplicate. Results of this analysis are discussed below.

4.2.1 Comparison of oil biodegradation in the presence of dispersants

The experimental results have shown that the effects of bio-dispersants, chemical dispersants and biological agents on biodegradation of crude oil vary over time. To better understand the biodegradation of biologically or chemically dispersed oil, a comparison of the percent crude oil remaining and the microbial population in each reactor has been made over time. Results are discussed below.

4.2.1.1 Treatment effects on biodegradation after 3 days incubation

Figure 4.7 shows the percent crude oil degraded after a three-day incubation period in the presence of biological dispersants or chemical dispersants or biological agents or combined biological dispersant and biological agents. After three days of incubation crude oil biodegradation was enhanced by six of the treatments while one treatment inhibited the natural oil biodegradation. A hydrocarbon reduction of 48.8% to 32.5% of

the initial amount was achieved by introduction of combined bio-dispersant and Degreaser, bio-dispersant and ASAP, Degreaser, bio-dispersant (1:4), ASAP and bio-dispersant (1:8) (Table 4.3). To compare the significance of the treatment effects to the control, analysis of variance (ANOVA) has been performed. All the treatments showed a significant difference in enhancing biodegradation of hydrocarbon with respect to the oil control as shown with p values (Table 4.3)

Chemical dispersion of oil using Corexit 9500 led only to 12.7% reduction of hydrocarbon. Its introduction significantly inhibited the biodegradation of hydrocarbon in comparison to natural (oil only) biodegradation ($\alpha = 0.05$, $p = 0.03$) indicating that the initial use of naturally or biologically dispersed oil is preferred over chemically dispersed oil (Figure 4.7 and Table 4.3). This result is supported by Daniel and Swannell (1998) who specified that chemical dispersants might provide alternative carbon sources readily used for microbial growth subsequently affecting the oil biodegradation process. Overall chemical surfactants have higher CMC values requiring the use of higher concentrations to increase their effectiveness, which in turn results in substrate competition between the surfactants and oil droplets. This could interfere with the hydrocarbon degradation process. But in this research due to an overall lower microbial population (Figure 4.8), there is no indication if hydrocarbon biodegradation has been inhibited due to alternative use of chemical surfactants as carbon sources. However the other possible reason could be toxicity of the chemical dispersants to the microbial community (due to increased lipophilicity of the chemical surfactants and solvents used in the formulation). This could have a significant effect for at least the first few days. Beyond this, a definite reason for why the system behaves like this cannot be confirmed since the experiment didn't

monitor other biodegradation controlling factors like pH, toxicity and dissolved oxygen. For day three, microbial data was not reported due to contamination of microbial plates.

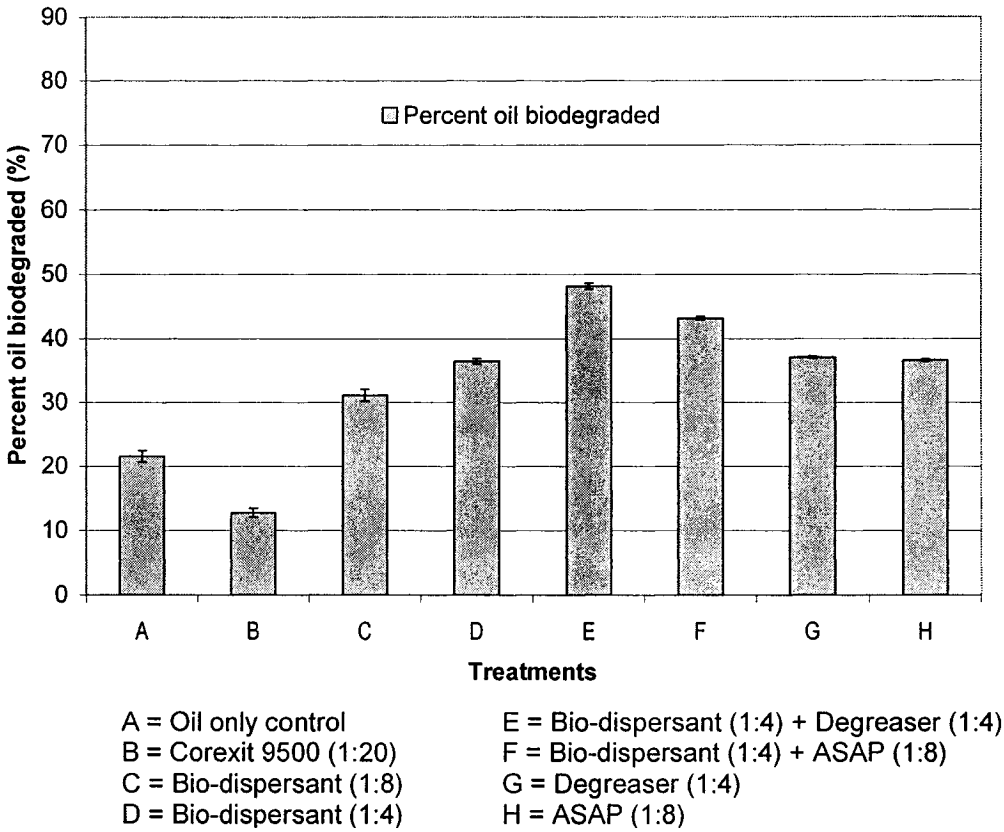


Figure 4.7 Biodegradation performance comparisons of different treatments using percentage crude oil remaining in the reactors; $100\% * (C_o - C_3)/C_0$

Table 4.3 Significance of treatments relative to control on crude oil biodegradation

Treatment	Percent oil biodegraded	*P* values[†]
Corexit 9500 (1:20)	13.7	0.032
Bio-dispersant (1:8)	37.1	0.009
Bio-dispersant (1:4)	36.6	0.003
Bio-dispersant (1:4) + Degreaser (1:4)	48.8	0.003
Bio-dispersant (1:4) + ASAP (1:8)	43.6	0.004
Degreaser (1:4)	37.2	0.007
ASAP (1:8)	32.5	0.007

[†] *P* values of less than 0.05 indicate significance difference between the amounts of oil degraded by the treatments relative to the oil only control

4.2.1.2 Treatment effects on biodegradation after 7 days incubation

Total petroleum hydrocarbon and microbial analysis of the reactors after seven days of incubation is shown in Figure 4.8. After seven days biodegradation of 36.6%, to 58.0% of weathered BRENT crude oil in the presence of Corexit, bio-dispersant (1:4), bio-dispersant (1:8), bio-dispersant (1:4), combined bio-dispersant and Degreaser, combined bio-dispersant and ASAP, Degreaser and ASAP treatments, was achieved (Figure 4.8). Looking at the results, an apparent relative enhancement of biodegradation is observed for three treatments: bio-dispersant (1:4) combined bio-dispersant and Degreaser and Degreaser. Statistical comparison (α=0.05) of the percent biodegradation values relative to the oil only control samples indicated that all the treatments don't have a significant effect in enhancing/ inhibiting the natural biodegradation process (p=0.820, 0.943, 0.287, 0.107, 0.416, 0.164 and 0.465, respectively).

The microbial population count shown in Figure 4.8 also depicted a similar trend to the hydrocarbon analysis. Regressing the logarithmic microbial data with the amount of hydrocarbon being degraded showed good correlation (R^2=0.82) supporting that loss of hydrocarbon from the reactors is mainly due to microbial consumption. After seven days incubation, the population of microbes counted have reached highest values about an order of magnitude 3.5×10^7 CFU/ml to 1.92×10^{13} CFU/ml. Comparing population counts between different treatments showed the lowest microbial population was in the reactors supplied with Corexit while highest value was obtained with reactors where a combined bio-dispersant and Degreaser was applied. This indicates that the addition of bio-dispersant enhances the biodegradation process by solubilizing the oil and makes it available for uptake by the micro organisms introduced through addition of the

Degreaser. As a result the amount of crude oil biodegraded was higher than the ones degraded by adding either bio-dispersant or Degreaser only.

On the other hand addition of bio-dispersants with the ASAP biological agents doesn't show enhancement in oil biodegradation relative to the ASAP only treatment. Reasoning on why this system (reactor treated with combined bio-dispersant and ASAP) behaved like this is difficult due to the dynamics within the reactor. It was discussed in section 3.2.2.1 that ASAP contains micro organisms, nutrients and synthetic surfactants. The synthetic surfactant Sodium Dodecyl Sulphate (SDS) is known to have an inhibitory effect on the biodegradation of crude oil (Tiehm 1994). Upon mineralization it might have released H_2SO_4 and acidified the system affecting both the performance of rhamnolipid biosurfactant and microbial community. Comparison of biodegradation between two different rhamnolipid biosurfactant (bio-dispersant) concentrations indicated that at higher rhamnolipid concentration (0.5%) the percent oil degraded and microbial growth was higher compared to the lower concentration (0.25%).

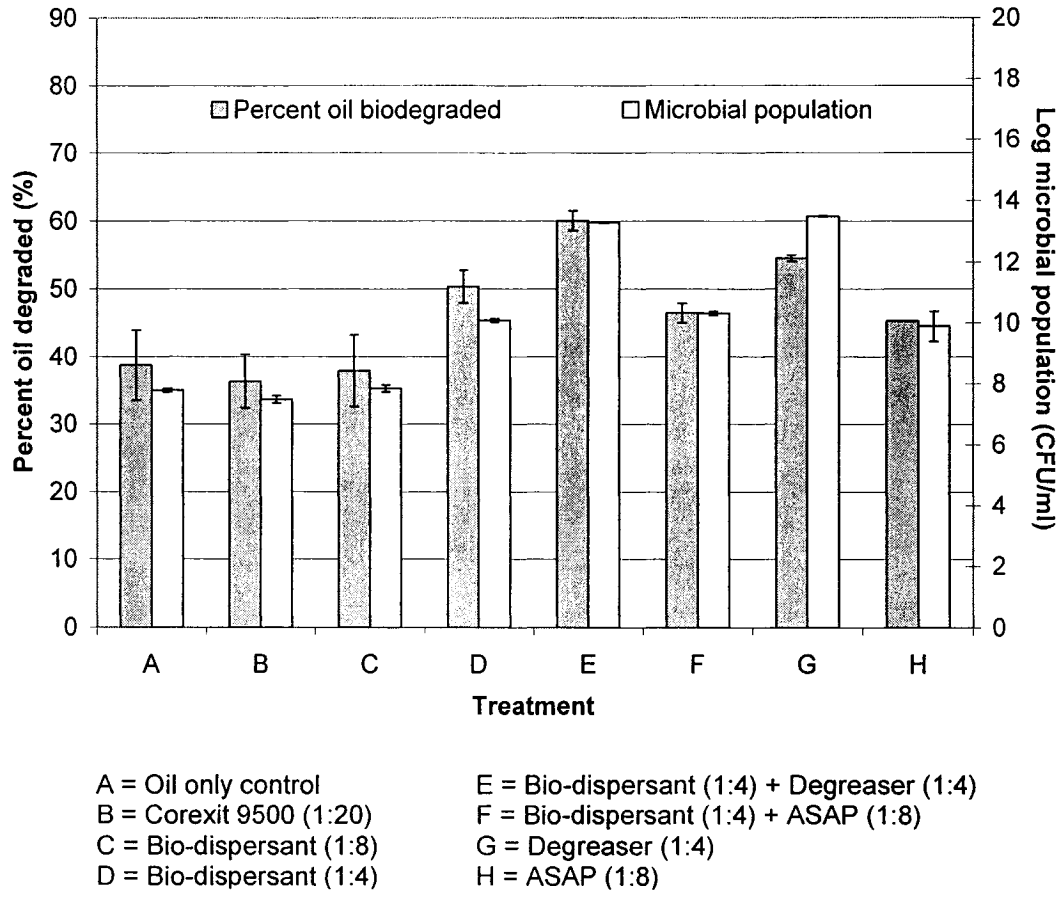


Figure 4.8 Biodegradation performance comparisons of different treatments using percentage crude oil remaining in the reactors; $100\% * (C_o - C_7)/C_0$ and microbial population in the reactors after 7 days (colony count)

4.2.1.3 Treatment effects on biodegradation after 14 days incubation

Figure 4.9 shows the total petroleum hydrocarbon and microbial analysis of the reactors after fourteen days of incubation. In terms of percent oil biodegradation, in all the reactors more than 50% of the crude oil has been degraded by the 14th day. Reactors treated with combined bio-dispersant and Degreaser showed highest degradation (62.1%) followed by Degreaser only (61.8%) and bio-dispersant only (58.2%). Statistical comparison (α=0.05) of the percent biodegradation values relative to the oil only control

samples showed no significance difference. Comparing the change in biodegradation between day 7 and day 14, reactors treated with Corexit, Bio-dispersant (1:8) as well the oil only control have shown tremendous change in the amount of oil degraded (from 36% to 55%). In contrast, the change in biodegradation between 7 to 14 days is lower for reactors treated with the other five treatments. This group has shown higher degradation on the 7^{th} day and it is suspected that after the 7^{th} day period, the more recalcitrant fraction of the crude oil remained in the flask, and thus, it was less readily degraded by the microbial cultures.

Microbial analysis of the flasks after the 14^{th} day incubation showed a decrease in the microbial population (Figure 4.9). The population of microbes counted decreased to values about an order of magnitude 1.06×10^{7} CFU/ml to 7.64×10^{12} CFU/ml. The highest microbial population is obtained in the reactors supplied with combined bio-dispersant and Degreaser followed by Degreaser only and bio-dispersant (1:8) only treatments. The decrease in microbial population can be due to the accumulation of toxic metabolites along with the depletion of the more readily degradable fraction of the petroleum hydrocarbons, which might have contributed to the decay of several microbial cultures present in the reactors. Performing regression analysis of the logarithmic microbial data with the amount of hydrocarbon being degraded showed poor correlation (R^2=0.3501).

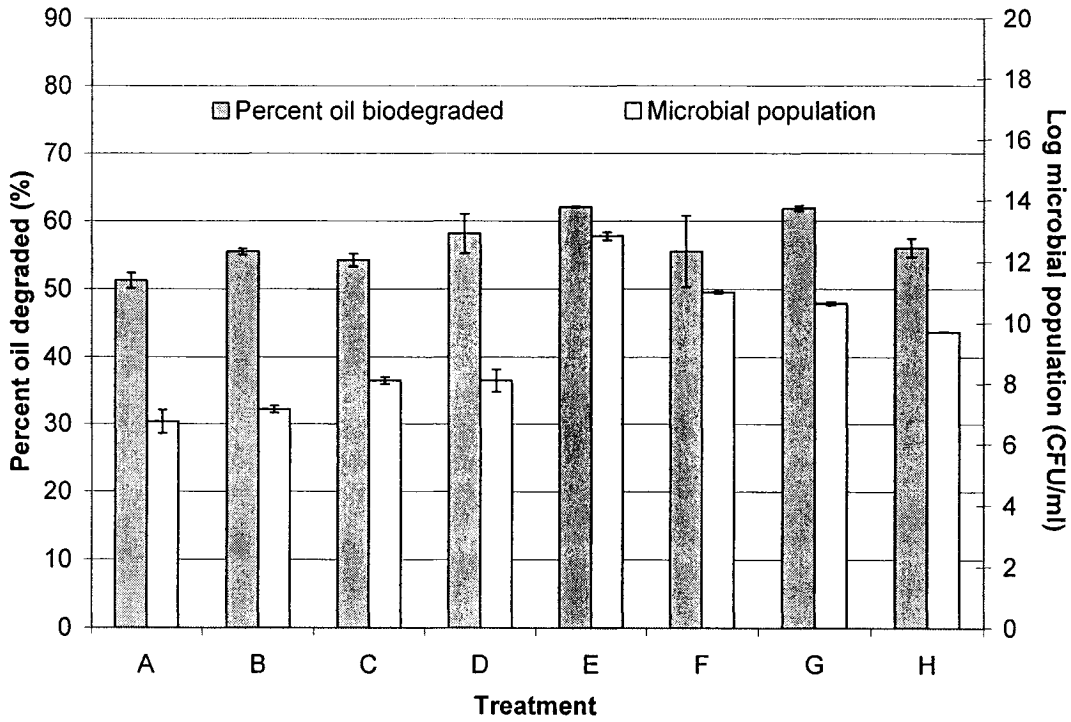


Figure 4.9 Biodegradation performance comparisons of different treatments using percentage crude oil remaining in the reactors; $100\% * (C_o - C_{14})/C_0$ and microbial population in the reactors after 14 days (colony count)

4.2.1.4 Treatment effects on biodegradation after 35 days incubation

Total petroleum hydrocarbon and microbial analysis of the reactors after 35 days of incubation is shown in Figure 4.10. By then a significant amount of the oil has been degraded. The highest percent oil biodegraded (i.e. 81.5%) has been observed with the reactor treated by combined bio-dispersant and Degreaser while reactor treated with Corexit has shown the lowest percent biodegradation. After 35 days of incubation the effect of biologically dispersing or chemically dispersing the crude oil as well

supplementing the reactors with biological agents is not pronounced on its biodegradability relative to the oil control (in which the oil has lost about 72.1%). Fresh BRENT crude oil is a light crude oil composed of 72% saturates 23% aromatics, 4% resins and 1% asphaltenes. It can be said that most of the fractions are biodegraded after 35 days (Qualitative result is shown in Figure 4.12). Regarding the microbial population no significant change was observed between days 14 to 35 in any of the reactors (Figure 4.9).

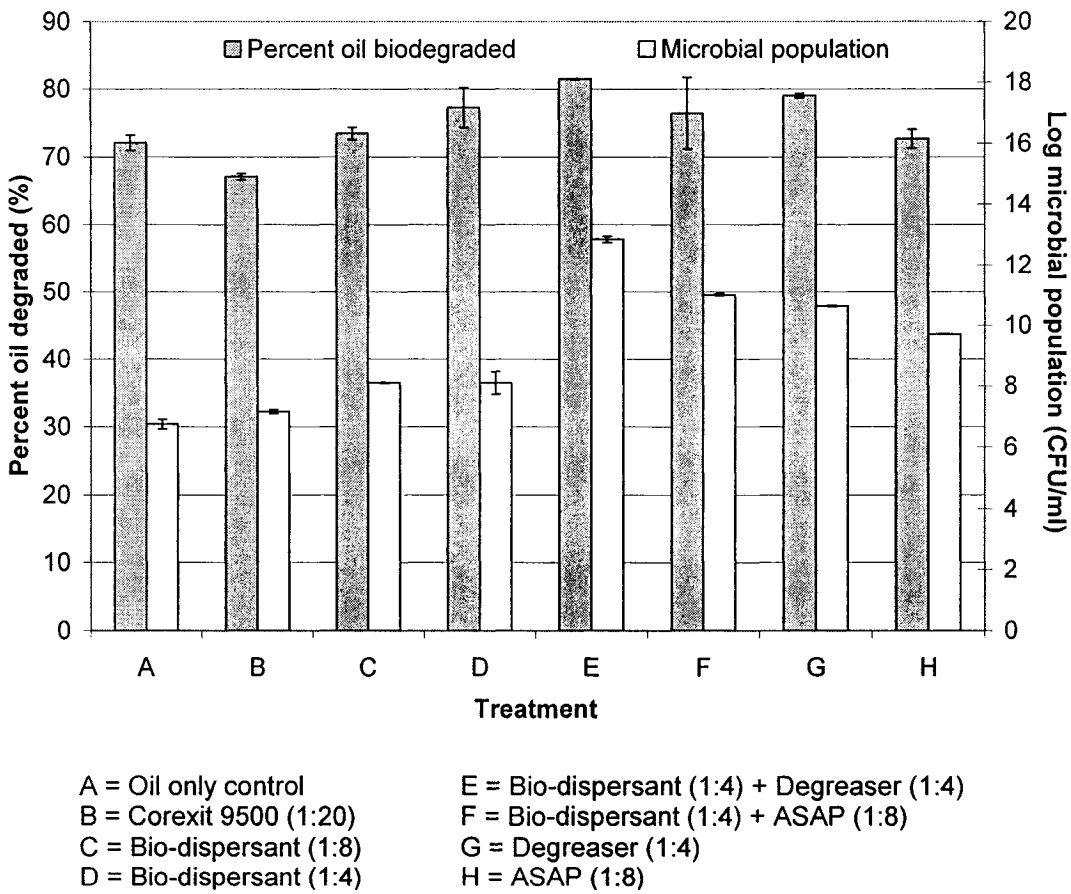


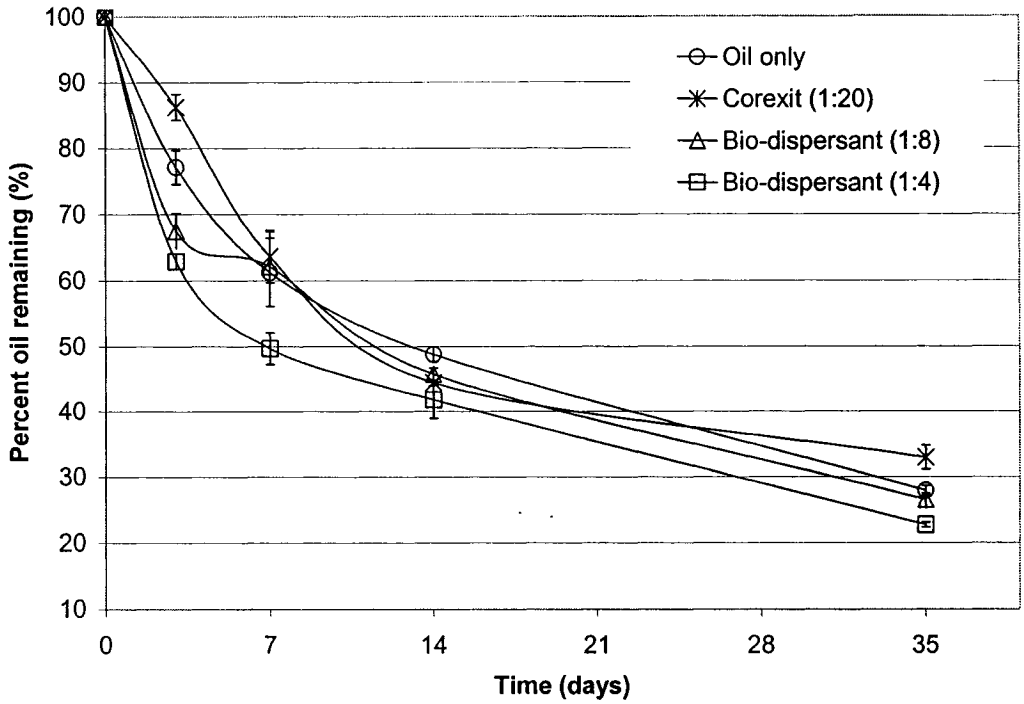
Figure 4.10 Biodegradation performance comparisons of different treatments using percentage crude oil remaining in the reactors; $100\% * (C_o - C_{35})/C_0$ and microbial population in the reactors after 35 days (colony count)

4.2.2 Biodegradation of crude oil over time

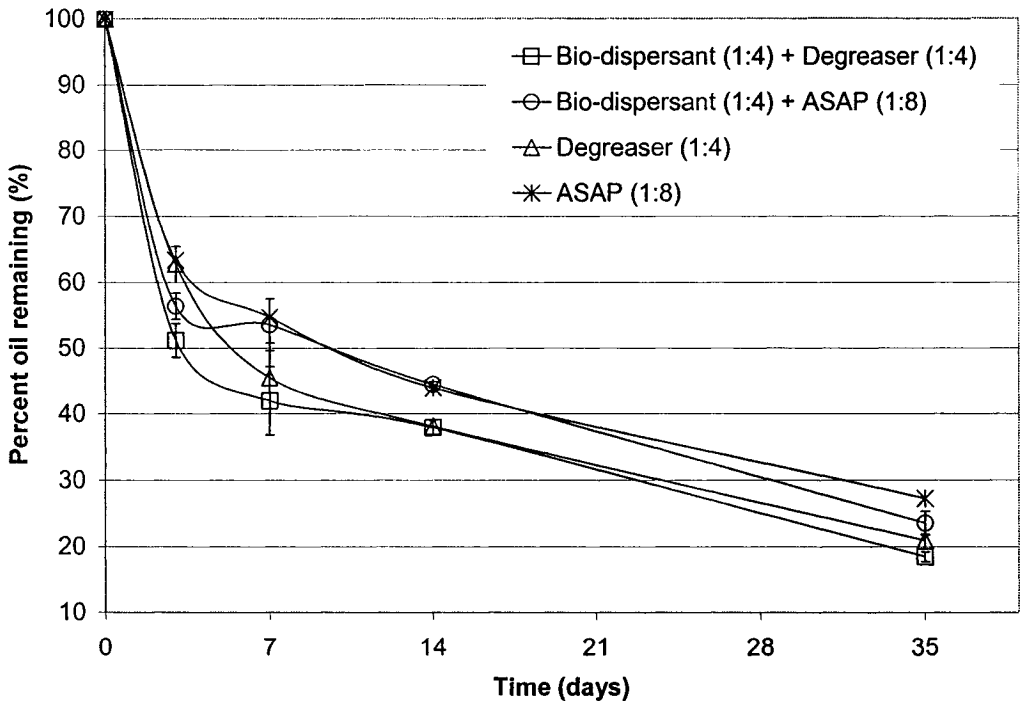
Total Petroleum Hydrocarbon: The biodegradation of weathered BRENT crude oil was measured by Total Petroleum Hydrocarbon (TPH) analysis remaining in the reactor. Figure 4.11 (a) and (b) show the biodegradation as a fraction of the initial concentration for the 7 treatments: bio-dispersant (1:8), bio-dispersant (1:4), combined bio-dispersant (1:4) and Degreaser™ (1:4), combined bio-dispersant (1:8) and ASAP™ (1:8), Degreaser™ (1:4), ASAP™ (1:8) and Corexit 9500™ (1:20) as well as an oil control over time. In general, it has been observed that introduction of various treatments resulted in biodegradation of crude oil within the range of 13.7% to 81% between 3 to 35 days. Overall, high biodegradability was observed during the first seven days of incubation, probably mainly related to biodegradation of lighter fractions of the hydrocarbon. However in the presence of some treatments the degradation extent was lower than the oil only control, for some it was not statistically significant relative to the oil only control and others have showed statistically significant enhancement relative to the oil only control. Details are described in section 4.3.2.

Qualitative analysis of residual oil remaining after 3, 7, 14 and 35 days of incubation in batch experiments was as expected, indicating a higher loss of lighter and medium fractions of the oil in comparison to the heavier (lower end) fractions of the hydrocarbon. Figure 4.12 shows example chromatograms of weathered BRENT crude oil and material recovered after 35 days of incubation corresponding to reactors treated with Corexit, bio-dispersant (1:4), and bio-dispersant (1:4) with Degreaser as well as an oil only control and the original oil sample. Chromatograms of residual oil remaining after 3, 7 and 14 days as well the Corexit , ASAP, Degreaser and rhamnolipid biosurfactant blanks are

shown in Appendix E. In most of the chromatograms while no intermediate compound formation has been observed, significant preferential biodegradation following Corexit dispersant use is observed (Figure 4.11 and Appendix E). This induced the residual oil to selectively become enriched in components of greater toxicity than those components biodegraded ultimately affecting the toxicity of the resulting oil residue (on an oil mass basis).



(a)



(a)

Figure 4.11 Weathered BRENT crude oil biodegradation over time: (a) reactors treated with bio-dispersant, Corexit and oil only control and (b) reactors treated with Bio-dispersant with Degreaser, Bio-dispersant with ASAP, Degreaser only and ASAP only

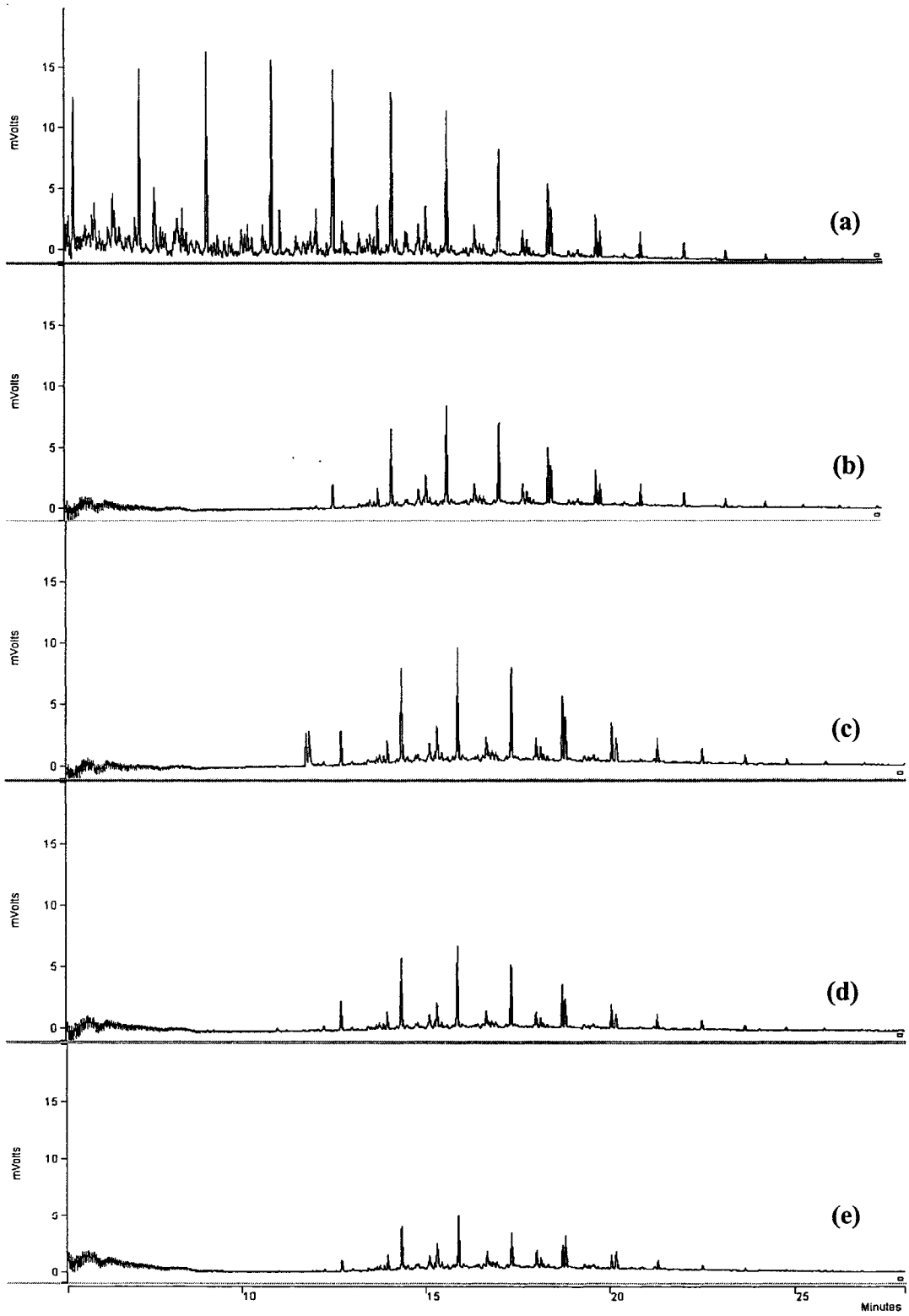
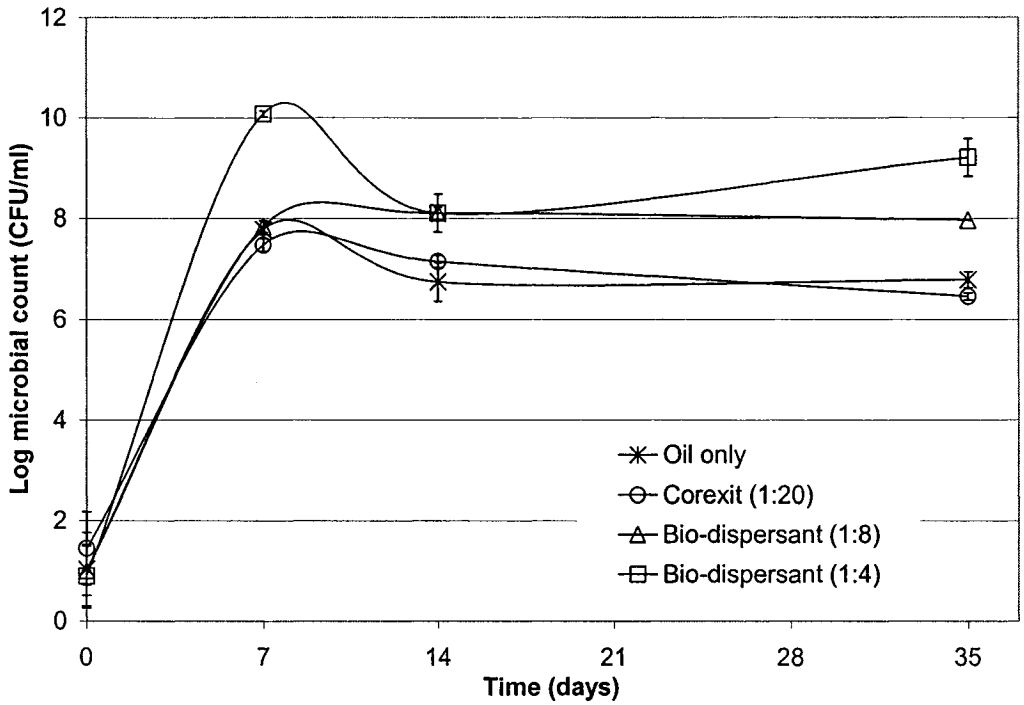


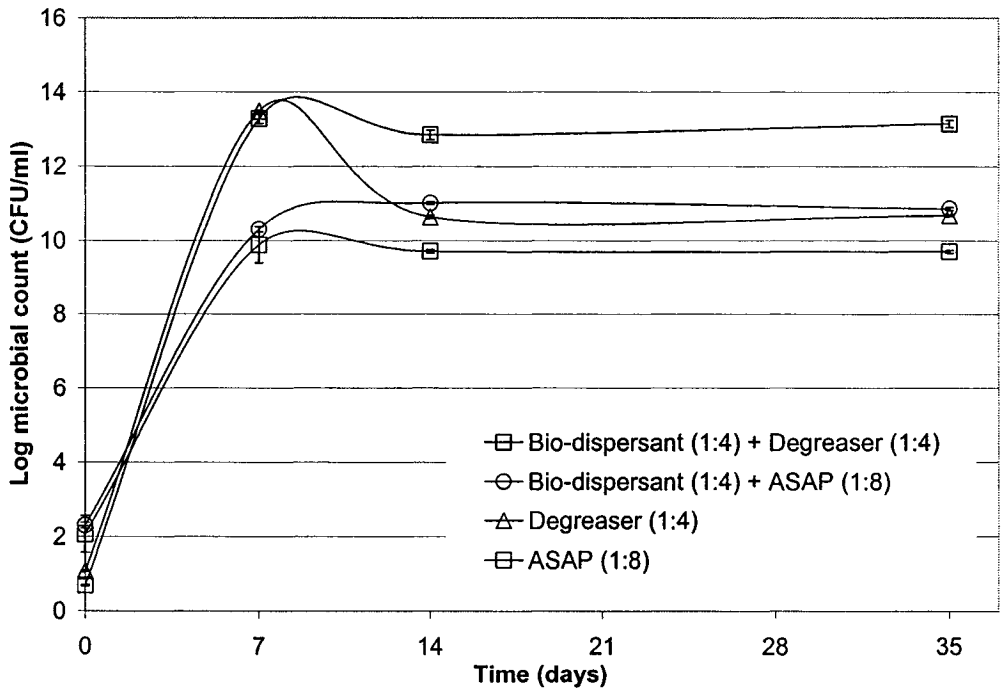
Figure 4.12 Chromatograms of weathered BRENT crude oil and material recovered from 35 days old flask batch reactors: (a) original oil sample, (b) oil only control, (c) Corexit 9500, (d) bio-dispersant (1:4) & (e) bio-dispersant (1:4) with Degreaser (1:4)

Microbial growth: To endorse the above biodegradation results, the reactor has been analyzed for microbial densities using the pour plate method (SMW 2000). Figure 4.13 (a) and (b) show the microbial growth after 7, 14 and 28 incubation time.

Most of the products showed microbial growth. However, in some cases the growth as well as the microbial oxidation is negligible relative to the control. In general the microbial growth of different treatments has shown similar trends. Between 0 to 7 days, an exponential microbial growth has been observed. The microbial population counted reached highest numbers after seven days followed by a decrease in microbial population (decay phase). It is thought that during the first 7 days the microbial population is growing by using readily degraded hydrocarbons. Once it is depleted, the micro-organisms are switching to the heavier fractions of petroleum hydrocarbons as a source of organic carbon. However not all the organisms followed this trend and the microbial population reduced. The decrease in microbial population can be due to the accumulation of toxic metabolites, or more recalcitrant ones, as well as the depletion of the more readily degradable fraction of the petroleum hydrocarbons which may have contributed to the decay of some microbial cultures present in the reactors. For day three, microbial data is not reported due to contamination of microbial plates. This made it difficult to accurately determine the lag phase.



(a)



(b)

Figure 4.13 Microbial densities over time: (a) reactors treated with bio-dispersant, Corexit and oil only control and (b) reactors treated with Bio-dispersant with Degreaser, Bio-dispersant with ASAP, Degreaser only and ASAP only

4.2.3 Crude oil disappearance kinetics

4.2.3.1 Crude oil biodegradation rate

The rate at which crude oil biodegrades is influenced among other factors by the population and type of micro organisms, bioavailability (dispersion) of crude oil, chemical composition of the oil, degree of oil weathering, oxygen supply, pH and temperature. To better compare the effects of different treatments on BRENT crude oil biodegradability, the biodegradation rate of the different treatments discussed above were estimated by fitting the degradation data using regression analysis (in an Excel® environment) to a standard first order biodegradation model:

$$\frac{dC}{dt} = -kC$$

$$\Rightarrow C = C_0 \exp^{(-kt)} \qquad \textbf{(Eqn. 4.1)}$$

Where C represents the hydrocarbon concentration (gL^{-1} i.e. g of hydrocarbon per litre of hexane), C_0 is the initial hydrocarbon concentration (gL^{-1}), k is the rate coefficient (day^{-1}) and t is time in days. The rate coefficient was obtained after fitting the degradation data, $\ln(C/C_0)$, versus time (0, 3, 7, 14 and 35 days), where the slope of the regression line provided the rate coefficient (k) (Figures 4.14). Using this procedure 16 k values (for the 8 treatments and 2 replicates) were calculated. Table 4.4 shows the average mean k values with their respective coefficients of determination (R^2) and coefficient of variation (CV). Also the half-lives of the crude oil fractions were determined (Table 4.4) using the following relation for the first order kinetics:

$$t = \frac{\ln(C/C_0)}{k}$$

$$\Rightarrow t_{1/2} = \frac{\ln 2}{k} = \frac{0.693}{k} \qquad \textbf{(Eqn. 4.2)}$$

Where $t_{1/2}$ = half life (days) and k = first order biodegradation rate coefficient, (day^{-1}).

First-order kinetics was used to model biodegradation of petroleum hydrocarbon compounds by previous biodegradation research (Page et al. 2002; Harris et al. 2002; and Aldrett et al. 1997). Although it is a common practice to fit oil degradation data to first-order kinetics, it is clear from figure 4.14 that the data points on the graph fits curve line better as oppose to the expected straight line for the $\ln(C/C_0)$ versus time plot. Theoretically, the $\ln(C/C_0)$ versus time plot produces a straight line indicating the rate of degradation is directly proportional to the concentration. Physically this shows that as the oil is consumed during the biodegradation process, the concentration drops so does the rate of biodegradation. This is true for an individual hydrocarbon compound where the rate depends mainly on its concentration. In case of crude oil, it is a complex mixture of hydrocarbons; the rate could be affected by the composition of the crude oil in addition to its concentration. To consider two factor dependency of a biodegradation process, Ikechukwu (2004), Karapanagioti et al. (2001) and Hutchins (1997) have used second order biodegradation models. Further, the degradation data were fitted to second-order kinetics using the following standard second-order model:

$$\frac{dC}{dt} = -KC^2$$

$$\Rightarrow C = \frac{C_0}{1 + Kt(C_0)} \qquad \textbf{(Eqn. 4.3)}$$

The rate coefficient was obtained after fitting the data to $(1/C)$-$(1/C_0)$, versus time (0, 3, 7, 14 and 35 days) plot, where the slope of the regression line provided the rate coefficient (K, $Lg^{-1}day^{-1}$) (Figures 4.15). Using this procedure 16 K values (for the 8 treatments and 2 replicates) were calculated. Table 4.5 shows the average mean K values with their respective coefficients of determination (R^2) and CV values. Similar to the first order crude oil biodegradation, the half-lives of the crude oil fractions were determined (Table 4.5) using the following relation for the second order kinetics:

$$t = \frac{1/C - 1/C_0}{K}$$

$$\Rightarrow t_{1/2} = \frac{1}{C_0 K} = \frac{0.2461}{K} \qquad \textbf{(Eqn. 4.4)}$$

Where $t_{1/2}$= half life (days), C_0 = the initial mean crude oil concentration, 4.06 gL^{-1} and K = second order biodegradation rate coefficient, ($Lg^{-1}day^{-1}$). Looking Figure 4.15 and Table 4.5 it is apparent that the data fits the second-order kinetics better. For the different biodegradation treatments, the rate constant derived from the first-order kinetics had statistically significant lower (p values from 0.05 to 0.001) R^2 values (from 0.40 to 0.72) from those of second-order kinetics (from 0.74 to 0.91). Similarly, the k values derived from first-order kinetics had statistically significant higher (p values 0.036 to 0.001) coefficient of variation, CV values (10.8% to 14.6%) from those of second-order kinetics (from 6.6% to 11.8%). In view of the above results, on the basis of R^2 and coefficient of variation (CV), it was concluded that the second order kinetics fits the oil biodegradation data better.

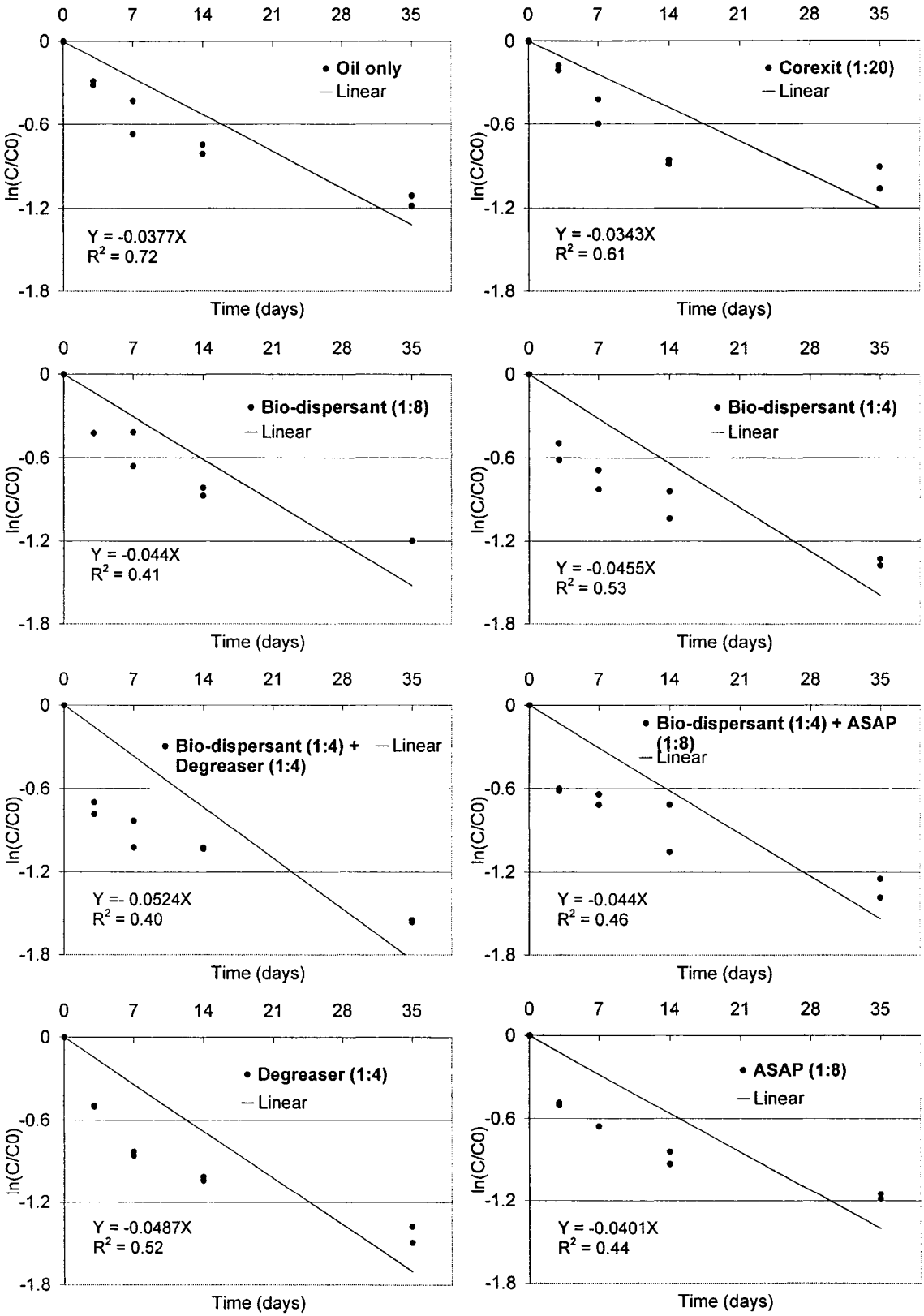


Figure 4.14 Concentration, $\ln(C/C_0)$ versus time plot

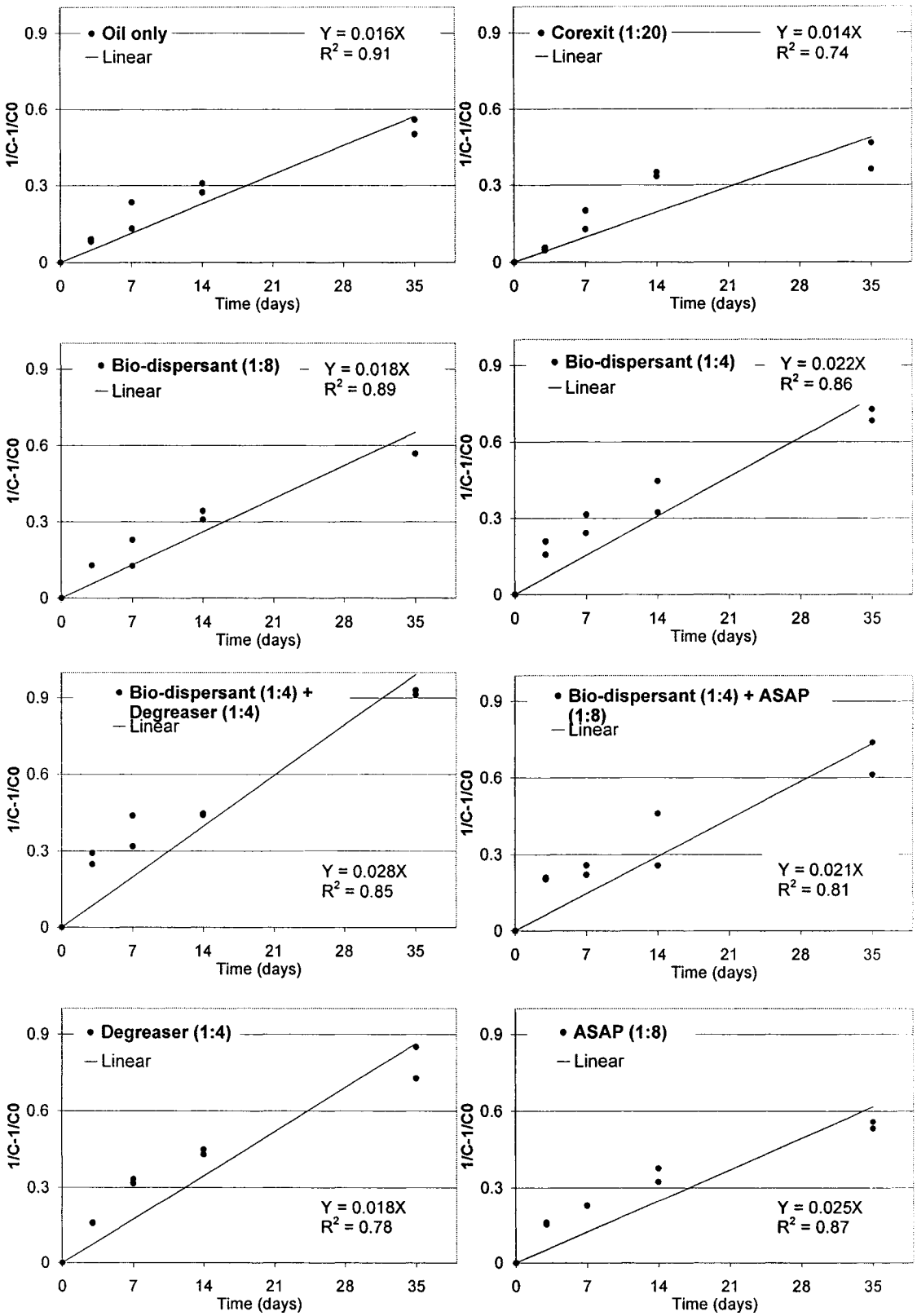


Figure 4.15 Concentration, $(1/C)$-$(1/C_0)$, versus time plot

In general the biodegradation rate coefficients and half lives obtained from both first-and second-order kinetics vary in the order of: combined bio-dispersant and Degreaser > Degreaser, bio-dispersant (1:4) > combined bio-dispersant and ASAP > bio-dispersant (1:8) > ASAP > oil only control > Corexit. The biodegradation rate constant of the biologically dispersed oil in the presence of exogenous micro-organism and nutrient (Degreaser), Degreaser alone and biologically dispersed crude oil at higher rhamnolipid concentration (bio-dispersant 1:4) are significantly different from the oil only control (at $\alpha = 0.05$, $p = 0.006$, 0.021 and 0.027 , respectively). The biodegradation rate constants of biologically dispersed oil at lower rhamnolipid concentration and its combination with the ASAP or ASAP alone didn't show significant differences relative to the oil control (at $\alpha = 0.05$, $p = 0.115$, 0.416 and 0.220, respectively). The biodegradation rate of the chemically dispersed oil was observed to be lower than that of the naturally dispersed oil, however statistically the difference was not significant (at $\alpha = 0.05$, $p = 0.115$).

Calculation of half lives of the crude oil fractions using second order biodegradation rate constant showed it takes between 8 to 17 days to biodegrade 50% of the oil. The shorter period (8.7 days) correspond to oil treated with combined bio-dispersant and degreaser whereas the longer period (17 days) correspond to corexit treated reactors. Comparison between half lives derived from 1^{st} and 2^{nd} order rate constants showed overall longer half life periods for first-order rates.

Table 4.4 First-order biodegradation rate coefficients for total hydrocarbons

Treatment	$k^{\dagger}$ (day^{-1}) Mean	Half life[¶] (days)	$R^{2\S}$	CV[#] (%)
Oil only	0.038	18.4	0.72	10.8
Corexit 9500	0.034	20.2	0.61	13.6
Bio-dispersant (1:8)	0.040	17.5	0.65	11.8
Bio-dispersant (1:4)	0.046	15.2	0.53	13.2
Bio-dispersant (1:4) + Degreaser, 1:4	0.053	13.2	0.40	14.6
Bio-dispersant (1:4) + ASAP (1:8)	0.044	15.8	0.46	14.1
Degreaser (1:4)	0.049	14.2	0.52	13.4
ASAP (1:8)	0.040	17.3	0.44	14.2

Table 4.5 Second-order biodegradation rate coefficients for total hydrocarbons

Treatment	$K^{\ddagger}$ (Lg^{-1} day^{-1}) Mean	Half life[¶] (days)	$R^{2\S}$	CV[#] (%)
Oil only	0.016	15.1	0.91	6.6
Corexit 9500	0.014	17.7	0.74	11.8
Bio-dispersant (1:8)	0.019	13.2	0.89	7.0
Bio-dispersant (1:4)	0.022	11.2	0.86	7.7
Bio-dispersant (1:4) + Degreaser, 1:4	0.028	8.7	0.85	8.1
Bio-dispersant (1:4) + ASAP (1:8)	0.021	11.8	0.81	9.3
Degreaser (1:4)	0.025	10.0	0.87	7.7
ASAP (1:8)	0.018	14.0	0.78	9.5

[†] k represents the first-order rate coefficient

[‡] K represents the second-order rate coefficient

[¶]Half life calculated using equations 4.2 and 4.4

[§] R^2 represents the coefficient of determination

[#]CV represents the coefficient of variation (100 * standard error/biodegradation rate coefficient (k))

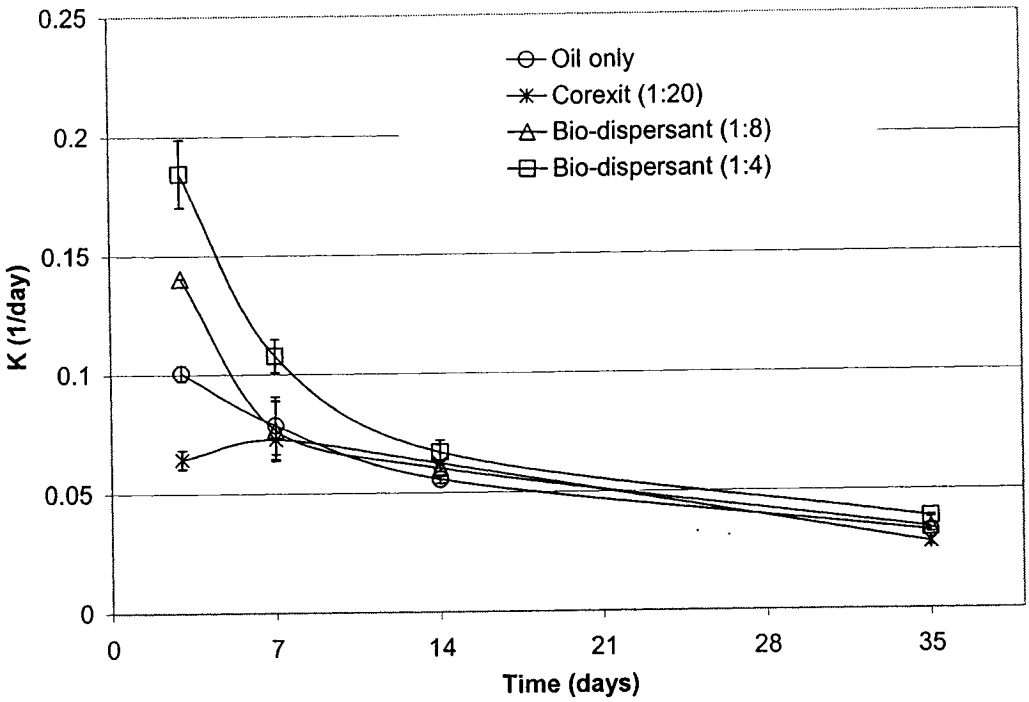
4.2.3.2 Biodegradation rate over time

To determine the time when the maximum biodegradation rate coefficient for each treatment has occurred, a rate coefficient for days 3, 7, 14 and 35 was calculated using the following first and second-order kinetics:

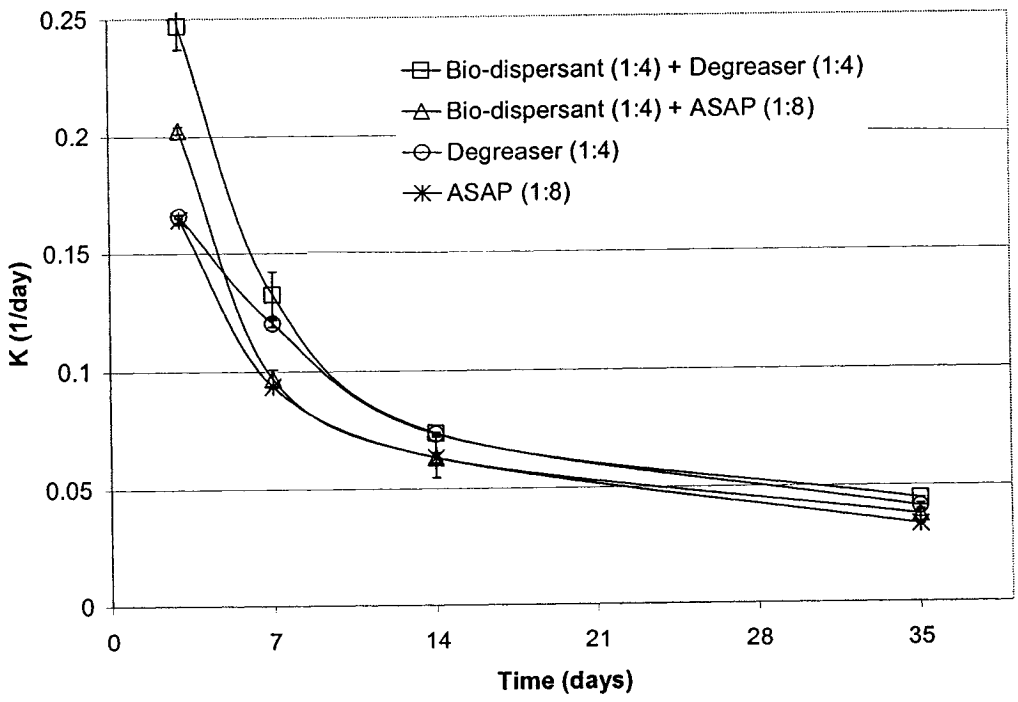
$$k_t = \frac{\ln(C/C_0)}{t}$$

$$K_t = \frac{1/C - 1/C_0}{t} \qquad \textbf{(Eqn. 4.5)}$$

Figures 4.16 and 4.17 shows the biodegradation rate coefficients of crude oil treated with bio-dispersant (1:8), bio-dispersant (1:4), combined bio-dispersant (1:4) and Degreaser™ (1:4), combined bio-dispersant (1:8) and ASAP™ (1:8), Degreaser™ (1:4), ASAP™ (1:8) and Corexit 9500™ (1:20) as well an oil control over time. As shown in Figures 4.16 and 4.17, an exponential increase in k values was observed for the first three days in most of the treatments (biological treatments) except Corexit and oil only control. In the case of Corexit the highest rate was observed after 7 days incubation. After 3 days incubation, the biodegradation rate coefficients range from 0.06 day^{-1} to 0.23 day^{-1} (first order) and 0.017 $Lg^{-1}day^{-1}$ to 0.095 $Lg^{-1}day^{-1}$ for (second-order), the highest rate was observed by oil treated with bio-dispersant and degreaser while the lowest rate was associated with Corexit. Also on the third day, the rest of the biological treatments have shown a considerably higher disappearance rate. The rate coefficients started to decrease exponentially after day 3 except for Corexit. The rate coefficient decreased at lower rate between days 14 and 35. In all treatments, the second order rate coefficients are lower than the first order rate coefficients.

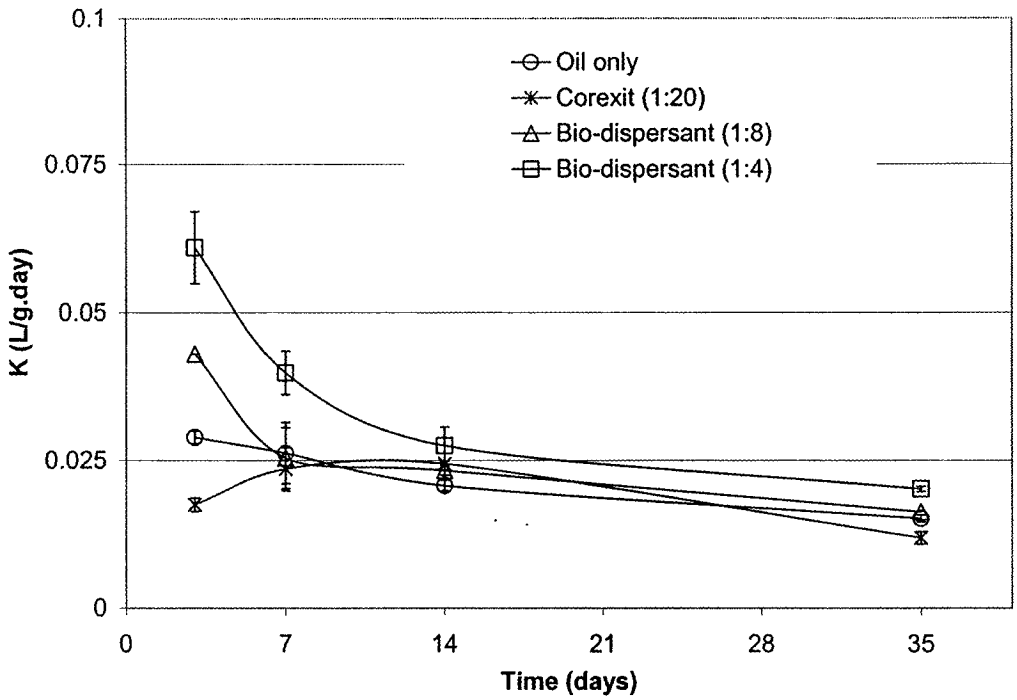


(a)

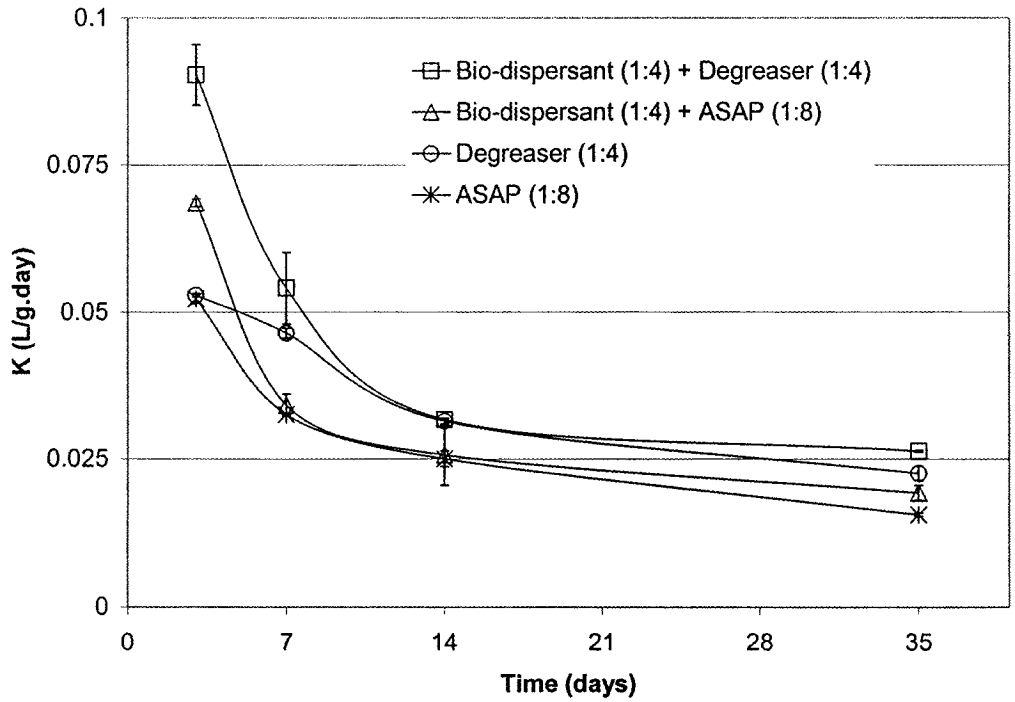


(b)

Figure 4.16 First-order biodegradation rate coefficients over time (a) reactors treated with bio-dispersant, Corexit and oil only control and (b) reactors treated with Bio-dispersant with Degreaser, Bio-dispersant with ASAP, Degreaser only and ASAP only



(a)



(b)

Figure 4.17 Second-order biodegradation rate coefficients over time (a) reactors treated with bio-dispersant, Corexit and oil only control and (b) reactors treated with Bio-dispersant with Degreaser, Bio-dispersant with ASAP, Degreaser only and ASAP only

4.2.4 Summary of biodegradation results

This study evaluated the capability of the rhamnolipid biosurfactant (JBR 425™) for enhancing biodegradability of crude oil spilled on surface water. Accordingly the biodegradation of weathered BRENT crude oil was evaluated and compared in the presence of biological and chemical treatment following the USEPA's biological effectiveness test method (USEPA 1996b). The experiment monitored change in microbial population and oil concentration as indicators of biodegradation. The results from the experiment were analysed and interpreted in different ways. One way looked at the differences in percentage of oil remaining and the change in microbial population of reactors treated with different treatments at a specific period of time. Another approach looked at an overall percentage of oil remaining and microbial population growth/ decay over a continuous period (0, 3, 7, 14 and 35 days). In the former case higher degree of biodegradation variability was observed at the 3^{rd} and 7^{th} days. At this time biological treatments showed significantly enhanced biodegradation of oil while chemically treated reactors showed a significant inhibitory effect. After the 14^{th} day no significant difference was observed between the chemically and biologically treated oil relative to the oil only control. In all the reactors microbial analysis followed similar trends as the hydrocarbon analysis, the highest population was observed at the 7^{th} day.

Often the biodegradation data has been fitted to either a standard first order equation or two parallel first order equations to evaluate the biodegradation rate coefficient and half life of crude oil for each reactor treated with different products. In this case the data was fitted primarily into a first-order kinetics which resulted poor model performance. The

use of two parallel first order equations to fit the data was not opted for due to the smaller size of biodegradation data. Further to improve the performance the biodegradation data was fitted to a second-order model. Overall, the result depicted that higher biodegradation rates by reactors with biological treatments. In general the biodegradation rate coefficients and half lives of hydrocarbon fractions vary in the order of: combined bio-dispersant and Degreaser > Degreaser, bio-dispersant (1:4) > combined bio-dispersant and ASAP > bio-dispersant (1:8) > ASAP > oil only control > Corexit. Also the calculation of the rate coefficient (k) shows highest degree of variability between treatments occurred during early days of incubation. Calculation of half lives using second order biodegradation coefficients showed, addition of combined bio-dispersant and degreaser treatment reduced the time required to biodegrade 50% of the crude oil by half.

Overall this has an important practical indication in that, most of the economical and environmental damage associated with oil spills occurred in the first few days. It is demonstrated by these results that application of the biological treatments has shown a tremendous biodegradation of crude oil for this first few days thus limiting possible environmental and economical damage. The results showed no difference in biodegradation after 14 days, between the biologically and chemically treated oil with respect to the oil only control. However in practical terms after 14 days the damage is already caused by the oil and the remaining oil in the open water can biodegrade by itself.

5 Conclusion and Recommendation

5.1 Summary and concluding remarks

The effectiveness of a commercial rhamnolipid biosurfactant (JBR 425™) on dispersion of BRENT crude oil spilled on surface water was evaluated following a modified swirling flask method (USEPA 1996a). Accordingly the effect of four different types of solvents, concentration of rhamnolipids, dispersant to oil ratio, temperature and degree of oil weathering on the efficacy of JBR 425™ for dispersing BRENT crude oil has been quantified. The study also compared the effectiveness of the biosurfactant as well the stability of the droplets formed by commercial chemical dispersant (Corexit 9500™) and natural dispersion.

The research addressed also the effect of the bio-dispersant (rhamnolipid biosurfactant solution in a pH 7.5 buffered distilled water) on the biodegradation of petroleum compounds. In addition to the bio-dispersant, the effect of the introduction of two biological agents: ASAP™ (commercial biological product containing bacterial consortium, surfactant and nutrients) and Degreaser™ (product containing bacterial consortium and nutrients), on crude oil biodegradation have also been evaluated. The biodegradation experiment was conducted following the USEPA's bioremediation effectiveness test procedure (USEPA 1996b) and the results were compared with biodegradation in the presence of the chemical dispersant (Corexit 9500™) and natural biodegradation. As indicators of biodegradation, the remaining amount of hydrocarbon and the change in microbial population were monitored over 3, 7, 14 and 35 days.

The following observations were made based on the dispersion results:

- Solutions of various dilutions of rhamnolipids in a water-based solvent (pH of 7.5) dispersed a tremendous amount of crude oil into the water column. In comparison to natural dispersion, the rhamnolipid biosurfactant enhanced dispersion of crude oil by more than 50 orders of magnitude. An overall increase in oil dispersion (10 to 82%) was observed with increasing rhamnolipid concentration (0.125% to 12%). Since the rhamnolipid was applied above its CMC value, it can be concluded that the main mechanism controlling dispersion by rhamnolipids was physical interaction between the micelles and hydrocarbon than surface tension reduction.

- For listing dispersants into the NCP schedule, a dispersant is required to have an effectiveness of at least 45%. It was found that the bio-dispersant can attain this range when delivered in the range of 1.5 to 2% rhamnolipid concentration to the oil-water interface. This can be delivered as a concentrate, for example 8% with a lower DOR (1:4), or more dilute (0.5%) with a higher DOR (1:0.125) without affecting its performance. The rhamnolipid if applied at this level can disperse the same amount of oil compared with Corexit 9500™ added at a DOR of 1:20. However rhamnolipid biosurfactant concentration used is relatively lower (2%) compared to the surfactant concentration used by chemical dispersants that makes use of 25% for water-based solvent and 65% for petroleum-based solvents.

- One of the problems of the commercially available dispersants is failure when applied on weathered oil and/or at lower temperature regions, resulting in a smaller window of opportunity of usage. Assessment of rhamnolipid biosurfactant effectiveness at a

lower temperature and on weathered BRENT crude oil, depicted insignificant reduction when compared with effectiveness results obtained on fresh oil and at moderate temperature, respectively. This indicated that the formulated rhamnolipid based bio-dispersant has a relatively wider window of opportunity of usage during response.

Subsequent to the biodegradation results, the following observations have also been made:

- Overall the biodegradation of biologically dispersed crude oil (using bio-dispersant) and/ or the reactors supplied with biological agents was higher relative to the oil only control. In contrast, biodegradation results for chemically dispersed crude oil was lower. These differences were especially apparent after incubation and analysis of the reactors for three to seven days. These findings suggested that biological treatments could reduce impact of spilled oils at its peak period i.e. few days after the spill, by dispersing and enhancing the biodegradation of the dispersed oils in the water column. On the other hand the chemical treatment (Corexit 9500) though very effective in dispersing the oil, showed an inhibitory effect on the biodegradation of crude oil for the first seven days.

- Addition of the bio-dispersant with Degreaser™ (biological agents containing nutrients and consortium of bacteria) showed the best performance in enhancing the biodegradation of the weathered crude oil. Therefore, a strategy of applying biological agents along with bio-dispersants may further stimulate biodegradation of petroleum compound.

- On the other hand, addition of the bio-dispersant to the ASAP™ or use of ASAP™ alone didn't show significant enhancement in biodegradation of the crude oil over the period of days. This could be explained by the presence of chemical surfactant in the ASAP™ mix and the product may not be recommended for use in an oil response.

- However application of the bio-dispersant at two different dispersant to oil ratios (1:4 and 1:8 of a 2% rhamnolipid solution) showed higher biodegradation in relation to higher concentration. Overall the amount of rhamnolipid used in the biodegradation experiment is relatively small i.e. 0.25% and 0.5%, but the above result could be considered as indicator to a possible increase in biodegradation with increase in rhamnolipid concentration.

- First and second order kinetics applied to model the rate of hydrocarbon disappearance (biodegradation rate coefficient and half life) showed higher rate of biodegradation on the 3rd day for reactor treated with the biological treatments, while 7th day for the reactor supplied with Corexit 9500. In general the biodegradation rate coefficients of hydrocarbon treated with different treatments varied in the order of: combined bio-dispersant and Degreaser > Degreaser > bio-dispersant (1:4) > combined bio-dispersant and ASAP > bio-dispersant (1:8) > ASAP > oil only control > Corexit.

Economically, the cost of delivering 2% rhamnolipid solution (which is equal to 8% of JBR 425 in pH 7.5 buffered distilled water) per mega ton of spilled oil is $698,784 USwhile Corexit at DOR of 1:20 is $138,552 US. To reduce the cost of JBR 425-based bio-dispersant formulation, an equivalent amount of 13.3% JBR 215 (contains 15%

rhamnolipid) can be used at a cost of $114,648 US per mega ton of spilled oil. Furthermore, the rhamnolipid biosurfactant has shown consistent dispersion performance irrespective of temperature as well as oil property changes due to weathering and also enhanced biodegradation of the crude oil. In addition it has added advantages of lower toxicity and stability against extreme salinity. This result agrees well with the already documented biosurfactant advantages for use in soils i.e. possible enhanced biodegradability, lower toxicity and stability against extreme salinity, temperature and pH (Mulligan and Gibbs 1994).

5.2 Recommendations for future work

The study presents an attempt to address a very wide problem but it by no means completely addressed this challenging problem and therefore future research is required. Based on the experience obtained in the present study the following recommendation can be made:

- In the present study the evaluation of bio-dispersant on oil spill dispersion as well biodegradation is limited to BRENT crude oil which is a light crude oil. Therefore further efficacy of the bio-dispersant for medium and heavy crude oils needs to be evaluated.
- Perform biodegradation study at higher (>0.5%) rhamnolipid concentration
- Application of a combined bio-dispersant and Degreaser mixture has shown a very promising result in enhancing biodegradation of crude oil. Thus feature work needs to be focused on the optimization of their concentrations for addition into the oil-water interface.

- In addition to the rhamnolipid biosurfactanct, testing the potential of other biosurfactants such as sophorolipids, either in combination with rhamnolipids or on individual basis, for oil spill dispersion.
- Another potential future research in this area could be integration of dispersion results to mathematical oil spill models that simulate different spill scenarios. The models estimate the effectiveness of the bio-dispersant for different scenarios.

6 References

Aldrett, S. Bonner, J.S., Mills,, M.A. Autenrieth, R.L. and Stephen, F. L. (1997). "Microbial degradation of crude oil in marine environments tested in a flask experiment." *Water Research*, Vol. 31, pp. 2840-2848.

Al-Sabagh, A, M. and Atta, A. M. (1999). " Water-based non-ionic polymeric surfactants as oil spill dispersants." *J. Chem. Technol. Biotechnol.,* Vol. 74, pp. 1075-1081.

American Society for Testing and Materials (ASTM). (2002). "F2059-00 Standard test method for laboratory oil spill dispersant effectiveness using the swirling flask." *ASTM 11.04*, West Conshohocken, Pa., pp. 1549-1552.

Atlas, R. M. (1995). "Petroleum biodegradation and oil spill bioremediation." *Marine Pollution Bulletin*, Vol. 31, No. 4-12, pp. 178-182.

Avmor Ltd. (2003a). ASAP™ bio-action floor degreaser technical datasheet. 950 Michelin, Laval, Quebec, Canada. www.avmor.com

Avmor Ltd. (2003b). Industrial degreaser and concrete cleaner™. 950 Michelin, Laval, Quebec, Canada. www.avmor.com

Avolizi, R. J. and Nuwayhid, M. (1974). "Effects of crude oil and dispersants on bivalves." *Marine Pollution Bulletin*, Vol. 5, No. 10, pp. 149-153.

Bai, G., Brusseau, M. L. and Miller, R. M. (1997). "Bacterial degradation of emulsified crude oil and the effect of various surfactants." *Can. J. Microbiol.,* Vol. 43, pp. 17-22.

Barkay, T., Navon-Venezia, S., Ron, E. Z. and Rosenberg, E. (1999). "Enhancement of solubilization and biodegradation of polyaromatic hydrocarbons by the Bioemulsifier Alasan." *Applied and Environmental Microbiology*, Vol. 65, No. 6, pp. 2697-2702.

Becher, P. (1965). *Emulsions, Theory and Practice*, 2nd edition, Reinhold Publishing, New York.

Blondina, G. J., Sowby , M. L., Ouano, M. T., Singer, M. M., and Tjeerdema, R. S. (1997). " A modified swirling flask efficacy test for oil spill dispersants." *Spill Science and Technology Bulletin*, Vol. 3, No. 4, pp. 177-185.

Brandvik, J. and Daling, S. (1998). "Optimising oil spill dispersants as a function of oil type and weathering degree: a multivariate approach using partial least squares." *Chemometrics and Intelligent Laboratory Systems*, Vol. 42, pp. 73-91.

Bruheim, P., Bredholt, H., Eimhjellen, K. (1997). "Bacterial degradation of emulsified crude oil and the effect of various surfactants. *Can. J. Microbiol.* Vol. 47, pp. 17-22.

Clayton, J. R., Payne, J.R., Farlow, J. S., and Sarwar, C. (1993). *Oil spill dispersants and mechanisms of action and laboratory tests*, CRC Press, Boca Raton, Fl.

Dahrazma, B. and Mulligan, C.N. (2004). "Extraction of copper from a low-grade ore by rhamnolipids." *Practice periodical of hazardous, toxic, and radioactive waste management*, Vol. 8, No. 3, pp. 166-172.

Environment technology centre. (2001). Environment Canada oil properties database, BRENT blend crude oil. www.etc.cte.ec.gc.ca/databases/ OilProperties/Default.aspx

Edwards, K. R. Lepo, J. E. and Lewis, M. A. (2003). "Toxicity comparison of biosurfactants and synthetic surfactants used in oil spill remediation to two estuarine species." *Marine Pollution Bulletin*, Vol. 46, pp. 1309-1316.

Etkin, D. S. (1999). "Historical review of oil spills from all sources." Proc., 1999 *Proc., 1999 Oil Spill Conf.,* American Petroleum Institute, Washington, DC, API pub. 4686B, pp. 1097-1102.

Fingas, M. F., Dufort, V. M., Hughes, K. A., Bobra, M. A., and Duggan, L. V. (1989). "Laboratory studies on oil spill dispersants." *Oil spill Dispersants: New Eccological Approaches, ASTM STP 1018*, L. M. Flaherty, ed., American Society for Testing and Materials, Philadelphia, pp. 207-219.

Fingas, M. F., Fieldhouse, B., Wang, Z., Sigouin, L., Landriault, M. and Mullin, J. V. (1999). " Analytical procedures for dispersant effectiveness testing." *Proc., 22nd*

Annual Arctic and Marine Oil Spill Program Technical Seminar, Environment Canada, Vancouver, pp. 231-241.

Fingas, M. F., Huang, E., Fieldhouse, B., Wang, L. and Mullin, J. V. (1996). " The effect of energy, settling time and shaking time on the swirling flask dispersant apparatus." *Spill Science and Technology Bulletin,* Vol. 3, No. 4, pp. 193-194.

Fiocco, R. J. and Lewis, A. (1999). " Oil spill dispersant." *Pure Appl. Chem.*, Vol. 71, No. 1, pp. 27-42.

Harris, B.C., Bonner, J.S., McDonald, T.J., Fuller, C. B., Page, C. A., Dimitriou-Christidis, P., Sterling, M.C. and Autenrieth, R.L. (2002). " Bioavailability of chemically dispersed oil." *Proc., 25th Arctic and Marine Oil Spill Program Technical Seminar,* Environment Canada, Ottawa, pp. 895-905.

Harvey, S., Elashi, 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.

Holakoo, L. (2002)."On the capability of rhamnolipids." MASc. Thesis, Concordia University, Montreal.

Hommel, R.K. and Ratledge, C. (1993). *Biosynthetic mechanisms of low molecular weight surfactants and their precursor molecules; in Biosurfactants, production, properties and applications*, pp. 3-64, Marcel Dekker, New York.

Hutchins, S.R. (1997). "Effects of microcosm preparation on rates of toluene biodegradation under denitrifying conditions." Journal of Industrial Microbiology & Biotechnology, Vol. 18, pp. 170-176.

Ikechukwu N.E. (2004). "Technical communication: modelling the rates of biodegradation, nitrogen fixation and uptake of fixed nitrogen during bioremediation of crude oil-contaminated environments." World Journal of Microbiology &

Biotechnology, Vol. 20, pp. 833-836

Jeneil Biosurfactant Co. (2002). *JBR 425 product data sheet*, Revision 4, Saukville, WI. http://www.biosurfactant.com

Karapanagioti, H.K., Gossard, C.M., Strevett, K. A., Kolar, R.L. and Sabatini D.A. (2001). "Model coupling intra particle diffusion sorption, nonlinear sorption, and biodegradation processes." Journal of Contaminant Hydrology, Vol. 48, pp. 1-21.

Lang, S. and Wagneer, F. (1987). *Structure and properties of biosurfactants; in Biosurfactants and Biotechnology,* pp. 21-45, New York, Marcel Dekker.

Lewis, A. and Aurand, D. (1997). "Putting dispersants to work: overcoming obstacles". *Proc., 1997 Oil Spill Conf.,* American Petroleum Institute, Washington, DC, publication No. 4620.

Lin, S. (1996). "Biosurfactants: Recent reviews." *J. Chem. Tech. Biotechnol.,* Vol. 66, pp. 109-120.

Mackay, D. (1995). "Effectiveness of chemical dispersants under breaking wave conditions." The use of chemicals in oil spill response. ASTM STP 1252, American Society for Testing and Materials, Philadelphia, Pennsylvania.

Macnaughton, S. J., Swannell, R., Daniel, F. and Bristow L. (2003). " Biodegradation of dispersed Forties crude and Alaskan North Slope oils in microcosms under simulated marine conditions" *Spill Science and Technology Bulletin*, Vol. 8, No. 2, pp. 179-186.

Matsfuji, M. Nakata, K. and Yoshimoto, A. (1997). "High production of rhamnolipids by *pseudomonas aeruginosa* growing on ethanol." *Biotechnology letters*, Vol. 19, No. 12, pp. 1213-1215.

Miller, R. M. and Bartha, R. (1989). Evidence from liposome encapsulation for transport-limited microbial metabolism of solid alkanes. *Appl. Environ. Microbiol.*, Vol. 55, pp. 269-274.

Moles, A.M., Holland, L. and Short, J. (2002). "Effectiveness in the laboratory of Corexit

9527 and 9500 in dispersing fresh, weathered and emulsion of Alaska North Slope crude oil under subarctic conditions." *Spill Science and Technology Bulletin*, Vol. 7, Nos. 5-6, pp. 241-247.

Mulligan, C.N. and Eftekhari, F. (2003). "Remediation with surfactant foam of PCP-contaminated soil." *Engineering Geology*, Vol. 2179, pp. 1-11.

Mulligan, C.N. and Gibbs, B.F. (1993). " Factors influencing the economics of biosurfactants; *in Biosurfactants, Production, Properties, Applications*, ed. N Kosaric Inew York: Marcel Dekker*)*, pp. 329-371.

Mulligan, C.N. and Gibbs, B.F. (2004). "Types, production and applications of biosurfactants."(2004). *Proc. Indian natn. Sci. Acad.*, B70, No. 1, pp. 31-55.

Myers, D. (1999). *Surfaces, interfaces and colloids. Principles and applications*, 2nd Ed., Wiley, New York.

National Research Council (NRC). (2002). *Oil in the sea III: Inputs, Fates and Effects,* National Academy of Sciences, Washington DC.

Nelson, E. C., Walter, M. V., Bossert, I. D., and Martin D. G. (1996). "Enhancing biodegradation of petroleum hydrocarbons with guanidinium fatty acids." *Environ. Sci. Technol.*, Vol. 30, pp. 2406-2411.

Oberbremer, A., Muller-Hurting, R. amd Wagner, F. (1990). Effect of the addition of microbial surfactants on hydrocarbon degradation in a soil population in a stirred reactor." *Appl. Microbiol. Biotechnol.,* Vol. 32, pp. 485-489.

Ordzie C. J. and Garofalo G.C. (1981). "Lethal and sublethal effects of short-term acute doses of Kuwait crude oil and a dispersant Corexit 9527 on bay scallops Argopecten irradiations and twp predators at different temperatures. *Mar. Environ. Res.*, Vol. 5, No. 3, pp. 195-210.

Page, C. A., Bonner, J. S, MacDonald, T. J. and Autenrieth, R. L. (2002). " Behaviour of chemically dispersed oil in a wetland environment. *Water Research*, Vol. 34, 2507-2516.

Prince, R. C., Lessard, R. R. and Clark, J. R. (2003). " Bioremediation of marine oil spills." *Oil and Gas Science and Technology-Rev.IFP*, Vol. 58, No. 4, pp. 463-468.

Rahman, K.S.M., Rahman, T. J., Kourkoutas, Y., Petsas, I.., Marchant, R. and Banat, I.M., (2003). "Enhanced bioremediation of n—alkane in petroleum sludge using bacterial consortium amended with rhamnolipid and micronutrients." Bioresource Technology, Vol. 90, pp. 159-168.

Schramm, L. L. (2000). *Surfactants: Fundamental and applications in petroleum industry*, American Chemical Society, Washington, DC.

SMW (Standard methods for the Examination of Water and Wastewater). (2000). "9215 B. Heterotrophic plate count using pour plate method, pp. 9-55 to 9-61." 17th Ed., American Public Health Association, Washington, DC.

Sorial, A., Venosa, A. D., Koran, K. M., Holder, E. and King, D.K. (2004). "Oil spill dispersant effectiveness protocol. I: Impact of operational variables." Journal of Environmental Engineering, Vol. 130, No. 10, pp 1073-1084.

Sorial, A., Venosa, A. D., Koran, K. M., Holder, E. and King, D.K. (2004). "Oil spill dispersant effectiveness protocol. II: Performance of revised protocol." *Journal of Environmental Engineering*, Vol. 130, No. 10, pp 1085-1093.

Swannell, R. P. J. and Daniel, F. (1999). " Effect of dispersants on oil biodegradation under simulated marine conditions." *Proc., 1999 Oil Spill Conf.,* American Petroleum Institute, Washington, DC, publication No. 212 (pp. 169-176).

Tiehm, A. (1994). " Degradation of Polycyclic aromatic hydrocarbons in the presence of synthetic surfactants. *Appl. Environ. Microbiol.*, Vol. 60, pp. 258-263.

USEPA (US Environmental Protection Agency). (1995). "Technical product bulletin #D-4." *USEPA, oil program center*, revised December 18, 1995. http://www.epa.gov/oilspill/ncp/corex950.htm

USEPA (US Environmental Protection Agency). (1996a). "Bioremediation agent

effectiveness test." *Title 40 code of federal regulations, Pt. 300, Appendix C*, Naragansett, R.I..

USEPA (US Environmental Protection Agency). (1996b). "Swirling flask dispersant effectiveness test." In : *Title 40 code of federal regulations, Pt. 300, Appendix C*, Naragansett, R.I., pp. 245-250.

Van Hamme, J. D. and Ward, O. P. (1999). "Influence of chemical surfactants on the biodegradation of crude oil by a mixed bacterial culture" *Can. J. Microbiol.*, vv. 45, pp., 130-137.

Varadaraj, R., Robbins, M. L., Bock, J., pace, S., and MacDonald, D. (1995). " Dispersion and biodegradation of oil spills on water". *Proc., 1995 Oil Spill Conf.*, American Petroleum Institute, Washington, D.C., pp. 101-106.

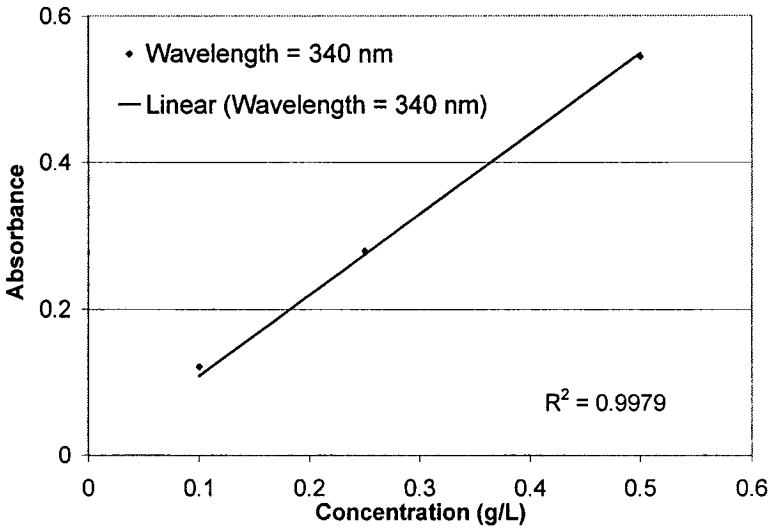
Venosa, A. D. and Zhu, X. (2003). "Biodegradation of crude oil contaminating marine shorelines and freshwater wetlands." *Spill Science and Technology Bulletin*, Vol. 8, No. 2, pp. 163-178.

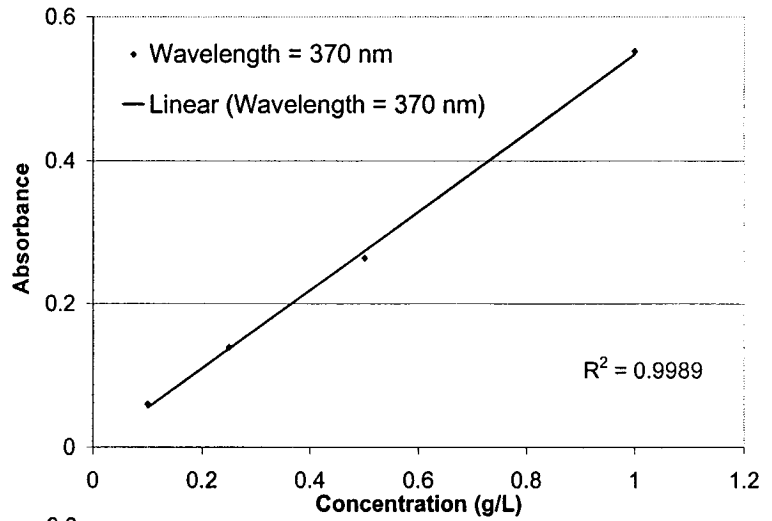
Vipulanandan, C. and Ren, X. (2000). "Enhanced solubility and biodegradation of naphthalene with biosurfactant." *Journal of Environmental Engineering*, Vol. 126, No. 7, pp. 629-633.

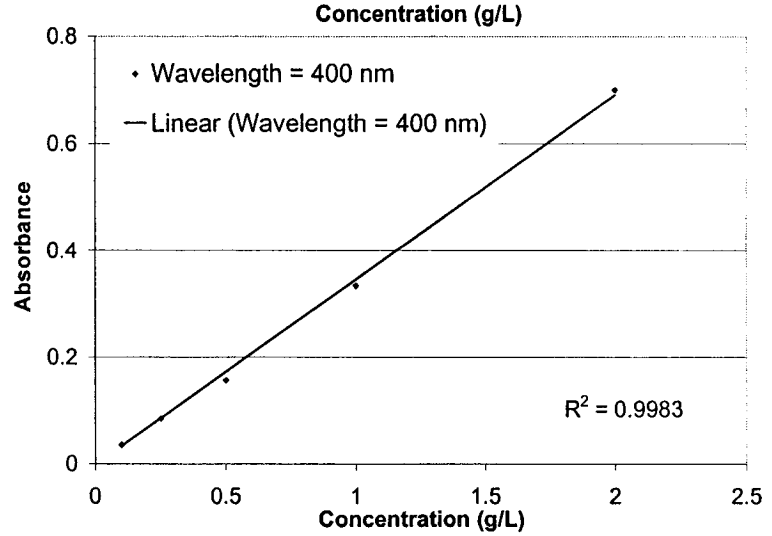
Zhang, Y. and Miller, R. A. (1992). " Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid biosurfactant." Appl. Environ. Microbiol., Vol. 58, pp. 3276-3282.

7 Appendices

Appendix A. An example spectrophotometer calibration curve



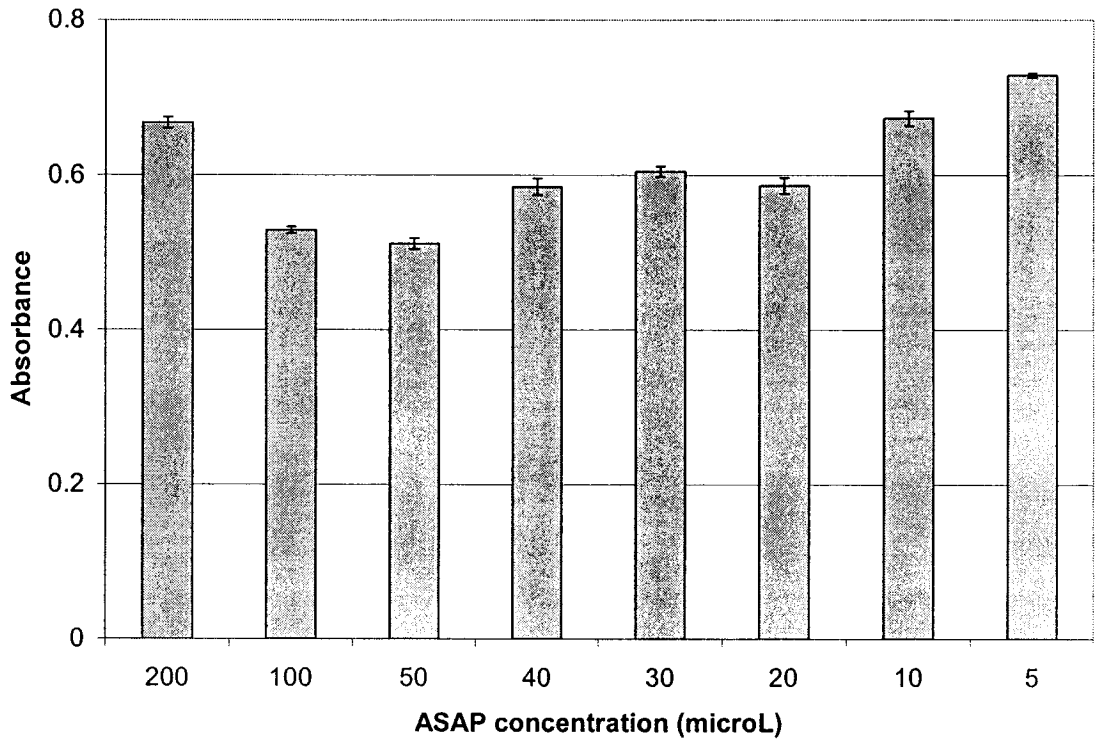




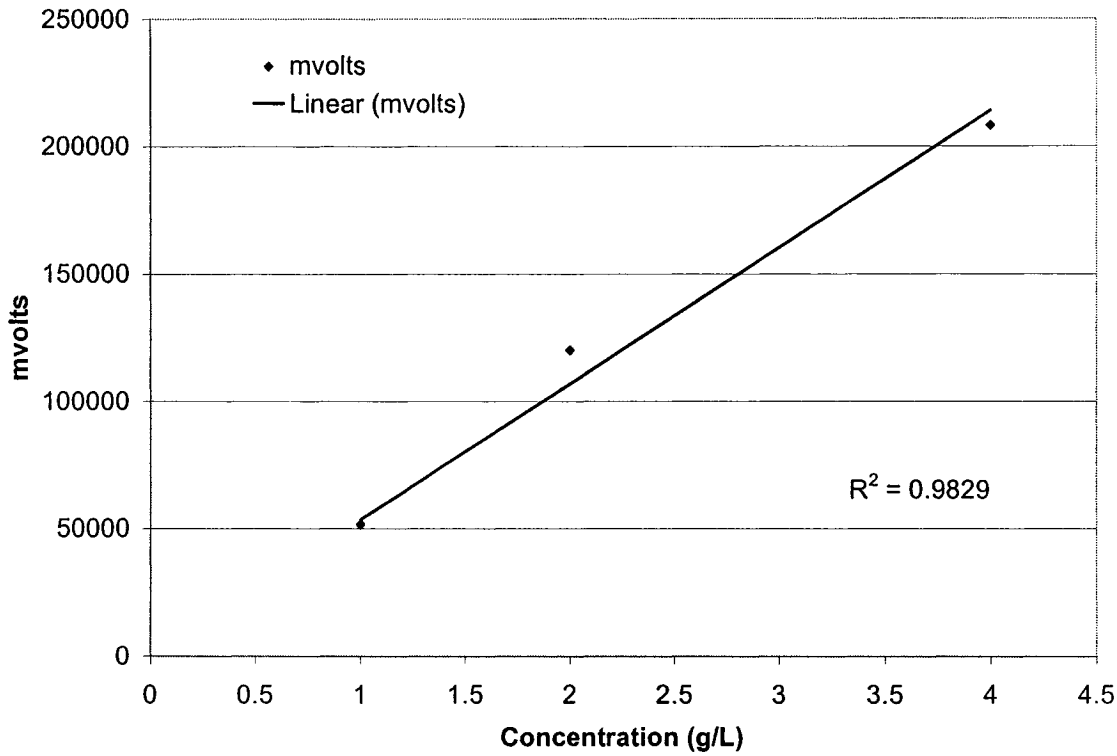
Appendix B. Matlab function used for ANOVA analysis

```
clc;
load filename.txt;
        %//** this is to load file to matlab environment**//

filename;

[p,tbl,stats] = anova1(filename);
        % //** this to run the one way anova**//

[c,m] = multcompare(stats)
        % //** this is for multiple mean comparison**//
```

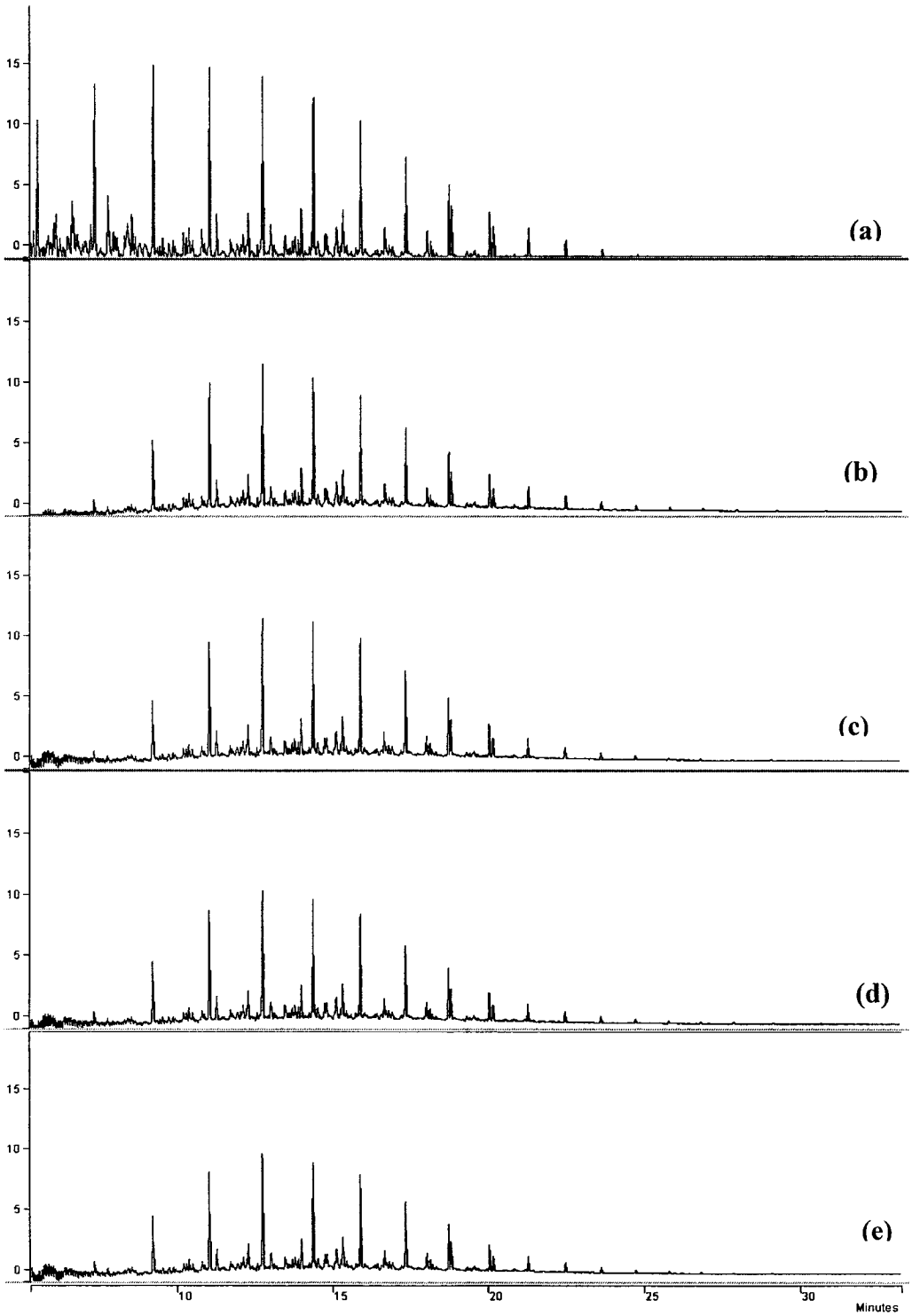
Appendix C. Effect of ASAP to oil ratio on crude oil biodegradation: A preliminary experimental result



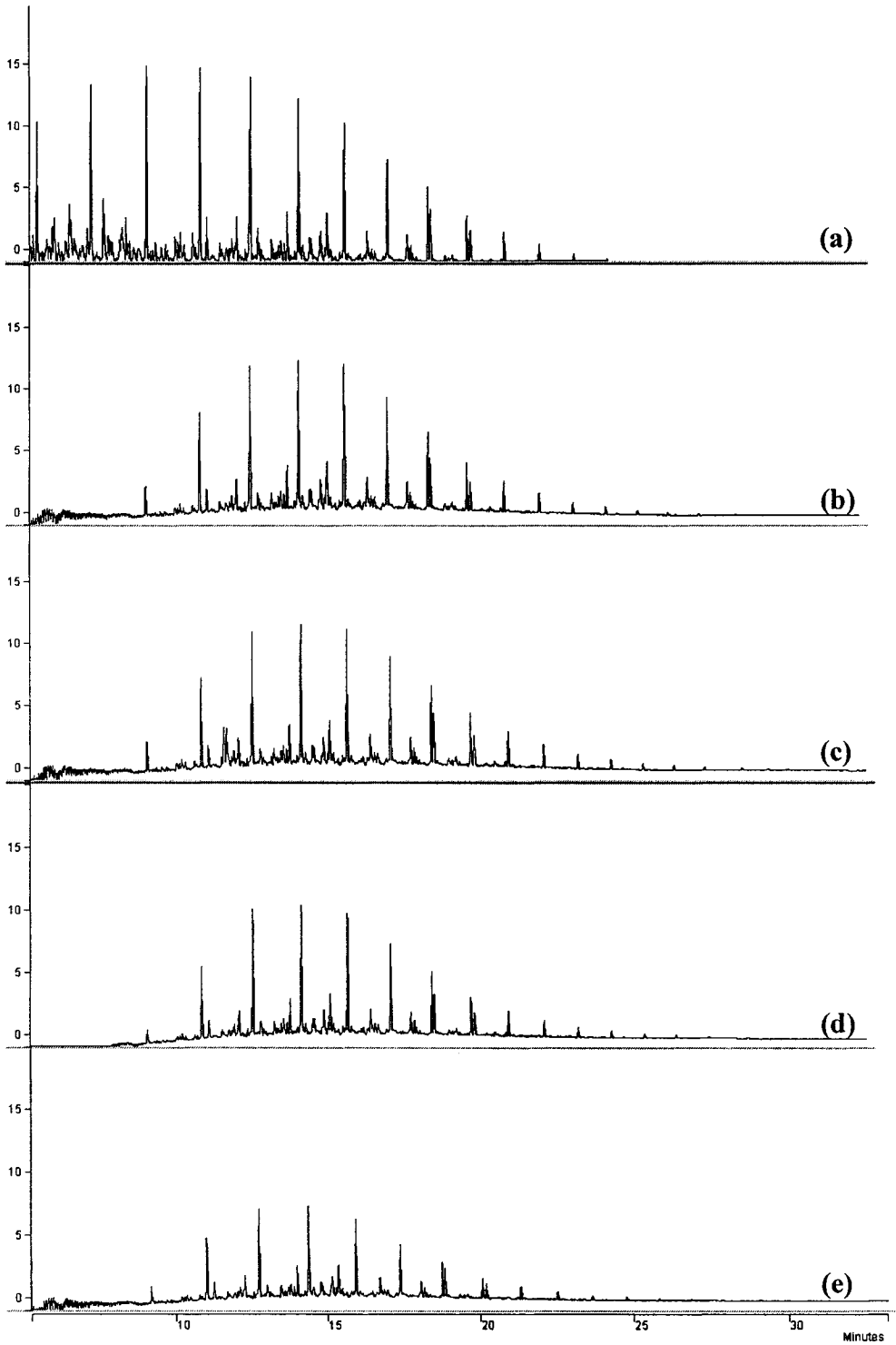
Appendix D. An example GC calibration curve



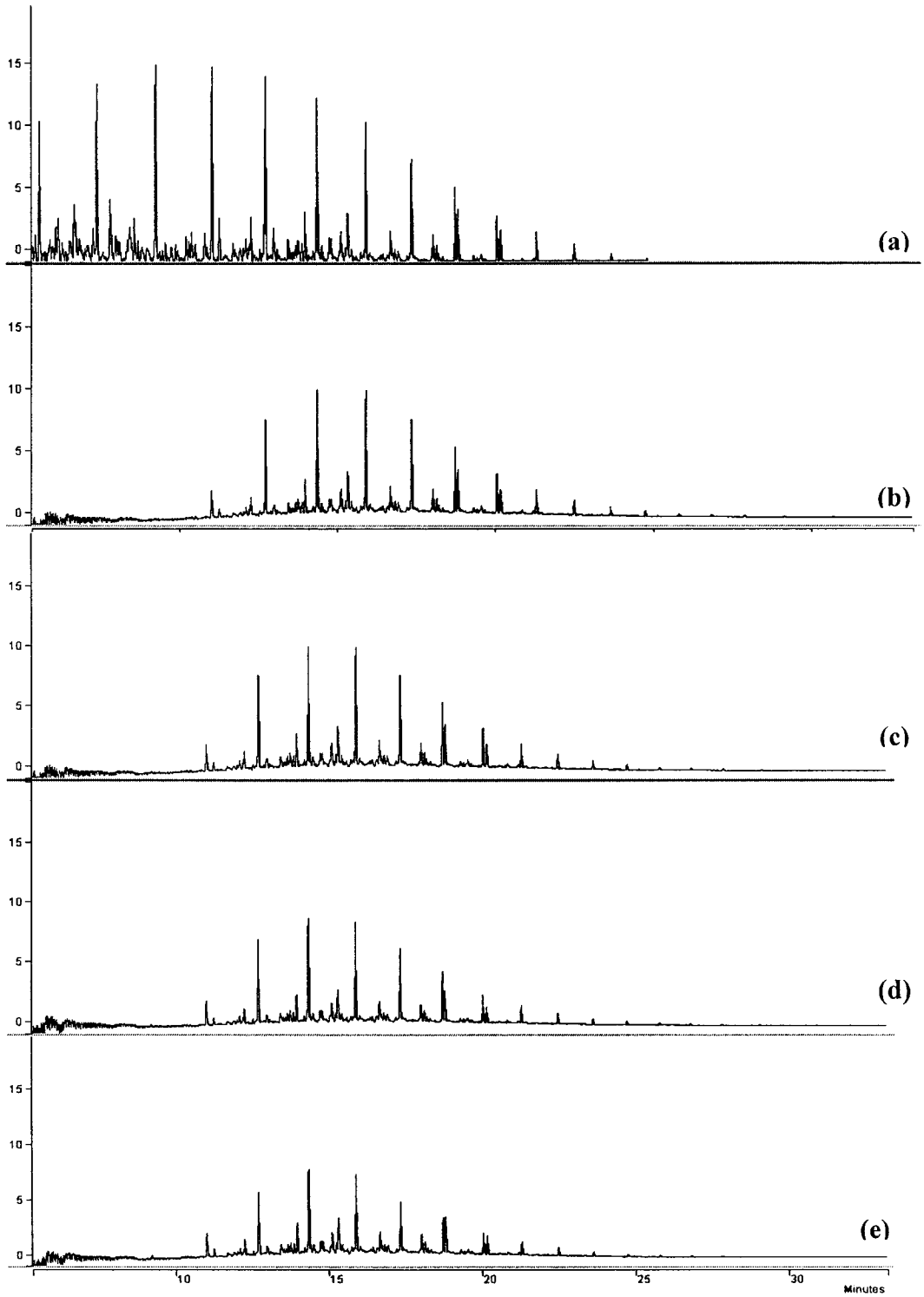
Appendix E.1 Chromatograms of weathered BRENT crude oil and material recovered from 3 days old flask batch reactors: (a) original oil sample, (b) oil only control, (c) Corexit 9500, (d) bio-dispersant (1:4) & (e) bio-dispersant (1:4) with Degreaser (1:4)



Appendix E.2 Chromatograms of weathered BRENT crude oil and material recovered from 7 days old flask batch reactors: (a) original oil sample, (b) oil only control, (c) Corexit 9500, (d) bio-dispersant (1:4) & (e) bio-dispersant (1:4) with Degreaser (1:4)



Appendix E.3 Chromatograms of weathered BRENT crude oil and material recovered from 14 days old flask batch reactors: (a) original oil sample, (b) oil only control, (c) Corexit 9500, (d) bio-dispersant (1:4) & (e) bio-dispersant (1:4) with Degreaser (1:4)



Appendix E.4 Chromatograms of (a) Fresh oil (b) weathered oil (c) Corexit 9500, (d) bio-dispersant, (e) Degreaser and (f) bio-dispersant