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The Effect of the Dispersant Corexit 9500 on the Biodegradation of Diesel Fuel at Low Temperatures

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Marit Larsen

The most exciting phrase to hear in science, the one that heralds new discoveries, is not "eureka!" but "that's funny..."

- *Isaac Asimov*

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Marit Larsen
15th June, 2015

ABSTRACT

Crude oil and different petroleum products are marine polluters and have become an important environmental issue in the last century. Hydrocarbons are inherently present in the oceans due to natural seeps, but the main source of petroleum pollution is anthropogenic sources. Biodegradation is an important process in oil spill remediation, and several chemical dispersants have been developed to aid the degradation process by rapidly dispersing the oil to increase its bioavailability. This study was performed to investigate the effect of the dispersant Corexit 9500 on biodegradation of diesel at low temperatures. Chemical and microbiological methods were used to analyse the biodegradation process at 3, 8 and 15°C over 60 days. Biological oxygen demand (BOD) analysis indicated an increase in the growth rates of the microbial community with increasing temperature and in the presence of Corexit 9500, while a total hydrocarbon analysis using GC-FID revealed that the amount of diesel degraded was independent of temperature and ultimately unaffected by the presence of Corexit 9500. Corexit did not affect the biodegradation of diesel at colder temperatures. Corexit itself was also biodegraded seemingly independent of temperature. Molecular analysis by denaturing gradient gel electrophoresis (DGGE) revealed changes in the intrinsic microbial community in the presence of Corexit 9500 and diesel, where the communities adapted depending on the substrate available. The microbial community was enumerated using Bushnell-Haas plates with Arabian crude oil, and due to large variations in the counts the method must be improved for more consistent and reliable results. Further research is recommended to better understand the effect of chemical dispersants on biodegradation at low temperatures to develop suitable methods for hydrocarbon pollution remediation. In addition, it is recommended to focus on improving methods for cultivation of hydrocarbon degraders.

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Abbreviations

APS - ammonium persulfate polymerising agent
ATP – adenosine triphosphate
BOD – Biological oxygen demand
CMC - critical micelle concentration
DGGE - Denaturing Gradient Gel Electrophoresis
DNA – Deoxyribonucleic acid
FID – flame ionization detector
GC – gas chromatography
GC clamp – guanine/cytosine clamp
Kbp – kilobase pairs
LD – lethal dose
NAPL – nonaqeous-phase liquid
ORR - US Office of Response and Restoration
OSCAR - The Oil Spill Contingency and Response model
PAH – polyaromatic hydrocarbons
PCR – polymerase chain reaction
POP – persistent organic pollution
RNA – ribonucleic acid
rRNA – ribosomal RNA
rrn – ribosomal RNA operon
THC - total hydrocarbon
UV – ultra violet
VOC- volatile organic compounds

1 INTRODUCTION

Crude oil is naturally occurring oil, which is often refined into different petroleum products. It consists mainly of hydrocarbons, asphaltenes and resins, paraffins, sulphurs and ash (Simanzhenkov & Idem, 2003). Hydrocarbons from crude oil and different petroleum products are naturally present in the environment through seeps from oil reservoirs, but it is production of oil and other anthropogenic activities that has lead to an accumulation of oil and petroleum in the environment (NRCC, 2003). This increased presence of hydrocarbons may cause long term toxic effects on the organisms living in the affected areas, or accidents and spills may cause acute effects such as death (Walker, 2006). In addition to natural seeps production of crude oil, marine vessel activities, industrial and municipal runoffs and planned or accidental releases all contribute to an increase in the amount of hydrocarbons present in the environment (Deppe, Richnow, Michaelis & Antranikian, 2005), where the majority of hydrocarbons released into the environment come from anthropogenic sources (Leahy & Colwell, 1990). Though oil spills are not considered to be the most important source of hydrocarbon pollution they usually have the most severe effects due to the high local concentrations of oil (Kaiser et al., 2005).

Oil spills in marine environments have severe and numerous consequences, where direct effects such as oily birds and fish death and more long term effects that may disturb feeding, reproduction and growth are serious issues (Walker, 2006). These effects may ultimately lead to changes in populations, communities or entire ecosystems (NRCC, 2003; Walker, 2006). Oil that reaches the marine environment is usually partially removed by conventional methods, such as containment of the oil using booms, burning of the oil and mechanical removal. Spilled oil is also subjected to the natural weathering process. This is a continuous process that starts immediately after oil reaches the water, where large part of the oil will evaporate and be weathered by wave and wind action. Oil will be dispersed, photooxidised and biodegraded at different stages of the natural process, and some of the oil will sink or spread and reach shore (Fingas, 2013). Marine environments are the largest recipients of hydrocarbon pollution, and it is important to examine how to effectively

manage the pollution issue (Atlas, 1981). Biodegradation is considered to be the major natural mechanisms for hydrocarbon removal in the environment, and involves a complex and diverse microbial community (Alexander, 1999). The degradation process is dependent on the microbial populations preferences of substrate, the bioavailability and concentration of the hydrocarbons, the temperature in the environment and the overall properties of the hydrocarbons (Atlas, 1981). To aid in oil spill cleanup, bioremediation has been used. This is a method where the intrinsic population of microorganisms present at a spill site are stimulated to increase the amount of hydrocarbons degraded, which is often performed by adding nutrients or oxygen (Filler et al, 2008). Increased dispersion of the oil by application of chemicals is another approach, where the aim is to make the oil more available for biodegradation and other natural degradation processes (Swannell & Daniel, 1999). Dispersants consist of solvents, surfactants and other compounds that separate oil (NRCC National Research Council Committee, 2005).

Major parts of the transportation of oil and petroleum products occurs in cold environments (Deppe et al., 2005), and as the exploration of oil is increasing in arctic areas research on hydrocarbon biodegradation in cold water is becoming more important (Symon & Skjoldal, 2010). Research on the use of dispersants and other remediation methods in the environment is therefore important to prevent harm to these vulnerable environments.

This study investigated the differences between the biodegradation rates at three different, but still relatively low, temperatures and if the presence of a chemical dispersant would increase the amount of hydrocarbons degraded compared to natural biodegradation where no remediation was initiated. This study also looked at the changes in the microbial population to investigate if the presence of a dispersant changed the structure of the microbial community compared to samples incubated with diesel. This was performed by incubating seawater samples at different temperatures with diesel as a hydrocarbon source and with and without a dispersant, and by looking at changes in the microbial community using denaturant gradient gel electrophoresis (DGGE).

2 BACKGROUND

Understanding the impact hydrocarbons have on marine and terrestrial ecosystems and how they enter the environment is important when discussing petroleum production and transportation. Degradation of hydrocarbons that have entered the environment through natural sources, spills or releases is depended on biochemical and environmental factors, the source and composition of the hydrocarbons in question and the execution of the clean-up process (Atlas, 1981). Biodegradation of petroleum hydrocarbons in marine environments depends on diverse factors, and an understanding of these processes is vital for aiding the rate of biodegradation and for developing reliable methods for bioremediation. Chemical dispersant are widely used to enhance biodegradation rates of spilled hydrocarbons, but as their effect and toxicity are not completely understood further research is required.

2.1 Petroleum hydrocarbons in the marine environment

Petroleum products end up in the marine environment from many sources, such as serious oil spills, natural seepage and leaks from marine vessels. According to the U.S Department of Energy, 4.9 million litres of petroleum hydrocarbons are spilled annually from vessels and pipelines into U.S waters alone (Symon & Skjoldal., 2010). A major oil spill could double this, and the total amount of petroleum hydrocarbons released into the oceans is most likely many times this amount. In addition, a chronic leakage of hydrocarbons affects organisms and environments in the long term (Walker, 2006).

2.1.1 Sources of petroleum that ends up in the marine environment

Continental shelves, rocky shores, coral reefs and other marine environments support a great diversity of living organisms in different ecosystems. Organisms living here are a part of a vulnerable and well-regulated community, where their flexibility gives rise to a complex system of feeding and recirculation. Disturbance, pollution and climate change

contribute to ecological changes in the marine ecosystems, where the human activities have had the most significant impact (Kaiser et al., 2005).

There are four major sources to petroleum oil pollution, disregarded accidents: a) natural seeps, b) releases that occur during extraction of crude oil, c) transportation of petroleum products and d) extensive consumption of oil and oil-based products (NRCC, 2003). Extraction of oil and gas causes discharge from platforms and other offshore installations and the release of volatile organic compounds (VOC) into the atmosphere. Spills include spills from offshore pipelines and platforms, the escape of volatile compounds from production, transportation and refining of hydrocarbons and discharges of produced water during production (NRCC, 2003).

2.1.2 The effects of petroleum hydrocarbons on aquatic life

Pollution released into the marine environment may lead to serious effects for both the marine life and the surrounding environment. Even though oil spills and the release of persistent organic species (POP) have been greatly reduced, pollution is still a major threat to the marine environment. Costal vegetation habitats, such as coal reefs and mangrove forests, function as buffers at the shoreline and are the home of thousands of species of fish and other animals, and these habitats are adversely affected by oil spills that reach the shoreline (United Nations Environment Programme, 2006). One of the more acute environmental effects is death, but a reduction in fitness, general disruption of the structure and function of the marine ecosystems and communities and other sublethal effects are also observed. Seen from an ecotoxicological aspect, the effects are generally divided into acute or chronic depending on the nature of the spill or discharge (NRCC, 2003; Walker, 2006)

Effects of hydrocarbons released in the marine environment are visible at four levels (United Nations Environment Programme, 2006):
1. Biochemical and cellular, where the effects may be a change in hormones, metabolism disturbance, or other critical effects in cells.

2. An effect in an organism, which may include behavioural, physiological and also biochemical consequences.
3. A change in population, where the most vulnerable effect is a change in the population dynamics by changing birth or death rates, affecting the lifespan of a population or otherwise changes in the structure.
4. A change in the structure of a community, where one species might be dependent on the other for survival, or the community is vulnerable for changes resulting in alterations in the structure and dynamics (NRCC, 2003; Walker, 2006)

There are many parameters to which the effects of pollution are measured. When pollutants that are slowly degraded, or not degraded at all, enter the marine environments they have the potential to travel over large distances. Most are lipophilic and are thus easily stored in the fat deposits of animals, while others are xenobiotic (Jeffrey, 1991). The compounds acting as toxins are usually metabolised into less harmful substances, but this metabolism process may also activate these molecules causing an interaction with other molecules or macromolecules present in an organism causing a toxic effect (Guengerich & Liebler, 1985). The problem with toxin storage in lipids in marine organisms is *biomagnification*, where pollutants become more concentrated from one trophic level to a higher in a food chain and eventually accumulates to high concentrations in the species at higher levels of the food chain (Walker, 2006). Some toxins act upon the mitochondrial membranes inhibiting the synthesis of ATP, while others act as carcinogens. Some substances act as neurotoxins and disturb the natural transmission of impulses in the nerves and synapses, or causes an enhancement of female hormonal processes disturbing growth regulation mechanisms, while other cause changes in behaviour and reproduction habits (Walker, 2006).

When pollutants affect an ecosystem the impact is not only seen on an individual level but also in the population dynamics where the number of some species might decline and level out to a lower number than previously, or a species could be locally extinct. Changes in population dynamics is often due to chronic pollution, and if this pollution persists the communities will often adapt and the population levels will be constant as long as the pollution rate does not change (Sheehan, 1984). The effects of an oil spill on different

environments are diverse, and affect the different organisms and habitats depending on where the spill occurs and the time of year. Fish are affected by aromatic hydrocarbons that have been dissolved in the waters, and lethal doses can occur in confined waters (Fingas, 2013). Slicks of oil from spills will affect the feathers of birds by removing their insulation and causing them to freeze to death, especially in cold environments (Symon & Skjoldal., 2010) and oil can be transferred and kill chickens in the eggs (Fingas, 2013).

2.1.3 Understanding the risk – the importance of prevention and contingency plans

Production and consumption of petroleum products are increasing and are considered a necessity in modern life. The majority of the oil and petroleum products are used as fuel, but they are also an essential part of the production of plastics, fertilizers and chemical feed stocks, leading to increased transportation of oil across the world's oceans which drastically increases the risk of oil spills (Fingas, 2013).

Oil spills are a frequent occurrence, but the accidental spills from tankers have decreased in the last 40 years (Eckle, Burgherr & Michaux, 2012). Although the major source of oil spills are from tanker accidents, these spills make up less than 5% of the total amount of oil polluting the environment, and according to a report published by the National Research Council in 2003, a total of 46% of the oil polluting the environment originates from natural seeps. Because these releases are slow the pollutants are usually degraded before causing any damage (NRCC, 2003). A rapid and effective response to oil spills results in less damage on the environment as the oil is removed before it reaches the shore or can be deposited on the ocean floor (Fingas, 2013).

Contingency plans are used for detailed planning, so that the response to an oil spill can be as quick and efficient as possible. According to the EPA Office of Emergency and Remedial Response (1999) a contingency plan covers different scenarios and outlines the hazards involved. It usually consists of a vulnerability analysis, a risk assessment and an outline of the response actions. It is impossible to know when a spill will occur, but it is possible to know what is being transported, what volumes a tanker contain and where it is going.

Response actions is a listing of who needs to be contacted in the event of an oil spill, and the major acts that will need to be set into action. Preparing for an oil spill minimises the possible harms to the environment and human health and safety, and well-designed contingency plans makes the coordination of personnel and equipment as simple as possible (EPA Office of Remedial and Response, 1999).

In order to protect the environment, the human population and the organisms living in the ocean and on the shorelines, avoiding pollution and having clear and updated contingency plans are important. Preventing pollution is the best strategy, but as most spills are accidental it is impossible to know when or where they will occur, and it can be hard to determine how severe the spill may be (EPA Office of Remedial and Response, 1999).

When it comes to prevention of oil spills, the most important thing is to keep the equipment and transportation vessels in good condition. Many pipelines used for oil and gas transportation are past their lifetime and have shown signs of erosion and the increase of tanker transport due to drilling in the Arctic areas are possible risk sources for oil spills. (Zlotnikova et al., 1999). Blowouts are quite rare but do also represent a spill source, and the modern engineering solutions have decreased the chances of serious oil spills in operational activities (Symon & Skjoldal, 2010). Regulating agencies are also a vital part of oil spill prevention where these, usually governmental, agencies can perform regular controls and unannounced inspections to ensure that the producers follow the regulations of health, environment, safety and production in conjunction with oil and gas activities (Cohen, 1987).

2.2 The fate and treatment of petroleum hydrocarbons released into the environment

An oil spill is the release of liquid petroleum hydrocarbons into the environment, particularly the marine environments, but spills may also occur on land. When oil reaches the marine environment it is subject to numerous processes that occur naturally in the marine environments. Fingas (2013) describes processes such as natural weathering of the

oil by waves, oxidation of the hydrocarbons, evaporation and decomposition by microorganisms, which are only a few of the processes that aid in an oil spill clean-up. These processes occur at different stages of the duration of the spill. Conventional cleaning is also used in an oil spill clean-up process. In conventional cleaning the spilled oil is physically removed from the waters and shorelines and kept from spreading by using oil containment booms to prevent oil from reaching the shore or vulnerable locations (Fingas, 2013). Spills may take weeks, months or years to completely clean and spills in colder climates are more challenging due to lower temperatures, which slow the natural processes and reduces evaporation (Atlas, 1991).

There have been many serious spills of oil, both from platforms and tankers transporting crude oil or refined oil products. One of the most well known accidents, even though it was far from the largest, is the Exxon Valdez oil spill off the coast of Alaska in 1989 that covered major areas of coastline and ocean. Research performed after this spill monitored the processes that occurred immediately after the oil was spilled and up to three years later (Wolfe et al., 1994). The more recent Deepwater Horizon spill in the Gulf Of Mexico in 2010 is ranked as the 5th largest oil spill recorded and has provided an opportunity to study the effect of dispersants and changes in microbial communities in the presence of oil (Krauss & Robertson, 2010; Kujawinski et al., 2011; Kostka et al., 2011). Figure 2.1 shows the long-term impact of the different weathering processes. From the figure it can be seen that floating barriers contained much of the oil at the start of the spill, and that biodegradation processes played a large part in degradation of the oil at a later time. A dispersant was applied to the spill, but due to a small amount of mixing caused by small waves the use was discontinued (Gilson, 2006).

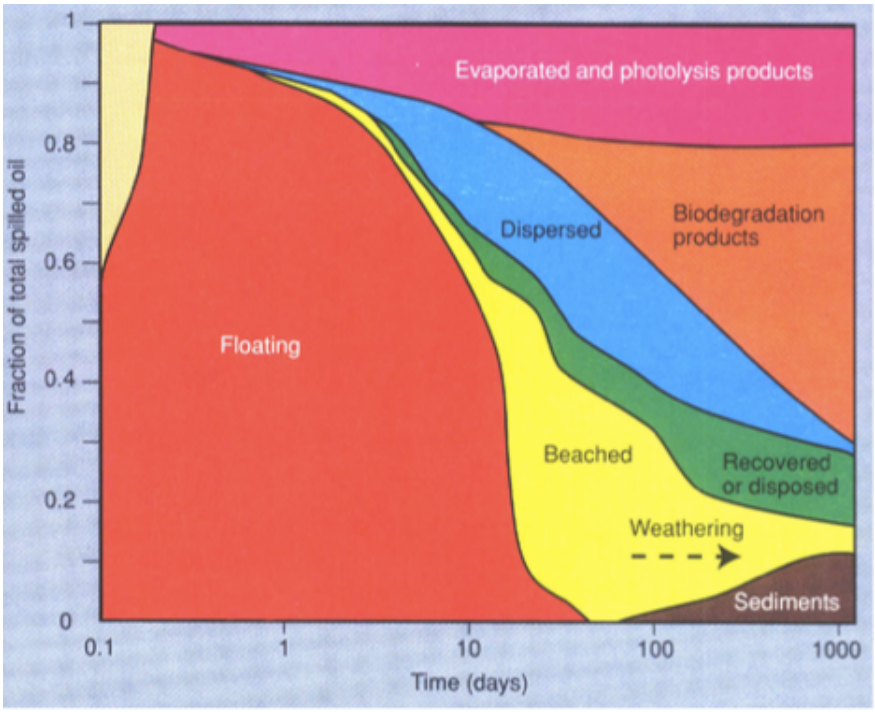

Figure 2.1 Oil degradation processes monitored after the Exxon Valdez spill on the coast of Alaska. After a tanker with crude oil hit a reef on the coast of Alaska, the processes of oil degradation were monitored over time to determine the impact of the different processes that are involved in an oil spill clean-up. It is apparent that floating and biodegradation are the two main processes (Wolfe et al., 1994).

Oil spills have a severe impact on the environment and life in the affected area. However, the occurrences of oil spills have provided a valuable opportunity to study the processes that aid in the degradation of the spilled hydrocarbons.

2.2.1 Oil spills in cold environments

With the increasing exploitation of oil production in cold areas, such as the Artic and the Barents sea, the issues and dangers that are associated with the increasing production, transport and storage of oil are becoming even more important to address (Yang et al., 2009). There has been indications that there is a higher risk of hydrocarbon pollution when transporting oil in areas with a cold climate (Energy Sector Management Assistance Program, 2003), and seen in context with the more difficult oil spill clean-up in these environments there is need for a safer and more efficient method of oil spill clean-up.

Biodiversity in cold environments is more sensitive to changes, and the same levels of contaminations that have a small impact in warmer climates may have a much greater impact on environments in cold regions (Snape, Riddle, Filler, & Williams, 2003). This is a consequence of the organisms' adaption to a very specific climate and environment found in cold regions, where minor changes in their habitats may have fatal consequences for the populations living there (Zacharias & Gregr, 2005).

Low temperatures, darkness and ice cover retard the loss of hydrocarbons by evaporation, microbial metabolism and photolysis causing the exposure duration to increase for organisms living in cold conditions (Symon et al., 2010). Microbial degradation is generally low in these areas (Collins, Racine & Walsh, 1993). As the temperature lowers, the microbial metabolism will slow even when oxygen and nutrients are present in adequate amounts (Atlas, 1977; Brakstad, 2008). Despite the general biochemical disadvantages caused by the cold, there are many organisms that thrive at low temperatures and microbial degradation of hydrocarbons occur event at temperatures as low as 0°C and down to -12°C (Margesin & Schinner, 1999). Adaption to low temperatures can have both genotypic and phenotypic modification reasons (Russell et al., 1990). It exists little evidence that the potential for microbial degradation of hydrocarbons is lower in cold regions compared to in warmer climates, but the annual window of opportunity is shorter in colder regions (Filler et al., 2008).

When oil is spilled into environments infested with ice, such as the Artic environment, the spreading, distribution and weathering process that contribute to the degradation of oil will differ significantly from the same processes in ice-free waters (Symon & Skjoldal, 2010). Figure 2.2 illustrates the ice and snow formations and how these can encapsulate spilled oil.

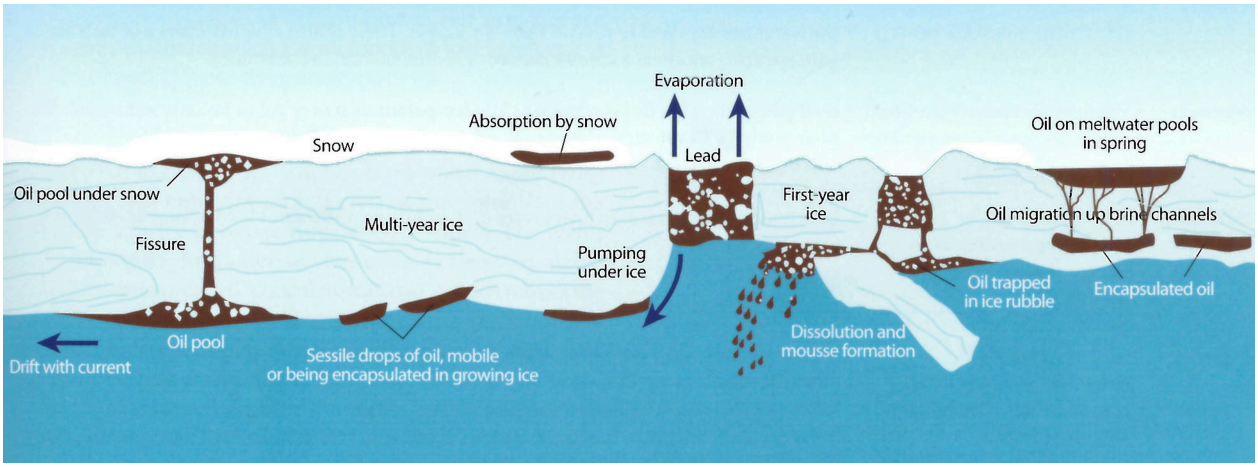

Figure 2.2 Movement of oil in cold and icy environments. Ice prevents oil from spreading, making it more difficult to degrade. Oil may also become trapped in the ice, restraining degradation. Oil can remain in melting pools, be pumped under the ice, encapsulated into the ice or be adsorbed by snow (Symon & Skjoldal, 2010).

Low temperature in the environment causes the degradation process to become slower. This is due to the increased viscosity of the oil, the effect of temperature on the involved enzymes and the effect on the microorganisms' membrane and general biochemistry (Margesin & Schinner, 1999).

2.2.2 The toxicity of diesel compared to crude oils and refined petroleum products

After a spill, diesel fuel will evaporate at the square root of time for the first few days, and the rate of evaporation will rapidly slow with time (Wolfe et al., 1994). Diesel fuels are highly degradable as they are largely composed of biodegradable saturated hydrocarbons and it usually takes only a few weeks for 50% of the diesel to biodegrade under optimum conditions compared to years for heavier oils (Fingas, 2013). Diesel fuels disperse significantly if the saturate content is high and the asphaltene and resin levels are low, and they usually disperse well both naturally and when a dispersant is added (Fingas, 2013).

As the biodegradation process begins, the diesel is rapidly degraded in the beginning before the rates slow significantly, and a study by Mukherji et al. (2004) indicated that the degradation of diesel is associated with the active log growth phase of the bacterial community. The study gave indications that the aliphatics were degraded first during the

first week of degradation, and the fraction could have been as high as 80% of the total weight of the degraded material. Aromatics were the second preferred species in the diesel even though it only constituted a mere 12.5% of the degraded material. The rate of degradation was much higher under aerobic conditions compared to degradation by microorganisms living in an anaerobic environment.

Diesel fuel shows the same toxicity rates as other crude oils with a similar density, but often contain additives that improve the properties of the fuel under different weather conditions that could possibly result in an increased toxicity for diesel fuels (Neff, Ostazeski, Gardiner, & Stejskal, 2000). Diesel is highly volatile, and if released in an environment with a high energy level and turbulent motion created by waves and current the long-term effects on the environment are considered few (Cripps & Shears, 1997). Diesel consists of a complex mixture, where the ideal composition is a high level of straight-chained alkanes. In addition to alkanes, diesel fuels also contain branched alkane compounds and aromatics with one or more aromatic ring, which increases the temperature that will evaporate the fuel (Knothe, 2010).

Small spills of diesel (between 2000 and 20 000 litres) will most likely evaporate, disperse and weather through natural processes, even in cold waters, as diesel oils spread quickly to a thin film that spreads on the water, making it more available for dispersion and degradation (Fingas, 2013). The US Office of Response and Restoration (ORR) (2015) reports that diesel is easily dispersed at the site of the spill when the winds reach 10 km/h or more, or if the waves break, and a specific gravity between 0.83 and 0.88, compared to 1.03, for seawater prevents the diesel from sinking and accumulating on the ocean floor. Diesel fuels do not attach themselves to the shoreline as is common with heavier crude oils, and when deposited onshore waves, tidal flushing and rain rapidly wash it away. Diesel is however considered one of the most acute toxic oil types, due to the amount of additives, and fish, invertebrates and seaweed are affected if these organisms come in direct contact with diesel (ORR, 2015).

2.2.3 Conventional clean-up of oil spills

Though it is important to focus on the prevention of oil spills, a rapid and effective response to oils spills will result in less damage to the environment (Fingas, 2013). According to Atlas (1981), no two oil spills are the same, and they act differently depending on the properties of the hydrocarbons spilled, the location of the spill, and temperature and weather conditions in the affected area. There are several methods involved in oil spill remediation that are used independent of the weather and environmental conditions. Oil will to a large extent break down by natural processes (see chapter 2.2.4), but in many cases the oil will reach shore and cause extensive harm to the shoreline and the organisms living there. It is therefore considered important to prevent as much as possible of the oil from reaching the shoreline. This is most commonly accomplished by the use of oil containment booms and collection of the oil on the water surface by skimmers, the use of dispersants to break up the oil and aid the natural biodegradation and controlled burning of the surface oil (Fingas, 2013).

2.2.4 Natural weathering of oil spills

When oil is spilled it undergoes weathering and breakdown. This process is natural, and consists of several different processes each of which have an impact on the amount of oil left in the environment. These processes have varying importance in the final breakdown of the oil spill in terms of the percentage of loss of oil from each mechanism. Weathering processes start immediately after oil enters the environment, but occurs at very different rates depending on the climate and environment of the location of the oil spill. The most important factor for determining the rate of weathering is the type of oil, where heavy crude oils are much harder to break down compared to lighter oils (Fingas, 2013; Atlas 1981). The natural weathering process is complicated, and Figure 2.3 illustrates the different processes that occur during natural weathering of oil spills.

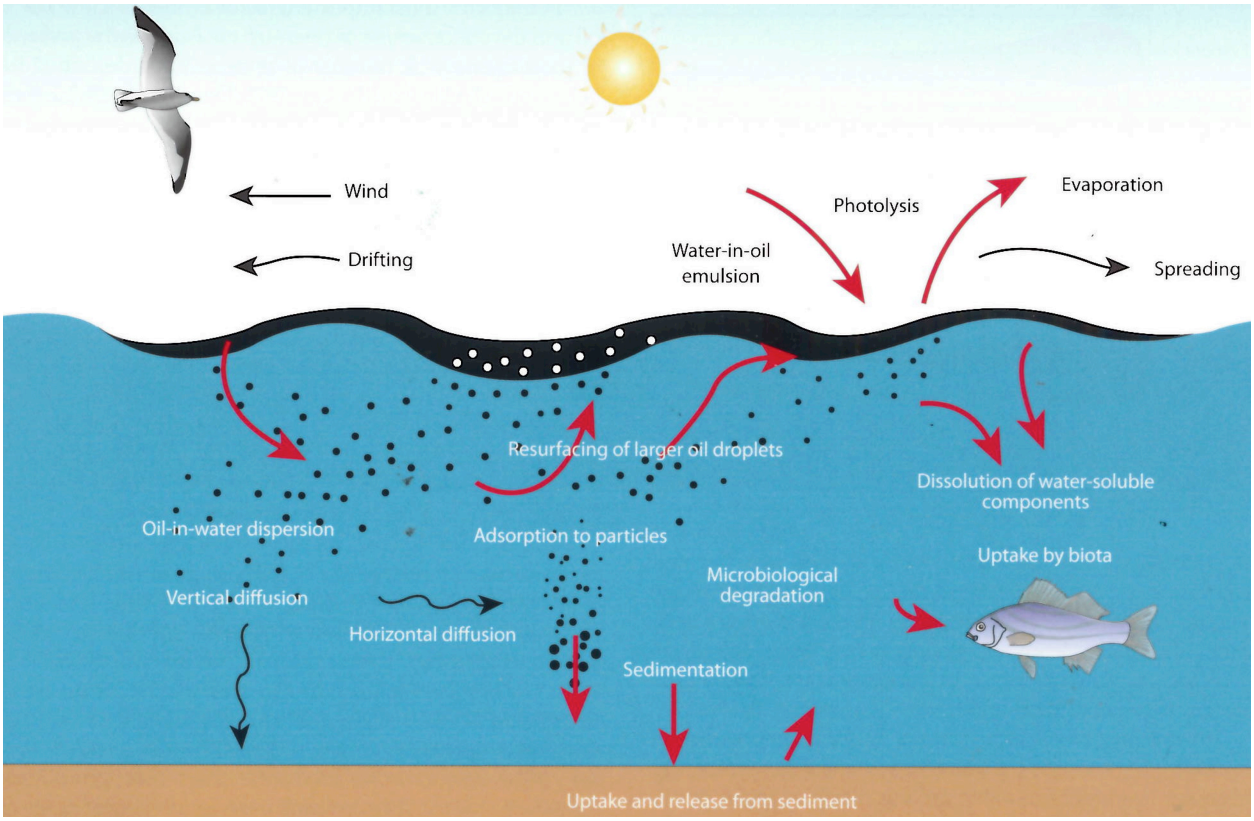

Figure 2.3 Natural weathering of oil spills. As oil is released into the environment, a natural weathering process starts immediately. Weathering consists of several mechanisms that work together to break down a large portion of the oil by processes such as evaporation, oxidation, sedimentation and biodegradation (Symon et al., 2010)

Evaporation of the oil has the greatest effect on the amount of oil that remains after a spill. The composition of the oil determines the rate of evaporation, where light fuels such as gasoline will evaporate off quickly at any temperature above zero where the oil will evaporate off at a high rate for the first few days, followed by a slower rate due to the heavier oil compounds which remains (Fingas, 2013). Evaporation is well documented as an important process in weathering, as it has been shown that the various compounds are depleted from the oil in accordance to molecular size rather than molecular structure and is usually quite rapid for the first three months (Ezra, Feinstein, Pelly, Bauman, & Miloslavsky, 2000). Evaporation does not noticeably change with increasing wind speeds, and an increased surface area does not affect the evaporation rate (Fingas, 1999).

Water and oil can mix and form an emulsion. Fingas (2013) describes several issues related to emulsions between oil and water, which impacts clean-up and weathering of oil: Formation of an emulsion is an important event in oil spill weathering, as it may increase the volume of the spill as the oil may contain 50% to 70% water. Depending on the emulsion that forms, the viscosity of the oil may increase, making the clean-up operation more difficult. Oil that has formed a stable emulsion is difficult or impossible to ignite (Putorti, Evans & Tennyson, 1994) and makes skimming and recovery difficult (Fingas, 2013). Emulsions slow biodegradation, and greatly reduce evaporation. In addition water uptake may occur, a phenomenon that is very similar to emulsions. During this process, water is not held long enough in the oil to form a stable emulsion, as there is not enough water mixed with the oil to do so. If the oil is very viscous water droplets can penetrate the oil when the sea is rough and exit the oil slick when the sea calms. Special chemicals can usually break down emulsions. Oil may also disperse, which is the process where droplets of oil enter the water by the disturbance of waves. Natural dispersion may be minimal, but in many cases it removes a part of the oil as it spreads in the water. Some of the lower weight aromatics can dissolve in the water and are removed from the oil and causing aquatic toxicity (Fingas, 2013; Kleindienst, Paul & Joyce, 2015).

A significant part of the oil will be oxidised, usually by photooxidation (Garrett, Pickering, Haith, & Prince, 1998). UV rays from the sun will cause the carbon and oxygen molecules to combine to new products that are usually more soluble in water (Fingas, 2013). Photooxidation is thought to selectively degrade alkylated aromatic compounds as compared to biodegradation which targets unsubstituted aromatic compounds (Garrett et al., 1998).

Fingas (2013) also describes what happens to oil that is not degraded at the water surface: Smaller portions of the oil will sediment and be deposited on the oceanic floor. This usually occurs when the oil reaches a higher density than water after interacting with a mineral-containing element that is present at the shoreline. Sedimentation of oil usually occurs close to shore, where the sediment oil degrades slowly and may harm the biota. If the oil reaches shore it will usually adhere to the surface, especially if it is has been weathered. Some of the

oil droplets will adhere to each other and create tar balls that can reach up to 10 cm in diameter. These configurations are taken by the water streams, and eventually deposited onshore.

Figure 2.4 shows the fate of a typical crude oil over time, where it is clear that evaporation, spreading and dissolution are the major factor in the early stages of an oil spill. Oxidation, sedimentation and biodegradation are present in later stages and determine the ultimate fate of the oil spilled (Symon & Skjoldal, 2010).

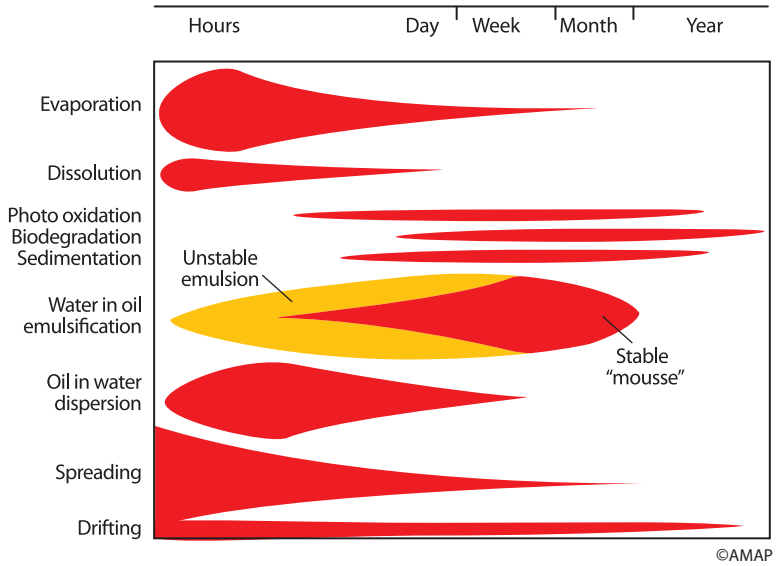

Figure 2.4 Fate of a typical crude oil. In the early stages a large portion of the oil will evaporate and spread out, being mixed with water. In later stages of weathering, the remaining oil will sediment, oxidise or form tar balls that ultimately wash up on shore (Symon & Skjoldal, 2010).

2.3 Biodegradation

Biodegradation is an important mechanism for the removal of hydrocarbons from the environment (Snape et al., 2001). Degradation may continue for a long time, dependent on the type of oil spilled, weathering conditions and temperature (see Chapter 2.2.4).

Many species of microorganisms have the ability to degrade hydrocarbons, and they exist just about everywhere in aquatic and terrestrial environments (Fingas, 2013). During

biodegradation hydrocarbon compounds are used as an organic carbon source where microorganisms break down the components to low molecular weight compounds that are excreted or further utilised by the organism (Leahy & Colwell, 1990). These organisms use hydrocarbons as an energy source, and bacteria, fungi and yeasts are a part of this large and diverse group. Even though these organisms are found everywhere, they are naturally more bountiful in areas where there are natural seeps of hydrocarbons (Atlas, 1981), but genotyping has shown that hydrocarbon degraders are present even in non-contaminated areas (Giudice, Bruni, Domenico, & Michaud, 2010). The rate of degradation depends on the type of hydrocarbons and usually increases with temperature. Biodegradation usually proceeds first with hydrocarbons of 12-20 carbons, and aromatics. Asphaltenes degrade very slowly, if at all. The rate of biodegradation also depends on the availability of oxygen, nutrients and the bioavailability of the hydrocarbons (Fingas, 2013).

2.3.1 Common hydrocarbon degrading microorganisms and the degradation pathway

It was Claude U. Sable that in 1946 first reported microorganisms' ability to utilize petroleum hydrocarbons as the primary source for energy. He found that these organisms are widely distributed in nature, and that hydrocarbon utilization depends on the chemical composition of the oil. Hydrocarbon degraders are a diverse group of bacteria and fungi with more than 100 species in 30 different microbial genera (Atlas, 1981). Both Gram-positive and Gram-negative bacteria have been found to be hydrocarbons degraders, but Gram-negative strains predominate (Margesin & Schinner, 1999). A vast variety of microbial groups have been reported, where the bacterial group include *Pseudomonas, Marinobacter, Alcanivorax, Microbulbifer, Spingomonas, Micrococcus, Rhodococcus, Cellulomonas, Dietzia* and *Gordonia* (Brito et al., 2006). Molds belonging to the species *Aspergillus, Penicillium, Fusarium, Amorphoteca, Neosartorya, Peacilomyces, Talaromyces* and *Graphium* have been shown to participate in hydrocarbon degradation together with the yeasts *Candida*, *Yarrowia* and *Pichia* (Chaillan et al., 2004). Other sources also report groups of *Vibrio, Corynebacterium, Acinetobacter, Achromobacter* and *Flavobacterium* as hydrocarbon degrading species (Atlas, 1981). The most relevant species globally, have been affiliated most with the Gammaproteobacteria, such as *Alcanivorax sp., Cycloclusticus sp.,*

Oleiphilus sp. and Planomicrobium sp. (Kleindienst et al., 2015). Despite the highly varied community of hydrocarbon degrading organisms that exists, *Pseudomonas sp.* dominate in oil contaminated environments as these bacteria often have the ability to degrade both linear and branched alkanes in addition to some aromatics (Karamalidis et al., 2010). It is also a microorganism with a highly adaptable metabolism, a feature that is not as prevalent in microorganisms inhabiting cold environments as these species are usually only able to utilize a narrow spectrum of hydrocarbons (M'rassi et al., 2015; Zhang et al., 2011). Some strains of *Pseudomonas sp.* have also been shown to degrade some xenobiotic compounds (Parales, Bruce, Schmid, & Wackett, 2002). Usually the microorganisms' ability to degrade hydrocarbon compounds decrease as the amount of carbons in the compound increases. However, ring structures with up to six rings have been reported to be degraded by some specific bacteria (Atlas, 1981).

Degradation of alkanes is initiated by the terminal oxidation of the alkane to 1-alkanol (an alkane-alcohol), catalysed by the enzyme *alkane monooxygenase* (also called *alkane hydroxylase*). This alcohol is further metabolised to an aldehyde by an alcohol dehydrogenase enzyme and then further to a carboxylic acid by an aldehyde dehydrogenase enzyme (Filler et al., 2008). This process is illustrated in Figure 2.5. Carboxylic acid goes into the fatty acid metabolism in the degrading organism, where it is degraded by β-oxidation to shorter fatty acids and also to Coenzyme A (Atlas, 1981). Alkane monooxygenase is the fundamental enzyme in the degradation of alkanes in bacteria, and it is one of three parts of the alkane hydroxylase complex. This complex consists of the integral-membrane alkane monooxygenase together with rubredoxin and rubredoxin reductase, which are soluble proteins (Filler et al. 2008). The bacteria that are capable of degrading a rather broad spectrum of alkanes, typically have several different homologs of the alkane monooxygenase complex, and in some instances toxic fatty acids have been shown to be accumulating in degrading organisms (Atlas, 1981; Filler et al., 2008).

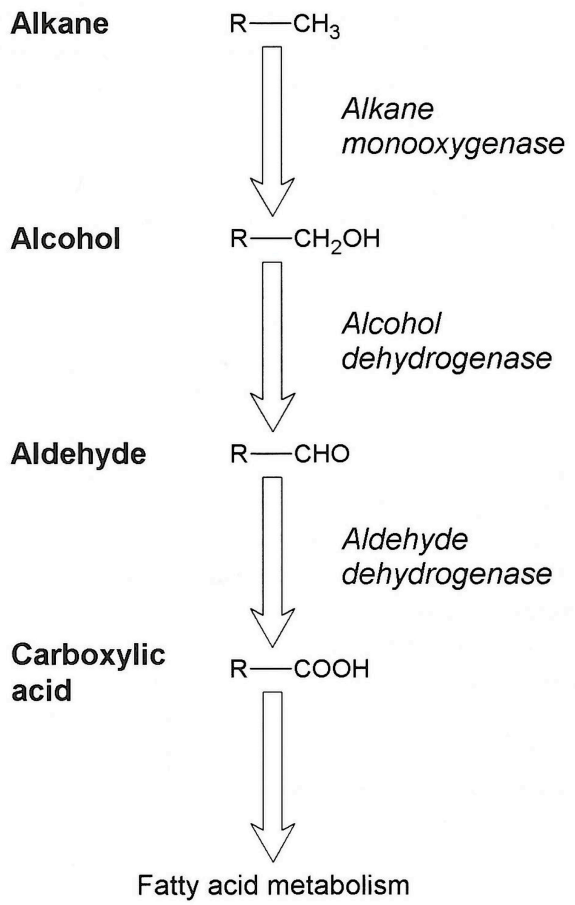

Figure 2.5 The general pathway of bacterial alkane degradation. Different enzymes participate in the degradation of alkanes, where they eventually are metabolised in the fatty acid cycle and participate in the production of the energy-rich molecule ATP (Filler et al., 2008)

Aromatics are common in most mixed crude oils, and in many refined petroleum products (Atlas, 1981). Degradation of aromatics is usually initiated by initiating two oxygen molecules on the aromatic ring by the help of the enzyme dioxygenase to produce cis-dihyrodiol, which is further dehydrogenated by a dehydrogenase enzyme to a catechol (Filler et al., 2008). From here, there are two possible pathways due to the multi-component systems of dehydrogenase enzymes as these enzymes consist of at least three proteins: The cathecol is cleaved by either a *meta*-cleavage dioxygenase enzyme or an *ortho*-cleavage dioxygenase enzyme to produce two different aliphatic intermediates that enter the central metabolic pathways where they are oxidised to provide energy or are used in biocellular processes (Atlas, 1981; Filler et al., 2008). The process is illustrated in Figure 2.6.

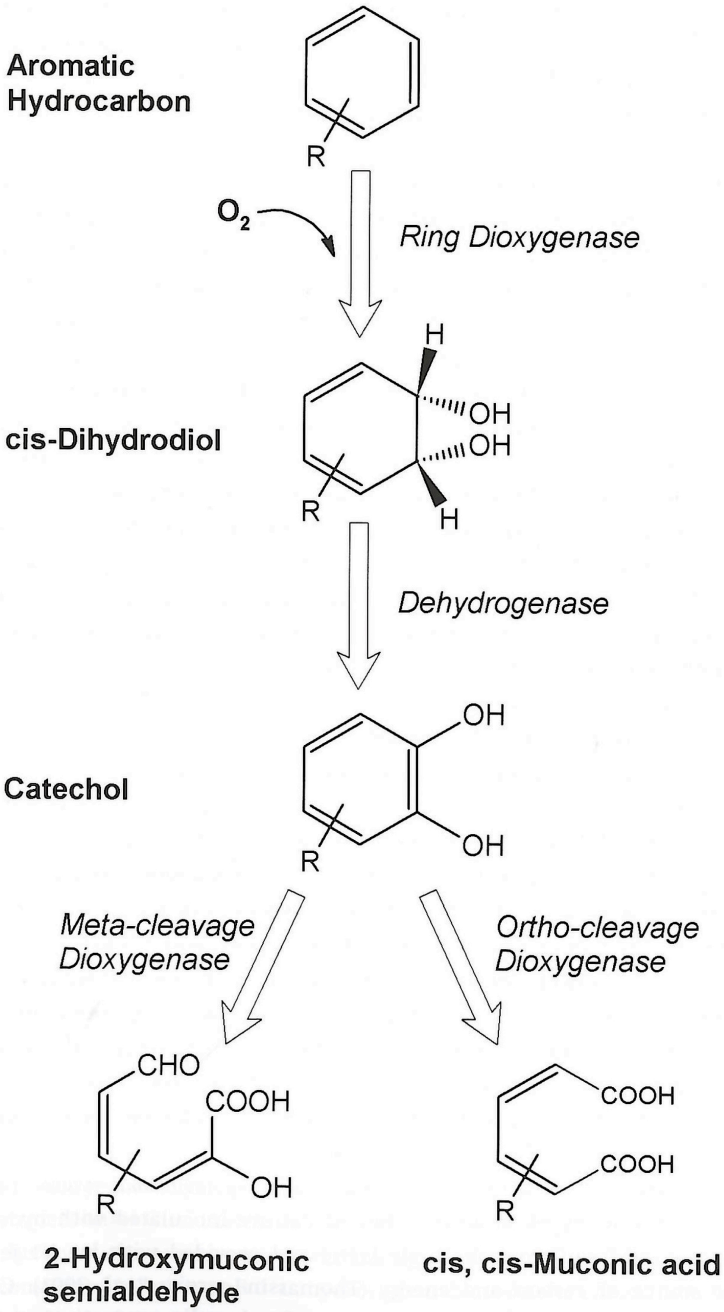

Figure 2.6 The general pathway of bacterial aromatic degradation. Different enzymes participate in degradation of aromatic compounds, creating aliphatic intermediates that enter the different metabolic processes to provide energy or function as substrates in biocellular processes (Filler et al., 2008)

It is the straight chained alkanes that are the easiest for microorganisms to degrade, and PAHs are often dependent on lighter substrates to induce the enzymes necessary for degradation (Atlas, 1991). Branching on alkanes interferes with the β-oxidation process and necessitates bypass mechanisms (Okoh, 2006). Usually it is saturated alkanes with 10-20 carbon atoms that are degraded first, followed by longer hydrocarbons containing 20-40 carbon atoms that are less soluble and have a lower bioavailability compared to shorter compounds (M'rassi et al., 2015). Two processes that are common during degradation of crude oil consisting of many different compounds of varying sizes are sparing and co-oxidation (Atlas, 1981). These processes affect the degradation of crude oil depending on the microbial population that is present. Sparing is carbon catabolite repression, where one substrate is preferred over another or where the presence of one substrate reduces the utilisation of another substrate. This process is a repressor-operator controlled system, where some enzymes favour the uptake of one particular substrate for metabolism, which may contribute to the lack of degradation of some compounds (Atlas, 1981). During co-oxidation microorganisms obtain the ability to utilise hydrocarbons that they are usually not able to degrade by taking advantage of the enzymes produced by other microorganisms (Atlas, 1981).

2.3.2 Cold-adapted, hydrocarbon degrading microorganisms

A about 85% of the biosphere is permanently exposed to temperatures below 5°C, and despite the cold temperatures these environments are successfully inhabited by cold-adapted microorganisms (Margesin & Schinner, 1999). Both psychrophiles and psychrotolerant microorganisms are abundant (Giudice et al., 2010). In Arctic environments, the *Pseudomnas*, *Rhodococcus* and *Sphingomonas* species are dominant psychophiles (Giudice et al., 2010).

2.3.3 Genetic and biochemical features of cold adapted hydrocarbon degraders

Local environmental conditions select for populations that have a low optimum temperature. The lowest temperature possible for bacterial growth is probably near -20°C as this temperature will affect the intracellular structures which will form ice crystals,

causing the cells to lyse (Margesin & Schinner, 1999; Price and Sowers, 2004). Lower temperatures may also indirectly alter the pattern of utilization of hydrocarbons, as the oils become waxy and less available, requiring some changes in the metabolic pathways and selecting for organisms capable of degrading the available hydrocarbons (Margesin & Schinner, 1999).

Cold adapted microorganism have many structural and physiological modifications that make them suitable for inhabiting cold environments, such as cold-active enzymes that function efficiently and are more flexible at cold temperatures and cold-shock and cold-acclimation proteins that make sure the protein synthesis functions as normal. In the membrane they often incorporate unsaturated fatty acids and carotenoids to ensure optimum fluidity and permeability, and in the cytosol these organisms often have cryoprotective substances ("anti-freeze") and extracellular compounds protecting the membrane (Giudice et al., 2010; Margesin & Schinner, 1999). Cold adapted enzymes generally have residues with greater polarity and less hydrophobicity, a low arginine/lysine ratio, additional glycine in the enzymes, less hydrogen bonds compared to more "regular" enzymes, no salt bridges, additional surface loops and reduced hydrophobic interactions between the sub-units of the enzyme, which contribute to the stability of the enzymes in cold environments (Filler et al., 2008)

Dziewit and Bartosik (2014) describe how cold habitats are vulnerable to changes in the environments and external influences, making them dynamic environments where the organisms living there can undergo changes in a rapid adaption to environmental impact. They state that such "short-term evolution" is possible due to the properties of plasmids and other mobile elements present in the cell, where these plasmids contribute to an expanded diversity of bacterial populations in these environments as these structures may carry additional genetic information and genes that gives an evolutionary advantage under more extreme conditions (Duilio et al., 2004). Dziewit and Bartosik (2014) also report that a majority of such plasmids have been found in Gram-negative bacteria, which might explain the abundant amount of Gram-negative bacteria in these environments compared to Gram-positive. According to the article, some of the plasmids have been identified to hold

several different genes, such as the genes coding for cold-shock proteins. The study showed that plasmids also code for enzymes that contribute to protein degradation in a higher degree than in more temperate environments, making these bacteria able to utilize amino acids as nutrients in their metabolism and to produce cold-shock proteins when needed. The study concluded that plasmids in these bacteria vary greatly in their genetic composition, where they code for functions such as transport of nutrients and adaption to nutrient deprivation, resistant to bacteriophages, heavy metals and also antibiotics resistance. All of these traits are though to help the cold-adapted bacteria to survive changes in the extreme environments they habitat (Dziewit & Bartosik, 2014).

2.4 Chemical and physical factors affecting hydrocarbon degradation

Biodegradation of oil depends on several factors, such as the solubility of the hydrocarbons and their molecular size and integrity, and also the surface area available for degradation and equilibrium partitioning. These factors determine what portion of the oil is actually bioavailable (Margesin & Schinner, 1999). To predict how spilled hydrocarbons will affect the environment it is important to have an understanding of these concepts.

2.4.1 Chemical composition of crude oil and diesel fuel

Crude oil is a complex mixture of many different hydrocarbon compounds, from simple unbranched alkanes to aromatics composed of many rings. Hydrocarbons degrade at different rates, depending on their molecular structure and the enzymes present in the microorganisms (Westlake, Jobson, Phillippe, & Cook, 1974). Petroleum hydrocarbons are usually divided into saturates, aromatics, asphaltenes and resins. The asphaltene class contains phenols, fatty acids, ketons, esters and porphyrins, while the resins are pyridines, quinolones, carbazoles, amides and sulfoxides (Simanzhenkov & Idem, 2003). Susceptibility for microbial degradation is highest for n-alkanes, where branched alkanes, low molecular weight aromatics and cyclic alkanes follows, respectively (Leahy & Colwell, 1990). Other organic compounds present in crude oil contains nitrogen, oxygen and sulphur (Speight, 1999).

Biodegradation depends on the composition of the oil; when light components are present these will evaporate off, leaving heavier components for biodegradation. Bacterial communities prefer to degrade less complex compounds first before degrading heavier compounds, and biodegradation is also dependent on the communities present, where different species of bacteria and fungi have different degradation abilities and substrate preferences (Atlas, 1981). Which compounds a microorganisms prefers, depends on their preferred metabolic pathways (see Chapter 2.3.1). In addition, the degradation of different classes of hydrocarbons may be carried out by completely different populations of microorganisms in different environments, further complicating the degradation of heavier, more complex hydrocarbons (Atlas, 1991).

Additives are usually present in diesel to prevent blocking of filters in vehicles at lower temperatures, as diesel is composed of heavier hydrocarbons compared to gasoline (Simanzhenkov & Idem, 2003). These additives are often more toxic to microorganisms than the diesel itself, and may contribute to a lower rate of biodegradation (Dillard, Essaid, & Herkelrath, 1997).

2.4.2 Concentration

Portions of the hydrocarbons in the degradation process dissolve and move into the aqueous phase, where the amount of hydrocarbons available greatly influences the rate of biodegradation. Hydrocarbons are soluble in water at very low concentrations, and in most cases oil is spilled in far greater amounts than can be dissolved (Atlas, 1981). It is usually assumed that growth occurs in accordance to Monod microbial growth kinetics (see Chapter 2.5.4), and thus the concentration of hydrocarbons in the aqueous phase influence the rates of uptake and mineralization of hydrocarbons by the microbial populations present at the oil spill site (Leahy & Colwell, 1990). Microbial degradation of hydrocarbons depends on the hydrocarbons solubility, equilibrium partitioning and the dissolved hydrocarbon concentration. Some bacteria are able to grow on the oil/water interphase, and other produce biosurfactants that makes the oil bore bioavailable (Krista Kaster, personal communication).

The equilibrium partitioning is a measurement of how the molecules distribute themselves among the different phases, such as the oil and water phase. In many situations this partitioning equilibrium is never reached, and for oil spills this is often due to variable weather conditions. The equilibrium partitioning coefficient is very useful for determining a compounds ability to accumulate in an environment, and it is often used to determine if spilled oil is still dissolving into the water phase some time after an oil spill (Schwarzenbach, Gschwend, & Imboden, 2005).

Water temperature also has an impact on the solubility of oils in water, and thus the concentration. Some petroleum products have increased solubility as the temperature decreases, but for most compounds the effects are opposite (Polak & Lu, 1973).

2.4.3 Physical bioavailability

Bioavailability describes how the microorganisms take up a particular component of oil, and how readily accessible an organic compounds is for uptake. Bioavailability of the compounds present in petroleum hydrocarbons depends on the surface area of the spill, where a greater surface area provides a larger space for attachment by microorganisms leading to an increased rate of biodegradation (Leahy & Colwell, 1990). The surface area of an oil slick may increase in aquatic environments where there is a large natural occurrence of high-energy waves and currents that disturb the oil and disperse and emulsify the oil in a higher degree into the water column (Leahy & Colwell, 1990). This again increases the bioavailability, because the surface area is connected to the transfer of mass between the oil and water phase, and can be explained by the following equation (Boyadjiev, 2011):

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

Where:
dC/dT = the rate of mass transfer of a certain component from oil to water
C = the concentration [g/m^3] of a specific compound in one phase that will travel to another phase (here from oil to water)
C_0 = the concentration [g/m^3] in the oil phase
D = the molecular diffusion coefficient [m^2/s] of the hydrocarbon in the oil
A = the total surface area [m^2] of the oil
L = the thickness of the liquid boundary layer (m) between the oil and water
V = the total volume of the oil [m^3]

Equation 1 illustrates how a larger surface area leads to a higher rate of mass transfer of hydrocarbons from the oil to the water phase, which gives a larger surface area for microbial attachment and a higher rate of biodegradation (Leahy & Colwell, 1990; Ulas, 2013)

2.4.4 Temperature

Temperature is an important factor as it influences all biochemical reaction and most chemical reactions. Biodegradation slows down in cold environments as this process is thought to obey the thermodynamic relationship between temperature and chemical reaction described in the Arrhenius equation, meaning that the rate of biodegradation decreases as temperature decreases. This is called the Q_{10} effect (Giudice et al., 2010).

The Arrhenius relation is shown in Equation 2 below:

$$k = Ae^{-Ea/RT} \qquad \text{Eq. 2}$$

k = the rate constant
A = the pre-exponential factor
E_a = activation energy
R = the gas constant (8.314 J/mol K)
T = temperature (K)

The relation studies the quantitative relationship between reaction rates and chemical equilibrium constants, and ultimately shows that the rate constant *k* can be expressed as a constant term that is proportional to temperature. The Arrhenius temperature-reaction rate correlation provides a general understanding of how temperature affects the biochemistry of cells, but fails to do so completely. The biochemical reactions are far more complex, where enzymatic catalysts provides a lowered activation energy, making the biochemical reactions dependent on both temperature and enzyme structure (Filler et al., 2008).

Low temperatures affect both biotic and abiotic factors involved in biodegradation. Temperature of the water on the surface has an effect on hydrocarbons that have not evaporated and affects microbial colonization, where low temperatures makes oil more viscous thus influencing the hydrocarbon degradation rate and the composition of the microbial communities (Atlas, 1981). It also influences the rates of mass transfer of the substrate and the electron acceptors utilised by the microorganisms (Yang et al., 2009). Biodegradation is a process that occurs at the later stages of hydrocarbon breakdown (see Figure 2.1), and as cold temperatures prevents extensive evaporation this will lead to a delay in the activation of hydrocarbon biodegradation (Atlas, 1981).

A decrease in temperature have also shown to cause a higher degree of crystal formation of the petroleum hydrocarbons, resulting in the hydrocarbons being less available for degradation by microorganisms (Margesin & Schinner, 2001). Most studies have shown that low temperatures do not affect the extent of biodegradation (Delille, Pelletier, Rodriguez-Blanco & Ghiglione, 2009; Mohn & Stewart, 2000; Prince et al., 2013). However, some studies have shown that some hydrocarbons are not as well degraded at low temperatures (Deppe et al., 2005; Margesin & Schinner, 2001; Whyte et al., 1998). Lower temperatures may also indirectly alter the pattern of microbial hydrocarbon utilisation, as heavier oils tend to become waxy in colder temperatures retaining precipitation of the oil and diminishing the bioavailability of the oil (Margesin & Schinner, 1999).

2.5.5 Nutrient availability

Nutrients and other organic compounds are required for growth, where exchangeable cations, nitrates and phosphates are some of the most important ones. There are descriptions of how the rates of biodegradation in coastal aquatic environments were affected by limited access to nitrogen an phosphorous (Atlas, Horowitz, & Busdosh, 1978), but there exist some controversy to weather biodegradation is limited by low nitrogen and phosphorous levels. Some researchers have reported that these factors are severely limiting for growth (Bartha & Atlas, 1973; Floodgate, 1979; Haritash & Kaushik, 2002), while other researchers have reached the opposite conclusion (Tyagi, Fonseca & Carvalho, 2011). As microorganisms incorporate both nitrogen and phosphorus into biomass, the availability of these within the same area as the hydrocarbons that are degraded is usually viewed as critical (Atlas, 1981). Biodegradation rates have also been shown to depend primarily on the amounts of nitrogenous nutrients that are present in pores in the bottom sediment (Margesin & Schinner, 1999). Diffusion of nutrients into an oil slick is often critical for biological growth, as this rate is crucial for establishing satisfying levels of nitrogen and phosphorus supporting microbial growth and metabolism (Atlas, 1981). Lack of phosphorous does not seems to affect biodegradation to the same extent as nitrogen depletion (Filler et al., 2008). When large quantities of hydrocarbons are spilled, the availability of inorganic nitrogen and phosphorous tends to deplete quickly, especially in cold areas that might already be deprived of nutrients (Yang et al., 2009).

2.5.6 Oxygen, alternate electron acceptors, salinity and pH

Oxygen is used as a terminal electron acceptor, and oxygen limitation is a reason why bioremediation fails in cold regions (see Chapter 2.6). In cold environments oxygen transport that has been given as being the rate-limiting step in aerobic degradation, as it may be consumed at a faster rate than what can be replaced by diffusion (Yang et al., 2009).

Hydrocarbons are subject to anaerobic degradation, providing that alternate electron acceptors such as nitrate and sulphate are available as terminal electron acceptors. Hydrocarbons degraded in anaerobic environments are usually insignificant, and the

hydrocarbons will usually persist indefinitely as contaminants (Atlas, 1981). When molecular oxygen is absents, some organisms utilize NO_3^-, Mn^{4+}, Fe^{3+} and SO_4^-as alternative terminal electron acceptors (Spence, Bottrell, Thornton, Richnow, & Spence, 2005).

The salinity of the world's oceans are quite stable, but in cold environments very saline pockets of water may form in ice formations allowing the water to remain liquid. This puts the organisms living in these pockets under stress, and the metabolism of these organism decreased as the salinity increased. This reduction in metabolism raises questions regarding the extent of hydrocarbon degradation in very saline environments (Atlas, 1981).

Biodegradations seems to be pH sensitive, as hydrocarbon mineralisation is favoured at a neutral pH (Margesin & Schinner, 2001).

2.5 Biological factors affecting hydrocarbon degradation

Both mechanism of adaptions to the environment and the hydrocarbons present and environmental factors affecting growth and health play a major part in the degree of hydrocarbon breakdown (Filler et al., 2008). Different species of microorganisms that are associated with hydrocarbon degradation in general and those isolated from cold environments are discussed in Chapter 2.3, where also metabolic processes of hydrocarbon degradation are outlined.

2.5.1 Mechanisms of growth and hydrocarbon utilization

Fingas (2013) state that organisms living in areas where natural seeps of oil occur will usually change and multiply in number when exposed to an increased presence of hydrocarbons, such as after an oil spill. Mechanisms for uptake vary amongst different hydrocarbon degrading species, but in general hydrocarbon compounds are transported from the environment and into the cell through the cell membrane, and from the cell and into the metabolic mechanisms of the cell (Lodish, 2000). There are three general theories regarding growth mechanisms and how microorganisms utilise hydrocarbons:

1. The microorganisms may utilise compounds that are solved in the water phase. This is limited by the partitioning rate of the different compounds, which influences the rate of biodegradation and also the rate of growth for the microorganisms. There are studies that have shown that growth rates may be more rapid than expected as seen in correlation to the partitioning rate (Bouchez, Blanchet, & Vandecasteele, 1997; Efroymson & Alexander, 1994), but in most cases the rate of biodegradation will depend on the partitioning coefficient. This has been confirmed by studies where an increased amount of hydrocarbons in the water phase lead to an increased growth of microorganisms due to the increased partitioning coefficient (Alexander, 1999; Wodzinski & Johnson, 1968).

2. Microorganisms may secrete a biosurfactant that facilitate desorption so that the growth rate exceeds the partitioning rate. Under such circumstances the growth rate is not limited by the partitioning coefficient, as produced biosurfactants could release hydrocarbons from the oil phase, cations may displace hydrocarbon compounds with a charge, extracellular enzymes may aid in releasing hydrocarbons from the oil phase or there might be an induced change in pH. The microbial growth will thus be limited by the amount of biosurfactant the microorganisms are able to produce (Alexander, 1999; Ron & Rosenberg, 2002).

3. For some organisms, adhesion to an oil/non-aqueous phase liquid (NAPL) surface may be required for degradation of hydrocarbons. Microorganisms may come in contact with hydrophobic liquid by adhesion to the surface, so that hydrocarbon compounds are directly utilised from the NAPL. When microorganisms come into direct contact with hydrocarbons, the hydrocarbons may penetrate the cell membrane directly without being in contact with the aqueous phase. (Alexander, 1999).

2.5.2 Growth kinetics

Kinetics is a valuable tool for evaluating how long pollution will remain in the environment. Once degradation of a compound is initiated, the kinetic rate will be a function of the compound being degraded, its concentration, the organisms involved and various environmental factors (Alexander, 1999). When evaluating degradation rates of a

compound, the enzymes involved in degradation determine the hydrocarbon degradation rate. Information on kinetics is important as it allows for the calculation of the remaining concentration of oil and makes it possible to evaluate whether the oil spilled will be degraded before it reaches shore or a particular habitat. Kinetics mainly focuses on the amounts of a compound degraded per unit of time. Models used to determine kinetics are either empirical or theoretical, and in order to use different models it is essential to have knowledge of how the models are used and the theoretical basis of them (Alexander, 1999; Reardon, Mosteller & Rogers, 2000).

Microorganisms that degrade organic substances such as petroleum hydrocarbons are utilising these compounds in different ways, usually as a source of carbon and energy (Chandran & Das, 2011). Monod mathematically formulated the relationship between the limiting concentration of a substrate to the growth rate, which is summarised in the Monod equation, where initially a very high concentration of the substrate will not further affect biodegradation rates (see Appendix A). The K_s values expressed in the equation depend on microorganisms and the compound being degraded, and it may also depend on whether the concentration of the compound is high or low (Alexander, 1999). A model, named *The Oil Spill Contingency and Response* (OSCAR) have been developed based on Monod kinetics, and is a model and simulation tool meant to predict the effect of an oil spill and to provide insight into the behaviour of oil during spills (Bagi, 2013; SINTEF, 2014).

The model describes the rate of the enzymatic degradation of a compound, where the enzyme (E) binds to the substrate (S) and creates a degraded product (P) (Chang, 2005). This relationship is illustrated in Equation 3:

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{\text{cat}}} E + P \qquad \text{Eq. 3}$$

The equation is used to evaluate how efficiently an enzyme converts a substrate into a product, and may be useful when evaluating degradation rates of hydrocarbons spilled in the environment. Enzymes are main contributors to hydrocarbon degradation, and thus the

rate they are able to convert a substrate into a product influences the overall degradation rate (Alexander, 1999; Margesin, Gander, Zacke, Gounot & Schinner, 2003).

2.5.3 Adaptation and metabolic limitations

Microbial population have the ability to adapt to changes in their environment relatively rapidly as microorganisms are usually very dynamic. Some of these functions are coded in plasmids present in different microorganisms, and other are attributed to normal mechanisms present in most individuals, giving the bacteria, fungi and other microbes present in a contaminated environment an opportunity to adapt to local levels of contamination (Margesin & Schinner, 2001). An increased amount of hydrocarbons in the environments will lead to a specialised population of microbes, but also to an increased rate of oxidation of hydrocarbons (Atlas, 1981; Leahy & Colwell, 1990; Margesin & Schinner, 2001). Cold-adapted microorganisms may be more vulnerable to changes of larger proportions, but usually these organisms will also adapt and degrade hydrocarbons in different environments (see Chapter 2.3.2).

Three mechanisms are associated with microbial adaptation to environmental changes (Leahy & Colwell, 1990):

1. Specific enzymes may be induced or supressed, depending on their function.
2. Genetic changes may induce needed metabolic capabilities
3. Specialisation of the population, enabling it to degrade the spilled hydrocarbons

Several reports have shown show that a community of hydrocarbon degrading microorganisms flourish and gradually take over as the majority of the present microbes in response to the presence of hydrocarbons (Atlas, 1981; Colwell, Walker, & Cooney, 1977). The organisms present usually reflect the degree of contamination in the ecosystem (Leahy & Colwell, 1990).

Mechanisms of adaption include genetic changes, and the principle of selective enrichments of a polluted community (Días, 2010). A combination of these two factors results in an

increase of the amount of hydrocarbon degraders, and thus an increased rate of biodegradation and an enhanced amount of degraded hydrocarbons (Leahy & Colwell, 1990).

Metabolic limitations are usually the result of enzyme-substrate interaction and the energy needed to perform these processes, in addition to the energy that is available for activating the metabolic process. The larger a compound is, the harder it is for the enzyme to bind and perform work. When steric hindrance is present, binding to a substrate will require more energy that may not be available to the cell (Yang et al., 2009). Different organisms are present in different environments, which may result in degradation of a compound in one environment but not in another (Alexander, 1999).

2.6 Bioremediation

Oil spills have become a widespread and serious issue in both marine and terrestrial environments, and techniques that stimulate biodegradation have become increasingly more relevant. Bioremediation is a promising option, and is thought to be more effective and economic than conventional oil spill clean-up (Yang et al., 2009). Bioremediation often consist of stimulating the indigenous population present at a spill site by modifying environmental factors such as nutrient conditions and the availability to molecular oxygen, where effective bioremediation converts a majority of the polluting hydrocarbons into water and carbon dioxide, in addition to non-toxic by-products (Atlas, 1991). Its economic advantages over other physiochemical remediation methods make it a preferred alternative when applicable (Leahy & Colwell, 1990; Silva et al., 2014).

2.6.1 Methods of bioremediation

Bioremediation has three general approaches (Suni, Malinen, Kosonen, Silvennoinen, & Romantschuk, 2007):

1. Natural (intrinsic) attenuation
2. Biostimulation
3. Bioaugmentation

Yang et al., (2009) describes natural attenuation as the process where naturally occurring microorganisms are allowed to degrade the spilled hydrocarbons without interference but under close monitoring to enhance the degradation by the indigenous microbial community and thus accelerating degradation rates by adding limited nutrients and/or by making the hydrocarbons more bioavailable so the microbes will be able to degrade the compounds more effectively. It is uncertain to what degree the access to oxygen and nitrogen limits the biodegradation of hydrocarbons, when the affected area needs to be re-fertilised and if one addition of fertilizer is enough (Yang et al., 2009). The addition of nutrients is not always beneficial, and the addition of nitrogen has previously shown to inhibit the mineralisation of aromatic and aliphatic compounds in laboratory scale tests (Ferguson, Franzmann, Revill, Snape, & Rayner, 2003; Morgan & Watkinson, 1989)

Bioaugmentation, also called seeding, aims to inoculate hydrocarbon degrading bacteria in areas where these are not indigenously present or only present in very small quantities. The overall effect of bioaugmentation has not been proven to be particularly great, even though the method shows increasing concentration of bacteria in the start of the inoculation (Margesin & Schinner, 1999). Bioaugmentation also apply selective pressure to the indigenous populations, which may result in a restrained growth of both the indigenous and foreign microbial strains. Inoculated microorganisms will most likely be outcompeted by indigenous ones (Yang et al., 2009).

Atlas (1991) briefly describes genetically engineered microorganisms highly adapted for hydrocarbon degradation as another approach of bioremediation. As many of the genes related to microbial hydrocarbon degradation are located on plasmids, the potential for creating microbial strains capable of degrading hydrocarbons at a high rate have been demonstrated through the use of genetic engineering of existing microorganism. There is considerable controversy to this approach, where releasing enhanced microorganism possessing artificially enhanced features into an environment where they are neither indigenous nor natural has been viewed with scepticism.

2.6.2 Nutrient requirements for bioremediation

It has been shown that fertilizer can enhance degradation of hydrocarbons, and the changes in the community was rapid and lasting when fertilizer was added (Röling et al., 2004). During bioremediation it is nitrogen addition that most often provides a degradation response. Proper nitrogen management may increase cell growth, decrease the microbial lag phase, and maintain high activity levels in active populations in addition to increasing the levels of hydrocarbon degradation (Zahed, Aziz, Isa, Mohajeri, & Mohajeri, 2010). This has been linked to nitrogen management, which shows that unless a proper amount is applied the treatment has little effect or may be counterproductive by inhibiting growth and vital metabolic processes (Filler et al., 2008). Limited phosphorous availability does not affect bioremediation in the same way as nitrogen deficiency, but may help stimulate growth during nitrogen addition (Chang, Weaver, & Rhykerd, 1996). Excess amounts of nitrogen and phosphorus may be limiting for growth, but it is usually an excess amount of nitrogen that has been shown to be inhibiting to hydrocarbon degradation (Mills & Frankenberger, 1994).

Most nitrogen fertilisers are composed of nitrate and ammonia compounds that easily dissolve in water, which increase the salt concentration in water and may inhibit microbial activity if the amounts of fertilizer are large. Nitrogen must therefore be properly applied to provide optimum conditions for bioremediation (Filler et al., 2008; Zahed et al., 2010).

2.6.3 Biosurfactants

A technique that is becoming increasingly more relevant for bioremediation is use of biosurfactants. They have the potential to play an important role due to their low toxicity and high biodegradability (Silva et al., 2014a). Biosurfactants are useful in oil spill remediation where they contribute to removal of hydrocarbons and microbial bioremediation by increasing the bioavailability of hydrocarbons ad they are composed of amphipathic molecules, where hydrophilic and hydrophobic parts make it possible for them to act in compounds of different polarities, reduce surface tension and increase the contact area for insoluble compounds (Silva et al., 2014b).

Biosurfactants are often products of low molecular mass with a low surface tension or heavier polymers, where different glycolipids, lipopeptides and phospholipids are the most common compounds. They are produced on the cell surface or are excreted from the cell (Kapadia & Yagnik, 2013). Bacteria, fungi and yeast all produce biosurfactants and *Pseudomonas sp.* is known for its capacity to produce extensive amounts of glycolipids (Silva et al., 2014b). Their role in oil spill remediation is usually to enhance the dispersal of oil, hence increasing the bioavailability of the hydrocarbons. They are also being used as an alternative method of oil recovery from productions sites, and can be used to clean off spilled oil from shorelines and from production equipment (Silva et al., 2014b).

The major advantages of biosurfactants are their high biodegradability, their inexpensive production and the easily accessible raw materials used for production. They are also simple to keep control of when released in the environment, as they are not directly toxic and have a high specificity for different substrates (Kapadia & Yagnik, 2013).

2.6.4 Challenges associated with bioremediation

Our current knowledge of bioremediation seldom allows for *in-situ* bioremediation, especially in cold environments. Yang et al., 2009 mentions that methods that are applicable in more temperate environments usually requires modifications to be useful in colder regions, and the nature and conditions of the polluted environments also determines to what extent the bioremediation methods will have an impact. Environmental factors like cold temperatures and ice prolongs biodegradation processes from years to decades, complicating bioremediation. Microbial communities also need to be better understood in order to effectively utilise bioremediation (Yang et al., 2009; Margesin & Schinner, 2001). In addition, biological decontamination cannot reduce the contaminant concentration to zero, and therefore conventional methods are still needed (Margesin & Schinner, 1997). The hydrophobicity and low solubility in water of hydrocarbons also poses a problem, as it results in a slow and often insufficient biodegradation. One of the most important issues to address is the limited access to the polluting hydrocarbons, preventing enzymes and

microbes involved in the bioremediation to utilise their full degradation potential (Silva et al., 2014a)

2.7 Dispersants

Treating spilled oil with specially prepared chemicals is another alternative in oil spill clean-up. Dispersant is a term used for chemicals that are used to promote the formation of small droplets of oil that will separate from a large slick of and help move the oil into the water phase. This resembles the natural weathering process where the movement of the water breaks the oil into smaller fragments. These agents are not always effective, and both the dispersant and the treated oil may be toxic to the aquatic wildlife (Kleindients et al., 2015).

2.7.1 Properties and applications

The term dispersant refers to a chemical that promote the formation of droplets that can move (disperse) throughout the water phase, and they consist of surfactants that gives them soap-like properties (Fingas, 2013). They key components of dispersants are surface-active agents that have a hydrophobic and a hydrophilic portion, making it possible to be part of both the water and the oil phase. Depending on their hydrophile-lipophile balance they either stabilise a water-in-oil emulsion or an oil-in-water emulsion (Griffin, 1954). Dispersants are also classified as being anionic, cationic, nonionic or zwitterionic depending on their charge (NRCC, 1989).

Fingas (2013) describes the common use of dispersants, where they are mainly used to aid the separation of oil into the water phase, and to keep the emulsion stable so that the hydrocarbons will be more bioavailable. They are usually applied undiluted directly to the oil slick by the aid of helicopters or planes, allowing the dispersant treatment to be applied to an oil spill within very short time of an accident. If the wave activity is sufficient, the dispersant will be effectively mixed with the oil. If the wave activity is low, there might be a need for mechanical mixing in order for the dispersant to work as desired. There are many dispersant that are used for different purposes; some are used for releasing oil from

shorelines and other affected areas, some are used to break or prevent emulsions, some cause the oil to sink while others solidify the oil so that it can be more easily collected. One common dispersant groups aids biodegradation by aiding growth or other parameters needed for an efficient biodegradation of hydrocarbons (Fingas, 2013; Lessard & Demarco, 2000).

Some criteria for what properties a good dispersant should possess was summarized by Philips and Stewart (1974):

1. A dispersant should be easily biodegradable
2. It should not be a preferred substrate when oil is present
3. It must not be toxic to indigenous bacteria and the environment

The essential element in dispersant treatment is to add enough dispersant to the affected area in order to stimulate the formation of droplets of the corrects size, as droplets larger than 1000 μm will break through the oil slick and collect the oil in smaller areas (Fingas, 2013). It is a necessity to have sufficient movement in the ocean in order to spread and mix the dispersant thoroughly into the oil-affected area, as mechanical mixing might not be possible. Some oils are too viscous to be dispersed, and an excessive use of dispersants may be counterproductive rather than helpful. The use of dispersants on diesel fuels and other light fuels may also ultimately be harmful, as these petroleum products usually contains a high level of aromatics that should not excessively enter the environment (European Maritime Safety Agency, 2009).

The toxicity of dispersant is an important issue to address, even though the dispersants used today are far less toxic compared to previously used chemicals. Both the acute toxicity and the lethal concentration that causes death in 50% of the population (LD_{50}) are common measurements of toxicity that are very relevant when dealing with dispersants, and even today the use of dispersants is the source of concern as their toxicity has not been completely evaluated in the field (Walker, 2006).

2.7.2 Dispersant-oil interactions

When a dispersant is added to water, their hydrophobic parts will seek to be together and they will orient themselves so that the hydrophilic parts is surrounded by the aqueous phase and their hydrophobic "head" form a closed hydrophobic environment where the oil droplet resides (Figure 2.7). This reduces the interfacial tension, and increases the amount of oil in the water (NRCC, 2005).

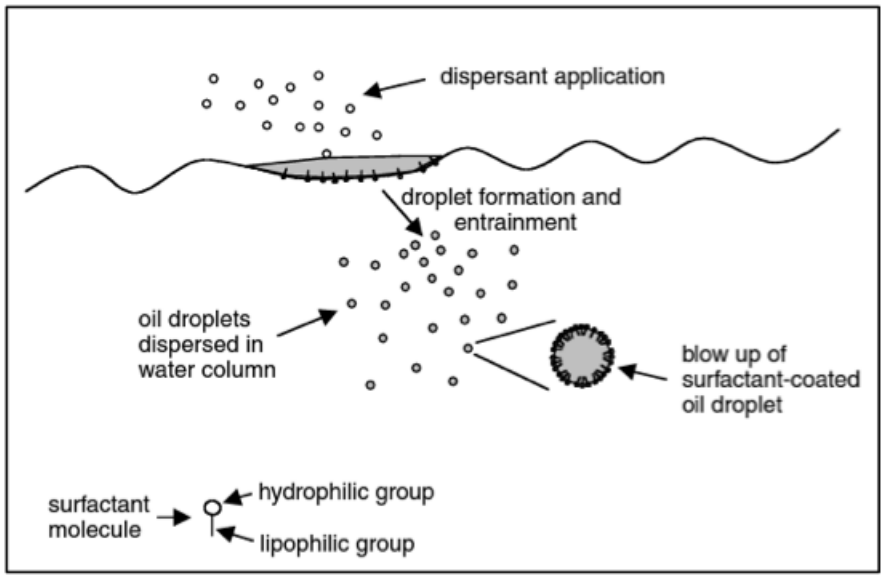

Figure 2.7 Mechanism of chemical dispersion. The dispersant accumulates at the surface of the oil and water, and promotes the formation of small droplets of oil that is dispersed into the water. These droplets can be colonised by microorganisms, and degraded (NRCC, 2005).

At low concentrations a dispersant is usually fully dissolved in the water phase, but when the concentration increases the molecules will aggregate and form small structures called micelles. The lowest concentration that leads to micelles is called the Critical Micelle Concentration (CMC) (Alexander, 1999; Berg, Tymoczko, Stryer & Gatto, 2012). Above the CMC level there is little change in interfacial tension, and new surfactant molecules will form new micelles. If the concentration is below the CMC level, the dispersant will accumulate on the water-oil phase without dispersing the oil (National Research Council Staff, 1989).

Dispersants contribute to entrainment and dispersion, where oil is transported from the oil slick and into the phase water by the formation of micelles that traps the oil in the hydrophobic regions and then spread it into the water, where it may be transported to different locations. In a dispersant, the solvents that are present are added mostly to promote the dissolution of surfactants and additives into a mixture with the water. Commercial dispersant are usually composed of two different surfactants with different solubility in water and oil (Kleindienst, 2015; NRCC, 2005).

Seven requirements have been suggested in order for a dispersant to enhance the formation of oil droplets (NRCC, 1989):

1. The dispersant must be added in a suitable dose.
2. Dispersant molecules must have enough time to penetrate and mix into the oil.
3. Surfactant molecules must form oil-containing micelles.
4. The oil-water surface tension must decrease, weakening the oil film.
5. Sufficient mixing energy must be applied by waves or wind action.
6. Droplets must be dispersed throughout the water column to prevent them from coming together and forming larger droplets again.
7. Droplets must be diluted to a concentration that is not toxic, and remain in the water long enough to be biodegraded.

Because oils vary a lot in composition, it is thought that certain dispersants could be more effective in the presence of one type of oil over another, and that a dispersant can be matched to a specific type of oil. Today, only a limited number of dispersants are available, making such a matching difficult (NRCC, 1989).

2.7.3 The effect on biodegradation

Predicting the effect of a dispersant on the biodegradation of hydrocarbons may be difficult as the physical and chemical condition of a dispersant, combined with its toxicity, determines its individual properties. In addition, the properties of specific hydrocarbons present in the oil contribute to the effect of the dispersant. It is not possible and very

impractical to attempt to evaluate every hydrocarbon and its effects in the different commercial dispersant without separating the different hydrocarbons into groups based on their chemical qualities (Mackay & Wells, 1983). A dispersants effect on biodegradation is also debated, as some studies show an increase in degraded hydrocarbons (Efroymson & Alexander, 1994; Rouse, Sabatini, Suflita, & Harwell, 1994; Swannell & Daniel, 1999), while others have shown that dispersants may inhibit degradation (Rouse et al., 1994). An ionic dispersant seems to have growth-inhibiting properties, while the inhibition by anionic dispersants is variable. It has also been shown that a concentration of the dispersant above the CMC value may cause inhibition of the biodegrading microorganisms (Rouse et al., 1994).

2.7.4 Corexit 9500

Corexit®EC9500A is an oil spill dispersant produced by Nalco Environmental Solutions LLC, and may cause skin and eye irritation if exposed. It is non-flammable, and has a pH of 6.2. The product is not thought to be a sensitizer, and it is considered to be non-toxic when used as recommended. The LD_{50} is considered to be 5.00 mg/kg, and it is expected to have a10-30% motility in water (LLC Nalco Environmental Solutions, 2014).

Studies have shown indications that Corexit has the ability to increase the surface area of crude oil creating small droplets of oil, thus increasing the microbial colonization on these droplets and increasing the number of droplets being degraded, contributing to an increase in the overall degradation of spilled oil (Swannell et al., 1997). In a study conducted by Lindstrom and Braddock (2002), the effects of Corexit 9500 on hydrocarbon mineralization were studied. It was concluded that Corexit 9500 did not lead to an increased carbon mineralization, as there was no evidence of differenced between degradation in samples with and without dispersant added. It was also shown that the population of microorganisms that readily degraded the dispersant rather than hydrocarbons increased and was usually higher that the population of hydrocarbon degraders. It could however seem that the dispersant induced a change in the microbial population, as different hydrocarbons were degraded compared to the control without dispersant (Lindstrom &

Braddock, 2002). This shows the need for further investigations on the effect of the dispersant Corexit 9500.

Corexit 9500 was also used extensively during the Deepwater Horizon spill to disperse the oil and aid in biodegradation (Bælum et al., 2012; Kujawinski et al., 2011).

2.8 Methodology

Several different methods are available for monitoring degradation of hydrocarbons in seawater, and both chemical and microbial analyses are frequently used for monitoring weathering and degradation processes of minor and major oil spills throughout the different environments around the world. The biological oxygen demand (BOD) is commonly used to monitor the microbial growth by analysing the oxygen consumption, giving an indication of the degradation of available hydrocarbons. Chemical analyses such as gas chromatography is used to monitor and determine the exact loss of hydrocarbons from start to finish of the degradation process. Bacterial enumeration is used to examine the total count of hydrocarbon degrading organisms during their growth process, and how the amount of microbes changes over time. Molecular analysis of the populations' DNA gives information of the structural changes of a community over time, and can give indications of the effect of hydrocarbons on a microbial indigenous population.

2.8.1 BOD analysis

The biological oxygen demand (BOD) is the amount of dissolved oxygen that is consumed by aerobic microorganisms during degradation of organic material over a given period of time. It is commonly expressed in mg/L, and is often used to determine the amounts of organic pollution present in water, or to give an indication of microbial activity (Speight & Arjoon, 2012). Two common ways of measuring the BOD values of a sample have been described in Speight & Arjoon (2012): 1) The dilution method, where a measured amount of an organism are added to the sample being tested and diluted with oxygen rich water. The amount of oxygen in the water is measured before the sample is sealed, and measured again

after a given time period to determine the BOD. 2) The manometric method, where the sample is kept in a closed container with a device that measures the pressure. A substance will consume the carbon dioxide produced during degradation, causing the pressure inside the container to drop. By analysing the drop in pressure, the amount of consumed oxygen can be calculated.

The analysis used in this study was a manometric measurement performed by the OxiTop® Control System (WTW). This is a closed bottle system with a fitted headspace that measures the drop in pressure inside the bottle (Figure 2.8). The BOD is inversely proportional to the pressure (see Appendix B), and the OxiTop system will measure the pressure change over time during the biodegradation of a hydrocarbon, such as the diesel fuel analysed here (WTW Weilheim, 2006). Blanks and positive controls are used to observe if any contamination occurs and minimise the influence of false positives or negatives, respectively.

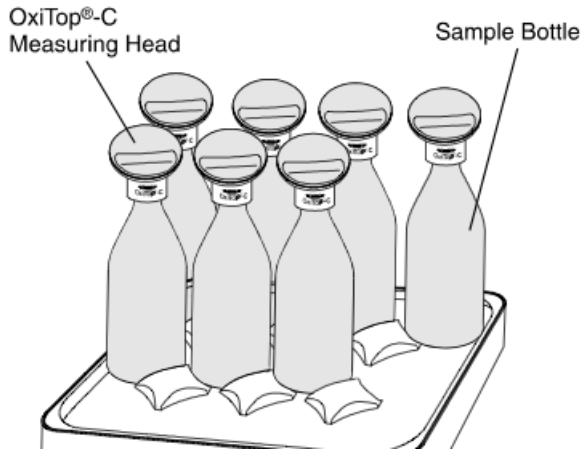

Figure 2.8 The OxiTop® system. The system consists of sample bottles containing different samples. The measuring head registers the drop in pressure, which relates to the BOD and makes it possible to monitor the growth of the microorganisms (WTW Weilheim, 2006).

The microorganisms in the sample bottle will draw oxygen from the air in the bottle to degrade the hydrocarbons present. Due to the reduction in the amount of oxygen that is present in the bottles the pressure will sink, and the change is detected and the information

is stored in the measuring head. The data can be transferred to a controller belonging to the system, and the BOD value will be calculated. The pressure sensor can register differences in the interval of 500 to 1350 hectopascal (hPa), with an accuracy of ±1% of the value (WTW Weilheim, 2006).

2.8.2 Chemical analysis

To evaluate the total hydrocarbon content (THC) in the samples at different times, gas chromatography was used to perform the analysis and determine degradation of hydrocarbons over time. To extract hydrocarbons from the sample a liquid-liquid extraction was performed according to the standard extraction method, which involves liquid extraction from an aqueous phase to a hydrocarbon solvent phase (e.g. pentane) (European Committee for Standadization, 2000). The compound of interest is separated into the phase where it is miscible by mixing the two phases in a separatory funnel multiple times, and extracting the phase of interest each time. When mixing the two phases, a compound will move into the phase where it is soluble, and stay in this phase (European Committee for Standardization, 2000).

After a liquid-liquid extraction, samples are usually up-concentrated to a known volume by evaporating off a majority of the solvent in which it has been extracted (Delille et al., 2009). The up-concentration is performed by an apparatus designed for this, which is part of the standard method for hydrocarbon analysis (European Committee for Standadization, 2000). In order to identify and quantify the different chemical compounds present in the samples, gas chromatography (GC) is the preferred method for analysis. The sample is vaporised and introduced into a chromatographic column, where each compound in the gas phase has a vapour pressure. Different compounds will partition between the gas phase and the stationary phase, and each time a molecule enters the gas phase it will be carried towards a detector by an inert mobile phase stream. The chemical components react in different ways to a stationary phase inside the column, and they are thus separated by their chemical affinity. Because of this, the different compounds with distinct chemical properties will reach the detector at different times and give a complete analysis of the chemical

substances present in a sample (Stuart, 2003). A GC equipped with a flame ionization detector (FID) was used in this study.

A FID detector is a commonly used detector for GC, and it is considered to be the best "universal" detector for organic analysis. The detector responds to the compounds in the sample as they evaporate and elute from the end of the chromatographic column. In a FID detector, the compounds that are eluted are passed through a hydrogen-air or hydrogen-oxygen flame that causes positive ions and electrons to form together with carbon dioxide and water. The ions will migrate between the electrodes in the detector causing an external current to flow that carries the evaporated compounds through the detector for identification and quantification (Stuart, 2003). GC-FID can be used to monitor the degradation of hydrocarbons, where the values are calculated as a percentage degraded relative to the abiotic control samples (Deppe et al., 2005).

2.8.3 Microbial community analysis

Enumeration of the microbial community can be used to monitor the growth in different samples, and to estimate population sizes. The method chosen for the enumeration in this study was agar plates designed to select for hydrocarbon degraders with crude oil as a hydrocarbon source, where the colonies were counted after a set incubation period. The enumeration methods are commonly used to monitor the number of hydrocarbon degrading microorganism present during an oil spill, and to isolate and analyse certain hydrocarbon degrading microorganisms (Vinas, Sabate, Espuny, & Solanas, 2005)

In order to investigate the population of microorganisms present in the samples, and the changes of this population over time during degradation of a substrate, molecular based community analyses are commonly used. To perform these analyses, DNA is isolated from the community as a whole and separated according to size to evaluate the structure of the community. Denaturing gradient gel electrophoresis (DGGE) is commonly used, and is a molecular based method that allows for separation of DNA fragments of nearly the same length. This method is useful for looking at differences in the population after a certain amount of time to evaluate the effects of e.g. hydrocarbon degradation, as DNA is extracted

form the entire population. DNA is amplified using PCR with primers containing a GC clamp (40-45 bp GC rich sequence). The GC clamp is located within the last five bases from the 3' end of the primers, and promotes a specific binding at this end due to the bonding between the G and C bases (Rettedal, Clay, & Brozel, 2010). The primers are designed to amplify the 16S ribosomal RNA (rRNA) sequence, which is useful when evaluating differences between communities. The 16S rRNA is a component in the small subunit of the ribosomes in prokaryotes, and is commonly used to evaluate differences in population due to its slow evolutionary rate (Woese & Fox, 1977).

The amplified 16S region PCR product is separated on a polyacramide gel containing increasing amounts of urea and formamide, which causes the DNA to denature in the gel. The denaturing point of DNA depends on its sequence and the content of GC base pair bindings, and denatured DNA is not able to travel in the gel. Based on their nucleic acid sequence, the amplified DNA fragments will denature at different places in the gel and then become stationary. Theoretically one band represents a population in the community, but in practice several different bands may represent one population (de Araujo & Schneider, 2008), and different populations may be represented by only one band (Vallaeys et al., 1997). This phenomenon occurs because several 16S gene sequences may be present within a single microorganism (Case et al., 2007). DGGE analysis is commonly used to analyse changes in microbial communities, but have some disadvantages to be aware of: It may be difficult to reproduce the results, as DNA is extracted from a diverse community of environmental microorganisms. PCR bias and artefacts is also an important factor to keep in mind when evaluating DGGE results (Acinas, Sarma-Rupavtarm, Klepac-Ceraj, & Polz, 2005).

2.9 Research objectives

The objective of this study was to evaluate the effect of the dispersant Corexit 9500 on biodegradation of diesel fuel in cold environments where the temperature is around 4-8°C during longer periods of time. The effect is monitored by incubation at low mesophilic and

psychrophilic temperatures where the indigenous population of microorganisms are allowed to populate. By comparative analysis the following issues were addressed:

- Does the dispersant enhance biodegradation rates?
- Does the selected dispersant work better at higher temperatures?
- Will the use of a dispersant alter the population of hydrocarbon degrading microorganisms?

In addition the degradation of Corexit 9500 itself was evaluated. The aerobic degradation of hydrocarbons was performed by using Shell Diesel Extra. The experiment was performed at 3°C, 8°C and 15°C, with and without the dispersant. The biological oxygen demand (BOD) was monitored over a 60 day period, where analysis was performed at time zero and after incubation for 30 and 60 days. The hydrocarbon content was analysed by liquid-liquid extractions and GC-FID gas chromatography and the microbial community was analysed by DGGE and enumeration. Use of Bushnell-Haas agar media with crude oil was also evaluated, where the focus was whether or not this method was suitable for enumeration of hydrocarbon degraders.

3 MATERIALS AND METHODS

In the following chapter, a detailed account of the techniques and materials used for the analysis of diesel fuel breakdown in cold environments will be explained. The execution and time schedule of the experiment will be explained to clarify when the different sections of the experimental work was performed, followed by a description of the different sections. The methodical execution will be explained in detail for each experimental setup.

3.1 Experimental overview

Analyses were performed at time zero, after 30 days of incubation and after 60 days of incubation. For each time period, DNA and remaining hydrocarbons were extracted from the seawater for the different temperatures (3°C, 8°C and 15°C) and dilution series of samples were plated on Bushnell Haas agar plates with light Arabian crude oil as a selective substrate media to determine the total count of hydrocarbon degraders at the different temperatures as the population had been allowed to grow over time. The experimental analysis consisted of three sections: a BOD analysis using the OxiTop® system to monitor growth, chemical analysis of the hydrocarbon content using GC-FID to determine the total degradation of hydrocarbons and a microbiological analysis of the biodegrading community to look at the numerical increase in the microbial populations using plating and the changes in population structure using denaturant gradient gel electrophoresis (DGGE).

The carbon source for the samples was Shell Diesel Extra for the test bottles and sodium benzoate ($C_7H_5O_2Na$) for the positive control bottles. One portion of the samples for each temperature were added diesel, another the dispersant Corexit 9500 and the remaining samples were added both diesel and Corexit in order to evaluate the effect of the dispersant on microbial hydrocarbon degradation (see chapter 3.2.3 for details). The purpose was to evaluate the degradation of hydrocarbons over time to investigate if the presence of Corexit 9500 increased the biodegradation of diesel in cold environments. For time zero the seawater sample was taken directly from the collection carboy, while for analysis of hydrocarbons and the microbial community after 30 and 60 days of incubation BOD bottles

were sacrificed. One set of bottles was sacrificed for hydrocarbon extraction and another set for enumeration and DNA extraction for experiments performed at time zero, and after 30 and 60 days. For time zero diesel, Corexit and a Corexit/diesel aliquot was added to the samples immediately prior to analysis. For analysis after 30 and 60 days, bottles were prepared and incubated before they were sacrificed for analysis.

To evaluate the total hydrocarbon content, the hydrocarbons were extracted in pentane using the liquid/liquid extraction method and cleaned up using fluorisil solid phase extraction. GC-FID was used to measure the total hydrocarbon content of the samples. The extracted DNA was amplified using PCR and analysed by running it on a DGGE gel to evaluate the changes in the microbial population. The overall results gave an indication of the total degradation of the diesel fuel in the samples, and the effect of the dispersant Corexit 9500 on hydrocarbons degradation and microbial population.

3.2 BOD analysis

Aerobic biodegradation of diesel in cold temperatures was monitored over a period of 60 days by monitoring the oxygen demand in a closed bottle system using the OxiTop® system. This system uses static respirometric measurements where the decrease in available O_2 was measured to determine the biological oxygen demand (BOD). Bottles were sacrificed after incubation for 30 and 60 days to isolate DNA and perform hydrocarbon extraction and evaluate the total hydrocarbon degrader count to obtain information of the biodegradation of diesel fuel. Bottles incubated at 3°C were sacrificed after incubation for 67 days rather than after 60 days. A sample was analysed directly from the sampled seawater for time zero.

3.2.1 Sampling

Three 5 L Nalgene bottles were autoclaved prior to sampling of seawater. The water was obtained 2nd of March 2015 from the facilities belonging to the International Research Institute of Stavanger (IRIS) in Mekjarvik, Norway through a pipeline system at 80 meters depth in an unpolluted fjord (59°1'N, 5°37'E). The temperature at the time of sampling was

8°C. The carboys were stored in cooling incubators (Termaks, B8420) for three days at 3°C, 8°C and 15°C with one carboy at each temperature. The density of seawater was assumed to be 1.026 kg/L and the salinity 3.5%.

3.2.2 Experimental setup

To perform the BOD measurements the OxiTop® Control system was used to monitor the changes in pressure in a closed bottle system. All equipment was sterilised prior to use.

Three modified (N-source) Bushnell-Haas inorganic nutrient solutions of pH 8.2 (solutions A-C) and one Balch trace solution (solution D) was prepared as described in Table 3.1. The solutions were used to provide nutrients for microorganisms growing in the BOD bottles, and functioned as a nitrogen and trace elements source. The solutions were filter-sterilised using a sterile 0.2 µm filter (PALL).

Table 3.1 Modified (N-source) Bushnell-Haas inorganic nutrient solution (A-C) and Balch trace element solution (D). The solutions were prepared to 1.0 L with dH_2O and filter sterilised using a 0.2µm filter.

Solution A		**Solution B**		**Solution C**		**Solution D**	
Concentration [g/L]		**Concentration [g/L]**		**Concentration [g/L]**		**Concentration [g/L]**	
K_2HPO_4	16.20	$NaNO_3$	25.00	$CaCl_2$	2.50	EDTA	0.50
KH_2PO_4	0.80	NH_4Cl	0.60	$MgSO_4$	1.50	$MnSO_4$	0.40
		$FeCl_2$	0.05			$MgSO_4 \cdot 7\ H_2O$	3.00
		EDTA	0.20			NaCl	1.00
						$FeSO_4 \cdot 7\ H_2O$	0.10
						$CoCl_2 \cdot 6\ H_2O$	0.10
						$CaCl_2 \cdot 2\ H_2O$	0.10
						$ZnCl_2$	0.10
						$CuSO_4 \cdot 5\ H_2O$	0.04
						$NiCl_2 \cdot 6\ H_2O$	0.02
						$AlK(SO_4)_2 \cdot 12\ H_2O$	0.02
						H_3BO_4	0.01
						$Na_2MoO_4 \cdot 2\ H_2O$	0.01
						$Na_2WO_4 \cdot 2\ H_2O$	0.01

200 mL of seawater was dispensed into each of the experimental bottles by weight using a density of 1.026 g/ml. 400 mL was added to the blank bottles. Nutrients were added to all bottles as listed in table 3.2. For the blanks, twice the amount was added to compensate for the double volume of seawater.

Table 3.2 Nutrients added to the BOD bottles with seawater. The solutions A-D, a vitamin solution and amino acids were added to the bottles. For the blank samples, the amount of each solution was doubled to compensate for the double volume of seawater.

Solution	Amount
Solution A	200 µl
Solution B	200 µl
Solution C	200 µl
Solution D	200 µl
Vitamin solution (Sigma Aldrich, MEM vitamin solution)	10 µl
Amino Acids (Sigma Aldrich, Amino Acids Mix, RPMI 1640)	10 µl

Diesel and Corexit 9500 was filter sterilised using a 0.2 µm filter prior to use. A 5% (v/v) Corexit in diesel aliquot was prepared by adding 0.05 ml Corexit to 0.95 ml of diesel. The diesel, Corexit and the aliquot were stored at room temperature for the duration of the experiments.

For each temperature (3°C, 8°C and 15°C) the following set was prepared, in all 57 bottles:

- Two blanks.
- Two positive controls.
- Five parallels with added 30 µl Corexit in each.
- Five parallels with added 30 µl diesel in each.
- Five parallels with added 30 µl 5% Corexit in diesel aliquot in each.

Two parallel bottles were prepared to perform extraction of hydrocarbons, Corexit and DNA at time zero. All samples were prepared in the same manner as for the incubation bottles. The blanks were used to monitor potential contamination of the samples, both after incubation and at time zero. One set of bottles was filtered trough a 0.2 µm filter and used for DNA extraction to determine the microbial community at time zero, while the other set was sacrificed for hydrocarbon extraction.

To the positive controls sodium benzoate (1.0 mL, [3.84 g/L]) was added to serve as a carbon source. To absorb carbon dioxide that was produced during biodegradation two pellets of sodium hydroxide was added to the rubber sleeve in the bottles of the OxiTop system. The bottles were fitted with pressure sensors (OxiTop-C measuring heads, WTW) that were sealed tightly to monitor pressure changes that occurred as oxygen was consumed during biodegradation. The bottles were left to equilibrate over night before they were started. Data was monitored and collected during incubation using the OxiTop controller and evaluated using the Achat OC software program.

After 30 days two bottles of each set incubated with diesel, Corexit and the Corexit/diesel aliquot were sacrificed for each temperature to extract hydrocarbons (for method see chapter 3.3) and to perform the bacteriological analyses (for method see chapter 3.4). Samples were taken again after 60 days to perform identical analyses as at time zero and after day 30. Before the bottles were set up, DNA was extracted from the seawater for all temperatures to monitor the microbial community over time (for method see chapter 3.4).

3.3 Chemical analysis of hydrocarbon content

The hydrocarbon content was analysed using GC-FID. Prior to GC analysis, hydrocarbons were extracted from the seawater by a liquid-liquid extraction using pentane (n-Pentane 99%, GC capillary grade, VWR) as the organic solvent during extraction. The hydrocarbons were extracted for analysis at time zero, and after 30 and 60 days to determine the degree of biological hydrocarbon degradation over time with and without Corexit 9500 addition. The samples were up-concentrated to a specific volume by evaporating the majority of the pentane prior to the GC analysis.

3.3.1 Liquid-liquid extractions

All glassware was washed with pentane three times before use. Extractions were performed in accordance with guidelines in the ISO 9377-2 standard provided by the European Committee of Standardization (2000). Extractions were performed with n-pentane as the hydrocarbon solvent. For extractions of time zero samples, bottles were prepared

separately using seawater incubated at 3°C, 8°C and 15°C. 200 mL of seawater was dispensed into each of the experimental bottles by weight using a density of 1.026 g/ml and nutrients, vitamins and amino acids were added in accordance with table 3.2. For extraction of hydrocarbons at day 30 and day 60 incubated bottles were sacrificed.

Extractions were performed by adding 20 mL of pentane to the BOD test bottle and stirring the content in the bottle for 30 minutes on a magnetic stir plate. The content of the bottle was poured into a 500 mL separator funnel and the funnel was turned 12 times while regularly releasing pressure. When there was no more pressure in the funnel it was left for 5 minutes to separate the water and solvent phase. If an emulsion formed, magnesium sulphate (2 g) was added to break the emulsion. The aqueous phase was carefully drained into a beaker making sure no pentane was drained out of the funnel. The solvent hydrocarbon phase was drained into a 250 mL separation funnel. The water was returned to the 500 mL separation funnel, and 20 ml of pentane was again added to the BOD bottle. The bottle was carefully rinsed to collect any residual oil and the pentane was poured into the beaker and swirled and then added to the water in the 500 mL separation funnel. The funnel was turned and pressure released as previously described, and the funnel was set to rest before the aqueous and solvent phases were drained into a beaker and 250 mL separation funnel respectively. The extraction was performed two more times using 20 ml pentane for a total of three times, draining the solvent phase into the 250 ml funnel. The water phase was discarded after the last extraction.

A glass column with a fritted funnel was prepared by adding Florisil adsorbent (Merck, column chromatography grade, 2 grams) evenly to the column and then sodium sulphate ($NaSO_4$, anhydrous, 2 grams) evenly on the top of the Florisil. The column was rinsed with pentane (10 ml) without agitating the surface. Any residual aqueous phase in the 250 mL separation funnel was drained and discarded prior to the fritted column filtration. When all the pentane had drained from the column, the hydrocarbon solvent phase was carefully added to the column for adsorption of any residual hydrophilic substances. The filtrated solvent phase was filtrated into a 50 mL glass bottle. Isooctane (1.5 mL, Merck) was added and it was sealed with a Teflon coated top and stored at 4°C until up-concentration.

3.3.1 Up-concentration of samples

All glassware used for up-concentration was washed with pentane three times prior to use to ensure the equipment was clean. From previous trials in the lab it had been experienced that a stirring speed of 150 rpm was needed for an effective evaporation that was finished in the course of 6-8 hours, depending on the efficiency of the evaporation wells.

The evaporation setup consisted of a Büchi Syncore evaporation apparatus connected to a circulating cooling bath (VWR, 1180S) that contained diluted anti-freeze (Biltema, standard), a vacuum pump (VWR, V-855) and a secondary condenser (Büchi, type S). The VWR circulating cooling bath was replaced by a recirculating cooler (Julabo, F250) after the time zero samples. The evaporation apparatus consisted of six evaporating wells with interchangeable special glass beakers for evaporation that was designed to allow evaporation down to 0.3 mL. Evaporation past this point was prevented by a cooling mechanism where a small volume of water at the bottom of the well holding the glass beaker was kept cool by the circulating anti-freeze, preventing the evaporation of the bottom portion of liquid in the glass beaker. The cooling bath was set to 0°C, and the anti-freeze fluid circulated throughout the evaporation system and into the secondary condenser. The vacuum pump was set to 900 mBar to suck up the evaporated pentane, which was led into the secondary condenser that cooled and condensed the evaporated pentane into a 2000 ml collection flask.

Pentane has a low boiling point (36.1°C), so the evaporation wells were tempered to 50°C and the cover rack to 55°C to ensure an effective evaporation of the pentane. Isooctane (1.5 mL, boiling point 99°C) was added to prevent the loss of extracted hydrocarbons that could occur due to excessive evaporation of the pentane.

When the liquid content in the glass beakers reached approximately 2-3 mL, the liquid for each sample was pipetted into 5mL volumetric flasks. The glass beakers were washed with a small amount of pentane (approximately 1 mL) that was collected to the volumetric flask. The volume was brought up to 5 mL and the samples were added to clear glass sample vials and stored at 4°C until analysed using GC-FID.

3.3.3 Analysis of hydrocarbon content using GC-FID

After up-concentration, the samples were chemically analysed using GC-FID to evaluate the effect on the dispersant Corexit 9500 on the degradation process. A pre-prepared hydrocarbon standard (ASTM® D5442 C12-C60 Qualitative Retention Time Mix) ranging from C_{12} to C_{60} was used to calibrate the instrument to identify the retention times of the hydrocarbons of interest from the diesel fuel and Corexit extracts. The GC instrument (Agilent 6890N) was equipped with a flame ionization detector (FID) and a Gerstel Multipurpose Sampler (MPS). A MSD ChemStation G1701DA was used to monitor and manage the results obtained in the GC-FID analysis. The samples (1 mL) were analysed in amber glass autosampler vials (2 mL) that were fitted with a Teflon septa and a fitted metal cap. Pentane was analysed as a blank sample for each sequence, and the analyses were performed according to the instruments operating instructions (Gerstel Supplies, 2000).

3.3.3.1 GC-FID method

The autosampler injector for liquid (Gerstel, MPS) was equipped with a glass syringe (Gerstel Australia, 10 µL). The liquid injector was set to sample 5.0 µL of liquid in three fill strokes for each sample injection, where the injection volume for analysis was 2.0 µL with a fill speed of 5 µL/s. The viscosity delay was two seconds. The sample vials were placed in an autosampler rack (Tray 2, VT98). Injection penetration depth was set to 30 mm with a syringe air volume set to zero. After injection, the syringe rinsed with cyclohexane with a fill/eject speed of 50.0 µL/s.

Only the back inlet was used, set to a split ratio of 10:1 with a split flow of 20.0 mL/min. To create a continuous gas flow, helium gas was used with a total flow of 25.1 mL/min. The gas saver flow was set to 15.0 mL/min at 1.50 min. The initial temperature for the inlet was 300°C with a pressure of 65.8 kPa. The column used (Colum 2, Agilent HP-5 19091J-413, back column) was a capillary column composed of 5% phenylmethylsiloxane, which was connected to the front detector (FID) and the back inlet. The maximum temperature for the column was 325°C, the nominal length was 30.0 m, the nominal diameter was 320 µm and

the nominal film thickness was 0.25 µm. The pressure in the column was kept constant, where the initial flow of helium was 2.0 mL/min with an average velocity of 33 cm/sec.

The instrument used a stepwise temperature setting, where the temperature settings were as follows: The temperature was increased to 50°C for three minutes with an equilibration time of 0.20 minutes. The temperature was then increased by 12°C per minute until the oven reached 350°C. This temperature was held for six minutes. The post temperature was set to 40°C. The front detector (FID) was set to a temperature of 325°C, where the hydrogen flow was set to 40.0 mL/min and the air flow was set to 450.0 mL/min. A makeup gas flow of nitrogen was set to 45.0 mL/min. The makeup gas flow was used to sweep the components trough the detector to minimise the band broadening of the spectrum (Froehlich, 2008). The flame and electrometer were turned on, and the lit offset was 2.0.

ChemStation was used to analyse the results, where the integration method was set to integrate the total area response between the selected retention times.

3.3.3.2 Calibrating the instrument

Calibration of the instrument was performed using the ASTM D5442 C12-C60 Standard Retention Time Mix (6.25% w/w, Sigma Aldrich). The standard was used for the GC-FID instrument to determine the retention time for individual hydrocarbons and the peak area response in accordance to the concentrations of the different compounds (complete list in Appendix C). The standard had been solved in 10 mL pentane. Retention times for each of the hydrocarbons were established based on their order of elution. Short chains will elude and reach the detector prior to longer chains. Linear calibration curves of response versus concentrations were made for each of the hydrocarbons. The standard curve and calibration of the instrument was performed to establish a reference to calculate the total area response between C_{12} – C_{60}.

3.3.3.3 Chemical analysis of diesel fuel and recovery

Diesel was diluted to concentrations of 25 g/L, 5 g/L, 1 g/L and 0.2 g/L of diesel in pentane to reveal its chromatographic content, and give an indication of its content for comparison to the profile of the extracted hydrocarbons. The dilutions were analysed using GC-FID to reveal its chromatographic content, and give an indication of its content for comparison to the profile of the extracted hydrocarbons. The amount of hydrocarbons present in the different samples was quantified using GC-FID analysis to determine the total removal of hydrocarbons from biodegradation in the samples.

THC was calculated as the mean total area response value for each sample, where the area was defined as the space above the baseline. The total hydrocarbon fraction removed from the samples was calculated on the basis of the response, rounded to the closest million. The percentage was calculated based on the difference in the total area of response at day zero compared to the total area of response for day 30 and 60. The results gave an indication of the total amount of hydrocarbons degraded in the samples for the different temperatures and for their content.

3.4 Community analysis of the hydrocarbon degrading bacteria

Bacteriological analyses were done at time zero, and after 30 and 60 days to compare changes that had occurred over time, and to enumerate the bacteriological community. By analysing at time zero and at the mid and end times of the experiment at the temperatures 3°C, 8°C and 15°C the possible changes in the biodegrading community could be analysed at different low temperatures. DNA was isolated from the bacteriological community and the 16S regions were amplified by PCR to analyse the population changes by DGGE. To enumerate the bacteriological community, the seawater was plated on saline Bushnell-Haas agar plates spread with filtered light Arabian crude oil. The oil plating method was quantified to determine its efficiency in cultivating and enumerating hydrocarbon degraders.

3.4.1 DNA extraction

Seawater was vacuum filtrated through a nitrocellulose filter (0.2 µm GSWP, Millipore) and DNA was extracted from the filter using the commercially available DNA extraction kit PowerSoil® DNA Isolation Kit (MoBio). DNA was extracted from the seawater for time zero for all temperatures to determine the initial microbial community. It was crucial to determine that a community of microorganisms were present in the seawater from the start, as this was a prerequisite for the experiment to perform as predicted.

For time zero, one litre of seawater was filtered for each temperature to verify the presence of DNA prior to starting up the BOD bottles and to get a profile of the initial community (see Chapter 3.2.3). The water was taken directly from the incubated collection carboys. BOD bottles were sacrificed as described in Chapter 3.1 after 30 and 60 days for DNA extraction, where the content of the bottles were filtered using the vacuum filtration system. The filter was cut into small pieces on a sterile surface and added to a PowerBead® tube provided in the PowerSoil® kit. DNA was extracted using the reagents from the kit according to the manufacturers instructions. Only 50 µL of the proved C6 solution was used to obtain a higher concentration of the extracted DNA. The presence of DNA was verified by running 5 µL of the sample with 2 µL 6x DNA Loading Buffer on a 1% agarose gel stained with GelGreen (VWR). The extracted DNA was stored at -20°C until analysed.

3.4.2 PCR amplification

The 16S rRNA regions of the extracted DNA were amplified using PCR and the universal forward primer 341F (5'-CCTACGGGAGGCAGCAG-3') and reverse primer SD907-r (5'CCCCGTCAATTCCTTTGAGTT-3') that had a GC-clamp attached to the 5' end (5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3'), targeting the V3-V4 hypervariable region of the 16S rRNA gene (Brakstad & Bonaunet, 2006).

The PCR reaction was performed in 50.0 µL volumes. The components of the reactions are listed in Table 3.3

Table 3.3 Components of the PCR reaction.

Component	Concentration	Volume
Double distilled H_2O	-	To 50.0 µL
10x PCR buffer (Sigma Aldrich)	10.0 mM Tris HCl, 50 mM KCl	5.0 µL
10 mM dNTPs	40.0 mM	1.0 µL
Forward primer	100.0 µM	1.0 µL
Reverse primer	100.0 µM	1.0 µL
Taq Polymerase Jumpstart Taq™ (Sigma Aldrich)	25 U/ µL	0.3 µL
$MgCl_2$	25 µM	3.0 µL
Template DNA	-	1.0 µL

The target sequences were amplified using a PCR instrument (Applied Biosystems 2720 Thermal cycler). The PCR reaction was run with denaturation at 95°C for 10 minutes, followed by 30 cycles at 95°C for one minute, 55°C for one minute and 72°C for 2 minute to replicate the 16S rRNA sequence, and completed with extended elongation at 72°C for ten minutes.

The presence of reaction product was verified by running the PRC product on a 1% agarose gel stained with GelGreen (VWR) and loaded with 6x DNA Loading Dye.

3.4.3 Denaturant Gradient Gel Electrophoresis (DGGE)

To evaluate the changes in the bacterial community, the PCR products were run through a denaturant gradient gel electrophoresis (DGGE). To run DGGE the amount of DNA present in the samples was estimated by evaluating the visibility of the bands in the agarose gel and comparing this to the band visibility of previously quantified samples. It was determined that a sample volume of 15.5 µL of sample from the time zero DNA extractions and a volume of 12.5 µL sample for the rest of the extractions would be sufficient. The samples were added loading dye up to 20 µL, and 5 µL of 6x loading buffer was added to each sample prior to loading the gel. The loading buffer was composed of 5ml TAE buffer, 5 ml glycerol (100%) and 0.5% bromophenol blue with xylene (200µL).

The glassware belonging to the DGGE apparatus was washed thoroughly with ethanol prior to assembly. A continuous gradient of 20-80% of the denaturing agents formamide and urea was used to make the DGGE gel, as the urea and formamide creates a denaturing environment separating the DNA fragments based on size and melting point. A 100% denaturant would correspond to 7M of urea and 40% deionized formamide. To prepare the DGGE gel, solutions of 20% and 80% formamide/urea were prepared as described in Table 3.4. All solutions were filtered through a 0.2 µm filter (PALL). As the solutions were light sensitive, they were stored at 4°C in amber glass bottles.

Table 3.4 20% and 80% DGGE solutions. The volumes of compounds were added to a 100 mL volumetric flask, and dH_2O was added up to 100 mL. The solutions were sterile filtered and stored in amber glass bottles.

Compound	20% DGGE solution	80% DGGE solution
40% Acylamide Bis	15.0 mL	15.0 mL
50x TAE buffer	2.0 mL	2.0 mL
Formamide	8.0 mL	32.0 mL
Urea	8.4 g	33.6 g
dH_2O	Up to 100.0 mL	Up to 100.0 mL

The PCR products were run on a 6% polyacrylamide gel prepared with the solutions in Table 3.4 to create an even gradient of urea and formamide. 30 µL of tetramethylethylenediamine (TEMED) was added to the DGGE solutions, and 300 µL of a 10% ammonium persulfate polymerising (APS) agent was added to both solutions shortly before pouring the gel. The gel was poured using a dispensing needle placed between the two glass plates of the apparatus, and the DGGE solutions were evenly dispersed using a gradient former connected to a persitaltic pump (Watson-Marlow Sci-Q 323) that ran at 7 rpms.

The 25 µL volume of sample and dye was loaded into the wells of the gel. The polyacramide gel was run for 18 hours at 90V using the IngenyPhor U2 system with 17 L of 1x TAE buffer heated to 60°C. A pump circulated the buffer. The gel was stained using 100 µL of GelRed

(VWR) to approximately 3 L of 1x TAE buffer by immersing the gel in the dye solution for one hour. The gel was visualised and images were taken using the BioRad Gel DOC XR Imagery System.

3.4.4 Bacterial enumeration

In order to determine the amount of hydrocarbon degraders, seawater was plated on saline Bushnell-Haas plates spread with light Saudi Arabian crude oil. This oil was light, easy to spread and the hydrocarbon degraders in the samples were able to degrade it well (Mulkins-Phillips & Stewart, 1974). The plates were made by dissolving Bushnell-Haas broth (3.75 g, Sigma Aldrich) and NaCl (25.00 g) to a total of 1.0 L with double distilled water. The solution was added agar (15.00 g, Merck) and heated to a boil before it was autoclaved. The agar was left too cool for a short while until it was cool enough to hold and Vitamin Solution (300 µL, Sigma Aldrich) and Wolfe's Trace element solution (1.00 mL, see Table 3.1) was added to the agar. The agar was poured into sterile petri dishes and left to solidify and dry. Saudi Arabian crude oil was filtered (0.2 µm, PALL) and 100 µl was plated onto each Bushnell-Haas plate. The plates were left to dry prior to being plated with seawater.

All samples for time zero, day 30 and day 60 were diluted in sterile seawater and plated on Bushnell-Haas oil plates (100 µl) with three parallels for each samples. All plates were incubated at 15°C for approximately 14 days, and the colonies were counted to determine the total cell count in the seawater. For time zero, the seawater was taken directly from the collection carboys for all three temperatures and diluted in in series from 10^{-1} to 10^{-6}. Dilutions and an undiluted sample were plated. For day 30 a dilution series from 10^{-1} to 10^{-8} was prepared from the sample removed from a sacrificed BOD bottle used for DNA extraction. For day 60, a dilution series from 10^{-1} to 10^{-8} was prepared and plated, and a sample was also taken from the blank sample bottles and diluted up to 10^{-4}. For the day 60 samples the plates for the 8°C and 15°C samples were incubated for 7 more days to evaluate if this would lead to an increase in the number of colonies.

4 RESULTS

Biodegradation of diesel fuel was investigated at three different temperatures, with and without addition of the dispersant Corexit 9500. Chemical and microbiological methods were used to analyse the biodegradation process and changes in the microbiological community. All relevant findings are presented in this chapter.

4.1 BOD analysis

Aerobic degradation of diesel was monitored continuously over a period of 60 days, and BOD values were sampled at a set interval by the OxiTop heads. Mean BOD values were plotted and standard error was calculated. A grey field in the graphs represents this standard error. Two parallels were used for the blank and positive controls and five parallels were used for the experimental bottles. Pressure change in the bottles was used to evaluate the oxygen requirements for microbial growth and biological degradation of hydrocarbons.

The BOD experiment ran for 60 days for the temperatures 8°C and 15°C. Due to a suspected low growth rate for the samples incubated at 3°C the samples bottles were restarted and incubated for seven more days in order to include a greater part of the exponential phase. The OxiTop bottles containing the 3°C diesel bottles stopped collecting data after day 30, thus this data is missing from the sample set.

BOD data for samples incubated with diesel, Corexit and the Corexit/diesel aliquot are presented comparatively based on temperature and content. Data for the blanks and positive controls are presented separately. Parallel bottles in a set had variations of the data points during the incubation time (Figure 4.1), and variations were calculated for using standard error calculations.

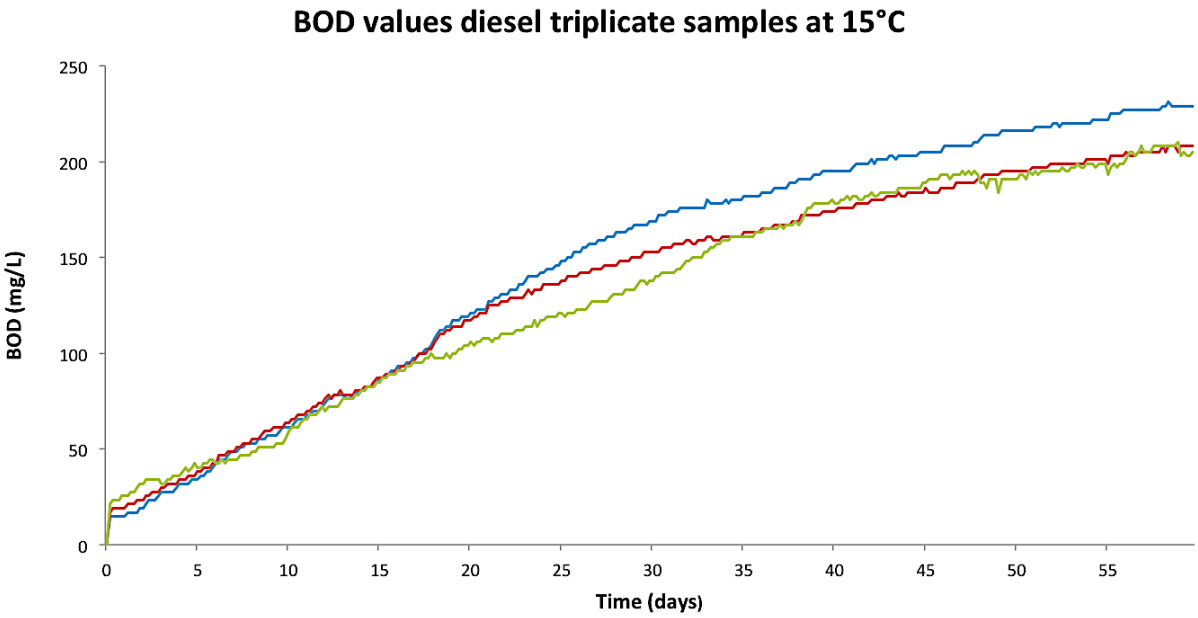

Figure 4.1 Representation of three parallel samples incubated at 15°C for 60 days with diesel. The graph visualises the differences between the BOD values for a typical set of samples incubated for 60 days.

The BOD values for the samples varied amongst the parallels (Figure 4.1). A variation in the values of around 20-25 mg/L occurs, depending on temperature and incubation time.

4.1.1 Positive controls and blank samples

The BOD curves for sodium benzoate at 3, 8 and 15°C traces the growth in the positive control bottles (Figure 4.2).

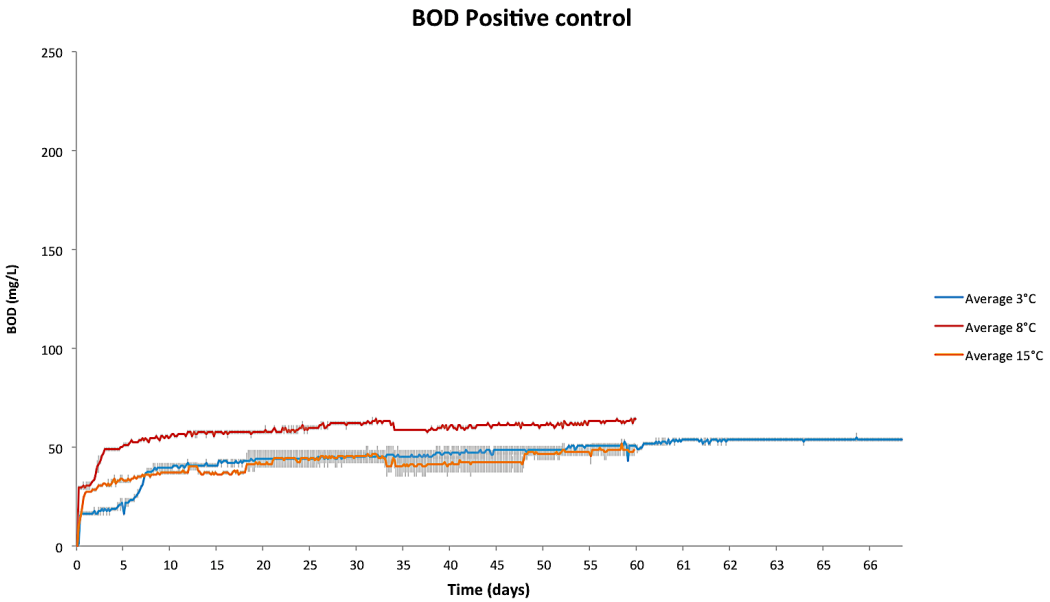

Figure 4.2 Average of parallels for the positive controls at temperatures 3°C, 8°C and 15°C. The samples incubated at 8°C separates themselves from the other samples by having higher BOD values. When including the standard error values, the degradation rate was approximately the same for all temperatures.

The positive controls performed as expected as approximately the same amount of sodium benzoate was degraded for all the samples and the growth rates of the samples were similar, demonstrating that the experimental setup was correct and indicating that the experimental bottles performed well. The exponential phase took place prior to day 15 for all samples, and the BOD values for the 8°C samples was higher than the other samples throughout the incubation period.

The BOD values for the blank samples were low, no greater than 15 mg/L (Figure 4.3), indicating that the samples were not polluted.

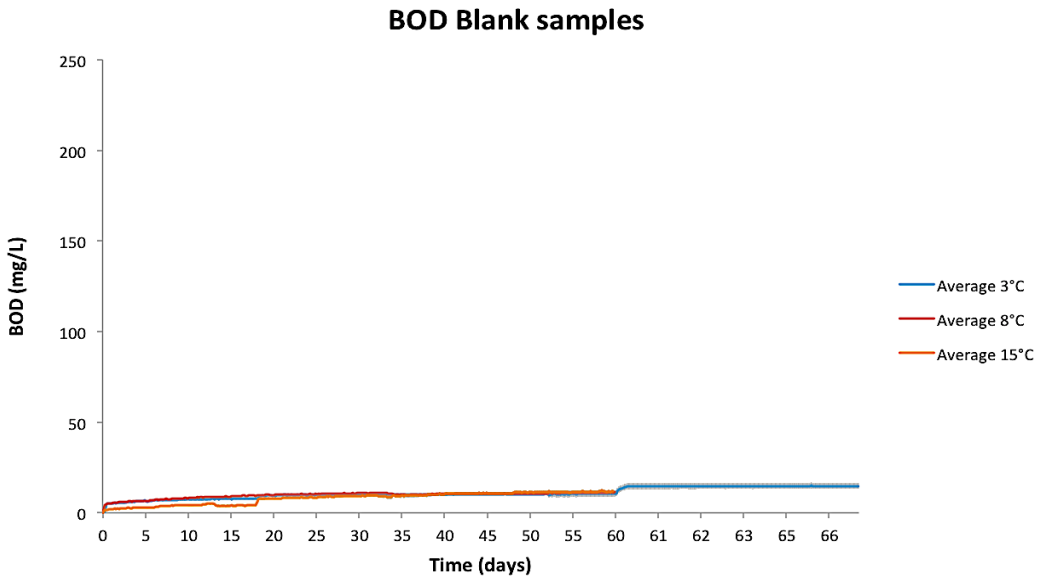

Figure 4.3 BOD values for blank samples at temperatures 3°C, 8°C and 15°C. The blank samples verify than the samples were not polluted. Some background growth was expected, as seawater does contain some organic carbons available for biodegradation.

Some growth was expected as trace elements, nutrients and amino acids was added to the bottles. Seawater usually contains 2-3 mg/L trace amounts of organic carbon available as substrate for the microorganisms (Ulas, 2013).

4.1.2 The effect of temperature on biodegradation of diesel

In general, a high biological oxygen uptake rate was seen with increasing temperatures for all treatments (Figure 4.4). However, in some time intervals at 8°C the biodegradation rate was higher than seen at 15°C initially, especially for samples incubated with only Corexit. The samples with diesel and the Corexit/diesel aliquot displayed greater difference between the temperatures compared to the samples with only Corexit, where the difference between 8°C and 15°C was minimal at the end of the incubation period. The growth was generally limited at lower temperatures, and the BOD values for the samples incubated with only Corexit were lower compared to the samples with diesel and the Corexit/diesel aliquot (Figure 4.4). Samples incubated with only Corexit had the same shape for the growth curve as for the other samples, but the BOD values were significantly lower.

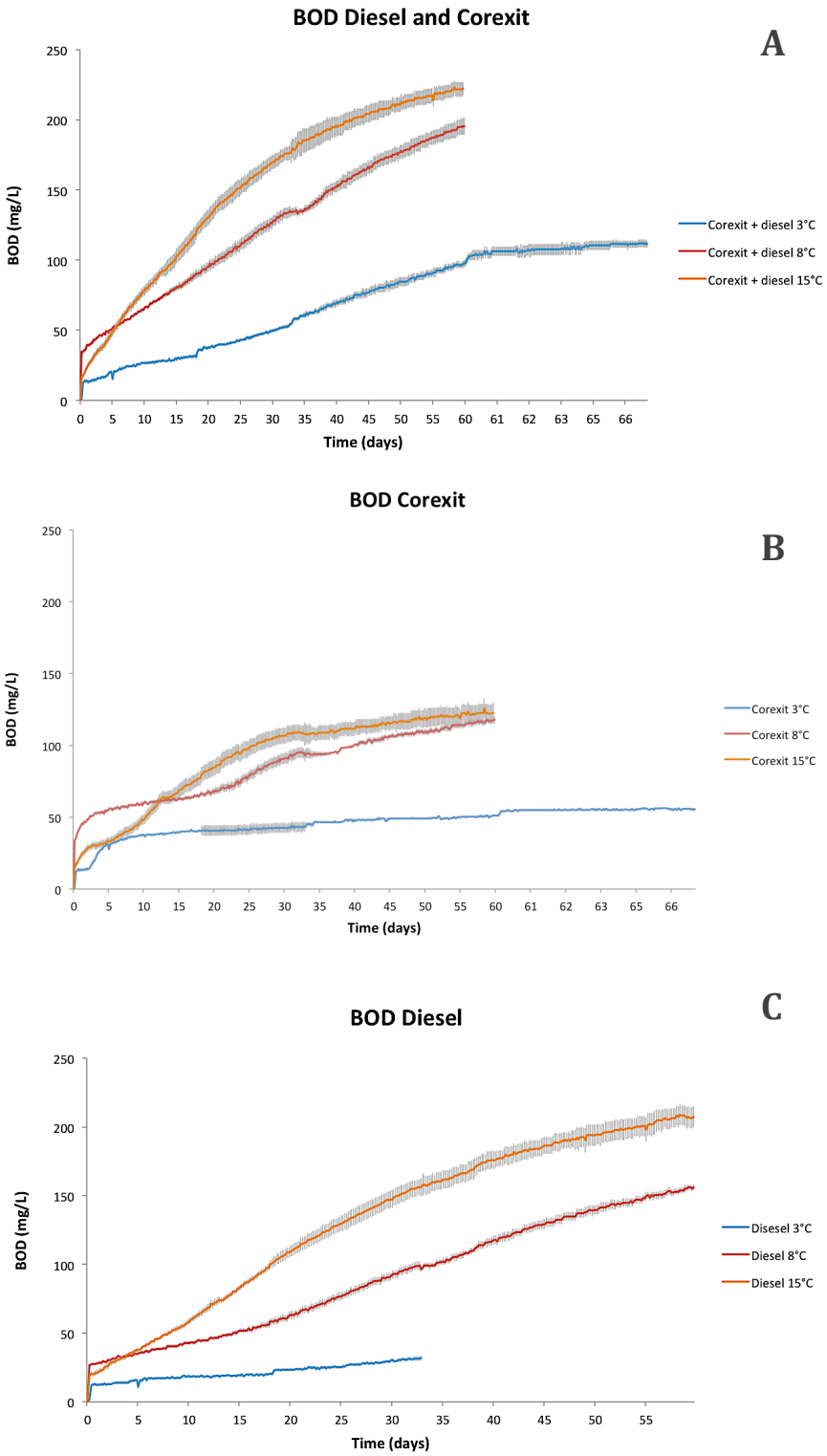

Figure 4.4 BOD values for samples with diesel, Corexit and a Corexit/diesel aliquot compared for temperature. The graphs indicate that the growth rate was highest for the samples incubated at 15°C, second highest for the samples incubated at 8°C and lowest for the samples incubated at 3°C.

Standard error for the samples increases over time, and was greatest for the samples incubated at 15°C. All samples had entered or were about to enter the stationary phase after incubation for 60 days. All samples had a very short lag phase. It appears that the samples containing only Corexit entered the stationary phase somewhat earlier than the other samples. Due to missing data for diesel at 3°C, few indications were given of the effect of temperature on the biodegradation of diesel alone (Figure 4.4C).

4.1.3 The effect of Corexit 9500 on biodegradation

The evaluation of the data for the BOD values shows that the dispersed samples have a higher biological oxygen demand (Figure 4.5). The exception is the samples incubated at 15°C, where the BOD values were approximately the same for the samples with diesel and the Corexit/diesel aliquot (Figure 4.5C). The premature shutdown of the data sampling for the diesel samples incubated at 3°C made it difficult to make assumptions of the effect of Corexit at this temperature (Figure 4.5 A). Data collected after restarting the bottles after 60 days showed little variations and revealed stable BOD values at this point, indicating that the sample had entered the stationary phase prior to the 60 day mark (data not shown).

The samples entered the stationary phase after approximately 60 days. The exception is the 3°C Corexit sample, which entered this phase at an earlier time (Figure 4.5A). The curves for the samples are steeper for the samples incubated at 15°C, showing that these samples had a more intensive microbial growth compared to the 8°C samples which have straighter curves and overall lower BOD values. The reaction rate was highest for the samples incubated at 15°C, with little variations in the data points over time.

The standard error was highest for the three 15°C samples (Figure 4.5C). The standard error values increased over time, with exception of the time period between day 20 and 35 for the samples incubated at 3°C where the standard error values were higher compared to the rest of the incubation period for the sample (Figure 4.5A).

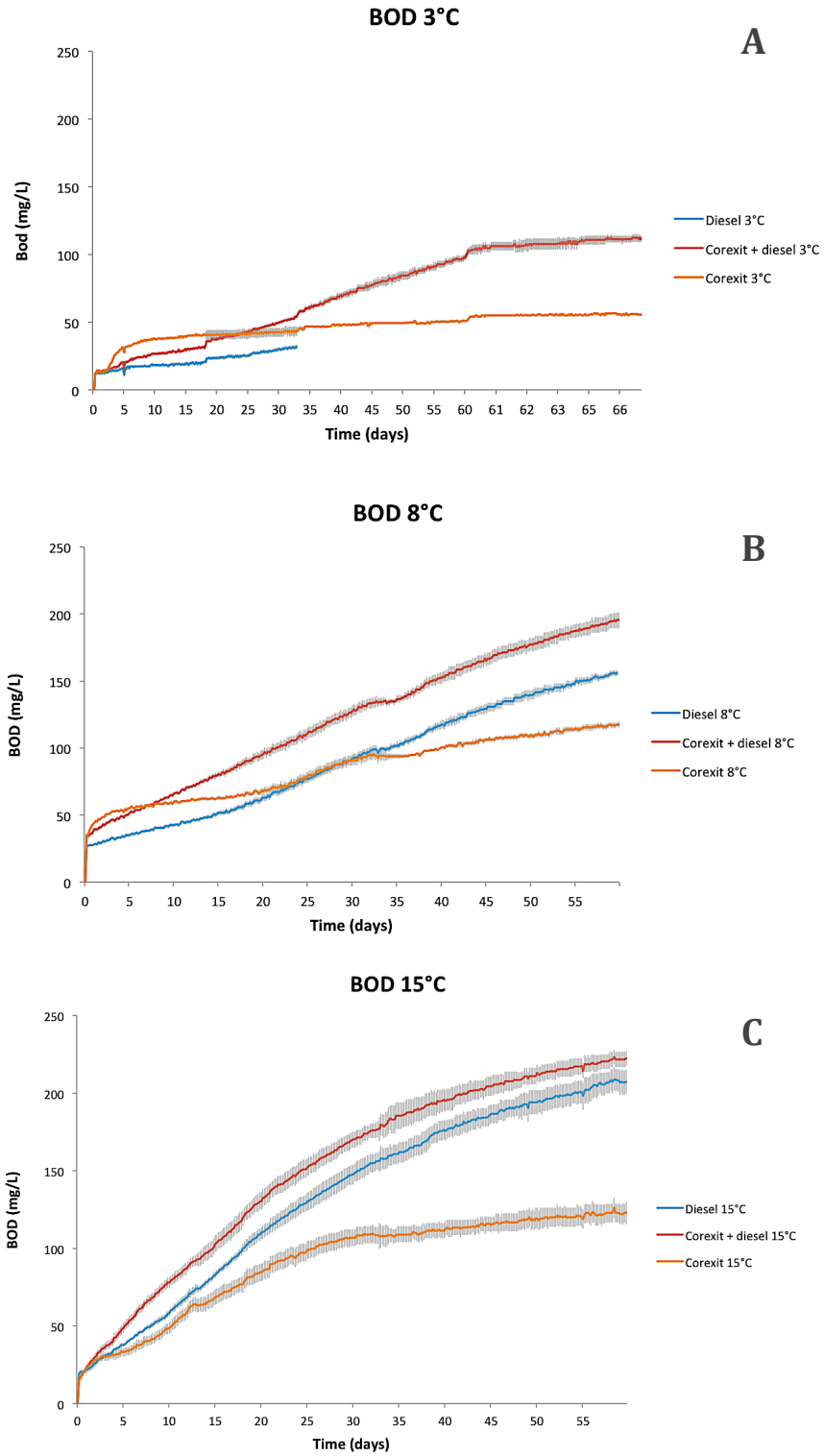

Figure 4.5 Effect of Corexit 9500 on biodegradation of diesel. The BOD values show that the sample containing both Corexit and diesel had higher growth rates and overall a higher biological oxygen demand. Assumptions for the degradation of diesel at 3 °C were difficult as the data was lost from day 30.

The addition of Corexit 9500 to samples containing diesel increased the biological oxygen demand at lower temperatures. The total amount of hydrocarbons degraded (Table 4.1) indicated that both diesel and Corexit were degraded rapidly, and that the addition of Corexit did not increase the degradation rate as both diesel and Corexit were degraded extensively during the first 30 days of incubation (see Chapter 4.2).

4.2 GC analysis

To determine the total hydrocarbon removal in the experimental BOD samples, gas chromatography equipped with a flame ionizer was used to calibrate reference hydrocarbons by establishing their retention times and to analyse the total hydrocarbon content of the samples extracted from seawater.

4.2.1 Calibrations

The GC-FID results were used to identify the total area of response in experimental sample chromatographs by establishing the time interval for hydrocarbons in the standard. The standard (ASTM® D5442 C12-C60 Qualitative Retention Time Mix) contained reference hydrocarbons used to establish retention times (Appendix C).

The retention times were used to determine the range of the hydrocarbons left in the extracted samples, and to look at the chromatographic profile of diesel and Corexit 9500.

4.2.2 Chemical analysis of diesel and recovery

The recovery of diesel and Corexit after extraction and up-concentration was based on a direct GC-FID analysis of diluted diesel and on extraction of diesel and Corexit from seawater. Diesel was diluted in pentane to concentrations of 25.0 g/L, 5.0 g/L, 1.0 g/L and 0.2 g/L and used as a standard for chemical analysis of the composition of diesel.

The composition of diesel was used to evaluate whether Corexit or diesel was left in the samples after incubation. To determine the percentage of diesel that had been degraded

and to determine whether Corexit had an effect on the overall degradation, the differences in response was evaluated.

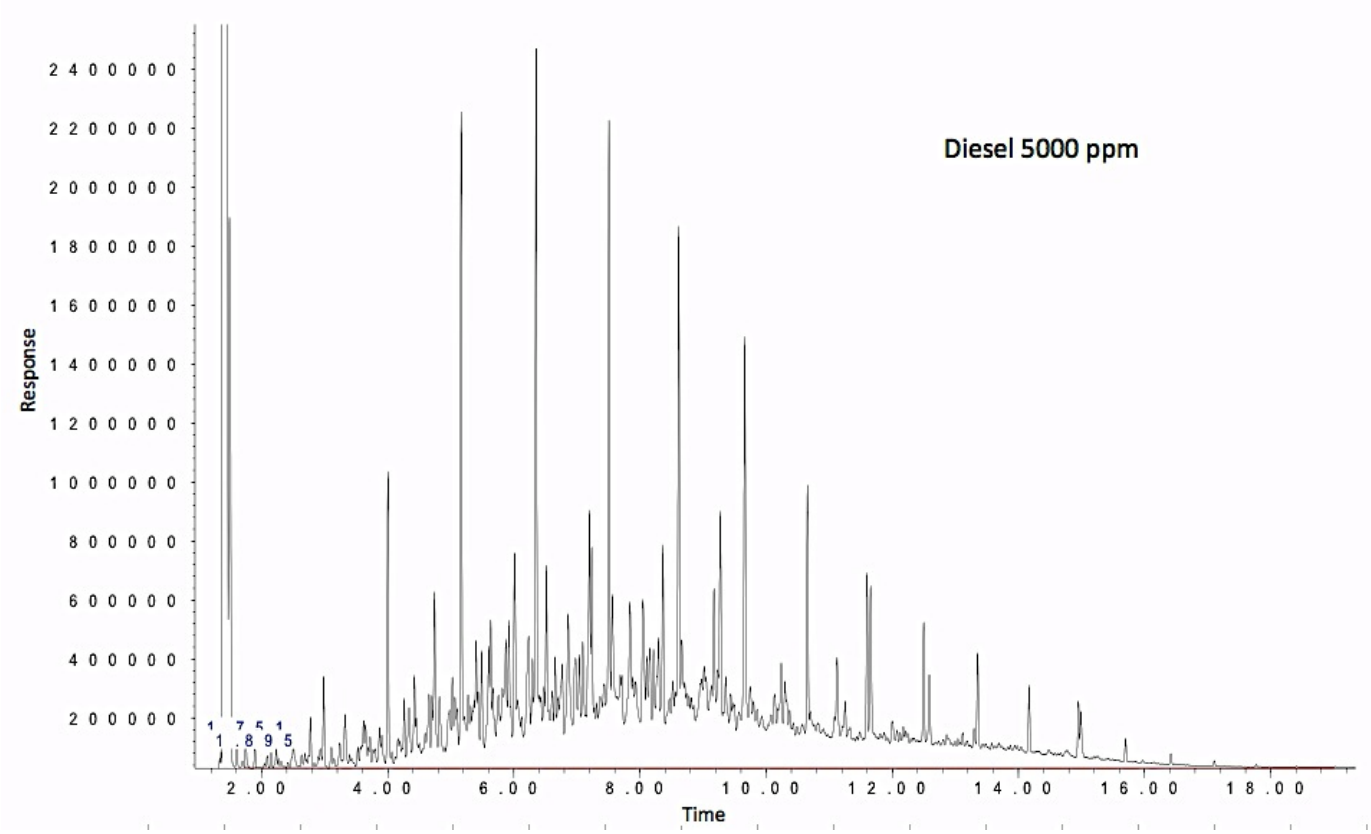

Figure 4.6 Chromatographic profile of diesel at a concentration of 5000 ppm (5 g/L). The chromatogram shows the retention times and response for the different compounds present in diesel. The area above the red integration line was integrated to get the total area response value. The carbon fraction of diesel was mostly between C_9 to C_{17}.

The chromatogram for diesel illustrates its composition and is shown with a red line representing the area used to integrate the total area response. The chromatographic profile shows that most compounds have a retention time between four and 14 minutes, showing that the most prevalent hydrocarbons were in the range from C_9 to C_{17} based on the retention times (data not shown).

The chromatographic profile of Corexit is very similar to that of diesel (Figure 4.7), but with a slightly narrower hydrocarbon range (around C_9 to C_{12}). Corexit is composed of a diesel fraction, and the coinciding chromatographic profiles for diesel and Corexit is as expected

(Roald Kommedal, personal communication). The first peaks in the chromatographic profile is the pentane and isooctane solvents, present at around time 2.00.

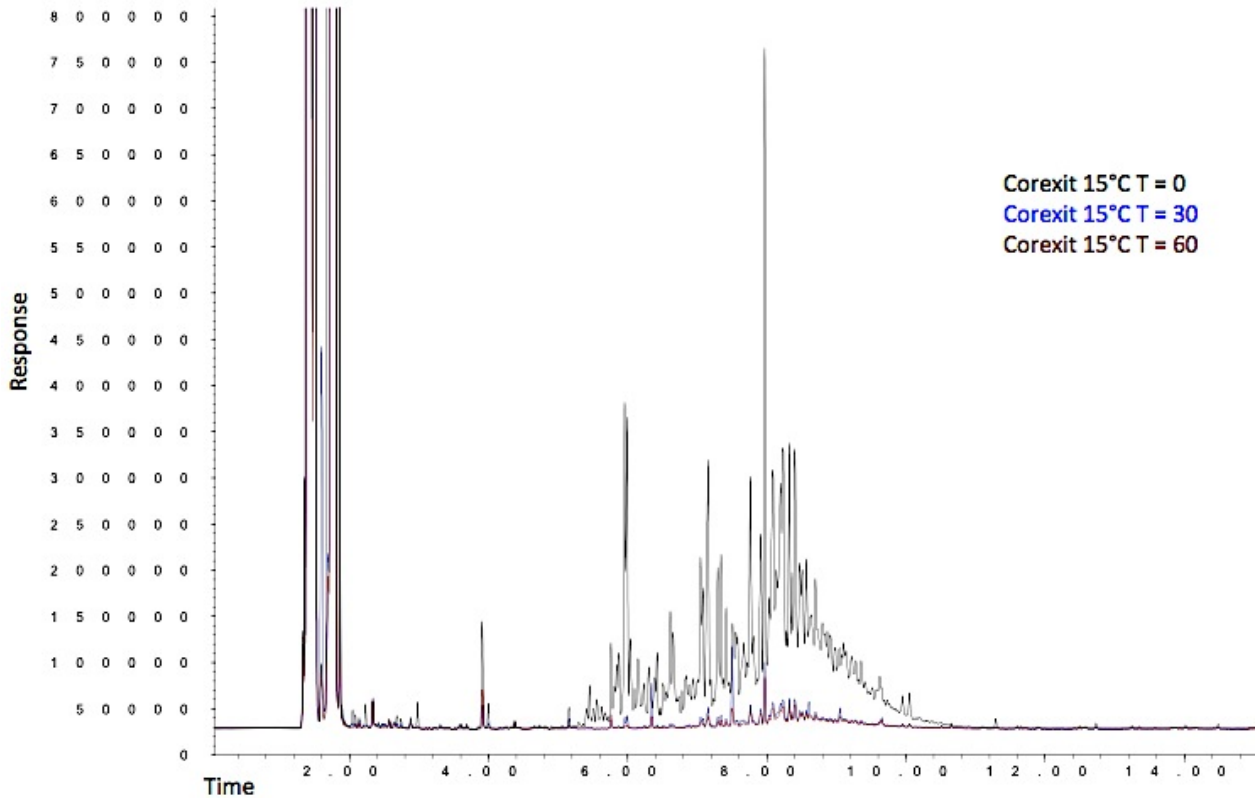

Figure 4.7 Chromatographic profile of Corexit 9500 at 15 °C. The chromatographic profile of Corexit is very similar that of diesel, with similar retention times. The hydrocarbon range of Corexit is slightly narrower than diesel, with an abundance of hydrocarbons ranging from C_9 to C_{12}. The peaks at time 2.00 were determined to be the pentane and isooctane solvents.

Diesel contained a broader spectrum of hydrocarbons than Corexit, where diesel had some lighter and heavier compounds compared to Corexit (Figure 4.6 and 4.7).

4.2.3 Total hydrocarbon analysis

Total hydrocarbon content (THC) analysis was performed for the samples from time zero and after incubation for 30 and 60 days to compare the total hydrocarbon removal caused by aerobic degradation of crude oil at 3°C, 8°C and 15°C with and without the presence of

the chemical dispersant Corexit 9500. The total removal of hydrocarbons was also investigated for Corexit alone. The chromatographic profiles for the analysed samples show that most of the diesel and Corexit had been degraded in the samples for all temperature, with an almost 100% degradation for diesel after 60 days at 15°C (Figure 4.8; Table 4.1).

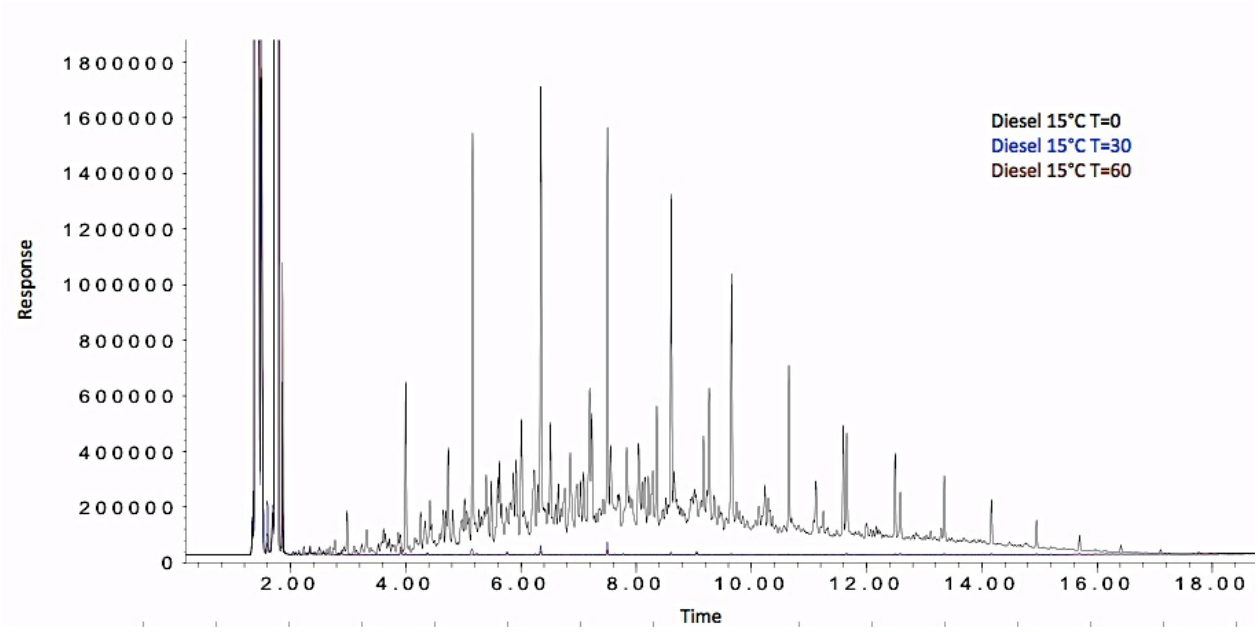

Figure 4.8. Chromatographic profile of diesel at point zero, after 30 days and after 60 days of incubation at 15°C. The chromatograph shows the initial amount of diesel at time zero in black, and the amount of diesel left in the sample after incubation for 30 and 60 days, where almost all the diesel has been degraded after 30 days.

After 30 days of incubation the diesel had been degraded almost completely for all samples. For diesel samples incubated at 15°C only the diesel from time zero is visible in the chromatographic profile (Figure 4.8). The same profiles were seen for 3°C and 8°C samples with only diesel. This profile was also seen for the samples containing both Corexit and diesel (Figure 4.9), and for the samples with Corexit alone (Figure 4.7) for all temperatures.

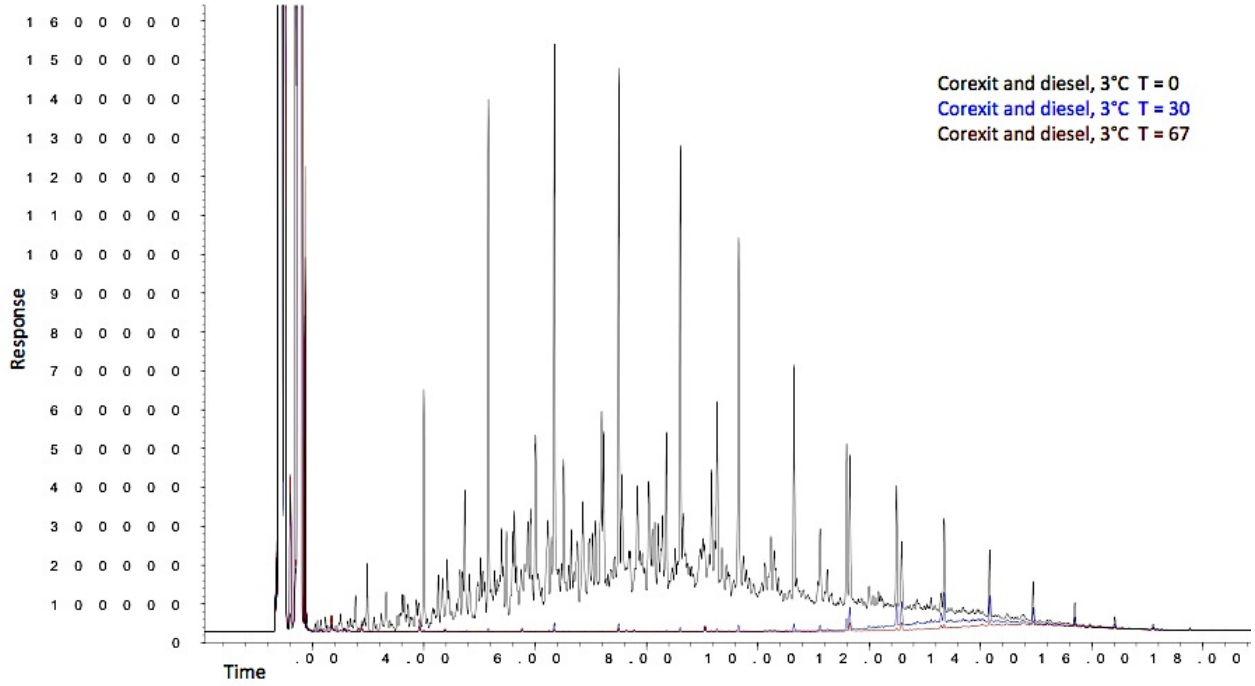

Figure 4.9 Chromatographic profile of samples containing Corexit and diesel incubated at 3°C. The samples incubated at 3°C generated the same profile as for the samples incubated at 8°C and 15°C, where the major hydrocarbon fraction from approximately C_9 to C_{17}, and where most of the hydrocarbons had been degraded after 30 days of incubation.

The chromatographic profiles shown in Figure 4.7, 4.8 and 4.9 are representative for all the analysed samples, where the hydrocarbon fractions left were similar for all the samples. All samples had a starting hydrocarbon fraction ranging from around C_9 to around C_{20}, with some variation depending on whether the fraction originated from diesel or Corexit.

For all temperatures, the degradation of diesel and Corexit was above or around 90% total degraded hydrocarbons (Table 4.1). The final result is based on the total area response in the sample chromatograph for samples at time zero and for those incubated for 30 and 60 days (67 days for samples incubated at 3°C). The blank mean response was calculated for the blank sample incubated at 15°C as this was the sample that integrated the best, and it was assumed that the other samples would show a similar value. The blank value was subtracted from each experimental sample mean response to eliminate the background noise found in the blank samples. The values show a total degradation of 92-99 % for diesel

after 30 days, and from 95-99% degraded after 60 days. The total amount of hydrocarbons degraded from the samples containing Corexit alone was the lowest of the values. The diesel samples incubated for 30 days for the temperatures 3°C and 8°C were lost during up-concentration, and are not included in the calculations.

Table 4.1 Removal of total hydrocarbon from BOD bottles over a period of 60 days. The extractions for 3°C were performed after incubation for 67 days. The table shows the degradation of total hydrocarbon from the incubated BOD bottles, analysed using GC-FID.

Sample	Temperature	% Biodegraded T=30	% Biodegraded T=60
Diesel		-	95
Diesel and Corexit	3°C	92	95
Corexit		76	88
Diesel		-	96
Diesel and Corexit	8°C	96	96
Corexit		76	92
Diesel		99	99
Diesel and Corexit	15°C	92	95
Corexit		93	93

GC-FID analysis revealed that the majority of the hydrocarbons of both diesel and Corexit had been degraded after 30 days (Table 4.1). For both diesel and Corexit the degradation could be considered to be complete after the 60 day incubation period, as some of the peaks in the chromatographic profile may be a result of the ongoing degradation of the microbial community itself (Roald Kommedal, personal communication).

Based on the chromatographic profiles for the samples, the effect of Corexit on the biodegradation of diesel was small or non-existing. Corexit was degraded approximately as much as the diesel, and the microbial community rapidly degraded both compounds during a 30-day period. There was also little difference between the different temperatures indicating that diesel was rapidly degraded even at lower temperatures, which may eliminate the need for a dispersant during diesel spills.

4.3 DGGE

Prior to the DGGE analysis, the DNA extracted from the BOD bottles was amplified using PCR that targeted the 16S rRNA bacterial gene. Gel electrophoresis with a 1% agarose gel confirmed that all products contained DNA. As there were some differences in the intensity of the bands (Figure 4.10), the gel was visually analysed to approximate the amount of PCR product sample that was needed to run the DGGE for the samples extracted at time zero, at day 30 and at day 60 (67). The intensity of the different bands can be seen in Figure 4.10.

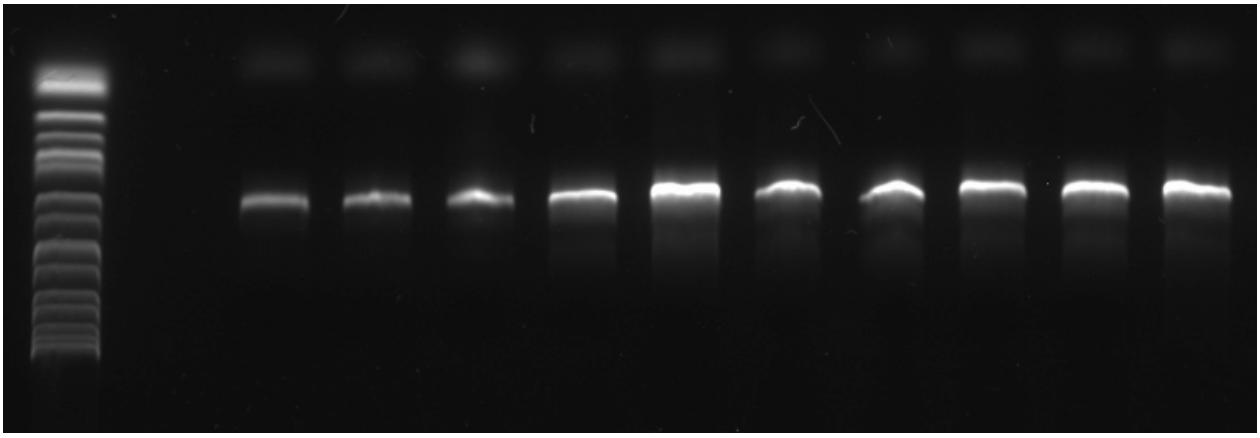

Figure 4.10 Section of agarose gel for PCR products. The first three bands after the marker is the DNA extracted at time zero, which contained less DNA from the extraction and gave a lower intensity in the gel. The figure is not labelled, as all wells contained DNA and the intensity of the bands were only of two varieties.

The DGGE results indicated a change in the bacterial community over time and with the different additions of Corexit and diesel and incubation at different temperatures (Figure 4.11). The figure is marked with C for samples added Corexit 9500, C+D for samples added the 5% Corexit/diesel aliquot and D for samples added diesel.

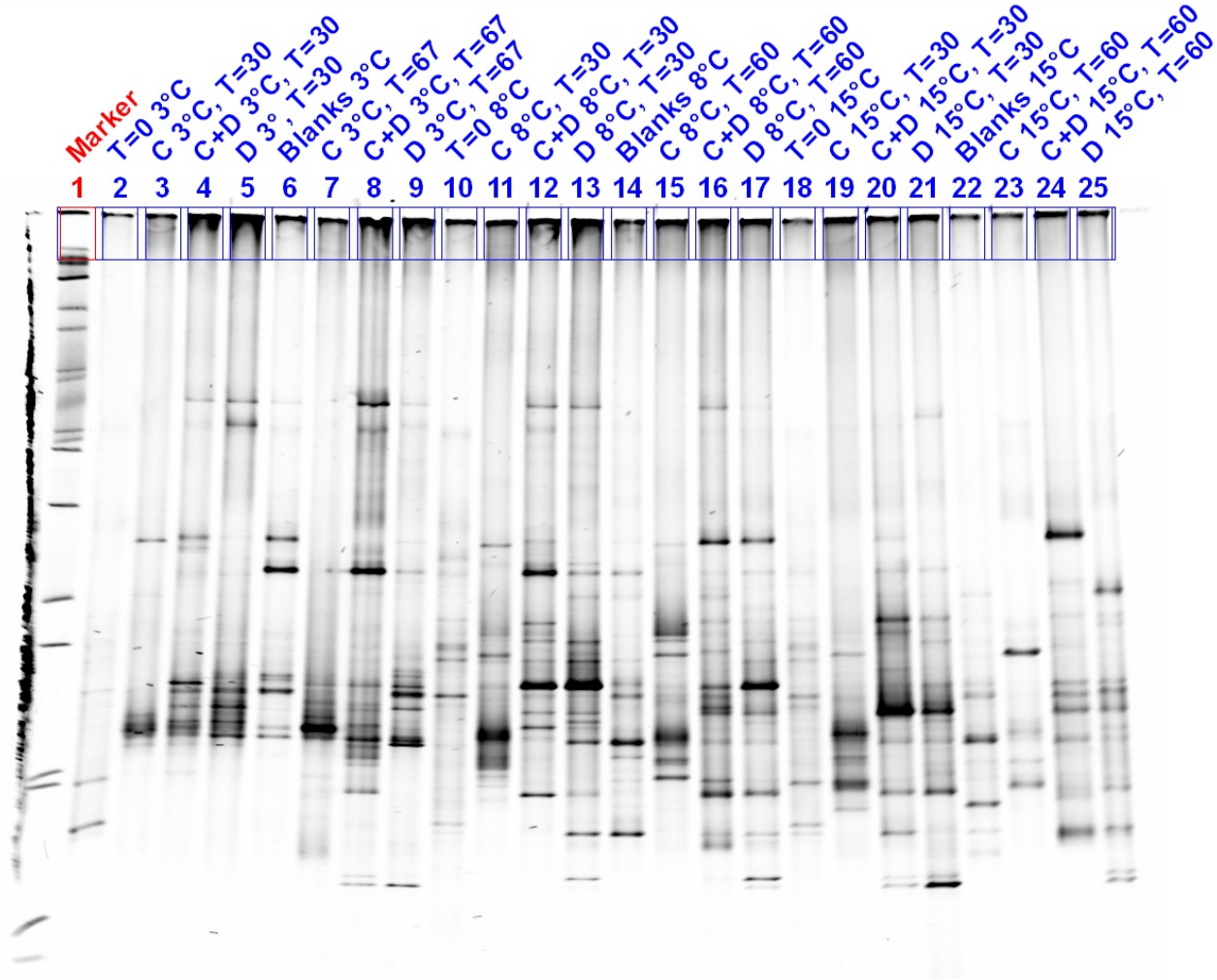

Figure 4.11 DGGE results. The extracted DNA was amplified with primers that targeted the 16S rRNA sequence of the microbial DNA. The PCR product was run trough the DGGE gel, and the lines shown in the figure represent the 16S rRNA for the community. There are clear differences between the samples, indicating a shift in the microbial community was caused by the presence of Corexit or diesel. The samples are sorted by temperature and incubation time.

At time zero, the microbial communities were similar, as all bands could be found at all temperatures. Some bands were harder to see than others, but by altering the exposure of the image all bands were found in the three time zero samples, indicating that the communities were very similar. There were some bands that were more visible for some of the samples than others; most band for the 3°C samples were weak. For the 8°C and 15°C samples, the bands were more visible and appeared to be identical to each other, but there

were a few more bands for the higher temperatures. The different intensities of the bands could indicate that the different bacteria were present in different amounts for the samples.

For the blank samples there were also many similarities between the three different temperatures. The lowermost band found in the 8°C lane was found weakly for the 15°C sample, and not at all for the 3°C sample. The lowermost band of the 15°C sample was not found in any of the other samples while the bands above these were similar for all three samples, except for some of the bands that had differences in intensity. The two upper bands that were visible for the 3°C and 8°C samples were not visible for the 15°C samples. The small differences in the bands indicated that the bacterial community was stable for the three samples, but did go through some changes over time depending on the temperature. The communities for 8°C and 15°C were the most similar as these samples had some bands that were not found for the 3°C samples.

When comparing the samples based on changes caused by the compounds added, it was clear that there were changes in the communities based on whether Corexit or diesel was added prior to incubation for all temperatures, and that the diversity of the communities became greater over time. For the 3°C samples, the two uppermost bands that were visible was present in all samples including the blank but not in the samples where only Corexit was added. All bands present in the blank sample were located in the other samples, and the band with a high intensity in the blank sample was also very visible for the Corexit/diesel aliquot sample incubated for 67 days, but was barely visible when the sample had been incubated for only 30 days. Many of the bands that were present in the samples were not present in the lanes containing only Corexit, indicating that Corexit caused a change in the composition of the bacterial community different from diesel. Many of the bands became more intense after 67 days and for many of the samples, such as for the Corexit/diesel aliquot, the amount of bands increased after the 67 days incubation period. For the samples containing only Corexit more bands appeared after 67 days, where one of them became more intense. A common feature was that some bands became more intense after 67 days incubation, while other bands became less intense after a prolonged

incubation period, showing that the community was continuously changing as the compound added was degraded over time.

The same community characteristics were seen for the sample incubated at 8°C and 15°C, where the addition of Corexit resulted in a shift in the microbial community different from that observed with addition of diesel and the microbial diversity increased. Samples incubated at 8°C showed the same characteristics for the uppermost bands as observed for the samples incubated at 3°C. For samples incubated with only Corexit, the band became more intense over time, but the community did not change much from 30 to 60 days. In the Corexit/diesel sample the amount of bands increased at the same time as the intensity of the bands changed, where some bands became more or less intense over time. For the samples incubated with only diesel, the same bands are visible except for the intense band at the top of the lane for the samples incubated for 60 days, which was not present after 30 days.

For the 15°C samples, the features were similar as previously mentioned. The diversity of the community was not as great as for the samples incubated at 3°C and 8°C, but a clear change in the communities was observed. All bands except for one found in the blank sample could be found in many of the other samples. For samples incubated with only Corexit, the bands became fewer and less intense. For samples incubated with the Corexit/diesel aliquot, the bands were very much similar after 30 and 60 days, except for an increased intensity in the uppermost band after 60 days. The bands found for the samples incubated with diesel were also the same, but an additional band was found at the uppermost section after incubation for 60 days.

When comparing the different samples with regards to the temperature and how this may have had affected the community, the diversity of the community became greater. The samples incubated with only Corexit had the same bands for the 8°C and 15°C samples, while the samples incubated at 3°C had less bands and lower intensity for some of the similar bands. This trend was visible for the Corexit/diesel aliquot and the diesel samples as well, where there were less bands for the 3°C samples and many of the same bands for the

other temperatures. tTemperature had a lower influence on the growth and developments of the community compared to the effect of the different compounds added. The DGGE samples visualises the changes that appeared in the communities over time and the differences between them, giving an indication of the effects of diesel and Corexit in the samples.

4.4 Microbial enumeration

After the plates had been incubated for approximately 14 days, the colonies present on the plates were counted and the method evaluated.

4.4.1 Colonies of hydrocarbon degrading microorganisms

The colonies of hydrocarbon degraders were usually clear or white in colour, and appeared both with and without clearing zones (Figure 4.12 and 4.13). On plates where the colonies grew very dense, some black and a few orange colonies were also found. The clearing zones were areas around the colonies where the oil had been degraded or where the microbes had produced a surface acting compound (Krista Kaster, personal communication). Clearing zones were of varying size (Figure 4.13).

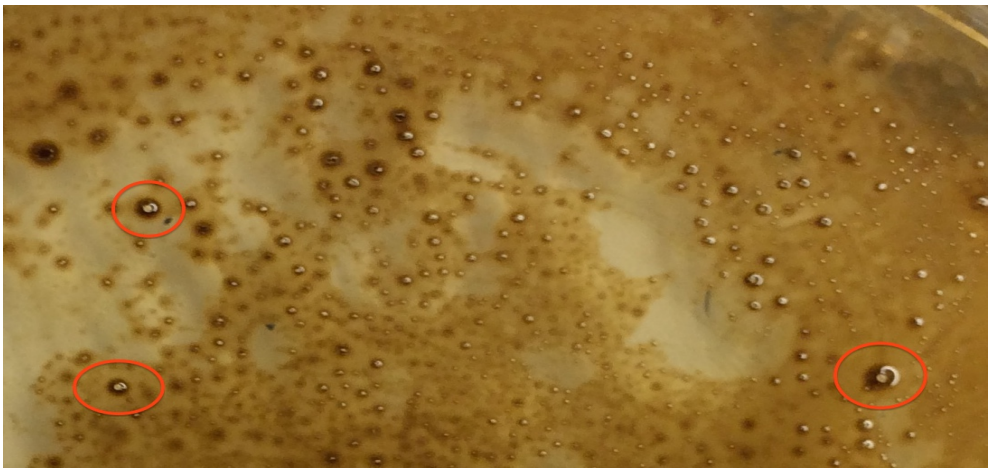

Figure 4.12 Colonies of hydrocarbon degraders. The bacteria able to degrade crude oil formed colonies on the Bushnell-Haas plates, and appeared mainly as clear colonies. The colonies are circled in this image to show an example how they appeared on the plate. Most of the smaller dots that are seen on the figure were smaller colonies.

Colonies were present in varying sizes, but most colonies were very small. There were no distinct differences between the sizes of the colonies from water samples neither from the different temperatures, nor from the samples incubated with Corexit, diesel or the Corexit/diesel aliquot. There were some large colonies present on the plates, but they were less abundant than the small ones. The white colonies were usually only seen on plates with dilutions from 10^1 to 10^3, and they appeared mainly in the clearing zones where they had degraded the oil. On these plates it was also difficult to distinguish colonies from bubbles that had formed in the oil when it was mixed with water during plating. Plates with dense growth had mostly white colonies in the clearing zones (Figure 4.12).

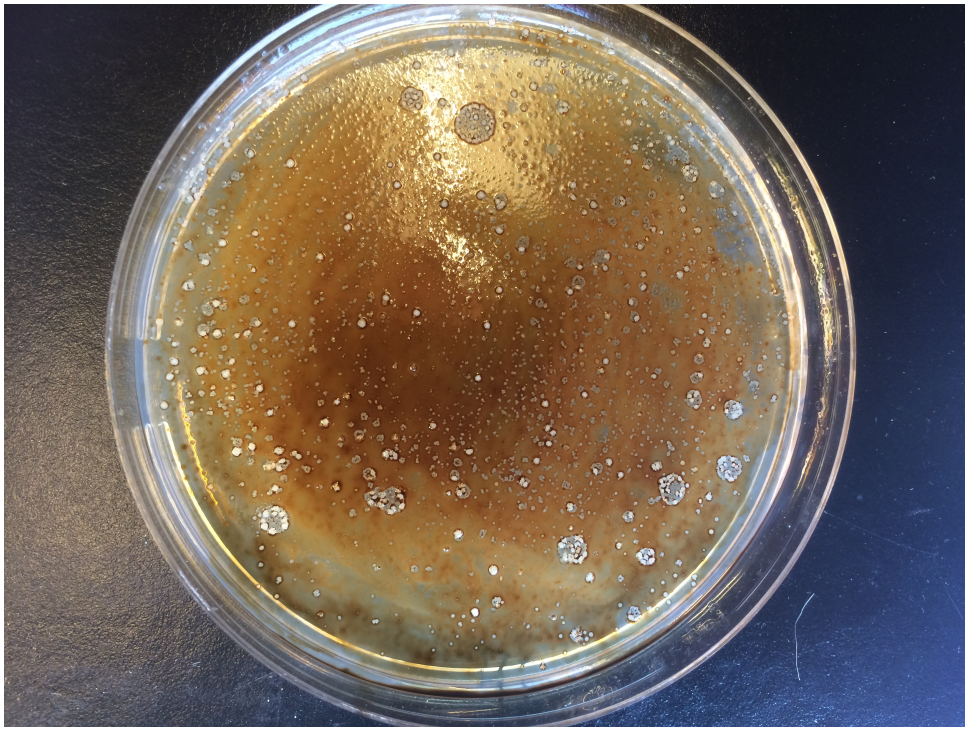

Figure 4.13 Plate with dense growth. Plates that had been plated with samples of low dilution, showed a dense growth of colonies. On many of these plates, the white colonies that grew in the clearing zones were abundant, and the clear colonies were small and hard to distinguish from bubbles.

On most plates, there was a great variation in the counts of colonies between the three parallels and small colonies were sometimes hard to find and count, especially for the plates for the day 60 samples.

4.4.2 Enumeration

The number of microorganisms present in the samples is reported as the average between the three parallels. Standard error is reported as the margin of error of colonies for the colony counts. The counts were used to analyse the growth in the incubated bottles over time, from time zero to day 60. At time zero a sample of seawater was taken directly from the sample carboys for each temperature. For day 30 and day 60, samples were plated from all three temperatures for samples added Corexit, diesel and the Corexit/diesel aliquot.

For time zero the number of microorganisms present in the seawater are presented in table 4.3.

Table 4.3 Enumeration of seawater samples for T=0 incubated for 14 days. Seawater was sampled directly from the sample carboy and plated into Bushnell-Haas plates spread with light Arabian crude oil in dilutions up to 10^{-6}. The numbers are reported as the number of microorganisms present in the sample per 100 µL .

	3°C	SE	8°C	SE	15°C	SE
Seawater time zero	5.3x104	±20	4.3x105	±4	6.3x105	±9

The water incubated at 3°C had a slightly lower count of bacteria present at time zero compared to the water incubated at 8°C and 15°C (Table 4.3). The plates for time zero had large differences between the plate counts, ranging from 20-50 colonies in differences between parallels of the same dilution, which may have caused the variation between 3°C count and the 8°C and 15°C count.

For day 30, the number of microorganisms present in the samples is presented for the three temperatures and for addition of Corexit, diesel and the Corexit/diesel aliquot (Table 4.4).

Table 4.4 Enumeration of samples at T=30 incubated for 14 days. Dilutions from 10^{-1} to 10^{-8} for samples at T=30 were plated on Bushnell-Haas plates with light Arabian crude oil. The numbers are reported as the number of microorganisms present in the sample per 100 µL.

	3°C	SE	8°C	SE	15°C	SE
Corexit	$5.0x10^7$	±10	$4.7x10^6$	±6	$6.1x10^7$	±11
Corexit/diesel	$5.0x10^7$	±3	$5.2x10^7$	±10	$1.0x10^9$	±5
Diesel	$5.4x10^7$	±7	$10.8x10^7$	±11	$5.3x10^7$	±12

At day 30 there was a significant increase in the number of microorganisms present in the sample compared to samples at time zero. There were some variations between the counts at 8°C and 15°C, while the counts at 3°C were all of the same order. For day 60 (67) the number of microorganisms present at the end of the degradation varied substantially in the counts for parallels of the same order, and between the samples in the dilution series (Table 4.5).

Table 4.5 Enumeration of samples at T=60 incubated for 14 days. Dilution from 10^{-1} to 10^{-8} for samples at T=60 were plated on Bushnell-Haas plates spread with light Arabian crude oil. The numbers are reported as the number of microorganisms present in the sample per 100 µL.

	3°C	SE	8°C	SE	15°C	SE
Blanks	$1.16x10^2$	±13	$6.8\ x10^1$	±4	$3.6\ x10^1$	±5
Corexit	$9.8x10^2$	±7	$8.6x10^4$	±2	$7.7x10^6$	±6
Corexit/diesel	$2.0x10^3$	±0	$6.8x10^4$	±4	$4.2x10^7$	±10
Diesel	$5.1x10^2$	±3	$4.1x10^6$	±19	$6.6x10^3$	±6

The numbers for these counts were significantly lower compared to the counts for day 30, and they can be compared for the counts performed for time zero. When comparing the growth to the BOD results (Figure 4.4 and 4.5), the low counts were unexpected as the BOD values indicated a continuous growth from day 30 to day 60. The plates for 8°C and 15°C were incubated for 21 days in total to investigate whether the colonies were only growing slow or if the numbers counted were the actual ones. There was no difference in the number of colonies on the plates from day 14 to day 21.

5 DISCUSSION

This chapter presents a discussion of the BOD results. The results will be compared with the results for the total utilisation of the hydrocarbons, and issues regarding the use of the dispersant Corexit 9500 will be discussed in view of the results from this and other research. A discussion regarding the changes in the microbial community and the use of DGGE for investigation of changes in microbiology will also be presented, in addition to a review of the effectiveness of the enumeration method using Bushnell-Haas plates spread with Arabian crude oil.

5.1 Analysis of diesel biodegradation (BOD)

The positive controls indicated that the BOD setup performed as expected (Figure 4.2). Depending on the temperature the controls had some differences in the BOD values prior to day 20, where the controls incubated at 8°C displayed a more rapid exponential phase resulting in a steeper curve. As 8°C was roughly the intrinsic temperature for the microbial community (see Chapter 3.2.1), this could possibly be due to the fact that the 8°C did not require time to adapt. Approximately the same amount of sodium benzoate was degraded in all samples and the stationary phase was reached after 20 days indicating that sodium benzoate had been degraded. High standard error values were most likely a result of the deviation between the two parallels; when only two samples are used a large variation between the two samples will result in a large standard error compared to when more parallels are used. It is common to observe larger variations towards the end of a biodegradation process, as the competition for resources and substrate increases as the substrate is degraded and the nutrients are diminished (Hibbing, Fuqua, Parsek, & Peterson, 2010). The blank samples indicated that the system was not polluted, and the blanks showed many of the same features as the positive controls in regards to the incubation temperature, but the samples incubated at 3°C and 8°C were parallel throughout the incubation period (Figure 4.3).

Samples incubated at 15°C displayed a more rapid growth rate and had higher BOD values in the end of the incubation period (Figure 4.4). This is an expected result as the metabolic rates of the microorganisms are slower at lower temperatures, leading to decreased rates of biodegradation (Venosa & Holder, 2007). Communities in the samples were in continuous growth during the entire incubation period, but the samples incubated at 8°C and 15°C seemed to be entering the stationary phase after 60 days, while the 3°C samples were entering the stationary phase at approximately the same time or a few days later (Figure 4.4), giving an indication that the temperature had little effect on the growth phases. Temperature did affect the amount of growth in the samples, and it also affected the growth rate as the samples incubated at 15°C had higher rates compared to the samples incubated at 3°C (Figure 4.4).

Corexit was degraded quickly as the exponential growth at the beginning of the incubation period was higher in the samples containing only Corexit compared to the samples containing both Corexit and diesel. Both of these conditions showed a greater exponential growth when compared to the samples containing only diesel (Figure 4.4). This could indicate that Corexit acted as a preferred substrate over diesel. Dispersants' ability to promote the growth of hydrocarbon degrading microorganisms have been shown in other studies (Swannell et al., 1997; Varadaraj, Robbins, Bock, Pace, & MacDonald, 1995), and it has been shown that the hydrocarbons present in Corexit 9500 can be utilised by a bacterial community (Bælum et al., 2012). If Corexit 9500 was utilised as a substrate in the BOD bottles, the dispersant does not meet the requirement set by Mulkin-Phillips and Stewart (1974), saying that the dispersant must not be a preferred substrate over the diesel. In some studies the number of dispersant degraders have been shown to be higher than the number of hydrocarbon degraders in nutrient rich water (Lindstrom & Braddock, 2002), indicating that Corexit may be the preferred substrate. When compared for temperature it was noticeable that Corexit 9500 was utilised as a substrate, as the growth curves for the different samples were comparable and the BOD values for Corexit were approximately half of the BOD values for the samples containing both Corexit and diesel (Figure 4.5). These values give the impression that Corexit was degraded at the same time as the different compounds found in diesel. In addition, the results from the DGGE (Figure 4.7) show a shift

in the different microbial community for samples containing Corexit only, supporting the claim that Corexit is utilised by the microbial community. The samples containing Corexit had a rapid exponential phase occurring almost immediately after incubation started, indicating that Corexit was rapidly degraded in seawater (Figure 4.5).

The addition of Corexit 9500 led to a significant increase in the rate of diesel biodegradation at 3°C and 8°C. The biodegradation rate of the samples where diesel was dispersed with Corexit 9500 show a significantly higher amount of bacterial growth when Corexit was added to the samples, compared to when diesel was degraded on its own (Figure 4.5). For the temperature 8°C, the growth rates were significantly higher, indicating that dispersion with Corexit lead to an increased rate of diesel degradation in the samples compared to when no dispersant was added. For 15°C the addition of Corexit seemed to have no significant effect on the rate of diesel biodegradation when including the standard error values the end BOD values were approximately the same for the two samples (Figure 4.5). As expected, the rate of diesel degradation was highest for the samples incubated at 15°C despite no apparent effect of the dispersant, likely due to the faster growth rate. The values for the samples incubated with Corexit and diesel at 15°C were slightly higher when compared for incubation at 8°C, where the values were 225 mg/L for 15°C and 195 mg/L for 8°C. An interesting observation is how the BOD values for the samples incubated at 15°C were similar to the 8°C samples for the Corexit/diesel aliquot, but when looking at the samples incubated with only diesel the BOD values for 8°C were significantly lower that the 15°C ones. For samples incubated at 3°C the data was lost between day 30 and 60, and therefore could not be compared to the other samples. BOD values for the samples incubated with only Corexit were the lowest for both 8°C and 15°C. The stand error for the graphs was relatively similar to each other, making the results comparable as they have similar variances in the measurements.

A masters' thesis written on the same subject compared the degradation of Ekofisk crude oil in the presence of Corexit 9500 to the degradation of the crude oil alone (Ulas, 2013). In this study the water was sampled from the same fjord at the same time of year (Chapter 3.2.1), and the experimental analyses were the same. During a 49 day incubation period, the

amount of crude oil degraded by the microbial community were the same for the samples incubated with Corexit and the crude oil as for those incubated with the crude oil on its own, indicating that a presence of Corexit did not lead to increased degradation of the oil in the samples. The effect of Corexit 9500 on biodegradation have been reported as variable, where some studies report an increase in biodegradation of hydrocarbons in the presence of a dispersant (Brakstad et al. 2014; Lessard & Demarco, 2000; Prince et al., 2013), while others report no change in the amount of hydrocarbons degraded in the presence of the dispersant (Foght & Westlake, 1982; Lindstrom & Braddock, 2002)

There seems to be limited research performed on the effect of dispersants on diesel alone, and thus few comparisons can be done to evaluate if the increased degradation of diesel in the presence of Corexit 9500 was caused by the dispersion of the diesel making it more available for biodegradation, or if other factors contributed to the results. It has been shown to be difficult to predict how dispersants will affect biodegradation in colder temperatures, but there has been shown to be decreased dispersant effectiveness on spilled oil that has been significantly weathered (Moles, Holland, & Short, 2002). Due to loss of data for the biodegradation of diesel at 3°C, it was also difficult to make prediction whether the addition of a dispersant to spilled diesel in cold environments can positively affect the bioremediation rates at this temperature.

The temperature, salinity and degree of weathering of the spilled petroleum hydrocarbons can affect the effectiveness of a dispersant (Moles et al., 2002). The effectiveness of a dispersant is also dependent on its “window of opportunity” that range from hours to weeks and is defined as the time frame between the spilling of the oil to the point where the oil is either too weathered or too viscous to be dispersed (Lessard & Demarco, 2000). Lab tests can provide an indication of the performance of dispersants and give and understanding on how different environmental factors and different types of hydrocarbons affect their performance. The indications should be evaluated using caution, as different environmental conditions affect their performance (Moles et al., 2002) and as the parameters of such small scale laboratory studies are under strict control, where stirring, temperature and other conditions are constant throughout the experiment period. In

addition, only one parameter is evaluated at a time and the scale of such laboratory studies is small compared to full-scale studies. When a dispersant is considered being used for oil spill treatments, it is important to assess its effect on the environment in addition to its ability to increase the biodegradation rates by increasing the bioavailability of the oil. The possible toxicity of dispersant and Corexit 9500 is briefly discussed in Chapter 5.4 in consideration to the results of the experimental analysis.

Possible error sources for the BOD experimental bottles were contamination of the seawater while preparing the bottles. Errors or strange values for the OxiTop measurements were attributed to faulty programming of the heads by the controller, causing loss of data or sudden rises in the measured values.

5.2 Analysis of hydrocarbon utilisation (THC)

Retention times for the hydrocarbon standard gave a basis to determine the range of the hydrocarbons in the samples. Both diesel and Corexit are light compounds, and the majority of the hydrocarbon fraction was between C_9 and C_{20} for diesel, with a narrower range of the hydrocarbons present in Corexit. Both the diesel and the Corexit in the samples was degraded almost completely after 30 days, and the temperature did not seem to have a significant impact on the amount of diesel degraded (Table 4.1). As diesel is a light fuel, it is naturally degraded rapidly. It spreads easily, and its light composition makes it very bioavailable (Lindstrom & Braddock, 2002). The majority of the Corexit seemed to have been degraded to the same extent as the diesel. The extensive degradation of Corexit in the samples violates the criteria that the dispersant should not be the preferred substrate in the presence of oil and petroleum products (Mulkins-Phillips, 1974).

When extracting hydrocarbons after 30 days, the samples incubated at 15°C had experienced a complete degradation of diesel, compared to some lower percentage for the samples incubated at 3°C and 8°C. This result is not surprising, as biochemical processes are temperature dependent and the rates are faster at higher temperatures (Gillooly et al., 2001). It is difficult to make conclusions regarding the effect of temperature on the

degradation, as the total hydrocarbon content was not analysed prior to 30 days of incubation. More frequent sampling is required to more precisely determine the effect of temperature on the total amount of hydrocarbons degraded prior to day 30. The Corexit was almost completely degraded for the 8°C and 15°C with a 92% and 93% degradation after 60 days, respectively. Corexit did not degrade as much at 3°C, where the total percentage of degradation was 88. Compared to the degradation of diesel, Corexit seemed to be degraded less at 3°C. Results of the GC-FID analysis showed that the temperature did not have a significant impact on the degradation of diesel, as the diesel was almost completely degraded at 3°C, 8°C and 15°C. Usually the temperature does affect the degradation of hydrocarbons (Giudice et al, 2010), but as diesel is a very light fuel the low temperature might not have been enough to slow biodegradation to a point where the degradation became unsatisfactory.

When comparing the results for degradation of diesel in the presence of Corexit, the amount of degradation of the hydrocarbon content was the same. When comparing the results for the samples containing diesel, Corexit and the Corexit/diesel aliquot, it is clear that addition of Corexit to the samples did not increase the extent of biodegradation of diesel, and neither the amount of diesel degraded. The Corexit itself was almost completely degraded in the samples, indicating that it was also a substrate for the hydrocarbon degrading community. Previous studies have indicated that Corexit have a remedial impact on the biodegradation of spilled oil (Bælum et al., 2012; Lessard & Demarco, 2000; Prince et al., 2013; Campo, Venosa & Suidan, 2013), while other studies indicate that the effect of Corexit 9500 is possibly inhibitory (McFarlin, Prince, Perkins & Leigh, 2014; Lindstrom & Braddock, 2002). The studies on effect of Corexit 9500 on Ekofisk crude oil under the same conditions (Ulas, 2013) gave the same indications as observed in this study: the amount of crude oil degraded was not affected by the presence of a dispersant, and the dispersant may have acted as a preferred substrate over the diesel. Ekofisk crude oil is a light oil (Ulas, 2013), which could explain why the same trend is seen for diesel and this oil. As short hydrocarbons are more easily degraded, the use of a dispersant may be superfluous for diesel and other light oils as they are so easily degraded and the biodegradation rate is so high that the dispersant do not make a significant difference. As neither the temperature

nor presence of a dispersant affected the biodegradation of diesel, a plausible explanation for the results are that diesel is very biodegradable so that the temperature and presence of a dispersant had little effect on the total amount of hydrocarbons degraded (Olsen, Mills, Herbert & Morris, 1999). In this study, the majority of the compounds in diesel were degraded during 35 days of incubation, indicating that diesel is very biodegradable and will be degraded fast by the microbial community.

Studies on the biodegradation of Corexit 9500 itself are limited, but there are studies showing that Corexit is mineralised by a hydrocarbon degrading community (Campo et al, 2013). A study performed by McFarlin et al. (2014) that measured the effect of a dispersant during relevant environmental conditions revealed some increased stimulation by Corexit 9500 initially, with minimal effects in long-term incubation. The study was performed during a 60 day period, and the results are thus comparable to the results obtain in the present study.

The BOD experiment in this study was performed under perfect conditions, where temperature was constant, there was an excess of nutrients in the samples and stirring of the sample was consistent and strong enough to sufficiently mixt the diesel and the dispersant. These conditions are rarely found naturally, and the results of this study are therefore only indications of what could be the expected results in natural environments. Though the indications of the experiments performed in this study were that Corexit did not enhance biodegradation or the percentage of diesel degraded, it is unlikely that large oil slicks of spilled oil will disperse without the use of a dispersant (McFarlin et al., 2014). Despite varying evidence of its effect, there is a growing expectation that dispersants can help counter the effects of an oil spill (Lessard & Demarco, 2000).

In order to determine whether the hydrocarbon fraction of the extracted samples consisted of after incubation for 30 and 60 days, GC-mass spectrometry (GC-MS) could be used to identify the different substances left in a sample to analyse whether it was Corexit or diesel that was left in the samples after 60 days. Loss of hydrocarbons in the extraction process was a likely error source. This could have been calculated for by extracting a known volume

of hydrocarbons, and analysing the amount of hydrocarbons lost compared to the original amount. This would have given a known percentage amount of hydrocarbons lost in the extraction process. Due to time constraints, this was not done for this study.

5.3 Microbial community analysis

The microbial community was analysed using denaturing gradient gel electrophoresis (DGGE) to investigate the changes that had occurred in the microbial community, and by enumeration on Bushnell-Haas agar plates with light Arabian crude oil to investigate the numerical increase in cultivated bacteria during the incubation period.

5.3.1 DGGE

DGGE analysis revealed that changes occurred in the microbial community with addition of diesel and Corexit to the seawater samples, and the analysis revealed that the Corexit and diesel communities were different from each other. The stained gel revealed differences in the placements and intensity of the bands representing the 16S genome of the different bacterial species that were present (Figure 4.11). The analysis also revealed that there was a continuous change in the bacterial community over time, as the intensity of some bands changed during the incubation time from 30 to 60 days for the same set of samples. The results indicate that the indigenous bacterial community changed to include microorganisms that had a preference for the substrates added to the sample, inducing a shift in the bacterial community when it was exposed to Corexit or diesel. The bands for the samples where Corexit, diesel or the Corexit/diesel aliquot were added were all different, but the samples for Corexit/diesel included bands found for both the Corexit and diesel indicating that Corexit induced a shift in the microbial community different from the diesel alone. These results are consistent with what has been found in other studies, where it was shown that a community of microorganism responded to pollution by increased growth of hydrocarbon degraders and changes in the structure of these communities where some strains became dominant (Bruheim & Eimhjellen, 1998; Chakraborty, Borglin, Dubinsky, Andersen & Hazen, 2012; Harayama, Kasai, & Hara, 2004).

The DGGE analyses revealed that the Corexit and diesel stimulated different microbial communities. The alterations in the community structure induced by the dispersant compared to those induced by the diesel may have been caused by microorganisms utilising the dispersant as a substrate that resulted in a different microbial community, or that the dispersant itself directly or indirectly prevented the growth of some microbial groups (Yoshida et al., 2006). Changes in abundance or activity of some species after the addition of a dispersant have also been reported from other studies, where studies from the field, in mesoscale closed systems (mesocosms) and laboratory trials (microcosms) confirm these results (Mulkins-Phillips & Stewart, 1974; Nyman, 1999; Yamada et al., 2003). By using Corexit as a substrate, a different community developed compared to the community present in the samples containing only diesel. The combined environment of these two observed in the samples containing both diesel and Corexit could be a result of rapid degradation of Corexit, which could support both the organisms feeding on Corexit and those utilising diesel as a substrate. These finding are supported by a study that investigated the effect on the community structure in the presence of an oil-dispersant mixture. In these trials, cell density increased during the first 24 hours and was followed by an increase in the abundance of certain organisms together with changes in the community structure. In the presence of an oil-dispersant mixture, the community developed a distinct structure after the first 6 hours, while this process took 24 hours for communities with only oil added (Yoshida et al., 2006). This supports the indications that Corexit changes the structure of the microbial community in a different way than diesel, and that these changes may be induced faster for Corexit. This may also affect the community structure, as the microorganisms utilising the dispersant may be dominating as this community may adapt faster to the new conditions and outcompete the organisms that use diesel as a substrate (Hibbing et al.,2010). As an overabundance of nutriens was added to the experimental bottles, nutrients were not a limiting to either of the diesel or Corexit degrading community. A Corexit degrading community could outcompete a diesel degrading community if nutirents are limited (Krista Kaster, personal communication).

The intensity of the bands also changed over time where some bands became more or less intense during the last 30 days of incubation, indicating a continuous change in the

community structure throughout the entire incubation period. The number of bands represents the microbial diversity in the sample and ideally they mirror all the different microorganisms that were present, while the intensity of the bands indicates semi quantitatively the relative abundance of a specific microorganism or genome. Ideally, the more intense the band is the greater abundance of this microorganism is present in a sample (de Araujo & Schneider, 2008). Increased intensity of some bands shows that these microbes experienced an increase in growth during later stages of the incubation period, while bands that decreased in intensity illustrate how some specific groups of microorganisms experienced a decrease in cell number at the same time. This was probably caused by utilisation of the products that were available after some incubation time, such as biodegradation by products, and that some microorganisms could utilise the remaining compounds better than others and in this way outcompete other microbes and alter the community structure so that it differs from the changes observed initially (Yoshida et al., 2006).

There were also some differences in relation to temperature between the samples, where some wells with samples incubated at 15°C generally had some lower intensity in the bands after 60 days compared to the samples incubated at 3°C and 8°C. The reasons for this were uncertain, but it is conceivable that it was caused by the incubation temperature as the samples incubated at 15°C were further away from their intrinsic temperature of around 8°C, which subjected the microbial community to greater alterations in its structure in order to adapt to the rather large temperature span. At 15°C mesophilic bacteria would also start to thrive, while psycrophilic and psychrotolerant bacteria would only be found in the 3°C and 8°C samples, which could explain the differences in the 15°C community profile. Alternatively, the microbial community had degraded the hydrocarbons after 30 days, which could also explain the changes in the microbial community from 30 to 60 days for the samples. A larger standard error was also seen for the BOD values of samples incubated at 15°C, which may correlate to the adaption behaviour of the samples (Figure 4.4). The suggested causes cannot be confirmed, but constitute some interesting observations.

DGGE analysis gives important and valuable results when evaluating changes in microbial communities of environmental samples, but the method has its limitations. When evaluating the results it is important to be aware of these to avoid making faulty conclusions regarding the presence of different species, and their abundance in the sample. The fundamental factor when performing these analyses is that the original diversity of the DNA samples are not altered during the PCR reaction (Jackson, Roden, & Churchill, 2000). When investigating the diversity of complex mixtures of microorganisms that are commonly found in environmental samples, the use of universal primers may limit the amplification of smaller fragments of 16S rRNA and may thus result in an underestimation of the total number of species present in a sample (Vallaeys et al., 1997). The thought that one band represents one genome belonging to a defined specie (de Araujo & Schneider, 2008) may not always be reliable, as the amplification of the 16S region may produce multiple bands in the gel for some organisms and give a higher number of bands relative to the organisms present in the sample (Schmalenberger, Schwieger, & Tebbe, 2001). The causes of this multiple band effect are usually that two slightly different versions of the gene exist in a single organism or that there are denaturation domains in the amplicons (de Araujo & Schneider, 2008). The opposite effect is also a common cause of bias in DGGE analyses, where the amount of fragments causes an underestimation of the actual diversity of the number of species present in the sample (Vallaeys et al., 1997). Co-migration of DNA from different species with a similar 16S genome sequence into the same band is also commonly observed, resulting in single bands representing multiple genomes (de Araujo & Schneider, 2008). Usually around 95% of all single base sequences are detected using DGGE, but a bias may occur when two sequences that differ with two base pairs are run in the same lanes. These sequences are often not separated, while sequences with only one base pair in difference is usually separated well (Jackson et al., 2000).

When using the DGGE method, it is in practice impossible to achieve a reliable and accurate representation of every organism in a community, and the results are usually difficult to reproduce when working with microorganisms from environmental samples due to the differences in growth that may occur when repeating an experiment. One of the major advantages with the method is that it makes it possible to detect organisms that are usually

not culturable, and it provides a convenient way to confirm if differences exist between communities (de Araujo & Schneider, 2008). Because of its convenient and visual representation of the structure of a community, it was the preferred method when analysing the microbial community in the samples incubated with Corexit, diesel and the Corexit/diesel aliquot at different temperatures to easily determine if the different compounds initialised changes in the intrinsic microbial community.

One of the most likely error sources for DGGE analysis is PCR bias. Several different bias sources have been identified when using PCR to analyse the 16S region of environmental communities, where the formation of PCR artefacts or chimeric molecules (combined sequence of different genes), inhibition of the PCR amplification and differences in the ribosomal RNA (rrn) operon heterogeneity may also create a bias in 16S analysis from environmental samples at it may become unclear weather the 16S sequence represents one sequence or the entire 16S rRNA operon of an organism (Wintzingerode, Göbel & Stackebrandt, 1997). Complementing 16S analysis with information based on *cpn60* sequencing may reduce community identification bias (Kryachko et al. 2015).

5.3.2 Enumeration

Accurate enumeration of hydrocarbon degrading microorganisms have previously proven to be difficult, and methods such as most probable number (MPN), plating on oil agar, silica-gel media and agar in inverted petri dishes with an oil saturated filter at the bottom of the petri dish have been investigated (Walker & Colwell, 1976). No standard accurate and reliable method for enumeration using media seems to have been developed. Real-time PCR is now commonly used for quantification and to evaluate the microbial community, as it is a reliable method for microbial community analysis (Powell, Ferguson, Bowman & Snape, 2006).

The Bushnell-Haas oil plate method was proven to be unreliable, as the results showed unsatisfactory variations between parallels of the same dilution, and between samples from time zero to 60 days (see Chapter 4.4.2 for results). Enumeration showed an increased

number of hydrocarbon degraders from day zero to day 30, but when counting plates incubated for day 60 the bacterial counts were identical and in some cases lower than the numbers observed at after 30 days. As the biodegradation rate was increasing in the BOD bottles after incubation for 30 days (Figure 4.4 and Figure 4.5), these results were unexpected and thought to be incorrect as both the BOD analysis and the total amount of hydrocarbons degraded indicated continuous growth throughout the incubation period. The plates counted for day zero and day 30 had comparable counts for the parallels. For day 60, the number of bacteria present on the plates varied greatly between the parallels for a dilution and for the different dilutions. When comparing the results to the BOD and THC analyses, it was thought that the issues that occurred during enumeration could possibly be contributed to evaporation of the oil in the plates, as they had been stored for a longer period of time when used for enumeration of the day 60 community. The crude oil used on the plates was a light Arabian crude oil, which is easily degraded (Mulkins-Phillips & Stewart, 1974), making it a good hydrocarbons source for the plate method, but also causes more evaporation of oil from the plates compared to heavier oils.

Several issues with the plating method could have contributed to the unreliable results. For future use, the plates should be spread with oil a short time before use and the oil should not be given time to dry or evaporate. Plates with wet oil were easier to plate evenly, as the oil blended well with the water phase over the entire surface of the plate. When the oil had been given time to dry on the plates it was moved around by the spreader and exposed areas on the plates were there was no oil and consequently the water accumulated there. Colonies that grew on the plates where the oil was wet during plating were easier to count, and they were larger and more distinguishable compared to colonies on dry plates. For some plates the colonies were small and grew very close and made it impossible to count the colonies precisely, resulting in counts that were a rough estimate at best. For plates with a high concentration of bacteria, it was difficult to count all colonies and count the correct number of colonies on the plates.

A majority of environmental microbes are difficult to culture or cannot be cultured in the lab (Vinas et al., 2005), and it is thought that 90-99% of the microbial species present in an

environmental sample do not form colonies when traditional cultivation methods are used (MacNaughton et al., 1999). Cultivation of hydrocarbon degraders have previously been performed using Bushnell-Haas media exposed to volatile gasoline hydrocarbons (Horowitz & Atlas, 1977). The reported numbers of actual hydrocarbon degraders differs with the methods used for enumeration, and a low percentage of the isolated strains actually demonstrate an ability to degrade hydrocarbons (Okoh, 2006).
These findings strengthen the claim that the number of bacteria present on the plates may not be the correct numbers. It is unlikely that all hydrocarbon degraders in the samples actually grew on the plates, making the enumeration method even more uncertain. To address these issues, amplification of the 16S rDNA together with analysis by DGGE and the measurement if phospholipid fatty acid biomarkers (PFLA) have been suggested as additional methods to enumeration to evaluate a microbial community involved in hydrocarbon degradation (Okoh, 2006). Real-time PCR is also a good method for evaluating and quantifying hydrocarbon degraders, as it has better sensitivity and reproducibility, and is also quicker, compared to conventional hybridisation- and PCR-based techniques (Zhang & Fang, 2006). The method allows for evaluation of 16S rRNA genes to measure cell populations numbers and for measurements of catabolic genes and other important ecological processes (Smits, Devenoges, Szynalski, Maillard & Hollinger, 2004; Powell et al., 2006).

In general, the Bushnell-Haas oil plate method described is useful for verifying an increase in the bacterial numbers in a sample for comparison. The method is not suitable for exact counting of hydrocarbon degraders, but can be used for isolation of strains for identification purposes.

5.4 Experimental summary

When viewing the results from the BOD analysis, the DGGE gel and the GC-FID analysis together, the results correspond to each other. It has been shown that microbial communities change depending on the substrate available for degradation (Chakraborty et al. 2012; Bruheim & Eimhjellen, 1998) which was also observed for the sample bottles

added diesel and Corexit 9500 (Figure 4.11). The communities had a distinct community profile for the samples containing only diesel when compared with the community profile for the samples containing Corexit only. When both of these compounds were present in the BOD bottle, the profiles for diesel and Corexit were overlaying indicating that a mixed community consisting of both of these distinct communities had grown, causing degradation of both diesel and Corexit. As the samples contained nutrients in excess the communities were not competing for resources, and because the microbial communities were utilising different hydrocarbon substrates the two distinct communities could exist together resulting in degradation of both diesel and Corexit. In natural conditions nutrients and other resources are often limited (Yang et al., 2009), and the presence of a community that degrades Corexit is undesirable as this community may reduce the degradation of diesel (Lindstrom & Braddock, 2002).

The results of the BOD analysis indicated that there was an increased growth of the microbial community in the samples incubated with both Corexit and diesel, compared to the bottles incubated with only one of the compounds (Figure 4.5). For the samples incubated at 15°C, the curves for diesel and the Corexit/diesel aliquot were the same, showing that for higher temperatures the diesel was completely degraded and the degradation rate in the samples with only diesel was at the same rate as the samples with both Corexit and diesel, indicating that Corexit was not required for biodegradation at 15°C (Figure 4.5C). For the samples incubated at 3°C and 8°C, the BOD values for the samples containing both Corexit and diesel were significantly higher compared to the samples with only diesel or Corexit (Figure 4.5A and 4.5B). When comparing the BOD results to the DGGE analysis, the higher BOD values for the Corexit/diesel samples compared to the other samples could be explained by the growth of two different bacterial communities degrading different compounds in the diesel. The microbial community in a sample will establish an equilibrium of growth and death as the community reaches the lag phase.

The results from the GC-FID analysis also support the indications of two different microbial communities. Virtually all the diesel and Corexit was degraded in the samples at all temperatures (Table 4.1), indicating the presence of a community that could degrade both

substrates. This is supported by the DGGE analysis that indicated a substrate dependent change of the microbial community. Based on the GC analysis, it was clear that Corexit was degraded. When the dispersant is degraded in the sample, it is unlikely that it had an effect on the degradation of diesel at other times than initially (McFarlin et al., 2014). The rapid degradation of diesel alone also indicated that the dispersant may have had limited influence on the biodegradation rate. This is difficult to determine without more frequent extractions of the hydrocarbons to evaluate when the different substrates were actually degraded.

5.5 The use of dispersants as an emergency response for oil spills

Twenty-three commercially approved dispersants are available on the market, including Corexit 9500 and Corexit 9527, which are the two most common dispersants used for oil spill bioremediation (EPA National Contingency Plan Product Schedule, 2010). Dispersant are routinely used as an emergency response to oil spills, but little have been concluded regarding their toxicity, how they affect microbial communities and whether or not they remediate the biodegradation of oil spills to such an extent that its release into the environment is justified (Kleindienst, et al., 2015).

It has been shown that a mixture of the surfactants commonly found in dispersants may affect structures in a cell, or synergistically influence cells internal communication (Kleindienst et al., 2015) and some studies have shown that dispersants may cause an increased PAH uptake in fish (Ramachandran, Hodson, Khan, & Lee, 2004). It seems that the toxicological mechanisms are unexplained, but dispersed oil may be less or just as toxic as oil alone (NRCC, 2005). It has also been shown that Corexit 9500 may increase the toxicity of oil that remains in the environment through the degradation of selected hydrocarbons by a specialised microbial community (Kleindienst et al., 2015). Dispersants may be used as a preferred substrate, and in this way may prevent or slow down bioremediation rather than providing an effective remediation of oil spills (Yoshida et al., 2006).

The impact of a released dispersant is unpredictable and the research on the subject is contradictive. When considering the use of dispersants as an emergency response the negative effects need to be considered and evaluated against the indications from different research. The experiments performed in this study gave no indication that Corexit 9500 significantly increased the biodegradation rate of diesel at cold temperature, but the results are not necessarily true for the relevant conditions of its use. The long term effects on the environment when a dispersant is used needs to be evaluated to determine if the chemical remain in a vulnerable environment for an unsatisfactory amount of time, and to determine whether or not the use is justified or actually necessary.

6 CONCLUSION

Aerobic biodegradation of diesel in seawater and the subsequent changes in the microbial consortium was evaluated in seawater at different temperatures with and without the presence of the dispersant Corexit 9500. The microbial consortium was analysed at the same time intervals by denaturing gradient gel electrophoresis of the 16S rRNA gene and Bushnell-Haas plates using light crude oil as the hydrocarbon source was used to enumerate the hydrocarbon degraders.

Temperature did not affect the total amount of hydrocarbons degraded. Diesel was completely biodegraded after 30 days at 15°C and only 90% after 30 days at 3°C, indicating a slight decrease in the biodegradation of hydrocarbons with decreasing temperature. The biodegradation rate increased with temperature as seen by the BOD values. Biodegradation rates increased in the presence of the dispersant Corexit 9500 for the temperatures 3°C and 8°C, and both diesel and the dispersant itself was biodegraded rapidly in all samples. Corexit 9500 did not seem to increase the total amount of hydrocarbons degraded, but could have had an undetected effect at early stages of biodegradation. The microbial community changed depending on the substrate present, with distinct microbial profiles for Corexit and diesel demonstrating that both compounds were bioavailable substrates and degraded by different communities. Corexit 9500 addition increased the biodegradation rates at 3 and 8°C, but not at 15°C. However, due to the possible toxicity and bioavailability of Corexit 9500, and its limited effect on biodegradation in laboratory studies, the use of dispersants must be evaluated for each situation. Corexit may be useful for dispersing large oil slicks during oil spills, but as the microbial community is able to degrade Corexit its presence can cause competition for nutrients between the hydrocarbon degraders and the microbes degrading Corexit, ultimately affecting the hydrocarbon degradation. Future research is required to evaluate the effect of dispersant.

7 FUTURE RESEARCH

Many studies are aimed towards solving issues related to the use of dispersants during oil spill clean-up. As petroleum related activity is increasing in the Arctic regions the concerns regarding drilling activity and oil spills in these areas are becoming increasingly important issues to address, both in conjunction with general environmental concerns but also regarding the widespread use of chemicals that is spread in these vulnerable ecosystems. Further investigations on the effect of temperature on the biodegradation of spilled hydrocarbons and the actual remedial effect of dispersants in cold environments are recommended.

Studies should be conducted during different seasons in the same regions, as most areas experience variations in temperatures between the summer and winter season. The experiments performed in this study should be repeated for the colder and warmer seasons in order to evaluate the significance of the potential changes that may occur in the microbial community during temperature shifts when dispersants are used to increase biodegradation. Different petroleum products common on the market today are often contaminants in conjunction with an oil spill, and they should also be investigated to evaluate if dispersants have a better effect on lighter fuels such as diesel compared to heavier compounds such as engine oil, and how temperature affects their degradation with and without a dispersant present. Degradation of dispersants themselves should be investigated to evaluate if they are a more preferred substrate compared to spilled hydrocarbons and seeing that degradation of the dispersant is needed to meet toxicological requirements.

If enumeration of the hydrocarbon degrading community by the use of media is desired, resources should be allocated towards developing standardized and reliable methods for use in research labs. The most common methods of cultivation used presently are adequate for determining whether or not bacteria are hydrocarbon degraders, but are not selective enough to be used for precise enumeration of a community (Okoh, 2006). Alternatively,

molecular methods could be used, such as real-time PCR for quantification of hydrocarbon degraders.

It should be aimed towards developing systems that can extrapolate the findings to natural surroundings. The biodegradation process is dependent on a complex composition of many factors, such as sunlight, temperature, wind and current strengths and the biodiversity of an ecosystem. The diversity of these influences makes it difficult to transfer findings from laboratory scale experiments to the varied natural surroundings, as small-scale experiments only provide an indication of what the actual effects may be. Assessing the environmental risks of releasing dispersants into the environments is also an important issue that should be a priority in further research, as many ecosystems in the Arctic regions are vulnerable. This is particularly important as oil and gas activity is increasing in these regions.

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APPENDIX

A Monod Kinetics

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

Where $S << K_s$ and

μ = the specific growth rate of the microorganisms

μ_{max} =the maximum specific growth rate of the microorganisms

S = the concentration of the growth limiting substrate

K_s = is the value of S when $\mu/\mu_{max} = 0.5$, the "half-velocity constant"

μ_{max} and K_s are empirical coefficients, and will differ between different species based on the ambient and environmental conditions.

B Relationship of BOD and pressure

OxiTop System Operating Manual (2006):

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

Where:

$MW(O_2)$ = Molecular weight (32 000 mg/mol)

R = Gas constant (84.14 L mbar/mol K)

T_0 = Refrence 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)

Appendix

C ASTM® D5442 C12-C60 Qualitative Retention Time Mix (GC standard)

Molecular formula	Compound
$C_{12}H_{26}$	Dodecane
$C_{14}H_{30}$	Tetradecane
$C_{16}H_{34}$	Hexadecane
$C_{18}H_{38}$	Octadecane
$C_{20}H_{42}$	Eicosane
$C_{22}H_{46}$	Docosane
$C_{24}H_{50}$	Tetracosane
$C_{26}H_{54}$	Hexaconsane
$C_{28}H_{58}$	Octacosane
$C_{30}H_{62}$	Triacontane
$C_{32}H_{60}$	Dotriacontane
$C_{36}H_{74}$	Hexatriacontane
$C_{40}H_{82}$	Tetracontane
$C_{44}H_{96}$	Tetratetracontane
$C_{50}H_{102}$	Pentacontane
$C_{60}H_{122}$	Hexacontane