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The effect of the dispersant Corexit 9500 on the biodegradation of North Sea crude oil at low temperatures

Mona Ulas*

Faculty of Science and Technology, Department of Mathematics and Natural Science
Master of Science Degree Program, Water Science and Technology
University of Stavanger, Norway

*Contact information:
Telephone: +47 986 46 768
E.mail: mona.ulas@gmail.com

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Abstract

Constituents from crude oil and petroleum are major sources of marine pollution and despite the natural presence of crude oil hydrocarbons in the marine environment; anthropogenic activity is a major contribution to the total release of hydrocarbons to the oceans. The removal of hydrocarbon pollution is based on the natural weathering mechanisms, specifically biodegradation and its enhancement. Chemical dispersants have been developed that serve to disperse spilled oil more rapidly and extensively into the water column as tiny oil droplets, increasing the surface area available for microbial biodegradation. This study was conducted to investigate the effect of temperature on biodegradation, as well as the effect of the chemical dispersant Corexit 9500 on the enhanced biodegradation of crude oil from the Norwegian oil field, Ekofisk. The biodegradation of the dispersant alone was also investigated. Both chemical and microbiological methods were used to analyse the effects on biodegradation at 3, 8 and 15°C. BOD analysis showed increased biodegradation rates with increasing temperature. Total hydrocarbon analysis via GC-FID revealed that between 82 and 95% of hydrocarbons in the size range between decane and tetracosane were degraded over a period of 46 days. The addition of Corexit showed an increase of hydrocarbon removal of 2% at 8 and 15°C, and 10% increased removal at 3°C. Molecular analysis revealed changes in the microbial community of samples containing crude oil. All samples, including blank samples showed a shift in the microbial community from the original community found in the source seawater, over the biodegradation period of 46 days. Based on the results of this study it was concluded that the addition of Corexit 9500 had little or insignificant effects on the rate of biodegradation of North Sea crude oil in seawater at all temperatures. Increased biodegradation rates were however clearly observed as temperature increased. Further research is recommended to better understand the effects of chemical dispersants on biodegradation in low temperature marine environments and to develop more successful methods for the remediation of hydrocarbon contamination.

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Abbreviations

BOD: Biological oxygen demand

GC: Gas chromatography

FID: Flame ionization detector

MS: Mass spectrometry

HC: Hydrocarbon

MPN: Most probable number

NAPL: Non-aqueous phase liquid

THC: Total hydrocarbons

PCR: Polymerase chain reaction

rRNA: Ribosomal ribonucleic acid

DNA: Deoxyribonucleic acid

IRIS: International research institute of Stavanger

DGGE: Denaturing gradient gel electrophoresis

1 Introduction

Hydrocarbons (HCs) are organic compounds consisting of a carbon skeleton with covalently bound hydrogens. Crude oil (naturally occurring raw oil) or petroleum (crude oil and its refined products) generally consists of hydrocarbons, asphaltenes and resins, paraffins (saturated HCs, or alkanes), sulphur and ash (Simanzhenkov & Idem, 2003). Hydrocarbons from crude oil are a major source of marine pollution (Deppe et al., 2005). There is a natural presence of crude oil HCs in the marine environment as a result of natural processes (Widdel & Rabus, 2001). However, anthropogenic activities increase the amount of HCs accumulating in the oceans, and accidental release can lead to acute toxic and even long-term effects. Among the anthropogenic activities contributing to the accumulation of petroleum products in the sea are industrial and municipal runoffs, effluent release, offshore petroleum activity such as petroleum transport and production, and accidental releases as a result of tanker accidents (Deppe et al., 2005). Nearly half of all crude oil produced worldwide is transported by sea (Harayama et al., 1999), and petroleum derived from tanker accidents are believed to account for 10-15% of the oil released into the environment each year (Kennicutt et al., 1991). Spills from tanker and production accidents are of special concern due to the resulting high local hydrocarbon concentrations at the spill site and locations (e.g. shorelines) in which the oil may reach (National Reasearch Council, 2003).

The marine environment is considered the ultimate and largest recipient of hydrocarbon pollution (Atlas, 1981), and thus attention to solve and combat the pollution problem is of high importance. The environmental threat caused by hydrocarbon pollution in the marine environment is currently severe and numerous environmental consequences following petroleum discharges and spills have been documented (Malins, 1977; National Reasearch Council, 2003; Walker, 2006). Release of petroleum into the marine environment can cause harm in various ways, as spilled oil may reach shorelines, affecting wildlife externally by preventing normal physical functions required for survival (e.g. a bird covered in oil) or preventing the use of a habitat. Biological uptake of hydrocarbon pollutants can cause severe disturbances in metabolic reactions, tissue, genetic material and hormone balance in

different species, depending on the type of pollutant, environmental conditions and the recipient species and its route of uptake (Lee & Page, 1997; Walker, 2006). Disturbances in biological reactions in individual organisms can lead to secondary effects such as behavioural changes, reproductive failure, movement impairment, retardation of growth and development, and ultimately death. These effects can further lead to changes in a species population or a community, and in the worst case, cause changes to an entire ecosystem. As widespread toxicological effects of petroleum pollution may threaten numerous levels of biological organization, it is critical to gain knowledge about the fate of hydrocarbons within the marine environment in order to control and combat the pollution.

When oil reaches the marine environment, it is subject to several physical, chemical and biological processes, such as evaporation, dissolution, emulsification, photo-oxidation, biodegradation, which naturally degrades the hydrocarbon components of the oil (Atlas, 1981; Brakstad & Bonaunet, 2006; Díez et al., 2007). Weathering processes are highly dependant on environmental factors such as temperature, ocean currents and weather conditions. Biodegradation is considered to be the major natural weathering mechanism for removing hydrocarbon components from the marine environment (Alexander, 1999; Atlas, 1995; Lindstrom & Braddock, 2002). Hydrocarbon biodegradation involves the uptake and utilization of organic chemicals (substrates) by hydrocarbon degrading microorganisms (Alexander, 1999). This is a complex process, which is highly influenced by factors such as hydrocarbon properties, concentration and availability, the environment (e.g. temperature) and the microbial community (Leahy & Cowell, 1990). To accelerate the biodegradation rates, and thus enhance oil spill removal, methods to overcome certain limiting factors have been used (discussed in section 2.5). Chemically enhanced dispersing of oil is such a method. Dispersants are chemicals generally composed of solvents, surfactants and other additives and are used to enhance dispersion of oil in water (National Research Council Committee, 2005). A chemical dispersant can accelerate weathering processes such as biodegradation, by making substrates in crude oil more available for microbial uptake (Swannell & Daniel, 1999). Understanding the mechanisms and processes of microbial degradation is important to develop efficient chemicals that positively affect these processes. Several dispersants are in large-scale use throughout the world. Studies done to investigate the effect of chemically dispersed oil on biodegradation have shown varying

results (Lindstrom & Braddock, 2002; Mulkin-Phillips & Stewart, 1974; Prince et al., 2013; Swannell & Daniel, 1999). Therefore, further research on chemical dispersants is valuable. Research on the biodegradability of dispersants alone is equally important to prevent the release of potentially reluctant chemicals to the environment.

Research on biodegradation in cold seawater is becoming increasingly important, due to increased interest for oil exploration in the arctic areas. More and more biodegradation studies are focused around cold seawater (Brakstad & Bonaunet, 2006; Lindstrom & Braddock, 2002; Whyte et al., 1998), but still the majority of research found on biodegradation have been studied at higher temperatures (Delille et al., 2009). Persistent pollutants are driven towards the north and south poles due to wind and water currents, and organic pollutants are typically more persistent in cold environments (Walker, 2006). Petroleum pollution has been identified as the largest environmental threat in the Antarctic region (Snape et al., 2001). Research and development of suitable bioremediation methods are therefore crucial in order to counter and prevent environmental harm in this fragile region.

The main research hypotheses to be investigated are whether or not biodegradation rates increases with increasing temperatures, increases in the presence of the dispersant Corexit 9500, and if the microbial community changes with substrate addition.

2 Background

Biodegradation of petroleum hydrocarbons in seawater are dependant on several complex and interrelated factors (Atlas, 1981, 1995). Understanding the natural process of biodegradation and the fate of petroleum hydrocarbons is vital in order to positively influence the rate of biodegradation as a bioremediation method. With chemical dispersants already in use on large scale, it is now important to document the effects of these chemicals, both on oil, the environment and on biodegradation. This background starts with a short introduction to the fate of oil in the environment, followed by an overview of the biodegradation of petroleum hydrocarbons in seawater. Various factors affecting biodegradation are described. Next, strategies of stimulating natural biodegradation are introduced and discussed, followed by background information on chemical dispersants properties and their effects on spilled oil and on biodegradation. The chapter is concluded with a brief description of the chemical and microbiological methods used in this study.

2.1 Fate of spilled oil and effects on the environment

Once oil reaches the marine environment, it is subject to numerous physical and biological processes, which affects the ultimate fate of oil. These processes are shown in figure 1. However they do not have equal impact. Some occur at an early stage following oil spill, while others occur later. Research done after the Exxon Valdes oil spill (Wolfe et al., 1994) monitored the effect of each of these processes over time on the spilled oil (Figure 2).

Figure 2 shows the long-term importance of the process of biodegradation, in relation to oil spills. Ultimately, biodegradation is one of the main processes involved in the conversion of oil products into less harmful compounds and removal pollutants from the marine environment by mineralization. Evaporation can only remove lightweight hydrocarbons from the oil, and photolysis only occur on oil exposed to sunlight and thus has limited effect.

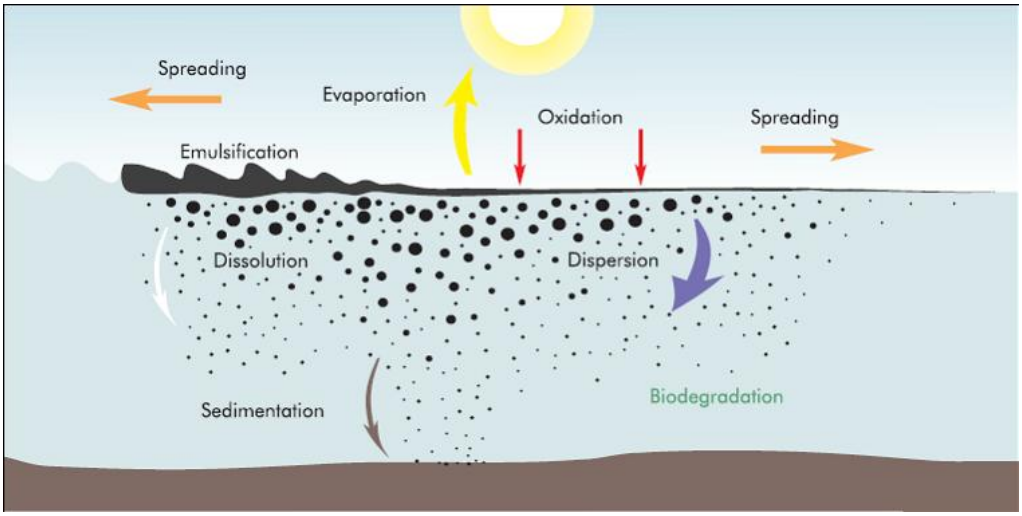

Figure 1: Fate of spilled oil in the marine environment.

Source: http://bushehrport.pmo.ir/en/maritimeenvironment/coastalmarine

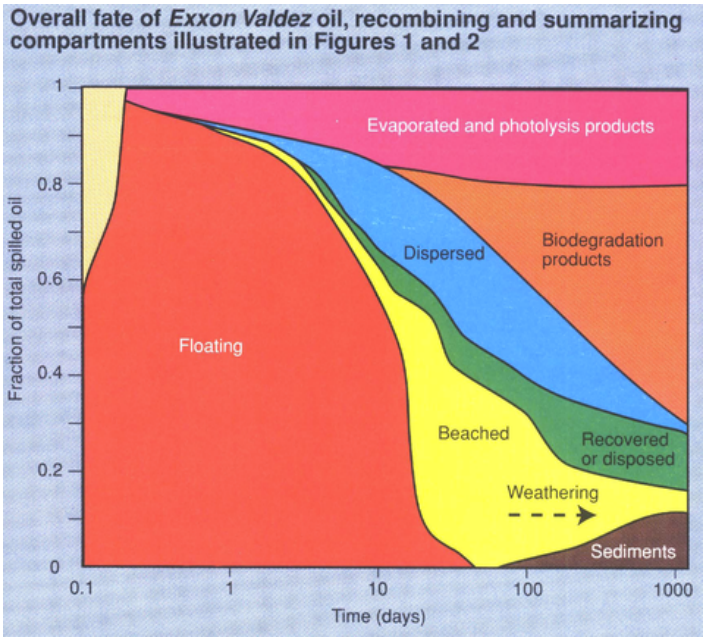

Figure 2: Overall fate of *Exxon Valdez* oil. (Wolfe et al., 1994).

Cold temperature regions can be more sensitive to oil spills. Pollutants tend to accumulate and degrade much slower due to the cold temperatures. Climate also drives environmental persistent pollutants towards the north and south poles due to global wind and water currents (Walker, 2006).

2.2 Biodegradation

Biodegradation is the process by which microorganisms utilize and transform organic chemicals into simpler non-hazardous substances through metabolic pathways (Margesin & Schinner, 2001). The process is of great importance in the attenuation of spilled petroleum hydrocarbons from the aquatic environment. After the 1989 Exxon Valdez oil spill, an estimated 50% of the spilled oil was biodegraded either in the water column or in the sediments (Wolfe et al., 1994). The rate of biodegradation is dependant on a number of factors. External factors (e.g. temperature) contribute to alterations of how available hydrocarbon substrates are for biological uptake as a result of external environmental factors. Internal factors depend on the degrading population or microorganism properties, including enzymatic and metabolic limitations, degrading mechanisms and population identity and size.

2.3 Chemical and physical factors affecting crude oil hydrocarbon biodegradation

The fate of hydrocarbons in crude oil is affected by several factors. All of which should be understood in order to predict and influence the fate of hydrocarbons pollutants in the marine environment. It is often assumed that bacterial degradation of petroleum hydrocarbons occurs on the dissolved HC fractions via Monod kinetics (Appendix A). However certain studies have brought the need for alternative explanations. Microbial biodegradation is nevertheless affected by numerous factors, including HC solubility, equilibrium partitioning, HC molecular size and the available surface area of oil.

2.3.1 Chemical Composition

Biodegradation varies according to the chemical composition and concentration of hydrocarbons in the aqueous phase. Crude oil is a complex mixture of hydrocarbons, which degrade at different rates depending on their chemical structure (Leahy & Cowell, 1990). Typically, biodegradation rate increases with decreasing molecular weight and chemical structure complexity of the hydrocarbon, and degrading communities preferentially degrades less complex compounds first (Atlas, 1995; Deppe et al., 2005; Whyte et al., 1998).

2.3.2 Concentration

Concentration of hydrocarbons in the aqueous phase will influence the rate of microbial uptake of organic compounds. The rate of mineralization is directly proportional to the substrate concentration (Leahy & Cowell, 1990), assuming that growth occurs on the dissolved hydrocarbon fractions in the water phase via Monod microbial growth kinetics (Appendix A). Hence, the microbial mineralization of crude oil is dependant on factors such as hydrocarbon solubility, equilibrium partitioning and the total dissolved hydrocarbon concentration in the aqueous phase.

2.3.3 Physical bioavailability

The physical state of oil affects the bioavailability of the organic substrates within the oil. Bioavailability is a term used to describe whether or not a compound is readily accessible for microbial uptake. The total surface area of the oil can largely affect the bioavailability, as the surface area is connected to the total mass transfer between the oil and water phase. The mass transfer rate between two phases can be explained by Eq. 1 (Boyadjiev, 2011):

$$\frac{dC}{dt} = \frac{D \cdot A}{L \cdot V} \cdot (C - C_0) \qquad \text{(Eq. 1)}$$

Where dC/dt is the mass transfer rate of a certain component from oil to water, C is the concentration [g/m^3] of the specific compound in the phase to which the component will travel (water phase), and C_0 is the concentration [g/m^3] in the oil phase. D is the molecular diffusion coefficient [m^2/s] of the hydrocarbon in the oil, A is the total surface area [m^2] of the oil, L is the thickness of the liquid boundary layer [m] between the oil and water, and V is the total volume of the oil [m^3]. The liquid boundary layer is a term from the Two Film Theory (Lewis – Whitman). The theory describes mass transfer between two phases using two films assumed to exist at the surface of each phase through which components must travel through and could be limited by. In this case the oil and water film. The only factor that is subject to change after an amount of oil reaches water is the surface area of the oil and is also therefore the only factor available for manipulation. According to Eq. 1, a larger surface area allows for a higher rate of mass transfer of components in the oil to the water

phase, leading to a potentially greater concentration of substrates available for microbial uptake in the water phase. Furthermore, it is assumed that a larger surface area allows for greater surface space available for microbial attachment, and thus allows for an increased rate of biodegradation. Increased surface area of oil can occur naturally in high-energy aquatic environments where sheer force of waves and currents cause the oil to disperse and emulsify in the water column. A larger amount of hydrocarbons in the oil is exposed at the oil water interphase, thus increasing their bioavailability (Leahy & Cowell, 1990).

2.3.4 Temperature

Temperature has in general an effect on the rate of chemical reactions, where higher temperature increases rates, and lower temperatures slows or retards chemical reactions. Consequently, biodegradation rates, which are dependant on metabolic reactions generally decreases with decreasing temperatures (Leahy & Cowell, 1990). This is consistent with the Arrhenius equation (Appendix A) that predicts exponentially decreased reaction rates as temperature decreases. As temperatures reach close to, and below 0°C, many hydrocarbons form crystal structures (Aislabie et al., 2006; Margesin & Schinner, 2001; Whyte et al., 1998), making the hydrocarbons less, if at all, available for microbial mineralization. Temperature can also affect the physical state and weathering of oil, the dissolution and bioavailability of hydrophobic oil fractions, resulting in lower rates of hydrocarbon utilization (Brakstad & Bonaunet, 2006; Margesin & Schinner, 2001). Oil viscosity increases and the volatility of hydrocarbons decreases with decreasing temperatures, both of which lowers the bioavailability of hydrocarbons in the oil (Margesin & Schinner, 2001; Whyte et al., 1998). Some studies show a correlation between decreased temperatures and decreased microbial degradation rates of hydrocarbons (Brakstad & Bonaunet, 2006; Deppe et al., 2005; Margesin & Schinner, 2001; Whyte et al., 1998), but DeLille et al. found that temperature did not have a significant effect on the degradation rates of crude oil in seawater (2009). Evidence indicates however, that regardless of the rate of mineralization, the final extent of mineralization does not seem to decrease with decreasing temperatures (Delille et al., 2009; Mohn & Stewart, 2000; Prince et al., 2013). The important factor to understand and influence is the rate of biodegradation. Having organic pollutants in an environment over longer periods of time as opposed to a short time, risks increased

environmental impact. Further studies on biodegradation rates will provide an answer to the conflicting results seen with regards to temperature effects.

2.3.5 Nutrient availability

Nutrient and oxygen availability affects the microbial degradation of hydrocarbons. Biodegradation rates have shown to be primarily limited by the availability of inorganic nutrients in low temperature marine environments (Atlas, 1995; Delille et al., 2009; Leahy & Cowell, 1990; Margesin & Schinner, 2001). Studies indicate that biodegradation of crude oil is enhanced in nutrient rich water (Rosenberg et al., 1993). Nutrient supplementation can thus be used as a tool to increase biodegradation rates in aquatic systems. Other factors such as oxygen availability, salinity, pH, pressure etc. also have effects on biodegradation but will not be addressed in this report.

2.4 Biological factors affecting crude oil hydrocarbon biodegradation

Biodegradation of crude oil constituents also depends on a variety of biological factors. The composition and biological nature of microbial populations affect the environmental recalcitrance of petroleum hydrocarbons (Leahy & Cowell, 1990). Factors such as the type of biodegrading populations, mechanisms of hydrocarbon utilization and adaptation are all important for understanding the biodegradation process.

2.4.1 Hydrocarbon degrading microorganisms

Hydrocarbon biodegradation in aquatic and soil environments is performed by a diverse populations of microorganisms, but it is generally bacteria which are primarily responsible for the degradation (Atlas, 1995; Leahy & Cowell, 1990). Fungi can also facilitate biodegradation, and can be very successful hydrocarbon degraders (George-Okafor et al., 2009; Leahy & Cowell, 1990). Hydrocarbon degrading microorganisms have the ability to adapt to ambient conditions, such as temperature. Hydrocarbon degraders which have adapted to a cold aquatic environment include psychrophilic or psychrotrophic microorganisms with potential growth temperature in the range of 0 to 20°C and 0 to 35°C, respectively (Margesin & Schinner, 2001; Whyte et al., 1998). In the Arctic and Antarctic

regions, it is typically the psychrotrophic populations who are the main contributors to biodegradation (Aislabie et al., 2006; Margesin & Schinner, 2001; Mohn & Stewart, 2000). These psychrotrophic species have advantages in their ability to adapt to cold environments, enhancing biodegradation (Whyte et al., 1998).

2.4.2 Mechanisms of growth

Microorganisms are considered to be present everywhere in the marine environments (Deppe et al., 2005). Hydrocarbon degrading species are present in variable amounts, but quantities are generally adequate for biodegradation (Delille et al., 2009; Leahy & Cowell, 1990), and local concentrations of HC-degrading microorganisms are thought to increase rapidly following oil spills.

The details around the initial phase of bacterial uptake of hydrophobic substrates in non-aqueous phase liquids (NAPLs) are somewhat unknown. Uptake mechanisms differ according to different species of hydrocarbon degraders. Three general theories exist, focusing on how the substrate is initially transported from the environment and to the cell wall, from where it is taken through the cell membrane and used further in metabolic processes (Alexander, 1999). The three theories are:

1. The microorganism may utilize substrates that are dissolved in the water phase. Microorganisms using this mechanism will be limited by the spontaneous partitioning of hydrocarbons into the water phase. Studies showing a correlation between increased growth and increasing hydrocarbon solubility in the aqueous phase support this theory (Wodzinski & Johnson, 1968). Some studies have shown that growth rates can exceed the rate of dissolution (Bouchez et al., 1997; Efroymson & Alexander, 1994; Osswald et al., 1996), bringing forth the next theories of initial uptake.

2. The microorganism excretes biological surfactants, which convert the substrates into droplets less than 1 micro meter in size, which can then be assimilated by the organism (Alexander, 1999). This process can be referred to as pseudosolubilization, as the substrate

is not truly dissolved in the water phase. Microorganisms using this mechanism would be limited by the amount of surfactants they can produce.

3. The microorganism may come in direct contact with the hydrophobic liquid by adhesion and colonization at the surface and utilize substrates directly from the NAPL. A limiting factor of this mechanism of utilization would be the surface area of the hydrophobic liquid.

For some organisms, attachment to the oil/NAPL surface is very important and may be required for degradation. For example, an *Arthrobacter* strain has been described that degrades hexadecane dissolved in a NAPL without excreting products that increase the water solubility of hexadecane. In this case, the spontaneous partitioning of hexadecane into the water phase can be ruled out because it is not detectable. Instead, the bacteria becomes attached to the NAPL-water interphase and is able to obtain the substrate directly from the NAPL (Efroymson & Alexander, 1991). The need for direct contact between the bacteria and the NAPL surface gained further support by observing that the addition of Triton X-100 (a surfactant that suppress cell adherence, but was not toxic to the bacteria at the concentration used) prevented mineralization of hexadecane dissolved in heptamethylnonane (Efroymson & Alexander, 1991). The same results were observed for the utilization of naphtalene dissolved in di(2-ethylhexyl)phthalate (Ortega-Calvo & Alexander, 1994).

2.4.3 Metabolism

A variety of hydrocarbons can selectively be metabolized by individual microorganism strains (Whyte et al., 1998). But mixed microbial cultures are required in to metabolize complex assortments of hydrocarbons (e.g. crude oil) (Deppe et al., 2005; Leahy & Cowell, 1990). Enzymatic pathways for hydrocarbon utilization are encoded on plasmid or chromosomal genes (Atlas, 1995; Leahy & Cowell, 1990; Rosenberg et al., 1993). Autochthonous species grow on hydrocarbon fractions due to increased numbers of hydrocarbon-utilizing plasmid genes in their populations (Atlas, 1995; Delille et al., 2009; Leahy & Cowell, 1990; Margesin & Schinner, 2001). Hydrocarbons can either be catabolized for energy or assimilated into protein biomass (Widdel & Rabus, 2001), preferably

aerobically, but also anaerobically at a much slower rate (Atlas, 1981; Leahy & Cowell, 1990). Figure 3 gives a simplified presentation of potential pathways of hydrocarbon utilization.

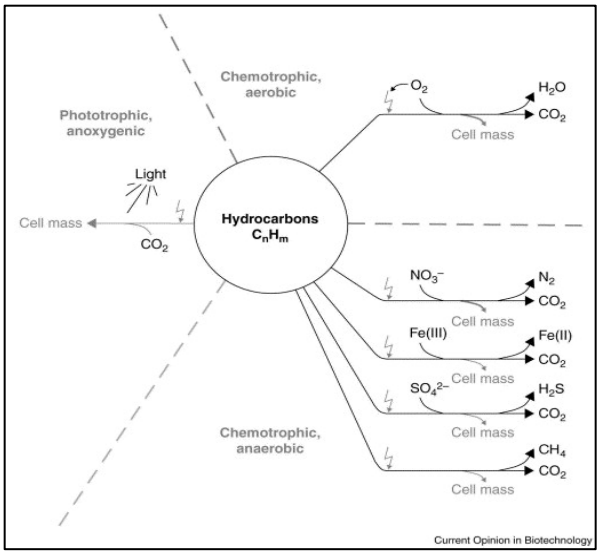

Figure 3: Potential pathways for hydrocarbon utilization by hydrocarbon degrading microorganisms (Widdel & Rabus, 2001).

2.4.4 Adaptation

Microbial populations have the ability to rapidly acclimate and adjust for environmental conditions and local contamination levels (Margesin & Schinner, 2001). Enhanced rates of HC oxidation have been observed in microbial communities exposed to significant amounts of hydrocarbon contamination (Atlas, 1981; Leahy & Cowell, 1990; Margesin & Schinner, 2001). In general, the mechanisms for adaptation involve gene or enzyme modifications and selective enrichment of the microbial community (Leahy & Cowell, 1990). Studies show that the rate of biodegradation is increased in previously exposed communities (Leahy & Cowell, 1990; Margesin & Schinner, 2001).

2.5 Stimulating biodegradation

Bioremediation is a term for strategies targeting the enhancement of the natural process of biodegradation of environmental pollutants. Bioremediation can be achieved by either bioaugmentation – the addition of microbial communities to a pollution site, or biostimulation – growth stimulation of indigenous microbial communities.

Bioaugmentation strategies involve the addition of foreign microbial communities to enhance the rate or extent of biodegradation (Atlas, 1995; Leahy & Cowell, 1990; Rosenberg et al., 1993). Foreign strains often have the disadvantage of being quickly outcompeted by indigenous microorganisms, due to being dependant on successful adaptation before growth processes can occur. Studies have also shown that locally adapted indigenous microorganisms tend to degrade substrates more effectively than foreign strains in aquatic soil systems (Margesin & Schinner, 2001). Scientists and industrial actors have tried developing genetically modified microorganisms for enhanced oil biodegradation. It has so far been unsuccessful due to adaptation challenges of foreign strains (Scragg, 2004).

Biostimulation can be used to enhance the biodegradation of environmental pollutants by positively influencing the growth conditions of native microorganisms. This can be achieved by adding nutrients to the natural environment, causing microorganisms to no longer be limited by nutrient availability, thus increasing and stimulating growth (Alexander, 1999). Some studies indicate that the presence of fertilizers will stimulate biodegradation (Delille et al., 2009). A fertilizer supplies nutrients, creating an optimal growth situation for the degrading microorganisms located at the oil-water interphase, resulting in increased biodegradation rates (Atlas, 1995; Leahy & Cowell, 1990). Addition of fertilizers is an ecological risk, because all organisms capable of utilizing the fertilizer will grow and increase the risk of eutrophication. Growth of unwanted organisms due to fertilizer addition may also outcompete the biodegrading population. Other methods used to enhance biodegradation include intense mixing or aeration, which has been investigated in laboratory studies and have shown to increase biodegradation (Alexander, 1999). However, such methods are practically very challenging to achieve on large scale in a marine oceanic environment.

An alternative biostimulation/chemical method is the addition of artificial dispersants. These chemicals, whose active agents are surfactants, serve to increase the surface area of the oil (National Research Council Committee, 2005), causing the oil to emulsify, which increases the potential surface area available for biodegradation (Leahy & Cowell, 1990; Margesin & Schinner, 2001). This eliminates the need for addition of nutrients because the background levels of biologically available nutrients, such as phosphorous and nitrogen,

become adequate for microbial growth when the surface to volume ratio of the oil increases (Prince et al., 2013). It is important though that the dispersant chemicals are environmentally safe, or at least poses a smaller environmental threat than the targeted environmental pollution will, if not removed.

2.6 Dispersants

Chemical dispersants are used to disperse oil more rapidly and extensively into the water column than the natural rate of dispersion due to physical weathering. This is believed to cause more rapid weathering of the oil as a result of increased biodegradation. However, research continues to determine how efficient these dispersant are at increasing biodegradation rates.

2.6.1 Properties and applications

A dispersant is generally a mixture of solvents, surfactants and other additives, that are applied to oil slicks to reduce the oil-water interfacial tension (National Research Council Committee, 2005). This promotes the formation of a larger number of smaller oil droplets in the water phase. Solvents in the dispersants are mainly there to keep the surfactants and additives dissolved and in a homogenous mixture. The interesting and most important part of a dispersant, are the surfactants, which are molecules with a hydrophobic and a hydrophilic portion. These are the chemicals responsible for the actual dispersion of the oil.

The use of an environmentally safe chemical dispersant on oil spills to enhance the rate of degradation has many benefits compared to other bioremediation methods. One is that it is easy to apply. When applied offshore, a boat or a helicopter can be used with equipment fitted to spray the dispersant on top of the oil slick on the surface of the exposed water (EUROPEAN MARITIME SAFETY AGENCY, 2009). There is no need for advanced mechanical removal equipment. Mixing is required though shortly after the addition of a chemical dispersant to an oil slick. If the wave energy is high enough, it will serve as adequate mixing force. Mechanical mixing might be necessary if natural mixing is not enough. The risk of eutrophication and unwanted growth of other organisms besides hydrocarbon degrading microorganisms is eliminated. Local concentration and presence of hydrocarbon pollutants

in the environment, and therefore the their ability to cause harm, is shortened. Additionally, the oils potential to reach shorelines is reduced, as a result of dispersion and increased biodegradation rates.

Despite the benefits of the use of chemical dispersants, there are also drawbacks and certain conditions that must be met to achieve benefits. Following an oil spill, there are several things that must be considered. First, not all oils will disperse effectively by use of chemical dispersants, for example heavy oils whose viscosity is simply too high (EUROPEAN MARITIME SAFETY AGENCY, 2009). Second, the use of dispersants on spills of light oils like diesel and other distillate fuels may cause more harm than benefits. They have high aromatic content, which is toxic to marine life and should therefore not be forced into the water column. These oils are typically very volatile and will naturally evaporate rapidly, which is environmentally safer than dispersing them into the water column. Refined oil products such as gasoline, contains toxic compounds such as benzene and should also not be forced into the water column. Refined oil products will naturally evaporate completely (EUROPEAN MARITIME SAFETY AGENCY , 2009). For a dispersant to be of environmental benefit it must also have certain properties to actually enhance biodegradation. Mulkin-Phillips and Stewart (1974) suggested three criteria for screening dispersants being considered for use in bioremediation of oil:

1. They should be biodegradable
2. They must not serve as a preferred substrate in the presence of oil.
3. They must not be toxic to indigenous bacteria.

In addition to these three criteria, the dispersant must also be able to disperse the oil under field conditions. It can be difficult to predict if surfactants will be toxic, biodegradable or a preferred substrate, except using previous experimental findings. Continuous research on commercial dispersants and surfactants is therefore important in order to extend the knowledge about commercial dispersants, surfactants and their effects, to be better able to predict which dispersant or surfactants will be suitable to the situation.

2.6.2 Dispersant-oil interactions

The mechanisms of oil dispersion by addition of chemical surfactants can be described using figure 4. The surfactant molecules lipophilic part will orient themselves into the hydrophobic liquid and surround a droplet of the liquid, with the surfactants hydrophilic part in the aqueous phase. As a result, tiny oil droplets are formed and transported into the water phase.

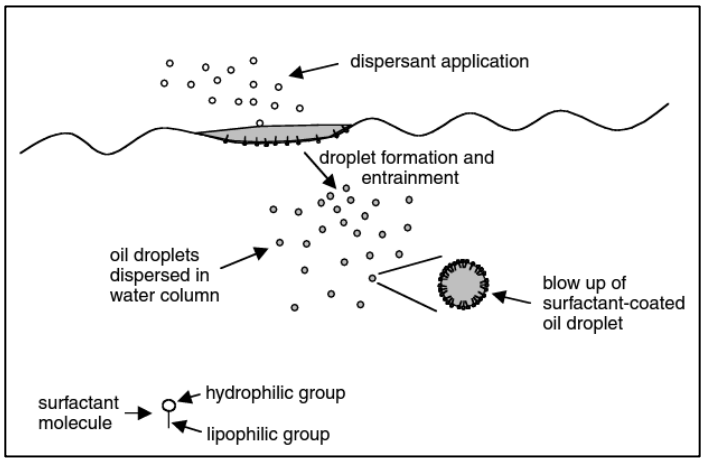

Figure 4: Surfactant accumulates at oil-water interphase, facilitating formation of small oil droples that become entrained in the water column ("National Research Council Committee," 2005).

2.6.3 The effect of dispersants on biodegradation

Predicting the effect of a given dispersant on biodegradation can be challenging. While the addition of some dispersants have been shown to increase biodegradation of hydrocarbons (Efroymson & Alexander, 1991; Nakahara et al., 1981; Rouse et al., 1994; Swannell & Daniel, 1999), others have been shown to be inhibitory (Rouse et al., 1994). There appears to be no theory or trend as to when and why certain dispersants inhibit bacterial growth, except that most ionic surfactants have showed inhibitory effects on growth, while non-ionic surfactants have varying results (Rouse et al., 1994). Also, research involving commercial surfactants and mixed microbial cultures have shown correlations between inhibited biodegradation of hydrocarbons and surfactant concentrations above the critical micelle concentration (CMC, the lowest concentration at which the surfactant molecules are able to form micelles) (Rouse et al., 1994). The challenge in predicting effects of dispersants and surfactants on biodegradation is likely due to the variety and complexity of microorganism

metabolism and cell properties. Further case by case research on is important to better understand the effect of putative dispersants and surfactants on biodegradation.

There is a general agreement that by the use of chemical dispersants (surfactants) which do enhance biodegradation, do so by increasing the partitioning rate of a hydrophobic substance from the oil phase to the water phase, or by increasing the interfacial area of the hydrophobic liquid (Alexander, 1999). The increase in interfacial area promotes a larger total mass transfer form the hydrophobic liquid to the water phase as well as allowing greater microbial colonization on the surface. In a biodegradation system, partitioning is dynamic. As dissolved substrate concentration is kept below the equilibrium concentration, the rate of mass transfer is increased (Eq.1). The concentration of the hydrophobic molecule in the water phase, and the total mass transfer can increase, as the surface increases and exposes more molecules to the oil-water interface, allowing them to transfer between the phases.

2.7 General methodology

There are numerous analytical methods available for monitoring oil hydrocarbon biodegradation in seawater. Biological oxygen demand measurements are used to monitor bacterial oxygen consumption as a result of growth on hydrocarbon substrates over time, giving an indication of the growth process. Chemical analysis at certain times during a biodegradation experiment can be used to examine the actual loss of hydrocarbons during the process. Bacterial enumeration can be used to examine the total microbial growth and molecular analysis can determine changes in the microbial community during the biodegradation process.

2.7.1 BOD analysis

Biological oxygen demand analysis is used to determine the oxygen requirements of microbial populations during biodegradation of organic chemicals. The chemical oxygen demand (COD) is similar, but measures oxygen demand for complete chemical oxidation of a compound. A BOD method typically involves measurement of the molecular oxygen

demand indirectly, either by quantifying molecular CO_2, which is produced proportionally to O_2 consumption (Brakstad & Bonaunet, 2006; Lindstrom & Braddock, 2002; Whyte et al., 1998), in the headspace of a closed bottle system, or by measuring the pressure changes in the headspace of sealed bottle. Biological oxygen demand is inversely proportional to pressure (Appendix A). To measure the pressure change over time during biodegradation of an organic substrate, instrumental sensors like the OxiTop control system heads are available (OxiTop System Control Operating Manual, 2006). In BOD analysis, both controls and blanks are required, as well as treatments (Whyte et al., 1998). Positive and negative controls are used to minimize the influence of false positives and negatives, while blanks are used to indicate contamination during the experiment.

2.7.2 Chemical analysis

Gas chromatography (GC) and total hydrocarbon (THC) analysis are both methods used to determine the carbon content of a sample. Both techniques are commonly used to evaluate chemical degradation of petroleum hydrocarbons. To prepare a sample for analysis via GC, liquid-liquid extraction followed by evaporation of organic solvent can be used. The liquid-liquid extraction is a method used to separate compounds according to their relative solubility in two immiscible liquid phases, most commonly water and an organic phase. Analytes can be recovered from either phase by mixing of the two immiscible liquids and multiple extraction of a single phase. This can be efficiently achieved using separatory funnels to separate the phases. The standard extraction method used in hydrocarbon analysis involves liquid extraction from an aqueous phase to a single hydrocarbon solvent phase (e.g. pentane) (EUROPEAN COMMITTEE FOR STANDARDIZATION, 2000).

Up concentration of the analytes to a known volume is needed for quantitative analysis via GC after liquid-liquid extraction (Brakstad & Bonaunet, 2006; Delille et al., 2009). The standard method for hydrocarbon analysis involves up concentration via an evaporation apparatus (EUROPEAN COMMITTEE FOR STANDARDIZATION, 2000). Gas chromatography is used to separate, identify and quantify the chemical constituents of volatile samples according to peak area responses. A sample is immediately vaporized after injection, and chemical components in the sample are carried through the GC column by an inert mobile

phase stream. Sample components interact variably with the stationary phase coating the inside of the column, and are thus separated by their chemical affinity. As components elute, detectors such as the flame ionization detector (FID), are used to quantify the carbon content, resulting in varying sized peak responses in a chromatograph according to the amount of carbon eluting at a given time. GC-FID responses can be used to monitor chemical degradation of hydrocarbons (Brakstad & Bonaunet, 2006; Deppe et al., 2005), and per cent biodegradation efficiency can be determined from peak area responses (Deppe et al., 2005).

2.7.3 Microbial community analysis

Microbial enumeration can be used to monitor microbial growth during biodegradation. Most probable number (MPN) can be used to estimate microbial population sizes in a liquid substrate media. It allows for the quantification of one particular metabolic group, such as hydrocarbon degraders. This approach is valuable for determination of the number of hydrocarbon degrading microorganisms locally present at the site of an oil spill, and has been used in studies to enumerate and analyse specific hydrocarbon degrading microorganisms (Haines et al., 1996; Vinas et al., 2005).

Molecular based community analysis is useful to investigate the microbial population characteristics and potential changes of the microbial community during biodegradation of different substrates. Denaturing Gradient Gel Electrophoresis (DGGE) is a molecular based method, which allows for the separation of identical or nearly identical length DNA fragments. To prepare a sample for DGGE, DNA is extracted from the whole community followed by PCR amplification of target sequences using primers containing a GC clamp (40-45 bp GC rich sequence). Variable regions of the 16S rRNA gene are used to assess microbial community changes.

The PCR products are separated on a polyacrylamide gel containing increasing concentrations of denaturants (urea and formamide). The denaturing point of a DNA molecule is dependant on its sequence, and migration of denatured DNA inside a gel is almost completely retarded. Amplicons will migrate distinct distances through the gel

based on their nucleic acid sequence before they reach their point of denaturation. Gel staining visualizes the resulting bands in the gel, which represent the community members. In theory, one band represents a single population within the community however in reality, one population may be represented by several bands or one band may be represented by multiple populations.

DGGE provides the ability to separate DNA sequences with as little as one base pair difference (Liu & Stahl, 2007), and has been used to study microbial diversity in different environments (Ferris et al., 1996; Nilsen & Torsvik, 1996; Teske et al., 1996). DGGE is useful for monitoring changes in community during the course of biodegradation, but has some disadvantages to be aware of. During sample preparation, it can be difficult to achieve reproducible and efficient extraction of DNA due to complex and diverse microbial communities in environmental samples. Also, it is important to keep in mind PCR artefacts and bias (Acinas et al., 2005). During DGGE, problems related to the co-migration of DNA from different species in the same band (Vallaeys et al., 1997) and the formation of multiple bands from the same species source are (Arújo & Schneider, 2008) important to be aware of.

2.8 Research objectives

The main objective of this study is to evaluate the effect of the dispersant Corexit 9500 on biodegradation of crude oil hydrocarbons incubated at low mesophilic and psychrophilic temperatures. By comparative analysis the following hypotheses were addressed:

- Does the selected dispersant enhance biodegrdation rates?
- Does the selected dispersant work better at higher temperature?
- Does the use of the selected dispersant change the biodegrading community structure?

In addition to the main hypotheses, the biodegradation of the selected dispersant alone was investigated. The aerobic biodegradation of hydrocarbons was investigated using crude oil from the Ekofisk oil fieild in Norway in seawater at different temperatures (3, 8 and 15°C), with and without the dispersant Corexit 9500 (Nalco). Biological oxygen demand (BOD)

was monitored over a 46-day period in closed bottle systems. At the beginning and end of the BOD experiment, hydrocarbon content was analyzed via liquid-liquid extraction in pentane and gas chromatography (GC) with flame ionization detection (FID). MPN and community analysis were performed to analyze the biodegrading community.

3 Materials and Methods

This chapter provides a detailed account of the techniques and materials used for the analysis of crude oil biodegradation. All experimental glassware were washed and dried before use, and safety precautions were observed at all times.

3.1 BOD analysis

Aerobic biodegradation of crude oil in seawater was examined over a period of 45 days. Oxygen demand was monitored over time using static respirometric measurements in closed bottle systems. Separate bottles were prepared for GC-FID, MPN and DGGE analysis at experiment start up (t=0), and designated BOD bottles were removed at experiment end (t=46). Samples used for GC-FID analysis were extracted in pentane, cleaned up using fluorosil solid phase extraction and up-concentrated to a defined volume.

3.1.1 Sampling

Seawater used in the experiment was obtained from the International Research Institute of Stavanger (IRIS) located in Mekjarvik, Norway, via a pipe intake system. The water was collected from a non-polluted fjord (59°1´N, 5°37´E) at a depth of 140 m. At the time of collection, the water temperature was approximately 8°C. The pipe intake system was 70 m. The water was collected in three autoclaved 10 L carboys and was stored at 3°C, 8°C and 15°C (one carboy at each temperature) immediately after collection. Sample seawater was used within three days after collection. The density of the seawater was assumed to be 1.026kg/L, and the salinity 3.5%.

3.1.2 Carbon Source

During the BOD experiment, the sole carbon source for microbial utilization was Ekofisk crude oil. The crude oil is a typical light crude and was provided by the University of Stavanger, from stock stored at 4°C. GC-FID analysis of the oil revealed that it consists mainly of low molecular weight saturated hydrocarbons. In the positive control samples, sodium benzoate ($C_7H_5O_2Na$, Merck) was used as the carbon source.

3.1.3 Experimental setup

Glassware, stir bars and rubber sleeve inserts (OxiTop) used for BOD analysis were washed, dried and autoclaved (Tuttnauer, 5075). Solutions were prepared in 510 mL amber bottles with stir bars (40 mm length, 7 mm diameter) and rubber sleeve inserts. Seawater was distributed into flasks by measuring mass difference on a weighing balance, accounting for seawater density (1.026 g/mL). A 5% Corexit aliquot was made by adding 0.5 mL Corexit 9500 to 9.5 mL crude oil. Crude oil, the 5% Corexit aliquot and Corexit solutions were sterile filtered into sterile 10 mL plastic containers using a 5 mL plastic syringe (BD syringe) and a syringe filter (0.2µm, PALL), before immediate addition to the designated bottles containing seawater. The sodium benzoate standard solution was made by adding 3.84 g sodium benzoate (AnalaR) to a 1 L volumetric flask and diluting to the 1 L mark with distilled water.

At each of the three temperatures (3, 8 and 15°C), 13 bottles were prepared, containing the following:

- Crude oil test flasks: 200 mL seawater, 30 µL crude oil. Three parallels.
- Dispersed crude oil test flasks: 200 mL seawater, 30 µL 5% Corexit aliquot. Three parallels.
- Dispersant test flasks: 200 mL seawater, 30 µL Corexit 9500. Three parallels.
- Positive control test flasks: 200 mL seawater, 1 mL sodium benzoate standard solution. Two parallels.
- Blanks: 400 mL seawater. Two parallels.

In addition to the contents above, 200 µL of nutrient solution A, B, C and D (provided by instructor), 10 µL vitamin solution (Sigma Aldrich, MEM Vitamin solution) and 10 µL amino acids (Sigma Aldrich, Amino Acids Mix, RPMI 1640) were added to all test flasks. To absorb carbon dioxide during biodegradation, two sodium hydroxide pellets (NaOH, Merck) were added in the rubber sleeve insert of all bottles. Each bottle was fitted with pressure sensors (OxiTop-C measuring heads, WTW) and sealed tightly to measure the pressure changes associated with oxygen consumption. Bottles were immediately placed in storage in incubators (Termaks, B8420) set to 3, 8 and 15°C, on inductive stirring systems (OxiTop, IS6). All bottles were incubated for 46 days. Data was monitored and collected using the OxiTop controller and evaluated using the Achat OC software program.

Additional samples used for time zero GC-FID analysis were prepared separately to determine the initial extract HC content, prior to biodegradation. Three bottles were prepared from each of the three seawater temperatures (3, 8 and 15°C), containing the following:

- Crude oil test flasks: 200 mL seawater, 30 µL crude oil.
- Dispersed crude oil test flasks: 200 mL seawater, 30 µL 5% Corexit aliquot.
- Dispersant test flasks: 200 mL seawater, 30 µL Corexit 9500.

Additionally, one blank bottle was prepared containing 200mL seawater (8°C). No nutrients were added to the time zero test bottles. All initial samples were analyzed immediately, without incubation time, by liquid-solvent extraction, up-concentration and GC-FID analysis.

3.2 Chemical analysis of hydrocarbon content

Before hydrocarbon content could be analysed via gas chromatography, seawater samples were extracted and evaporated to specific volumes. Liquid-solvent extractions in pentane were performed using a Florosil/sodium sulphate column. Up-concentration of each sample was achieved using an elaborate evaporation apparatus setup.

3.2.1 Liquid-solvent extraction and up concentration

All glassware used in the extraction were washed, dried and rinsed with pentane before use. The extraction was performed according to the guidelines of the European Committee for Standardization (EUROPEAN COMMITTEE FOR STANDARDIZATION, 2000). Pentane (n-Pentane, VWR) was used as the hydrocarbon solvent during extraction. Extraction was performed on following samples, on day 0 (prepared separately) and day 46 (BOD bottles):

- Crude oil - 3°C, 8°C and 15°C.
- Crude oil + Corexit - 3°C, 8°C and 15°C.
- Corexit - 3°C, 8°C and 15°C.
- Seawater - 8°C (day 0), 3, 8 and 15°C (day 46).

At day 0, seawater was extracted at only 8°C (taken directly from the collection carboy), while seawater blank BOD bottles for temperatures 3, 8 and 15°C were used in extraction at day 46.

For each sample flask, three extractions were performed. To each test bottle, 20 mL of pentane was added and the solution was stirred for 30 minutes on a magnetic stir plate. All liquid content was then poured into a 1 L separatory funnel. Extraction was performed by turning the funnel upside down in 12 repetitions, occasionally releasing pressure. The funnel and its contents were left to rest for 5 minutes, to allow separation of the phases. If an emulsion was formed in the oil phase, a small amount of magnesium sulphate (approximately 1 g, $MgSO_4$, VWR) was added. The denser aqueous phase was drawn from the bottom, into a clean 1 L beaker. The oil phase was drained into a 50 mL separatory funnel. The water phase was returned to the 1 L separatory funnel. In the second and third extraction, 20 mL pentane was added to the test bottle and swirled gently to collect residual oil phase, before being poured into the water collection beaker and swirled carefully to wash and collect residue. Finally, the pentane was added to the water phase in the 1 L separatory funnel, and extraction was performed as described, collecting the oil phase in the 50 ml separatory funnel after each extraction. The aqueous phase was discarded after the last extraction.

Residual liquid in the 50 mL separatory funnel was drained and discarded. A glass column with a fritted funnel was prepared by adding first 2 g Florisil (Merck, 0.150-0.250 mm, column chromatography grade) to the column, then 2 g of sodium sulphate (Na_2SO_4, Merck) on top of the florisil. The column was carefully rinsed with approximately 10 mL of pentane without agitating the surface. The extracted oil phase was slowly added to the column for removal (adsorption) of hydrophilic residuals (normal phase extraction) and the filtrate was collected in a 50 mL glass bottle. The column was flushed with approximately 10 mL of pentane, which was also collected in the glass bottle. To each bottle, 1.5 mL Isooctane (Merck) was added before they were sealed with a Teflon coated cap and stored at room temperature. The contents were up concentrated within 24 hours after extraction.

3.2.2 Up-concentration

All glassware used in this process were washed, dried and rinsed with pentane prior to use. The evaporation procedure was optimized using test extractions of hydrocarbon standard (HC) solutions by changing various parameters (e.g. temperature, pressure and stirring force) in the evaporation apparatus. Pentane (n-Pentane, 99.6%, VWR) and Isooctane (99.5%, Merck) were used as solvents during up-concentration.

The complete evaporation setup consisted of a Büchi Syncore evaporation apparatus, connected to a circulating cooling bath, a vacuum pump (VWR, V-855), and a secondary condenser (Büchi, Type S). The Büchi Syncore evaporation apparatus contained a six-position rack with six interchangeable tubes (Crystal R6) allowing evaporation down to 0.3 mL. Evaporation past this volume was prevented as the tubes featured cooling zones submerged in the cooling bath liquid. The circulating bath (VWR, 1180S) consisted of water and antifreeze (Biltema, standard). The secondary condenser was fitted with a 2000 mL round-bottom collection flask. During the evaporation procedure, the cooling bath was set to 0°C, rack temperature to 50°C, rack cover temperature was set to 55°C and stirring was set to 100rpm. The vacuum pressure was kept at 900 mbar.

To prevent potential loss of hydrocarbon content due to too much pentane evaporation (due to pentanes low boiling point, 36.1°C) 1.5 mL of isooctane (bp 99°C) was added to each

of the extract samples bottles, before pouring the samples content into the evaporation tubes. The Büchi system was sealed and evaporation procedure described above was started. Evaporation was continuously monitored and when the liquid content in the evaporation tube reached approximately 2 mL, the liquid was transferred to a 5 mL volumetric flask using a one-time use glass pipette. The tube was washed with approximately 1-2 mL of pentane, which was also collected in the volumetric flask. The content was then diluted to the 5 mL mark with pentane and shaken lightly. Solutions were added to amber glass auto sampler vials (2 mL) fitted with Teflon sealed silicon septra with polypropylene screw caps for GC-FID analysis.

3.2.3 GC-FID analysis

After up concentration, samples were chemically analysed via GC-FID. In order to correctly identify and quantify reference hydrocarbons in the crude oil extracts, calibration was done using a hydrocarbon standard dilution series.

The GC (Agilent 6890N) was equipped with a flame ionization detector (FID) and a Gerstel Multipurpose Sampler (MPS). MSD ChemStation G1701DA was used to monitor and manage results from the GC-FID analysis. Samples were analysed in glass amber autosampler vials (2 mL) fitted with Teflon septa and polypropylene caps. A pentane blank was included in each sequence. The analysis was performed according to the instrument operating instructions (MultiPurposeSampler Operation Manual, 2000).

3.2.3.1 GC-FID method

The instrument method used for hydrocarbon analysis was optimized and established by instructor prior to this experiment. Below, the instrument parameters used for the analysis of experimental samples are presented.

The liquid injector (Gerstel MPS) was equipped with a glass syringe (Gerstel Australia, 10 µL). The liquid injector performed 3 fill strokes of sample liquid at a volume of 5.0 µL prior to each sample injection. Sample vials were placed in an autosampler rack (Tray 2, VT98). Sample injection volume was 2.0 µL, with a fill speed of 5.00 µL/s, an injection speed of

50.00 µL/s and a viscosity delay of 2.0 s. Syringe air volume was set to 0 and injection penetration depth was set to 30 mm. After injection, the syringe rinsed with cyclohexane with a fill/eject speed of 50.00 µL/s.

In this method, only the back inlet was used, in split mode, with a split ratio of 10:1 and a split flow of 20.0 mL/min. A constant helium gas flow was used at a total flow of 25.1 mL/min. Gas saver flow was on and saver flow was set to 15.0 mL/min at 1.50 min. The initial temperature of the inlet was 300°C and pressure was 65.8 kPa.

Only column 2 (Agilent HP-5 19091J-413, back column) was used in this method, and was a capillary column composed of 5% Phenyl Methyl Siloxane, connected to the front detector (FID) and back inlet. The column features included a max temperature of 325°C, nominal length of 30.0 m, nominal diameter of 320.00 um and nominal film thickness of 0.25 um. Pressure was kept constant in the column and the initial nominal flow volume of helium was 2.0 mL/min with an average velocity of 33 cm/sec.

The oven used stepwise temperature settings. Initial temperature was 50°C and was held for 3.00 min with an equilibration time of 0.20 min. The oven temperature then increased with 12°C/min until it reached 350°C, which was held for 6 min. Post temperature was set to 40°C.

The front detector (FID) was set to a temperature of 325°C, with a hydrogen flow of 40.0 mL/min, an air flow of 450.0 mL/min and a makeup gas flow of nitrogen at 45.0 mL/min. Flame and electrometer were turned on and lit offset was 2.0.

For analyzing results, ChemStation computer interface was used. For experimental results, the integration method was set to integrate total area response between selected retention times, by using the following integration parameters: Initial Area Reject: 0 (initial), Initial Peak Width: 0.028 (initial), Shoulder detection: OFF (initial), Initial Threshold: 18 (initial), Integrator OFF (0.001min), Integrator ON (6.65min), Baseline Hold ON (6.7min), Area Sum ON (6.7min), Area Sum OFF (22.5min), Integrator OFF (22.5min).

3.2.3.2 Calibrations

To perform a calibration, a dilution series of a hydrocarbon standard, containing known concentrations of selected hydrocarbons dissolved in pentane was prepared in glass volumetric flasks. The standard solutions were analysed via GC-FID to establish retention times for the individual hydrocarbons and peak area response according to concentrations of the compounds.

Selected saturated hydrocarbons (C10-C40, refer to Appendix B) were calibrated at 100, 20, 4, 1 and 0.2 mg/L from dilution of the hydrocarbon standard (500 mg/L). The hydrocarbon standard was prepared in a 50 mL volumetric flask. Solid hydrocarbons were dissolved directly into the solution (50 mg). For the liquid alkanes, 50 µL was added via glass syringe. The concentrations of each of the liquid alkanes in solution were calculated separately, accounting for each alkanes density. The hydrocarbons were diluted with pentane to the 50 mL mark in the volumetric flask. The solution was then sonicated for 20 minutes at 50°C to ensure dissolution of solid hydrocarbons. The alkane standard solution was then used to make a dilution series of 100, 20, 4, 1 and 0.2 mg/L. The standard solution itself, along with the dilutions and a blank pentane sample, were added to glass amber autosampler vials and analyzed via GC-FID.

Retention times were established for each of the hydrocarbons based on the order of elution, where shorter chained hydrocarbons elute first. Linear calibration curves of response versus concentration were generated for each of the hydrocarbons. The main purpose of the calibration was to establish the retention times of selected reference hydrocarbons to properly calculate total area response between C10-C40 (THC). Calibration of hydrocarbon concentrations were done additionally in case of its usefulness, but were not needed.

3.2.3.3 Chemical analysis of crude oil and recovery

Crude oil used in the experiment was diluted 100 times in pentane and analysed via GC-FID. This revealed the chromatographic pattern of crude oil and provided an indication of its contents. Recovery of crude oil during the extraction process was also analysed. Four samples containing 0.1 mL crude oil and 200 mL of seawater were extracted, up-

concentrated and analysed via GC-FID. The total area response was calculated based on the integration method (see section 3.2.3.1) used in the experiment for both extracted and up-concentrated crude oil samples and for crude oil diluted in pentane. The difference in the total area response between the two was calculated as the total hydrocarbon recovery.

3.2.3.4 BOD experiment hydrocarbon analysis

Total hydrocarbon content in the extracted, concentrated experimental samples were quantified using GC-FID analysis. This was done to determine the total hydrocarbon removal during biodegradation of crude oil in seawater. Samples were analysed and results were treated according to the GC method described in section 3.2.3.1.

3.3 Bacteriological analysis of the biodegrading community

Bacteriological analysis was performed on the samples to enumerate microorganisms and investigate the bacterial community composition. By analysing at time zero, and at the end of the experiment for each of the three different temperatures, any potential changes in the biodegrading community could be observed. DNA extraction was performed to isolate DNA from the bacterial community in each sample, followed by PCR to amplify the 16S gene from the isolated DNA, and finally DGGE for community analysis. To enumerate the bacterial content in a sample, MPN analysis was performed at time zero and at the end of the experiment.

3.3.1 DNA extraction

All filtration equipment, including filters, metal tweezers and metal scissors were washed, dried, and autoclaved before use. DNA extraction was performed using the commercially available PowerSoil® DNA Isolation Kit (MoBio).

Seawater was filtered using a vacuum filtration setup with nitrocellulose membrane filters (0.22 µm GSWP, Millipore). For time zero analysis, 1 liter of seawater, taken directly from the carboy (refer to section 3.1.1) for each temperature (3, 8 and 15°C), was filtrated. For end experiment analysis, designated BOD flasks were sacrificed in parallel pairs and their

content filtered through the system. The filters containing cell material were carefully removed with metal tweezers and placed on a sterile surface. The filter was cut into small pieces, before adding them to the PowerBead tubes provided in the PowerSoil® DNA Isolation Kit. DNA was then extracted using the PowerSoil® kit as described by the manufacturer (available at http://www.mobio.com/images/custom/file/protocol/12888.pdf, last visited 8.4.2013). Samples were stored in a freezer (-20°C) between analyses.

3.3.2 PCR amplification

The PCR of the bacterial 16S rRNA genes was performed using the universal primers 341F (5'-CCTACGGGAGGCAGCAG-3') and SD907-r (5'- CCCCGTCAATTCCTTTGAGTT-3') with GC-clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') (Brakstad et al., 2008) targeting the V3-V4 hypervariable regions of 16S rRNA gene were used. Each PCR reaction was carried out in 50 µL mixtures containing 1 µL template DNA, 1 µL dNTPs (40 mM), 1 µL of each primer (100 µM), 0.3 µL taq polymerase (25 U/µ, Jumpstart Taq™, Sigma Aldrich), 5 µL PCR buffer (10 mM Tris-HCl, ph 8.5; 50 mM KCl, Sigma Aldrich) and 3 µL $MgCL_2$.

DNA target genes were amplified using a PCR apparatus (Applied Biosystems 2720 Thermal cycler). The PCR mix was heated (95°C; 10 min) followed by 30 cycles, each consisting of denaturation (95°C; 1min), annealing (55; 1 min) and DNA synthesis (72°C, 1 min). The reaction was terminated with a final extension (72°C; 7 min). After the last cycle, the PCR products were cooled to 4°C.

3.3.3 Denaturant Gradient Gel Electrophoresis (DGGE)

To ensure that the same concentration of DNA was loaded in each well the relative amount of DNA was estimated using a Nanodrop (Thermo scientific nanodrop 2000 sprectrophotometer). A continuous gradient of 20 – 80% of the denaturing agents urea and formamide were used for DGGE (100% denaturant corresponds to 7 M urea and 40% deionised formamide). PCR products were run on a 6% polyacrylamide gel using the IngenyPhorU-2 system in 17 L 1 x TAE running buffer at 60°C for 18 hours, at 90V. The gel

was stained using GelRed (VWR) solution for 1 hour. Images were taken in a BioRad GelDOC XR Imagery system.

3.3.4 Most probable number (MPN)

Marine Bushnell-Haas Broth (Broth) was made by adding 3.27 g Bushnell-Haas Broth (Fluka), and 30 g NaCl (Merck) to ultra distilled water and diluting to 1 litre in a 1000 ml volumetric flask. The solution was then autoclaved. Fluorecein diacetate (FDA, Alfa Aesar) solution was prepared by adding 100 mL Acetone (Merck) to 200 mg FDA. FDA was stored in a freezer (-20°C).

Broth (1.8 mL) was added to all wells in a 24-well sterile culture plate (Multiwell™). From the sample, 200 µL was added to the first dilution wells using pipette with sterile tips. Tip was changed and mixtures were mixed 10 times using the pipette. From these wells, 200 µL were transferred into the next row of wells, and mixed again, changing tips after each addition. The process was repeated until the original sample was diluted from 10^{-1} to 10^{-9}, with three parallels of each dilution. A blank was made by not adding culture to one of the wells for each MPN series. 20 µL of sterile filtered (0.2 µm Supor® Membrane, PALL®) crude oil was added carefully to the top of each sample well. All plates were incubated at 15°C for 14 days. After incubation, 50 µL thawed FDA solution was added to all wells and the plates were left on the lab bench for 1-2 hours. Wells developing a bright green colour were scored as positive for growth. Enumeration was done using an MPN-table (Figure 18). At time zero, a sample was collected straight from the carboys (see section 3.1.1) at each temperature and analysed in triplicates. For analysis at the end of the experiment, designated BOD flasks were sacrificed and sample was drawn from each sample bottle.

4 Results

Biodegradation of crude oil was investigated at different temperatures, with and without the addition of the chemical dispersant Corexit 9500. Chemical and microbiological methods were used to analyse the biodegradation process. This chapter provides information on all relevant findings.

4.1 BOD analysis

Aerobic biodegradation of crude oil was continuously monitored at various temperatures via BOD analysis. Pressure changes in closed bottle systems were used to quantify the oxygen requirement for biological degradation. Sample flasks were used to measure oxygen consumption by microorganisms during biodegradation of hydrocarbons in Ekofisk crude oil. Positive controls were used to measure the BOD of sodium benzoate at the different temperatures to verify the biodegradation protocol (check for heterotrophic growth potential). Blanks were used to indicate potential contamination during the experiment. Negative controls were not included in this experiment due to inadequate amount of bottle and OxiTop control heads available.

The BOD experiment ran for a total of 46 days, where all OxiTop control heads were set to BOD14, BOD7 or BOD5 to get denser measuring points through the process. BOD data was collected, heads were opened and pressure was equalized before restarting the OxiTop heads after each BOD time period. Original BOD data was therefore justifiably manipulated in the end to account for pressure equalization and head opening during the total experiment period. Due to technical difficulties, no BOD measurements were acquired between day 18 and 25. The starting point of BOD data following this time gap were manually estimated based on the BOD rate of change before and after the gap. BOD data from blank flasks were terminated after day 25 for temperatures 8 and 15°C. To compensate, BOD values were assumed stable at the last BOD value measured for the remaining duration of the experiment. Blank values were subtracted from each sample at their corresponding temperature. Final BOD values were plotted as mean BOD values.

Standard error (standard deviation divided to the root of n, where n is the number of parallels) was calculated for each data point in all parallels of all samples including positive controls and blanks, and is presented as a grey field around each BOD curve (see e.g figure 6). Positive control and blank bottles were run in two parallels, while all sample bottles were run in 3 parallels. Sample BOD data (BOD data from flasks containing crude oil, Corexit 9500 or both) are presented comparatively based on temperature and content, while positive control and blank BOD data are presented separately. A typical set of triplicate BOD curves used as a basis for the mean BOD curves and standard error is presented in figure 5.

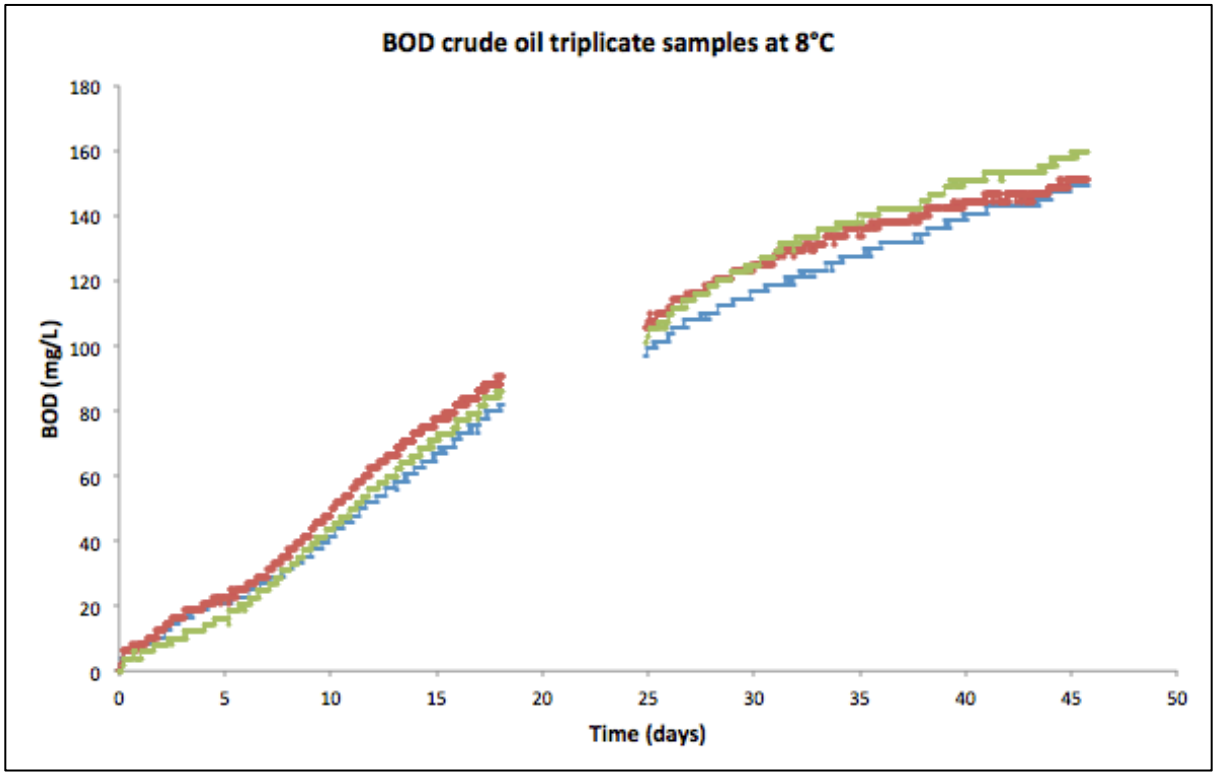

Figure 5: Typical variation in BOD curves per triplicate sample. This figure shows the BOD curves of three parallel crude oil samples at 8°C.

4.1.1 Positive control and blank flasks

BOD values for positive control and blank flasks were adjusted only slightly to yield a smooth curve as they were subject to pressure fluctuations during the first few BOD measurements following the initial sealing of the BOD control heads.

Figure 6 illustrates the mean BOD curves for sodium benzoate at 3, 8 and 15°C. The exponential phase for bacterial growth occurred between day 6 and 8 at 3°C, day 3 and 5 at 8°C and day 1 and 2 at 15°C. At temperatures 8 and 15°C, the parallel positive control flasks had larger error values compared to positive control bottles at 3°C.

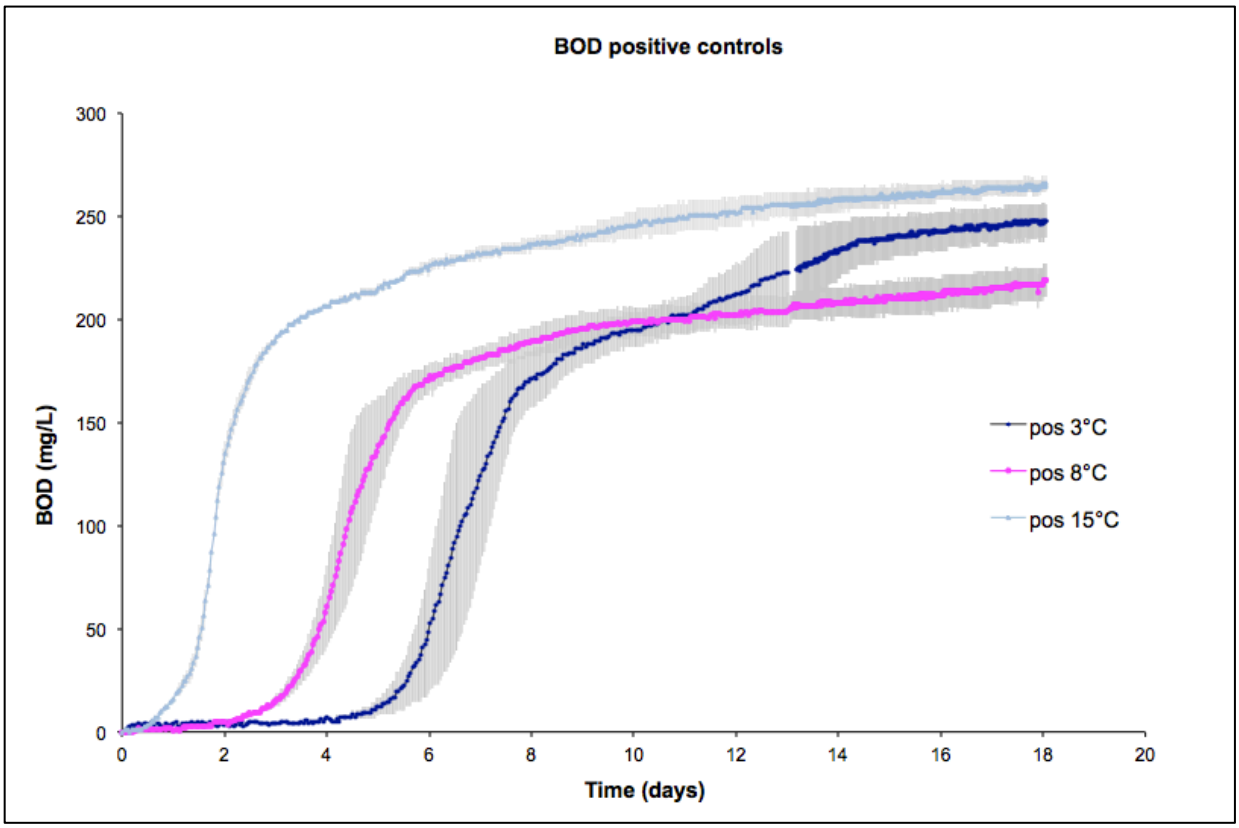

Figure 6: BOD curves based on mean BOD values for positive control flasks at 3, 8 and 15°C. Standard error is represented by a grey field around each curve.

The mean BOD data for blank bottles are presented in figure 7. BOD values were assumed constant at the last point measured until the end of the experiment. This was done to provide blank values for background compensation of sample BOD values at the corresponding temperatures after day 18. For blank bottles at 3°C, no BOD data was recorded between day 18 and 25. The first data point following day 25 was estimated based on the rate of change before and after the gap, and BOD data between day 25 and 46 were added to this value.

All blank mean values had low standard error. However, the blank bottles at 8°C shows higher activity than the other blanks. This can indicate contamination, or, since both blanks at this temperature showed almost identical trend, it can be ambient microbial activity as a result of these bottles being kept at the in situ temperature. Typically 2-3 mg/l trace amounts of organic carbon are present in the sampled seawater (Roald Kommedal, personal communication), hence some background growth can occur.

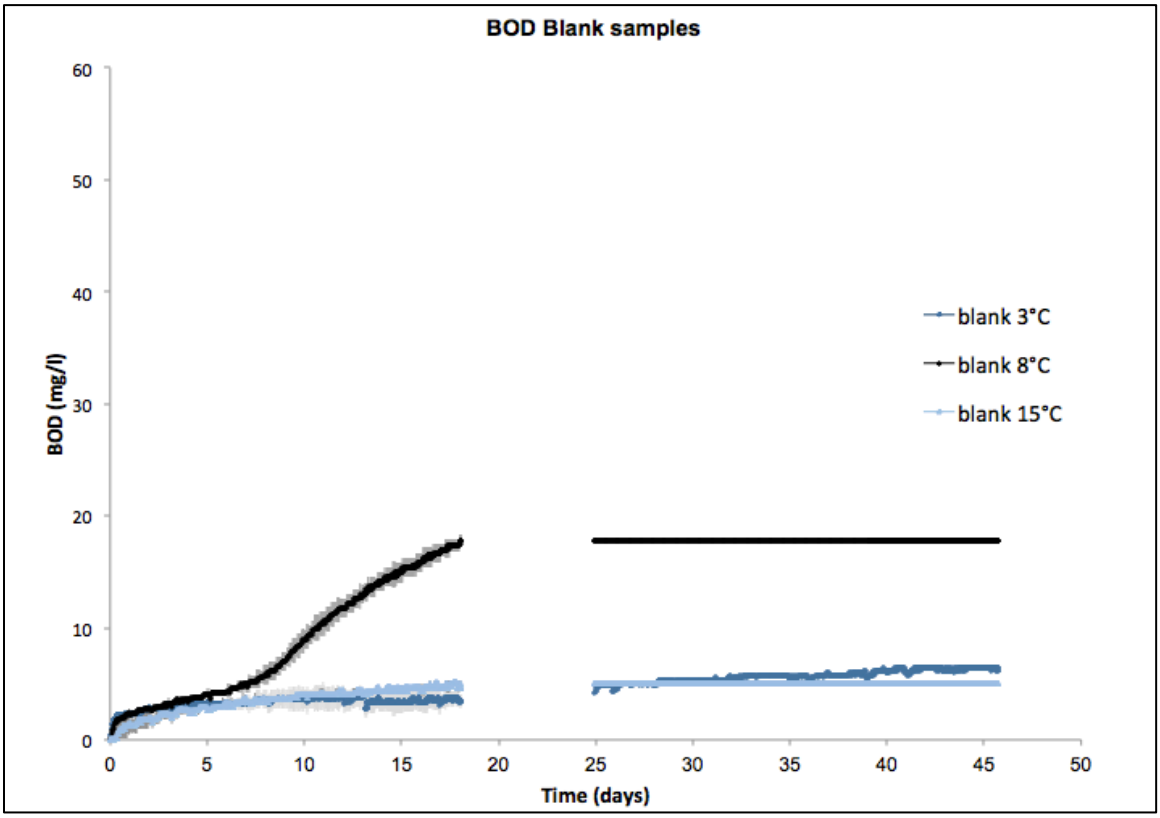

Figure 7: BOD curves based on mean BOD values for blank flasks at 3, 8 and 15°C. Standard error is represented by a grey field around each curve.

4.1.2 Effects of temperature on biodegradation

Differences in biological oxygen uptake rate based on temperature were observed in all samples. However, the standard error values for samples containing crude oil, and crude oil with Corexit 9500 at 15°C were very large compared with samples at 3 and 8°C.

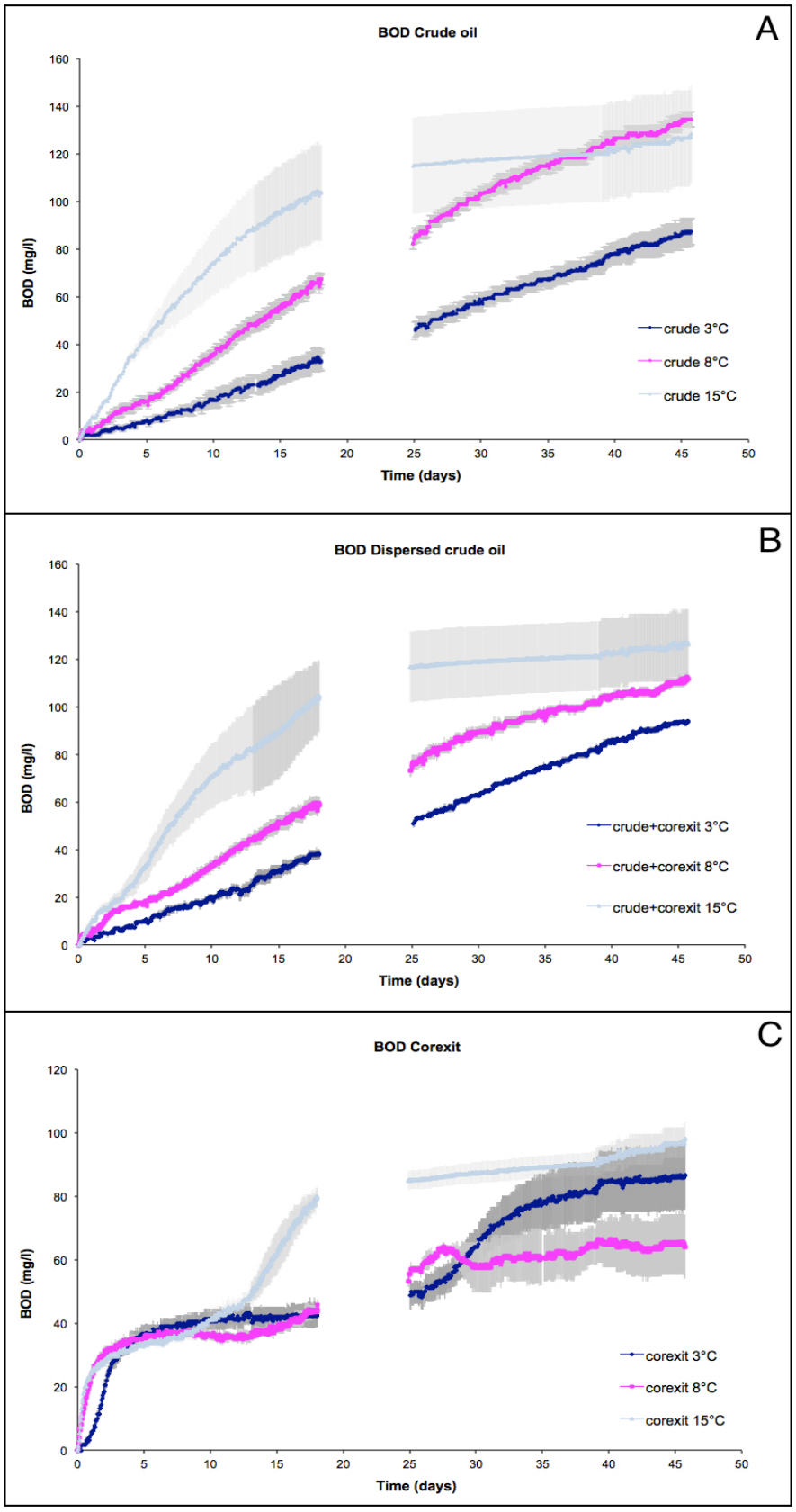

Figure 8: BOD curves based on mean BOD values for triplicate samples of A: crude oil, B: crude oil with Corexit and C: Corexit at 3, 8 and 15°C. Standard error is represented by a grey field around each curve.

Figure 8 A shows the mean BOD curves for the biodegradation of crude oil at 3, 8 and 15°. A clear difference in oxygen consumption rate was observed at each temperature, indicating more rapid biodegradation of crude oil with increasing temperature. Large standard errors were observed at 15°C. This was due to one of the three parallel flasks having much higher activity than the others after day four. The standard error for crude oil samples at 3 and 8°C remained relatively low and constant for the duration of the experiment.

BOD curves for samples containing crude oil and Corexit are presented in figure 8 B. Trends seen here were very similar to trends seen in the BOD curves for crude oil samples. A large standard error was observed at 15°C, while samples at 3 and 8°C had relatively low and constant standard error. The large standard error at 15°C for crude oil with Corexit was due to one of the three parallel flasks having lower activity compared with the others, which became significant at around day four.

BOD mean curves from the microbial degradation of Corexit 9500 at 3, 8 and 15°C are presented in figure 8 C. There seems to be an exponential growth phase occurring at all temperatures within the first day, with no lag phase at 8 and 15°C, and only a small delay at 3°C. Standard error values are very low in all Corexit samples the first three days, before they start to increase at 3°C. After 10 days, the standard error values become larger and somewhat variable due to variations in the individual BOD measurements, with the largest values occurring towards the end of the experiment period in temperatures 3 and 8°C. The difference in microbiological activity based on temperature was less clear in these samples. There seemed to be a second high rate growth phase occurring after the initial exponential growth phase on all three curves. This is most apparent at 15°C between day 14 and 18. The curve at 8°C can seem like it is about to enter a period of higher rate just before the period of lost data, but this remains unclear. The BOD curve at 3°C has a period of higher rate between day 27 and 32.

4.1.3 Effects of the dispersant on biodegradation

According to the BOD curves from this experiment, the addition of Corexit 9500 did not result in more rapid biodegradation of crude oil at any temperature. At 3°C (Figure 9 A), the BOD curves for crude oil samples with and without Corexit are almost identical, with relatively low and constant standard error values. Only a slight increase in average rate of oxygen consumption is observed in samples containing crude oil and Corexit, but the BOD curve is still just within the standard error field for BOD curve for crude oil. A very slight increase in reaction rate is observed in oil sample with Corexit at around day 6-7 (Figure 9 A). For the sake of simplicity, standard error field was not added to the BOD curve of Corexit samples in figures representing the different temperatures.

At 8°C (Figure 9 B), the BOD curve for crude oil also lies very close to the BOD curve of crude oil with Corexit. This time however, the BOD curve for crude oil has a higher oxygen uptake rate compared to the BOD curve for crude oil with Corexit. Additionally, both curves are outside each others standard error field after day 26. The two curves start to deviate from each other slightly right before day 18, and the data from the BOD curve of crude oil shows slightly increased rate compared with BOD curve of crude oil with Corexit after day 25. The BOD curve for crude oil with Corexit (Figure 9 B) shows a slightly increased rate at day two, before it drops back around day four. The BOD curve for flasks containing Corexit at 8°C reached a plateau at day 30.

Figure 9 C shows the BOD mean curves of experimental samples at 15°C. The large standard error fields at 15°C for samples containing crude oil and crude oil with Corexit, are very similar. The BOD curves for crude oil and crude oil with Corexit are close to identical. A slight drop was observed in the BOD curve for crude oil with Corexit around day three (Figure 9 C). All BOD curves at 15°C had reached a plateau at day 25.

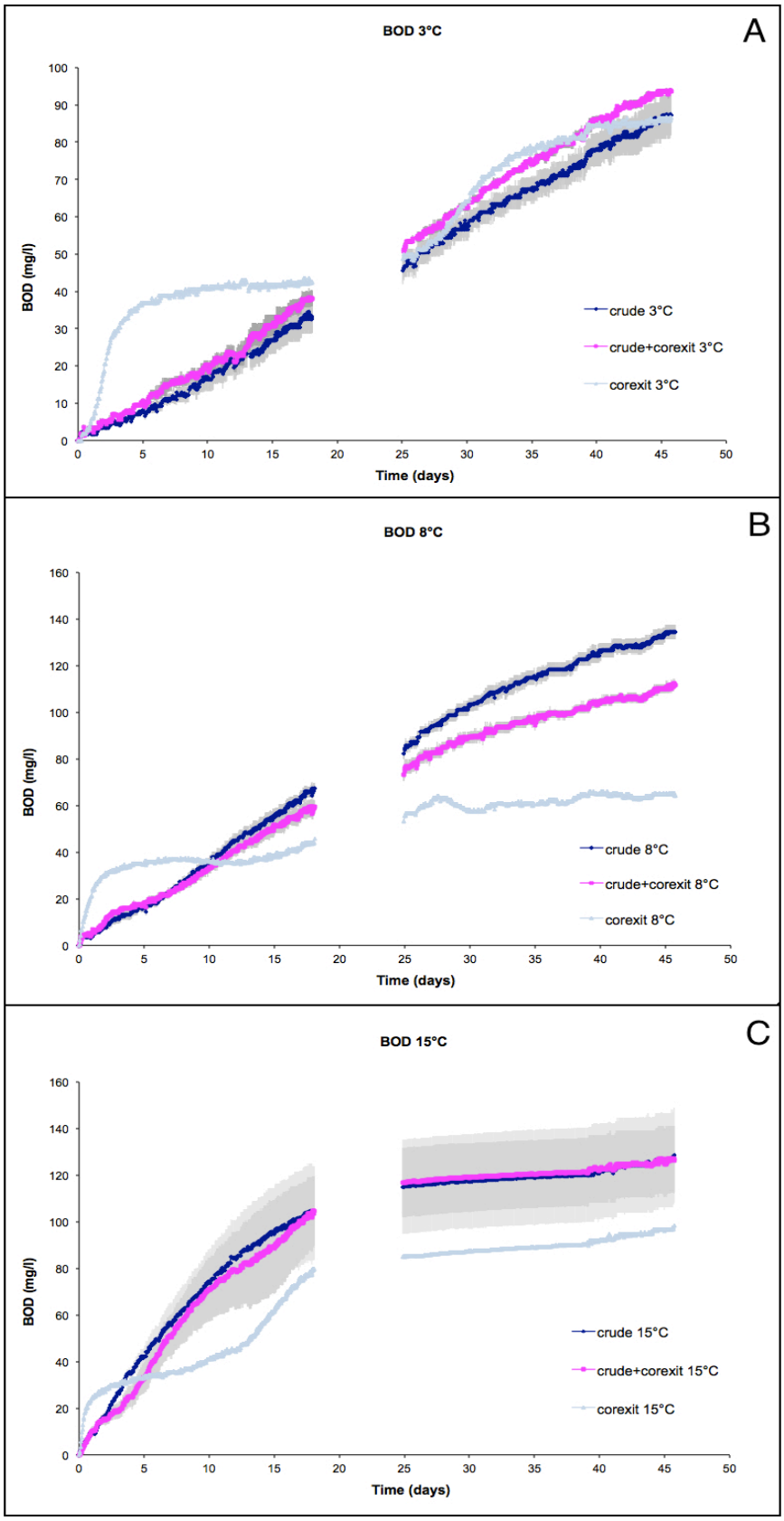

Figure 9: BOD curves based on mean BOD values of triplicate samples containing crude oil, crude oil with Corexit and Corexit at A: 3°C, B: 8°C and C: 15°C. Standard error is represented by a grey field around each curve.

4.2 GC analysis

Gas chromatography was used to calibrate reference hydrocarbons and establish their retention times in order to determine the total hydrocarbon removal in experimental BOD samples.

4.2.1 Calibrations

GC-FID calibration results were used to identify the time interval to be used for total area response in experimental sample chromatographs. This would yield the total hydrocarbon response in each sample. Retention times for each of the reference hydrocarbons are presented in table 1.

Table 1: Retention times for each reference hydrocarbon used in calibration.

Formula	Chemical	Retention time (min)
C9H20	Nonane	5.234
C10H22	Decane	7.049
C11H24	Undecane	8.676
C12H26	Dodecane	10.131
C13H28	Tridecane	11.455
C14H30	Tetradecane	12.683
C15H32	Pentadecane	13.832
C16H34	Hexadecane	14.915
C17H36	Heptadecane	15.940
C18H38	Octadecane	16.913
C19H40	Nonadecane	17.840
C20H42	Eicosane	18.727
C22H46	Docosane	20.380
C24H50	Tetracosane	21.905
C28H58	Octacosane	24.625
C32H66	Dotriacontane	27.000
C36H74	Hexatriacontane	29.195
C40H82	Tetracontane	32.000

4.2.2 Chemical analysis of crude oil and recovery

Crude oil recovery during extraction and up-concentration was calculated based on direct GC-FID analysis of crude oil diluted in pentane (1 mL crude oil, 99 mL pentane), and on extraction of crude oil from seawater followed by up-concentration and GC analysis. Four samples were prepared with 0.1 mL crude oil added to 200 mL seawater. Each were extracted and up-concentrated separately, and analysed via GC-FID. The up concentrated volume was 5 mL, leaving extraction samples at a concentration of 0.1 mL crude oil per 5 mL pentane, or 20 mL crude oil per litre pentane, assuming 100% recovery. Direct crude oil GC response was based on a concentration of 10 mL crude oil per litre pentane. The GC-FID response was therefore multiplied by two in order to get the correct concentration relationship between the raw and up concentrated samples. Recovery was calculated to be 89%, +/- 14%. The error was calculated based on the standard deviation value for the mean GC response value of the four extraction and up-concentration samples.

4.2.3 Total hydrocarbon analysis

Total hydrocarbon (THC) analysis via GC-FID was performed on experimental samples at the start of the experiment and at the end of the experiment to compare total hydrocarbon removal during aerobic biodegradation of crude oil at different temperatures, with and without the addition of Corexit 9500. The total hydrocarbon removal was also investigated for Corexit alone. THC response was determined based on the total area response between decane and tetracosane, at retention times 6.7 min and 22.5 min. The chromatographic profile of crude oil is shown in figure 10, along with a representation of the area used to integrate the total area response.

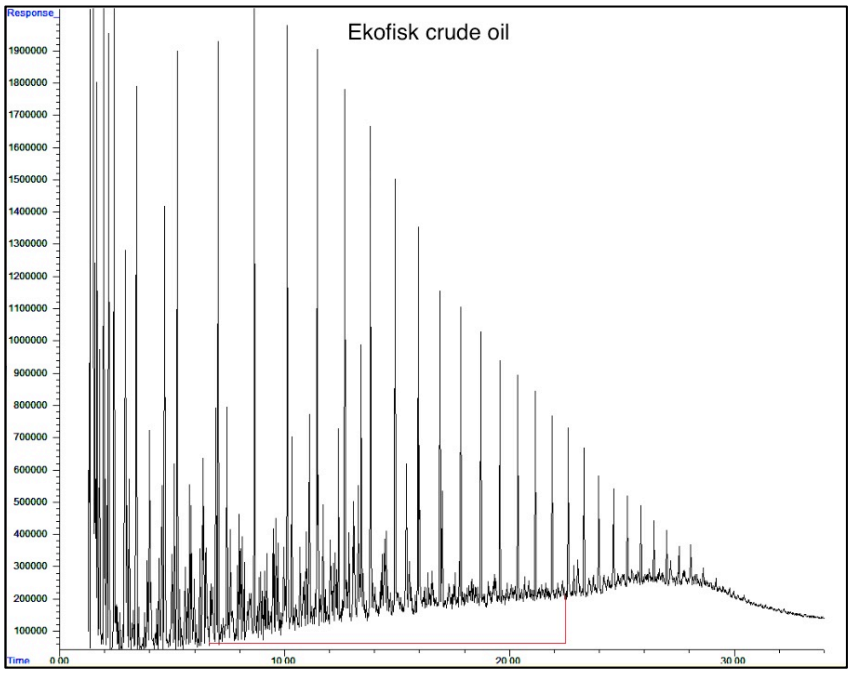

Figure 10: Ekofisk crude oil chromatographic profile. The integration line is showed below the chromatograph. The area above the integration line was integrated to get the total area response value.

Figure 11 shows a typical comparison of a GC profile of an experimental sample before and after the biodegradation process. For simple presentation of GC responses, initial and final GC results for each sample are presented in a single picture.

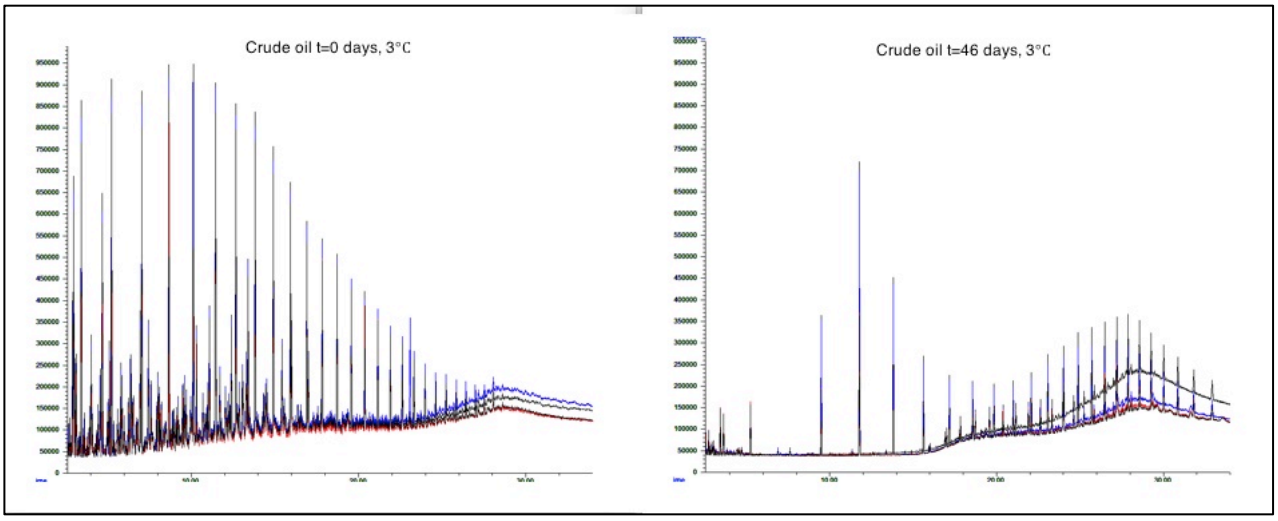

Figure 11: Experimental crude oil sample (3°C) chromatograph at day 0 and day 46.

Figure 12, 13 and 14 shows stacked comparative chromatograph responses based on four parallels per sample.

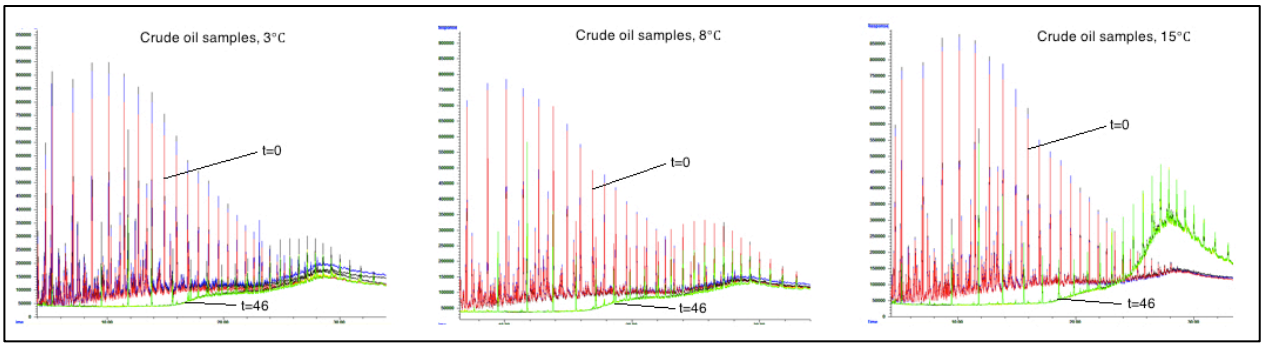

Figure 12: Chromatographs for crude oil sample initial (t=0 days) and end (t=46 days) analysis for temperatures 3, 8 and 15°C.

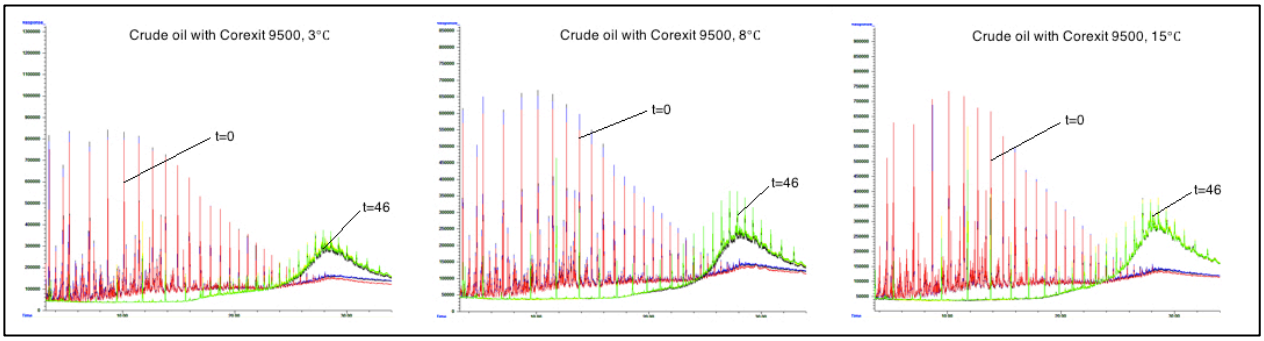

Figure 13: Chromatographs for crude oil with Corexit sample initial (t=0 days) and end (t=46 days) analysis for temperatures 3, 8 and 15°C.

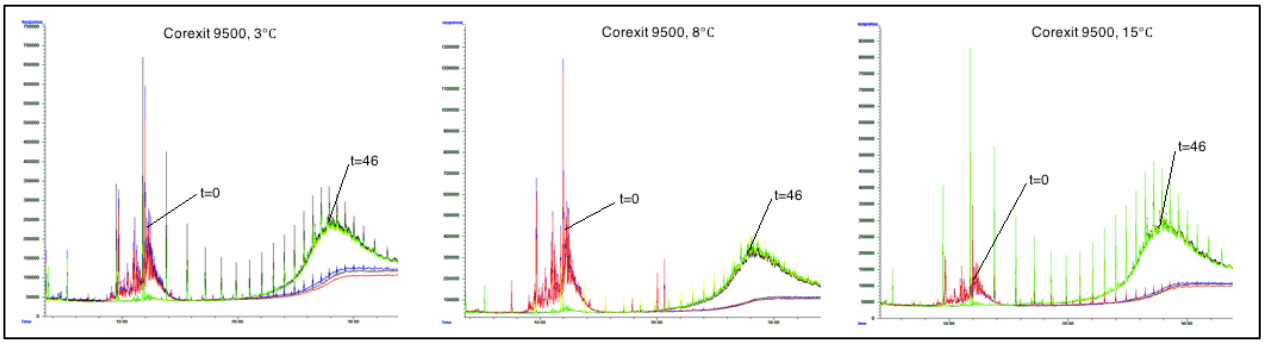

Figure 14: Chromatographs for Corexit sample initial (t=0 days) and end (t=46 days) analysis for temperatures 3, 8 and 15°C.

THC was calculated as the mean total area response value for each sample based on four measurements. The total hydrocarbon removal was then calculated based on the difference in total area response at day 0 and day 46. These results indicate the extent of biodegradation of crude oil and Corexit over the duration of the experiment. The total hydrocarbon removal during the experiment was initially intended to be analysed via total area response between C-10 and C-40. However, GC-FID analysis at the end of the experiment revealed a large hump in the chromatograph baseline for samples containing Corexit and in the crude oil sample at 15°C (Figure 12-14). This hump was found between retention time 22.5 min and 33 min. Due to inability to understand or explain this phenomenon, total area response was adjusted down to 22.5 min, which gave a total area response between decane (C10) and tetracosane (C24).

Final results are presented in table 2 as per cent degraded based on the total area response (refer to section 3.2.3.1 for details on integration method) in the sample chromatograph at t=0 and t=46. Blank mean response was subtracted from each experimental sample mean response. The error was calculated based on the standard deviation of each samples mean total area response value, which was then used to create upper and lower limits of the total per cent oil biodegraded. Each sample was extracted from a single BOD bottle (two of the three BOD bottle parallels were used for DNA extraction), but GC-FID analysis was run in parallels of four. The error is therefore connected to random errors in the GC instrument. The results in table 2 show that between 80 and 92% of the crude crude oil, with or without the addition of Corexit 9500, is biodegraded in seawater during a time period of 46 days, at temperatures 3, 8 and 15°C. Samples prepared with only Corexit shows almost complete removal of the dispersant at 3 and 8°C, while only 19% was degraded at 15°C according to the findings in this experiment.

Table 2: Per cent removal of total hydrocarbons from sample BOD bottles over a period of 46 days.

Sample	Temperature	% Biodegraded	Error
Crude oil	3°C	80	+6/-7 %
Crude oil with Corexit 9500		82	+/- 2 %
Corexit 9500		97	+/- 3 %
Crude oil	8°C	86	+/- 2 %
Crude oil with Corexit 9500		92	+/- 1 %
Corexit 9500		95	+1/-2 %
Crude oil	15°C	84	+/- 1 %
Crude oil with Corexit 9500		86	+/- 2 %
Corexit 9500		19	+10/-11 %

The blank response used to subtract from sample total responses were based on blank samples run at the time of extraction. Blank values from the beginning of the experiment were based on the extraction and up concentration of 200 ml source seawater (temperature comparison was disregarded at this point), which was analysed via GC-FID in two parallels. The GC sequence was set to run four parallels but due to gas flow being accidentally shut down, the sequence was interrupted. Blank responses at the end of the experiment were a result of extraction, up concentration and GC-FID analysis of the blank BOD bottles after their content had been filtered for DNA extraction. The resulting blank area response values were much higher than the blank values from the start of the experiment. Analysis of solvents used in the extraction (pentane and isooctane) revealed contamination in both solvents that had not been present at the start of the experiment. . Therefore, the blank value subtracted from t=0 samples and t=46 days were two different values.

4.3 MPN results

MPN analysis was performed on source seawater in triplicates per temperature at t=0, and for all BOD experimental sample flasks, one blank flask per temperature, and positive control flasks at 3 and 15°C. Source seawater was sampled for MPN analysis two days after collection and storage at 3, 8 and 15°C.

MPN was determined based on an MPN table (Appendix A, Figure 18). The MPN value was used to represent MPN/ml rather than MPN/100ml. This is justified due to the difference in dilution used in the experiment (0.1, 0.01 and 0.001), compared to the table (10, 1 and 0.1). Results are graphically presented in figure 15. T=0 data is based on triplicate MPN analysis of raw source seawater at each temperature, and is only presented in each individual temperature group for comparison purposes. Error is represented as a vertical line through the bars and is based on the standard error for each triplicate (experimental samples) sample. Error calculations were not available for blank and positive control samples at t=end.

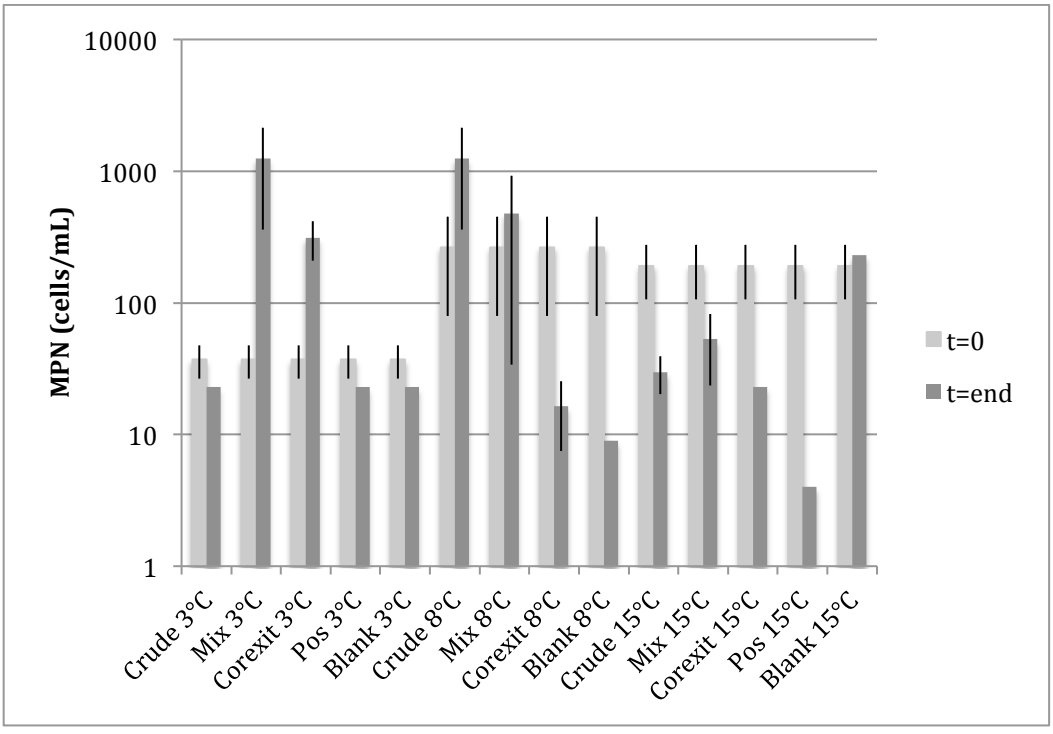

Figure 15: MPN Results. Mix=crude+Corexit samples. Pos=positive controls. A vertical line through the bars represents the standard error. Values were represented in a logarithmic scale of MPN/ml due to large variations in MPN values between samples.

Results presented in figure 15 shows the highest growth of hydrocarbon degrading bacteria in samples containing crude oil at 8°C, crude oil with Corexit at 3 and 8°C, and Corexit at 3°C. The rest of the samples show similar or reduced bacterial mass according to the MPN results.

4.4 DGGE Results

Prior to DGGE analysis of experimental samples, DNA was extracted from the liquid contents of the experimental BOD flasks. Gel electrophoresis (1% agarose gel) revealed that all samples contained DNA extracts. PCR amplification of the 16S rRNA bacterial gene was done for all DNA extract samples. Gel electrophoresis (1% agarose gel) revealed successful amplification in all samples (Figure 16). Labeling was excluded in the picture as all samples revealed equal intensity bands.

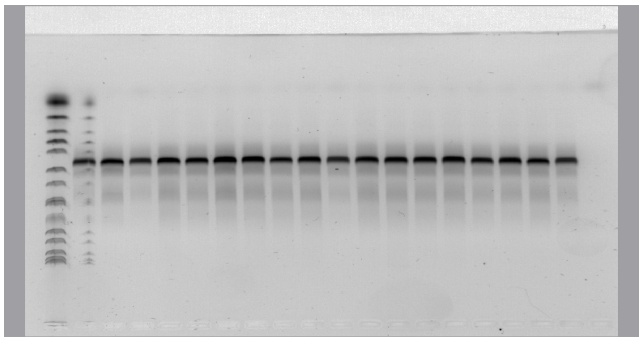

Figure 16: Gel containing PCR product samples after running gel electrophoresis for approximately 1 hour.

The DGGE results (Figure 17) show a change in the microbial community both over time at the different temperatures and with the different experimental treatments (Figure 17). At time zero the microbial communities were quite similar, showing the same band pattern, except for one band at 3°C not found at the other temperatures. The highest density of bands was found in approximately the same area in all samples. Samples from t=0 analysis showed low distribution of bands, with the highest band intensity at 8°C (the in situ temperature). Samples analysed at the end of the experiment all revealed larger band distribution in comparison with the initial samples, including the blank samples.

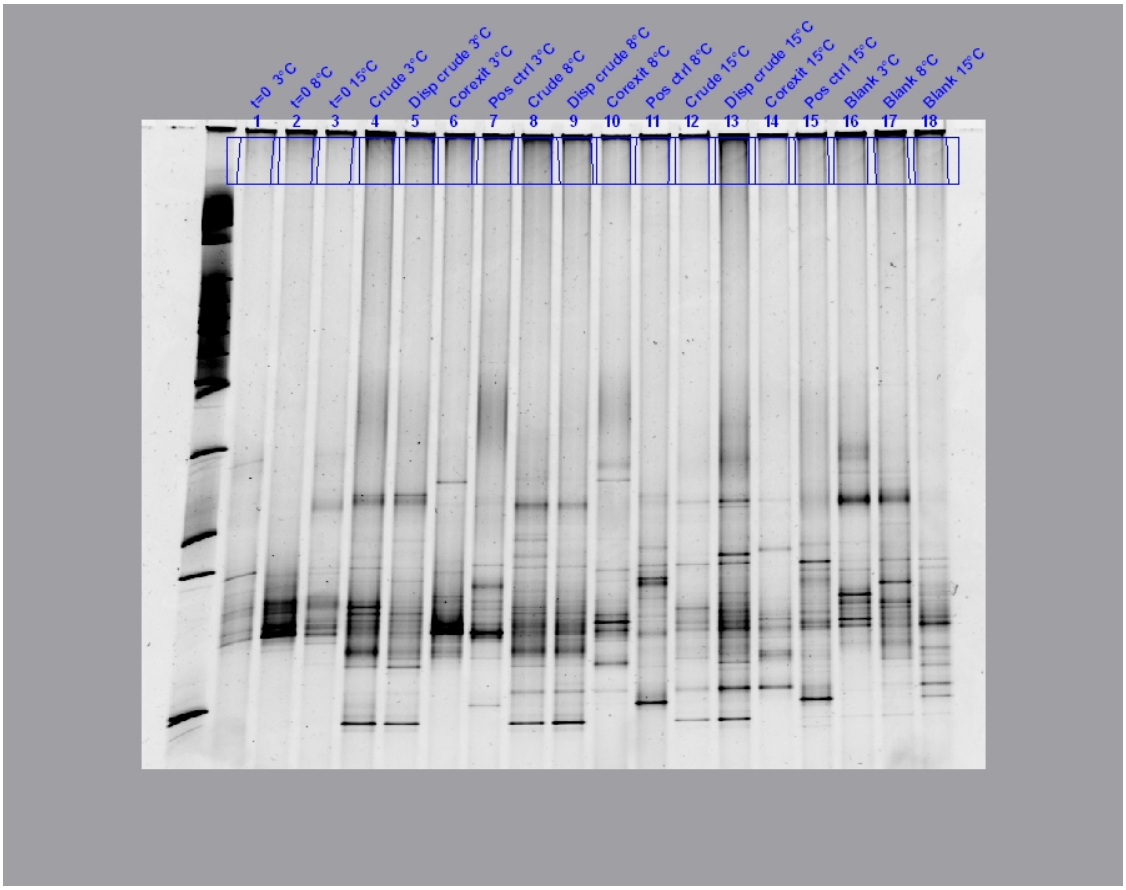

Figure 17: DGGE results. T=0 represent analysis at the start of the experiment. All other samples were result of experiment end analysis.

All samples containing crude oil or crude oil with Corexit showed a similar distinct band pattern at all temperatures. One band (on the bottom of the band profile) stood particularly out in samples containing oil and was not found in other samples. These samples also showed two bands in the upper edge of the band distribution area, which did not seem to be present in other samples except very faintly in Corexit at 15°C, and strongly in blank 3 and 8°C. Samples containing only Corexit all showed similar band patterns, but one band was found at 3 and 8°C (the upper most band) which is not found at 15°C or other samples except very faintly in 3 and 8°C blank samples. The blank samples all had a different band profiles but the blank sample at 15°C showed a completely different band profile compared to the other two temperatures.

5 Discussion

This chapter presents relevant experimental results and discussion of the findings, as well as possible errors during the experiment.

5.1 Analysis of crude oil biodegradation (BOD)

Biodegradation responses in BOD curves for the positive control flasks indicated parallel variations in reaction rate. The bacterial growth lag phase increased with decreasing temperature, and reaction rate increased with increasing temperature (Figure 6). The final extent of biodegradation seemed to be same for the positive controls at 3 and 8°C, but with an increased reaction rate in the endogenous phase at 3°C. Increased standard error values during this increase however, suggests that his is not a general trend. It is common to observe larger variations towards the end of a biodegradation process in batch systems due to increased competition for substrate, endogenous respiration etc. With only two parallels for each experimental condition, a deviation can have a large effect on the mean BOD curve. Overall, temperature positively affected the biodegradation rate in the positive control systems and verified the biodegradation protocol.

Biodegradation rates in crude oil experimental flasks with added Corexit showed great similarity to the crude oil experimental flasks at all temperatures. A slightly higher reaction rate was observed in flasks with crude oil and Corexit at 3°C compared to bottles with just crude oil. Whereas at 8°C, the opposite was observed (Figure 9 A and B). At 15°C, the BOD curves for crude oil with and without Corexit are essentially the same (Figure 9 C). This leads to the conclusion that dispersing crude oil with Corexit did not have any effect on biodegradation rates, or extent. The extent is assumed to be the same based on the similarity of the curves.

The BOD curves for the flasks containing only Corexit might provide some insight as to why Corexit did not affect biodegradation. At all temperatures, there is an exponential reaction rate period in the BOD curves for Corexit samples immediately, which lasts for a couple of days (Figure 8 C). Only at 3°C, a very small lag phase is observed. This suggests that the

dispersant was degraded very rapidly in seawater. This would only be important however, if Corexit acted as a preferred substrate over hydrocarbons in the crude oil. Looking at the BOD curves for crude oil samples with added Corexit (Figure 8 B), a small increase in reaction rate is revealed around day 6-7 at 3°C, day 3 at 8°C, and a small drop in degradation rate is observed around the same time at 15°C. This change in activity might have been the result of Corexit being degraded early on together with components in crude oil. This would mean that the dispersant does not meet one of the three requirements proposed by Mulkin-Phillips and Stewart (1974), that the dispersant must not act as a preferred substrate over the oil (Section 2.6.1.). Whether or not the dispersant actually dispersed the oil was not investigated here, but even if Corexit actually did have time to disperse the oil prior to being degraded, it did not result in increased biodegradation rates of crude oil. In such a small scale laboratory experiment, oil droplets could accumulate again once the dispersant is consumed and biodegradation would carry on as if the oil was never chemically dispersed in the first place. In the natural marine environment however, the likeliness of the dispersion reversing could be lower, depending on spreading, the oil/seawater volume ratio, water currents etc. Further studies are necessary to confirm whether or not Corexit is a preferred substrate, and if the oil stays dispersed once the dispersant is degraded. Future experiments should also include variations in oil and dispersant concentrations to determine if that has any effect.

Biodegradation rates were proportional to temperature in crude oil test flasks at temperatures 3 and 8°C. At 15°C, crude oil biodegradation rate showed a different trend where the degradation rate decreased towards the end (Figure 8 A). Overall, increased temperature showed increased biodegradation rate of crude oil. Large standard errors at 15°C were a result of one test flask deviating from the other two parallels but the curve trend was the same in all three flasks. It could be argued that the deviation is a rare exception from the general trend for biodegradation of crude oil at 15°C, but the same large deviation was found in one of the three parallel flasks containing crude oil with Corexit at 15°C (Figure 8 B). This indicates that there might be larger variations in reaction rates at higher temperatures compared to low temperatures.

Overall for the BOD experiment, temperature had a positive effect on the rate of biodegradation. The addition of the chemical dispersant Corexit did not affect the biodegradation rates and presumably not the final extent of degradation. The effect of Corexit 9500 on biodegradation have been variable (Lindstrom & Braddock, 2002), although certain studies have concluded positive effects of the presence of the dispersant during oil biodegradation (Prince et al., 2013). Temperature effects on the biodegrdation of crude oil concluded in this report are however quite consistent with findings in recently published studies (Brakstad & Bonaunet, 2006; Mohn & Stewart, 2000).

It is obvious to note that the absence of negative controls is not recommended and should be included in BOD analysis.

5.2 Analysis of hydrocarbon utilization (THC)

Prior to chemical analysis of experimental samples, calibration of the GC-FID method was performed using standard solutions of known alkane content and concentrations. Retention times were established for each reference hydrocarbon to identify the time range in which to calculate the total area response. The total area response was initially intended to be calculated between decane and tetracontane. Sample chromatographs showed however an unexplainable increase in response towards the end in samples containing Corexit. This was not found in any samples at t=0 analysis or samples with only crude oil, with the exception of crude oil at 15°C (Figure 12). Total extent of hydrocarbon removal based on the initially intended time period resulted in very low, and sometimes negative removal. The choice was therefore made to decrease the time range included in the total area response (from t=6.7-33min to t=6.7-22.5min), in order to exclude the "hump" from calculations. The sample chromatographs during end analysis (Figure 12-14) showed a general decrease in response before 22.5min, compared to initial samples. It could be interesting to analyse samples via GC-MS to identify which components contributed to this "hump" response and if those components were present initially. This could help explain the phenomenon. Unfortunately, there was not enough time to perform additional GC-MS analysis during this study.

GC-FID analysis of crude oil, and extracted crude oil revealed some hydrocarbon loss during the extraction and up concentration method. Analysis of the evaporated pentane during the up-concentration further revealed insignificant amount of hydrocarbon content, which led to the conclusion that hydrocarbon loss occurred during extraction. This hydrocarbon loss would have little effect on the THC analysis, as the loss would have been the same at both initial and end analysis, leaving the relative relationship between initial and end samples the same.

Hydrocarbon removal from crude oil over a time period of 46 days based on the integration method used was between 80 and 92% (Table 2). For crude oil, removal was 80% (+6/-7%), 86% (+/- 2%) and 84% (+/-1%) for 3, 8 and 15°C, respectively. For crude oil with Corexit, hydrocarbon removal was 82% (+/- 2%), 92% (+/-1%) and 86% (+/- 2%) for 3, 8 and 15°C, respectively. Temperature had little effect on the total hydrocarbon removal. Slightly higher removal was observed at 8°C compared with 3°C for crude oil with and without Corexit. The presence of Corexit increased the total hydrocarbon removal by 2% at 3 and 15°C, and 6% at 8°C. Taking error estimations into account, the increase was not significant at 3 and 15°C. Based on these results, the addition of Corexit 9500 to crude oil had an insignificant effect on the total hydrocarbon removal.

Hydrocarbon removal for Corexit was 97, 95 and 19% for temperatures 3, 8 and 15°C respectively. The low removal found for Corexit at 15°C might be a result of the high responses found in the corresponding chromatograph for Corexit during end analysis of samples (Figure 14). At the same time, the peak at the t=0 mark, appears to be smaller at 15°C compared with 3 and 8°C (Figure 14). Both of these factors contribute to a smaller difference between t=0 and t=end results. The differences in the largest peak reponse of the initial samples of Corexit at the different temperatures could be a result of inadequate quantitative transfer of Corexit to the sample seawater. The increase in peak responses at day 46 is possibly a result of contamination during extraction or up-concentration of the sample. An increase in peak responses was also found during end analysis (day 46) of the Corexit sample at 3°C (Figure 14). Overall, the same contamination occurred variably for samples during end analysis. The blank samples showed the same type of contamination and the total area response in blanks were subtracted from experimental sample responses

prior to hydrocarbon removal calculations. This would remove the contribution of this contamination during end analysis.

Errors were calculated based on the standard deviation of the mean response value for each sample, which was then used to create upper and lower limits of total hydrocarbon removal. Higher error was found for the crude oil sample at 3°C and the Corexit sample at 15°C. Since responses are based on extraction and up-concentration from a single BOD flask per sample, the errors were connected to random errors in the GC instrument.

5.3 Microbial community analysis

DGGE analysis revealed differences in banding pattern between experimental samples with different substrate contents (Figure 17). The banding pattern for both the bottles amended with crude oil and those with crude oil and Corexit were similar and certain bands were only found at these two experimental conditions. This indicates that the indigenous bacterial community performed selective enrichment of hydrocarbon degraders, causing a shift in the bacterial community profile when exposed to crude oil. This is consistent with other studies, which show changes in the microbial community following oil spill (Harayama et al., 2004). Due to the similarity of the banding patterns, the bacterial community can be assumed to have changed minimally with the addition of the dispersant. The community in samples containing only Corexit however, were different from other community profiles in the experiment. By using Corexit as substrate, an entirely different bacterial community developed. Sampling and community analysis of bottles with crude oil and Corexit early in the biodegradation process could reveal if the community was more similar to the community found in Corexit samples at that point. Since Corexit was consumed rapidly, it is possible that a potential different community was quickly outcompeted or developed into the community observed in crude oil samples. A microbial community study conducted with oil and a chemical dispersant in seawater samples supports this. The study showed that a distinct community developed after 6 hours in the presence of an oil-dispersant mixture, whereas with just oil the primary community was maintained the first 24 hours (Yoshida et al., 2006). Microbiological communities in both the oil-dispersant mixture and oil developed the same community structure after 24 hours

(Yoshida et al., 2006). The band profile for the crude oil sample at 15°C showed much lower band intensity compared to other crude oil samples. This could be due to quantity differences the samples, or inadequate sample volume transfer to that particular well mix. All blank samples developed a different community profile compared to the initial samples but a larger difference was observed at 15°C compared to the other two temperatures. Perhaps the community was subject to greater alterations and need for adaptation due to a larger temperature change compared to the in situ temperature and 3°C. Ultimately, it cannot be confirmed what has caused this change in community, but it is an interesting observation in relation with the large standard error seen on the BOD curves for flasks containing crude oil at 15°C (Figure 9 C). Perhaps these deviations in growth rate could indeed be a result of variations in population development.

MPN was used to enumerate hydrocarbon degraders in a liquid sample. This was done to observe whether or not the hydrocarbon degrading community increases during crude oil biodegradation. From the results (Figure 15), it was observed that the amount of hydrocarbon degraders in seawater increased over a period of 46 days, in flasks containing crude oil at 8°C, crude oil with Corexit at 3 and 8°C, and Corexit at 3°C. The amount of hydrocarbon degraders decreased in all experimental flasks at 15°C, in flasks containing Corexit at 8°C and crude oil at 3°C. The BOD curves for flasks containing crude oil at 15°C stagnates after approximately day 17 (Figure 9 C). This is typical when a growth process reaches the endogenous phase and could explain the loss of hydrocarbon degraders seen from the MPN results at 15°C. Due to the rapid biodegradation of Corexit in the experimental flasks with only dispersant, it is possible that an increase in hydrocarbon degraders occurred earlier in the biodegradation process. This particular hydrocarbon degrading community, if present, would have been subject to endogenous respiration earlier than the communities in samples containing crude oil, resulting in the loss of hydrocarbon degraders. Figure 8 C shows that the BOD curve for flasks containing Corexit at 8 and 15°C reached a plateau typical for the endogenous phase several days before the end of the experiment, consistent with the loss of hydrocarbon degraders seen in these samples (Figure 15). A decrease in hydrocarbon degraders was observed in positive controls and blanks, with the exception of a small increase in blank at 15°C. This is expected

due to the lack of hydrocarbon as a substrate; hence hydrocarbon degraders will not grow and the few present initially would be subject to endogenous respiration and death.

Based on the results found in this study, it is recommended to sample BOD bottles for enumeration of hydrocarbon degraders during the experiment to better observe growth trends during the biodegradation process. This would reveal what happens during the exponential growth phase and during the endogenous respiration phase. It would also show the hydrocarbon growth development in samples containing only Corexit. Data at only the beginning and the end of the experiment provide limited information. A previous study on biodegradation of crude oil in seawater showed an increase in the number of hydrocarbon degraders the first 10 days, followed by a decrease in numbers (Brakstad & Lødeng, 2004). Hydrocarbon degraders in crude oil samples and samples containing crude oil with Corexit could have been subject to endogenous respiration and loss of hydrocarbon degraders earlier than day 46, and earlier sampling could investigate this. Overall, MPN analysis showed that the amount of hydrocarbon degraders increased in most samples where hydrocarbons were available, except at 15°C, where the hydrocarbons degraders were subject to endogenous respiration.

5.4 Experimental errors

Errors during the biodegradation experiment, chemical and microbiological analyses were classified as either systematic or random errors. Potential sources of errors in solution preparation included inadequate quantitative transfer of ingredients. Errors in BOD analysis included cross contamination and loss of BOD data. Errors in the liquid-solvent extraction included loss of total hydrocarbon content. Possible errors in GC-FID analysis included potential contamination from column residuals and solvents. Potential errors during DGGE and sample preparation include cross sample DNA contamination during DNA filtration and extraction, PCR bias, band co migration and multiple bands per population. Sources of errors during MPN analysis include inadequate quantitative transfer of MPN solutions and cross contamination between MPN wells.

Standard error was calculated for each of the BOD and MPN triplicates sample averages. For the GC-FID analysis, no error calculation was possible for the sample flasks as they were analysed as one sample per triplicate. However, of each sample flask, four parallels were analysed via GC-FID. Average response and standard deviation for each sample based on random errors in the GC instrument were calculated.

6 Conclusions

The aerobic biodegradation of a light crude oil was evaluated in seawater at different temperatures, with and without the presence of the chemical dispersant Corexit 9500. Biological oxygen demand was monitored over a period of 46 days in sealed systems. Total hydrocarbon removal was determined by initial and end analysis of total hydrocarbon content via liquid-liquid extraction, up concentration and gas chromatography analysis. Microbial community profiles at the start and at the end of the experiment were investigated using DNA extraction, PCR amplification of the 16S rRNA bacterial gene, followed by denaturing gradient gel electrophoresis. Most probable number was used to enumerate hydrocarbon degraders before and after the biodegradation process. The chemical and microbiological analyses of crude oil biodegradation were performed to increase the understanding of crude oil biodegradation and the effects of chemical dispersants on biodegradation at different temperatures.

The primary conclusions related to the research hypotheses (see section 2.8) are as follows:

1. Biodegradation rates decreased with decreasing temperatures and the effect of Corexit on biodegradation did not change at different temperatures.
2. Biodegradation rates did not increase in the presence of the chemical dispersant Corexit. The dispersant was degraded rapidly in seawater and acted as a preferred substrate over crude oil based on the BOD results.
3. Microbial communities changed when substrates changed. A distinct microbial community was developed where Corexit was the only carbon source. Microbial communities that were observed in the presence of crude oil were similar regardless of

the temperature and addition of Corexit. The amount of hydrocarbon degraders increased when exposed to crude oil at 3 and 8°C, but decreased at 15°C due to endogenous respiration.

6.1 Further investigations

Further investigation on both temperature effects and the effect of dispersants on biodegradation are recommended. Bacterial enumeration should be performed at time intervals during the BOD experiment, and not only at the start and end of a biodegradation period, to monitor the bacterial growth over time and uncover growth trends of hydrocarbon degraders during biodegradation. Preferably, sampling of BOD flasks should be performed during the biodegradation process, for total organic carbon analysis and to uncover trends in hydrocarbon utilization. The effect of Corexit 9500, and other commercially available dispersants should be studied further to investigate the significance of the early and rapid degradation of the dispersant.

6.2 Implications

Laboratory experiments should be developed to ultimately extrapolate findings to natural systems. However, a synergy of complex factors (sunlight, water currents, predation etc.) makes biodegradation in nature complicated and difficult to study and compare with laboratory experiments. Despite these complications, an obvious recommendation is to decrease petroleum outputs to the ocean. It is also correct to assume that, despite local variations that may occur, crude oil degradation is generally slower in cold temperature marine systems, as the effect of temperature on oil and petroleum degradation is a factor heavily agreed upon, and findings (decreased biodegradation rates with decreasing temperatures) are similar in many studies, including this one. As a result, petroleum offshore activity in the arctic regions comes with great risk. Further analysis is required however, for proper environmental risk assessments.

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Appendix A – Theoretical information

Monod kinetics:

$$u = u_{max} \frac{S}{K_s + S}$$

Where $S << K_s$

Arrhenius equation:

$$k = Ae^{-Ea/RT}$$

k	Rate constant
A	Pre-exponential factor
E_a	Activation energy (J/mol)
R	Gas constant (8.314 J/mol ⋅ K)
T	Temperature (K)

Relationship of BOD and pressure ("OxiTop System Control Operating Manual," 2006):

$$BOD = \frac{MW(O_2)}{R \cdot T_m} \cdot \left(\frac{V_t - V_l}{V_l} + \alpha \frac{T_m}{T_0}\right) \cdot \Delta p(O_2)$$

$MW(O_2)$	Molecular weight (32 000 mg/mol)
R	Gas constant (83.14 L ⋅ mbar/mol ⋅ K)
T_0	Reference temperature (273.15 K)
T_m	Measuring temperature (K)
V_t	Bottle volume (mL)
V_l	Sample volume (mL)
α	Bunsen absorption coefficient (0.0310)
$\Delta p(O_2)$	Difference in oxygen partial pressure (mbar)

No. of tubes giving positive reaction out of			MPN index	95% confidence limits	
3 of 10 mL each	3 of 1mL each	3 of 0.1 mL each	per 100 mL	Lower	Upper
0	0	0	<1		
0	0	1	3	<0.5	9
0	1	0	3	<0.5	12
1	0	0	4	<0.5	20
1	0	1	7	1.0	21
1	1	0	7	1.0	23
1	1	1	11	3.0	36
1	1	0	11	3.0	36
2	0	0	9	1.0	36
2	0	1	14	3.0	37
2	1	0	15	3.0	44
2	1	1	20	7.0	82
2	2	0	21	4.0	47
2	2	1	28	10.0	150
3	0	0	23	4.0	120
3	0	1	39	7.0	130
3	0	2	64	15.0	380
3	1	0	43	7.0	210
3	1	1	75	14.0	230
3	1	2	120	30.0	380
3	2	0	93	15.0	380
3	2	1	150	30.0	440
3	2	2	210	35.0	470
3	3	0	240	36.0	1300
3	3	1	460	71.0	2400
3	3	2	1100	150.0	4800
3	3	3	>2400		

Figure 18: MPN Table. For 0.1, 0.01 and 0.001 combination, 100 times the value given in the table should be used. Source: http://www.eplantscience.com/botanical_biotechnology_biology_chemistry/enivronmental_science_engineering_laboratory_methodology/preparation_of_reagents_and_media.php.

Appendix B – Experimental information

Table 3: Chemical properties of saturated hydrocarbons used in calibration solutions.

Formula	Chemical	Supplier	Density (kg/l)	State at (20°C)
C9H20	Nonane	Merck-Schuchardt	0.718	Liquid
C10H22	Decane	Merck-Schuchardt	0.729	
C11H24	Undecane	Aldrich	0.740	
C12H26	Dodecane	Riedel-de Häen	0.750	
C13H28	Tridecane	Aldrich	0.755	
C14H30	Tetradecane	Fluka	0.762	
C15H32	Pentadecane	Aldrich	0.769	
C16H34	Hexadecane	Riedel-de Häen	0.770	
C17H36	Heptadecane	Aldrich	N/A	Solid
C18H38	Octadecane	Aldrich	N/A	
C19H40	Nonadecane	Aldrich	N/A	
C20H42	Eicosane	Fluka	N/A	
C22H46	Docosane	Aldrich	N/A	
C24H50	Tetracosane	Fluka	N/A	
C28H58	Octacosane	Fluka	N/A	
C32H66	Dotriacontane	Fluka	N/A	
C36H74	Hexatriacontane	Fluka	N/A	
C40H82	Tetracontane	Fluka	N/A	