Effect of low temperature on hydrocarbon biodegradation in marine environments

by

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Thesis submitted in partial fulfilment
of the requirements for the degree of
PHILOSOPHIAE DOCTOR
(PhD)

University of
Stavanger

2013

Faculty of Science and Technology
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Treat the Earth well. It was not given to you by your parents, it was loaned to you by your children. We do not inherit the Earth from our Ancestors, we borrow it from our Children.

~ Ancient Indian Proverb ~

Acknowledgements

Summary

Understanding the fate of oil discharges in cold marine environments has become a key issue as oil and gas industry is expandig exploration activities into frontier regions such as the Arctic Ocean. The aim of this research project was to assess a currently applied modelling approach for predicting crude oil biodegradation rate in the sea and evaluate its limitations. Furthermore, a relatively new static respirometry method was adapted for studying hydrocarbon (HC) biodegradation in seawater and to examine the effect of geographical origin of seawater on inherent biodegradation rate and temperature response.

The Oil Spill Contingency and Response (OSCAR) model, the industry standard in Norway to evaluate possible effects of accidental oil releases, takes biodegradation into account based on experimentally determined rate coefficient values. These values are adjusted to local ambient conditions by temperature compensation. Based on literature data and experimental observations, the compensation method implemented in the OSCAR model has been found to be debatable. Literature data and experimental results did not corroborate the rule of thumb approach used in the OSCAR model. The 32 different temperature compensation factors (Q_{10} values) calculated from published HC biodegradation rates ranged from 1.1 to 16.2 with an average of 2.8. The Q_{10} values for crude oil biodegradation obtained during laboratory tests were 2.6 and 9.9 at 5 and 0.5 °C, respectively. Crude oil type, incubation temperature range and other experimental factors were identified as important factors causing the high variation observed among Q_{10} values. Results showed that substrate limitation (reduced bioavailability) occurs at low temperature due to the effect of temperature on physico-chemical properties of oil. This temperature dependence of bioavailability implies that properties of crude oil influence the Q_{10} value. Hence, applying the same Q_{10} value for different oil types is not suitable. Besides bioavailability, initial number of bacteria participating in biodegradation is potentially affected by temperature, representing the second major cause of variation in observed Q_{10} values.

The OxiTop®-C system was found to be useful a tool for measuring HC biodegradation and for assessing the effect of different experimental and

environmental factors on biodegradation kinetics. Seawater sample pre-treatments (e.g. filtration and aging) and dilution of seawater has been tested to understand their influence on kinetic parameters. Dilution of the seawater affected all parameters: lag time, maximum oxygen consumption rate and pseudo first order degradation rate. Filtration and aging of seawater samples did not influence pseudo first order rate coefficient, the parameter most often reported for HC and crude oil biodegradation, while results were inconclusive in terms of lag time and maximum oxygen consumption rate. The OxiTop®-C system is suggested to be a suitable tool for kinetic model development and validation, especially due to high resolution of measurement data.

Using naphthalene as a model compound, biodegradation rate, temperature response and bacterial community composition of seawaters from two geographically and climatically different areas (the North Sea and the Arctic Ocean) were studied and compared. Three times higher pseudo first order degradation rate coefficients were measured for naphthalene in arctic seawater samples compared to temperate, at all incubation temperatures. Calculated at *in situ* temperatures, rate coefficients of the two seawater types were similar (0.048 d^{-1} for temperate and 0.068 d^{-1} for arctic). Moreover, temperature responses were also found to be comparable with Q_{10} ratios of 3.3 and 3.5 for temperate and arctic seawater, respectively. Temperate and arctic untreated bacterial communities were different at genus level, as revealed by pyrosequencing, and dominating genera during naphthalene biodegradation was also distinct in the two seawater types.

Inherent biodegradation capacity in arctic seawater was shown to be comparable to temperate, implying that biodegradation capacity can be similar in cold compared to warm environments. Overall, approaches like a coupled physical-metabolic model for predicting HC biodegradation rates based on exponential growth of bacteria could represent better alternative compared to temperature compensation when estimating biodegradation rates in the sea.

Abbreviations

bp	boiling point
BTEX	Benzene, Toluene, Ethyl-benzene, Xylene
COD	Chemical Oxygen Demand
CREST	Classification Resources for Environmental Sequence Tags
DGGE	Denaturing Gradient Gel Electrophoresis
DO	Dispersed Oil
GC	Gas Chromatography
HC	Hydrocarbon
ITOPF	International Tanker Owners Pollution Federation
NRC	National Research Council
mp	melting point
OC	Oxygen Consumption
OECD	Organisation for Economic Co-operation and Development
OSCAR	Oil Spill Contingency and Response
OTU	Operational Taxonomic Unit
PAH	Polycyclic Aromatic Hydrocarbon
PCA	Principal Component Analysis
PCR	Polymerase Chain Reaction
SO	Sediment Oil
ThOD	Theoretical Oxygen Demand
vp	vapour pressure
WAF	Water Accomodated Fraction
ws	water solubility

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Introduction

"Humankind has not woven the web of life. We are but one thread within it. Whatever we do to the web, we do to ourselves. All things are bound together. All things connect."

~ Chief Seattle, 1854 ~

The 21st century society heavily relies on petroleum products as energy sources. Crude oil, the raw material of petroleum products, has been formed during geological times from organic material under high pressure and high temperature conditions of subsurface environments. Oil reservoirs in various parts of the world have been and are being continuously discovered and exploited to meet increasing demands. During the last four-five decades, offshore oil production has gained importance, especially on the Norwegian continental shelf. Due to depletion of petroleum resources in temperate areas, exploration and exploitation activities are now also targeting arctic areas. Oil production poses risk of accidental releases and also controlled effluents of hydrocarbon (HC) containing waste streams into marine environments.

Oceans cover approximately 70% of the Earth's surface providing habitat for a vast diversity of plant and animal life. Oceans have an important role in maintaining the homeostasis of Earth partially due to their role in the global carbon cycle. The ecological importance of oceans has recently been recognized in relation to research directed to study the effects of global warming. Oceans are also important from the anthropogenic point of view as they serve as a major source of food supplies. Protecting marine environments is therefore very important. The average oceanic surface temperature north of the 60° latitude is below 10 °C, and constantly less than 5 °C below the north Atlantic thermocline. The effects of accidental releases or introduction of constant production effluents of HCs into permanently cold seawater is yet to be fully explored.

In some oceanic regions crude oil migrates or erupts to the bottom of the ocean through cold hydrocarbon seeps. The Gulf of Mexico is one of the most active seep areas of the world, estimated to receive 500 barrels per day from natural sources. On a global scale, the amount of oil entering the sea from natural sources is between

200.000 and 2.000.000 tons per year, accounting for almost 50% of all oil entering the sea. The other half originates from anthropogenic activities, such as crude oil production, processing and shipping.

Despite the fact that HCs, the major constituents of most crude oils, are natural compounds, most of them have recognized toxic effects on higher level biota. The impact of oil spills have been sadly demonstrated through several accidents, most recently by the Gulf of Mexico blowout. Marine animals suffer visible and obvious harm as a consequence of such events (e.g. oil covered birds etc.). However, the less visible long term effects are also important to consider as they can be similarly or even more severe. Following a spill event, contingency operations (e.g. dispersant use, skimming, burning etc.) need to be carried out immediately to reduce environmental impact to minimum. Hence, appropriate contingency planning prior to any operation is necessary. Comparing and evaluating alternative response scenarios is carried out using numerical models. These models rely on our understanding of the fate of pollutants in the sea and therefore are continuously being developed according to new knowledge. Studying behavior of HCs in marine environments is important for the development of these numerical models in order to be able to predict HC fate accurately and plan response strategies more effectively.

Scope and Structure

Scope

This PhD thesis focuses on the effect of temperature on marine hydrocarbon biodegradation and the temperature compensation method implemented in the Oil Spill Contingency and Response model for estimating hydrocarbon biodegradation. The dissertation presents the adaptation and evaluation of a static respirometry technique for obtaining quantitative data on hydrocarbon biodegradation. Temperature response and biodegradation capacity of different seawater types is compared based on static respirometry data. Additionally, the effect of bacterial community composition on temperature response and biodegradation capacity is experimentally studied and discussed.

Structure

The thesis is divided into seven chapters. Chapter 1 describes the background knowledge related to oil fate in seawater and introduces key issues which underlie the temperature compensation method used for estimating hydrocarbon biodegradation rates. Major findings related to the mechanisms of temperature control and factors affecting biodegradation rates are discussed. Chapter 2 presents methodological considerations regarding static respirometry technique and the two nucleic acid-based microbial community composition analysis methods, i.e. denaturing gradient gel electrophoresis and pyrosequencing. In chapter 3, 4 and 5, the three major topics of the research are presented in a publication format including separate introductions, methodologies, results and discussions, and conclusions. Major conclusions and achievements are summarized in chapter 6. Finally, suggestions for future research are presented in chapter 7.

Chapter 1. Theoretical background

1.1   Oil in the sea

Whether from accidental spills or operational releases, presence of oil in marine environments originating from anthropogenic sources is an environmental problem due to the well-known acute and chronic toxic effects of oil components (NRC, 2003). Therefore, environmental regulations oblige industrial developers to conduct environmental risk analysis before operations in order to evaluate possible impacts of accidental oil releases (Thérivel and Minas, 2002). Assessment of these impacts requires thorough understanding of behavior of spilled oil in seawater (Zobell, 1963; Atlas and Bartha, 1973; Brakstad et al., 2009). In the present chapter, oil weathering processes that determine behavior and fate of oil in seawater are discussed. First the relationship between composition, properties of oil components, and oil characteristics are introduced, as physical, chemical, and biological weathering processes greatly depend on these. The impact of oil characteristics and ambient conditions on weathering processes are then presented.

1.1.1   Characteristics of crude oil constituents

Crude oil is considered to be the most complex naturally occurring chemical mixture on Earth containing more than 17000 distinct compounds (ITOPF, 2002). Discovering the structural diversity of its constituents created a novel field of science, i.e. petroleomics (Marshall and Rodgers, 2004). Due to this complexity, it is not possible to consider characteristics and fate of individual oil components. Instead, characteristics of the four major compound groups, i.e. saturated and unsaturated hydrocarbons (HCs), asphaltenes and resins are discussed (Leahy and Colwell, 1990).

The most relevant properties for the environmental behavior of organic compounds are those that determine physico-chemical state, (e.g. vapor pressure, boiling point, density, viscosity) and those which govern their interaction with the several other phases present in the environment (aqueous solubility and octanol-water partitioning coefficient, i.e. the $logK_{ow}$).

Table 1.1-1 Composition of crude oils, average values from Hyne (2001) and values for a heavy fuel oil and Alaska North Slope crude oil from Wang et al. (2003). Wax content given in brackets.

HC group	Weight %		
	Average	Heavy fuel oil	Alaska North Slope
Saturates	79	45.0 (2.5)	77.6 (2.6)
Aromatics	15	29.0	15.0
Asphaltenes	6	13.0	4.0
Resins		15.5	6.1

Saturated HCs (e.g. n-alkanes, branched alkanes and cycloalkanes) can account for more than 79% of the crude oil (Table 1.1-1). These compounds are rather non-reactive, and non-polar due to the strong non-polar σ-bonds between their carbon atoms (Wilkes and Schwartzbauer, 2010). Small saturates (up to C_4) are gaseous, medium sized (C_5-C_{17}) are liquid, while large ones ($>C_{18}$), also called waxes, are solid at room temperature. Boiling point of n-alkanes and cycloalkanes with less than 10 carbon atoms remains below 200 °C, implying that they can be expected to evaporate under ambient temperatures. In general, boiling point, viscosity and density of saturates increases with increasing number of carbon atoms. As a result of their non-polar nature, saturates, especially longer chain alkanes, are practically insoluble in water. Aqueous solubility of higher chain-length members ($>C_{10}$) does not exceed 0.1 mg L^{-1} in distilled water (McAuliffe, 1987). Solubility in seawater is expected to be below concentrations measured in distilled water (Sutton and Calder, 1974). Due to their low solubility in water, alkanes are expected to partition into the non-aqueous phase liquid (NAPL) of an oil/water mixture and have low bioavailability. This is also shown by their high octanol-water partition coefficients ($logK_{ow} > 3$ for alkanes $>C_4$). As a result, environmental toxicity regarding saturates is of less concern compared to aromatics, though it is important to note that saturates are not completely harmless, e.g. membrane toxicity observed with bacteria (Sikkema et al., 1995; Barron et al., 1999; Donlan et al., 2005).

The unsaturated fraction of petroleum HCs constitutes mainly of aromatic compounds. Other types of unsaturated HCs such as alkenes are generally unstable

due to the reactivity of the double C-C bond and therefore appear to play minor role in crude oil (Wilkes and Schwartzbauer, 2010). Olefins are usually present in concentration below 1% by weight. Aromatic and polyaromatic hydrocarbons (PAHs) are chemically stabile due to the resonance stabilization of their benzene ring structure. Most aromatic compounds, though existing in liquid and solid form, have relatively high vapor pressure and low boiling point, consequently readily evaporate from crude oil under ambient temperatures (NRC, 2003). Compared to saturates, unsaturated HCs are more water soluble, especially the small, single benzene-ring compounds such as benzene (1785 mg L^{-1}) and toluene (530 mg L^{-1}). Water solubility decreases with increasing number of fused benzene rings, and degree and extent of alkylation. This is illustrated by the radically limited solubility of a two ring PAH, naphthalene (30 mg L^{-1}) compared to benzene (Scurtu, 2009). Similarly to water solubility, $logK_{ow}$ values increase with increasing number of benzene rings, illustrated by values 2.13 for benzene, 3.34 for naphthalene and 4.21 for fluorene (Jonsson, 2003). PAHs receive prominent attention in risk assessment due to their known toxic and mutagenic effects on aquatic biota (McAuliffe, 1987; Cerniglia, 1992; Donlan et al., 2005).

Asphaltenes and resins are not defined based on structural similarities, but according to their solubility in organic solvents (Akbarzadeh et al., 2007). Both groups are polar, and contain aromatic hetero-compounds with aliphatic substitutions. Hetero-atoms can be oxygen, sulphur, nitrogen, and trace metals (nickel and vanadium). The smallest polar compounds are known as resins (e.g. pyridines, quinolines, carbazoles, sulfoxides, and amides), while larger ones are known as asphaltenes (e.g. phenols, fatty-acids, ketones, esters, and porphyrins) (NRC, 2003). Asphaltenes are particularly heavy compounds, their density can reach 1.2 g mL^{-1}. Hence, oils with high asphaltene content are usually heavy oils. An important characteristic of asphaltenes is their tendency to precipitate (Goual and Firoozabadi, 2004; Goual, 2012). Resins play a crucial role in keeping asphaltenes in dispersion within the context of the crude oil, hence preventing precipitation of asphaltenes to occur (NRC, 2003). The relative amount of asphaltenes and resins has an important role in the formation and stabilization of water-in-oil emulsions due to their surface active

properties (Fingas et al., 2001). These two groups represent the most polar fraction of crude oil, hence the most water soluble and probably among the most toxic. However, relatively little is known about the toxic effects of this unresolved fraction of crude oils. It is important to note, that analysis of crude oil composition is fairly easy in case of saturates and aromatics, while highly advanced gas-chromatography is required for detailed analysis of resin and asphaltene fractions (Akbarzadeh et al., 2007).

1.1.2 Physico-chemical properties of oil

Composition of crude oils originating from different sources varies (ITOPF, 2002). The relative amount of the four groups described above determines the macroscopic physico-chemical characteristics of individual crude oils (e.g. solubility, specific gravity or density, and viscosity). It is important to consider these properties when assessing the behavior of oil in seawater.

Aqueous solubility determines the amount of HCs dissolved in the water phase, i.e. the biologically available fraction, which affects both toxicity and biodegradability of components. Solubility of oil is usually below 100 ppm and mainly BTEX compounds, naphthalenes and small PAHs are present in the water accommodated fraction (WAF) of crude oil (Barron et al., 1999; Brakstad and Faksness, 2000). Due to their toxicity, small amount of WAF can be sufficient to cause significant impacts on marine organisms. On the other hand, greater solubility is associated with increased biodegradation rates due to enhanced bioavailability of HCs for degrading bacteria (Yassine et al., 2013).

Depending on composition, crude oil density can vary from 0.7 to 0.99 g cm^{-3} at 15 °C (NRC, 2003). Compared to seawater, which has a density of 1.03 g cm^{-3}, most oils are lighter, therefore float on the surface. A commonly used indicator of density is the American Petroleum Institute gravity (API gravity) calculated as:

$$API\ gravity = \frac{141.5}{s_{g,15.5°C}} - 131.5 \qquad (1.1\text{-}1)$$

Where $S_{g,15.5°C}$ is the specific gravity of the crude oil at 15.5 °C. Higher API gravity oils are assumed to contain more of the low density, light compounds (e.g. small

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saturated and aromatic HCs), while low API gravity oils are considered to be enriched in higher density asphaltenes. Density is often used as an indicator for the weathering state of crude oil which in turn reflects the rate of further weathering that can be expected (weathering potential). Heavy oils are predicted to weather more slowly compared to light oils (ITOPF, 2002; Wang et al., 2003).

Viscosity depends on the relative amount of light and heavy fractions. The higher the percentage of light components such as low molecular weight saturates or aromatics, and the lesser the amount of asphaltenes, the lower the viscosity is. Oils that contain a high proportion of long-chain alkanes (i.e. waxy oils) flow less easily and are more likely to solidify at low temperatures compared to oils with low wax content (NRC, 2003). In general, viscosity influences spreading and mixing of oil in water, consequently affecting evaporation, dispersion, emulsification, and sedimentation.

1.1.3   Weathering of oil

When crude oil enters seawater, it remains in a separate phase initially, i.e. in a non-aqueous phase liquid (NAPL) called a slick. As soon as slick formation occurs, several physical, chemical and biological processes begin to change the distribution, composition, and characteristics of the oil (Figure 1.1-1). Significant spreading, evaporation, dispersion, and dissolution are generally observed within the first day following oil release in seawater (ITOPF, 2002). This is later followed by photo-oxidation, emulsification and biodegradation.

Ambient conditions such as wind speed, wave energy, and currents, considerably affect horizontal and vertical transport of oil. Therefore, weather conditions greatly affect the fate of oil during the initial phases of weathering. In general, stormy conditions with high wind speed and breaking waves represent a high mixing energy system, where most weathering processes are enhanced.

Spreading, the horizontal increase of the surface area of the spill, facilitates those weathering processes which depend on surface area (e.g. evaporation, photo-oxidation, dissolution, dispersion and biodegradation). In general, low viscosity light oils spread into larger areas forming thinner layer, while more viscous, heavy oils tend to form thicker layer or break up into patches (ITOPF, 2002). Moreover, slicks

tend to spread non-homogenously, producing thicker and thinner zones depending on local oil viscosity (Reed et al., 1999a). Light oils therefore will have larger surface area in contact with seawater aiding their relatively fast weathering.
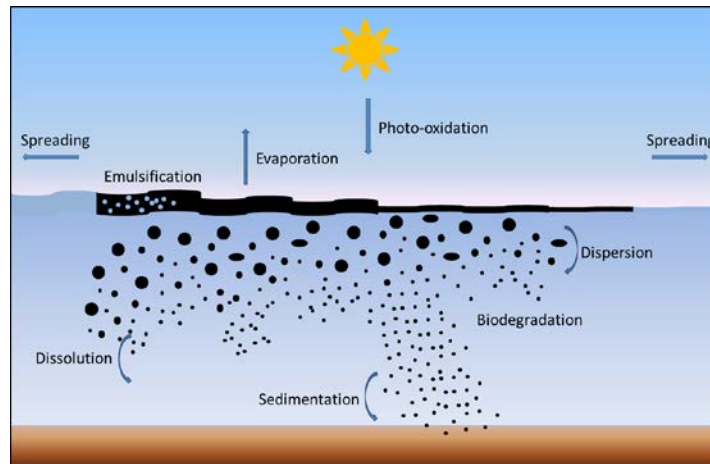
Figure 1.1-1 Processes occurring after oil spill event (ITOPF, 2002).

The larger the surface area, the more quickly volatile compounds can evaporate, causing the loss of up to 75% of the initial oil volume in case of light oils (Fingas, 1999). In contrast, heavy oils may only lose 10% of the original volume by evaporation. Since most volatile HCs are highly toxic, evaporation is a favorable process removing potentially harmful compounds from the seawater. High wind-speed facilitates faster evaporation. However, evaporation results in significant increase in viscosity, up to 1000-fold compared to the initial viscosity of the oil (Zhong and You, 2011).

Viscous oils are more difficult to disperse and are more prone to the formation of emulsions. In general they continue weathering more slowly. Viscosity and density of spilled oil also increases due to formation of water-in-oil emulsion and entrainment. A minimum of 0.5 % asphaltene content is necessary for the formation of water-in-oil emulsions (Fingas, 1995; Fingas et al., 2001). This process, mixing of water into the oil phase, is not characteristic to light oils, while oils with wax and asphaltene content of at least 5% are likely to form water-in-oil emulsions (Zhong and You, 2011).

Emulsification is generally an undesired process as stable emulsions are persistent and undergo slow weathering.

The opposite progression, mixing of oil into water by formation of oil droplets, is called dispersion. Low viscosity oils were found to disperse more rapidly, compared to heavy oils with high viscosity, especially under stormy conditions and breaking waves (Reed et al., 1999a). Dispersion increases the volume of water exposed to oil and the contact area between oil and seawater. The smaller the droplets are, the bigger the contact surface becomes. Such increased surface can enhance dissolution rate of water soluble components. This is advantageous for microorganisms capable of utilizing HCs (Macnaughton et al., 1999, 2003; Venosa and Holder, 2007). However, for higher level marine biota dissolved form of HCs is more toxic.

Solubility of oil can be enhanced by photo-oxidation, light-catalyzed reactions that oxidize the reduced carbon in HC molecules (Garrett et al., 1998; Dutta and Harayama, 2001; Lee, 2003; Prince et al., 2003). Products of photo-oxidation (e.g. polar ketones, aldehydes, carboxylic acids, and esters) are more water soluble and also more toxic than the initial compounds (Maki et al., 2001; Barron et al., 2003). Alternatively, light-reactions can also lead to formation of heavier, asphaltenic components, increasing the heavier fraction by condensation reactions.

All physical and chemical processes introduced until now contribute to increased density of weathered oils compared to fresh crude oils. Weathered oil can become so dense that it sediments by its own weight. Alternatively, interaction of weathered oil with particulate organic matter can further increase the density of dispersed or emulsified fractions to the point where it excesses that of seawater. When that occurs, droplets or emulsions sink into the sediment. Components of sedimented oil can only be removed by biological transformation.

There is a versatile group of microorganisms capable of utilizing HCs as carbon and energy source for their growth (Zobell, 1946). Biodegradation, i.e. microbial transformation of contaminants into less harmful compounds, occurs spontaneously and can be exploited as a cost-effective and environmentally friendly clean-up strategy (Atlas, 1991; Swannell et al., 1996; Atlas and Hazen, 2011). Depending on

the composition of oil and environmental conditions, biodegradation can remove or transform over 95% of light crude oil (Prince, 1993). Physical and chemical transformations of oil affect biodegradation as they determine the biologically available fraction of components (Uraizee et al., 1998). Dissolved compounds are readily degraded, while oil covered with a layer of highly oxidized asphaltenes may persist for a long time. Therefore, photo-oxidation is unfavorable for biodegradation. Dispersion on the other hand is favorable for biodegradation as it creates large surface area for dissolution or adhesion of microorganisms (Baldi et al., 1999). Biodegradation, the only process that completely eliminates HCs from the environment, is introduced in detail in the following paragraphs.

1.2   Hydrocarbon biodegradation

The ultimate fate of crude oil spilled in seawater is to enter the carbon cycle of marine environments through microbial uptake and transformation. The ability to transform and recycle organic material into the microbial loop is a common trait of all heterotrophic bacteria. The capability to gain energy and assimilate carbon from non-conventional substrates or xenobiotics is restricted to specific groups of microorganisms. Biodegradation is considered to be the transformation of organic compounds by microorganisms or "biologically catalyzed reduction in complexity of chemicals", a process that ideally transforms a potentially toxic compound into a non-toxic one (Alexander, 1994). Biodegradation is nothing more than a natural part of microbial metabolism. It is perceived as a beneficial activity in case it contributes to disappearance of anthropogenic pollution (NRC, 2003). Biodegradation of spilled HCs in the water column occurs predominantly under aerobic conditions, therefore only aerobic HC biodegradation is discussed herein.

1.2.1   Pathways and enzymes

The capacity of certain marine bacteria to utilize HCs as energy and carbon source probably evolved as a consequence of large amounts of HCs entering the oceans from natural seeps. HCs are potentially energy rich carbon sources (LaMontagne et al., 2004; Muyzer and van der Kraan, 2008). Bacteria growing near HC seeps were likely

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the first in seawater to develop strategies to cope with the metabolic challenge of channeling apolar and unreactive HCs into their metabolism (Widdel and Musat, 2010). Despite the diversity of HCs and enzymes involved in their transformation, there are general trends in the mode of their enzymatic conversion. Similarly to conventional substrates HCs follow the universal bifurcate substrate flow into cell synthesis (anabolism) and energy generation (catabolism). However, as they are rather inert compounds, they must undergo a preprocessing before entering common intermediary catabolic pathways (Figure 1.2-1).
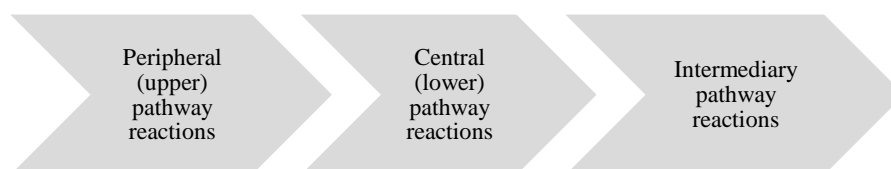
Figure 1.2-1 General scheme of hydrocarbon metabolism (Widdel and Musat, 2010).

During peripheral (upper) pathway reactions HCs are converted into more reactive intermediates generally by the addition of functional groups (Van Hamme et al., 2003; Head et al., 2006; Wilkes and Schwarzbauer, 2010). Activated HCs then go through further structural rearrangements in the central (lower) pathway reactions. Finally, the metabolic products of these reactions are channeled into the intermediary metabolic pathways (e.g. glycolytic pathway, tricarboxylic-acid cycle, fatty acid metabolism and biosynthesis of amino acids) (Wackett and Hershberger, 2001; Diaz, 2008; Fuchs et al., 2011).

Under aerobic conditions the primary attack of HCs occurs by making use of oxygen in reactions catalyzed by oxygenase enzymes. These enzymes are multicomponent protein complexes, generally containing a metal center (Rieske-type Fe_2S_2, other Fe_xS_x or rubredoxin) and electron transfer proteins. It is interesting to note that the functions of these enzymes are by far not limited to HC activation in bacteria. Thousands of oxygenases are known to carry out wide range of catalytic reactions also in plants and animals (Wackett and Hershberger, 2001).

During the first steps of HC transformation, attack of C-H or C-C bonds takes place in the reaction center of oxygenases, where reactive oxygen species are generated utilizing electrons channeled from NADH-H$^+$ coenzymes (Widdel and Musat, 2010). Oxygenase enzymes are able to convert several structurally similar compounds but can exhibit very narrow substrate specificity as well (Rojo, 2010). In general, the two major structurally different groups of HCs, alkanes and aromatics, are activated by different oxygenase enzymes. The major difference between the mode of action of oxygenases on saturated and aromatic HCs is attributed to the primary attack of C-H bond in the former and the primary attack of C-C bond in the latter.

Table 1.2-1 Enzyme classes involved in alkane activation (Rojo, 2010).

Enzyme class	Characteristics	Substrate length	Host
PRM, propane monooxygenase	Non-heme iron monooxygenase similar to sMMO[1]	C_3	Bacteria
sBMO, butane monooxygenase	Non-heme iron monooxygenase similar to sMMO[1]	C_2-C_9	Bacteria
pBMO, butane monooxygenase	Copper-containing monooxygenase similar to pMMO[2]	C_2-C_9	Bacteria
CYP153	Soluble cytochrome P450	C_5-C_{12}	Bacteria
CYP52	Membrane-bound cytochrome P450	C_{10}-C_{16}	Yeasts
AlkB-related	Non-heme iron monooxygenase	C_3-C_{13} or C_{10}-C_{20}	Bacteria
AlmA	Flavin-binding monooxygenase	C_{20}-C_{36}	Bacteria
LadA	Thermophilic flavin-dependent monooxygenase	C_{10}-C_{30}	Bacteria
Dioxygenase	Copper flavin-dependent dioxygenase	C_{10}-C_{30}	Bacteria

[1] soluble form of methane monooxygenase (sMMO)
[2] membrane-bound particulate form of methane monooxygenase (pMMO)

Alkanes are activated by monooxygenanses (hydroxylases), which incorporate one oxygen atom into the HC upon C–H bond cleavage at the terminal carbon and substitution of the hydrogen with oxygen. Alkanes with different chain length are oxidized by different monooxygenase classes (Table 1.2-1). Hence, bacteria possessing different type of monooxygenases are capable of degrading alkanes in a certain range of carbon numbers. For example, bacteria degrading medium-chain-length alkanes (C_5-C_{11}) or long-chain alkanes (>C_{12}), were frequently found to possess integral membrane non-heme iron monooxygenases related to the well-known

*Alk*B hydroxylase (van Beilen et al., 2001). Following the activation step, subsequent dehydrogenation of activated alkanes (e.g. alcohols) yields fatty-acids, which are further degraded in β-oxidation reactions.

Aromatic HCs are activated by dioxygenases, which incorporate both atoms of the dioxygen. The peripheral pathway reactions of aromatic HC transformation are carried out by ring-hydroxylating oxygenases (RHO), which form *cis*-dihydrodiols in the first step, followed by the activity of dehydrogenases converting these dihydrodiols into central intermediates, such as gentisate and catechol. There is no universal way for classification of RHOs (Peng et al., 2010). Schemes were proposed based on a) the composition of the electron transport chain (ETC) of RHOs (Batie et al., 1991), b) sequence homology score with toluene dioxygenases (TodC1) and benzene dioxygenases (BedC1) (Nam et al., 2001), c) analyzing the terminal oxygenase and the ETC components as a whole including phylogeny of the oxygenases (Kweon et al., 2008), and based on d) evolutionary and functional behaviours of RHOs, in relation to structural configuration of substrates and preferred oxygenation site(s) (Chakraborty et al., 2012). Despite the lack of a widely accepted grouping system, similar general rules apply for degradation potential of bacteria possessing certain types of RHO as outlined in case of alkanes, i.e. utilizable substrate range is determined by the RHO type present. Lower pathway reactions are also performed by dioxygenases. However, these enzymes carry out ring-cleavage instead of oxygenation. Intradiol dioxygenases facilitate *ortho*, while extradiol oxygenases facilitate *meta* cleavage of the aromatic ring.

Complete degradation of a HC requires a sequence of reactions to occur in a concerted manner. Regulatory genes, genes encoding transport proteins and genes encoding upper and lower pathway enzymes are usually arranged in clusters or operons and are expressed simultaneously. Genes coding for central pathway reactions are usually found on chromosomes, while those encoding peripheral reactions can be found either on chromosomes or on plasmids (Habe and Omori, 2003; Diaz, 2008). While the overall organization of catabolic processes is well-conserved among wide variety of HC-degarding bacteria, the pathways can have genus-specific variations attributed to enzyme distribution, regulation and gene

organizations (Diaz, 2008). This implies that two alkane degrading genera may have the same capacity for alkane utilization, though possessing different genetic background for it. Also, differential regulation of dioxygenase encoding genes implies that degradation activity may depend on the type of degraders present (Master and Mohn, 2001). Bacteria can carry multiple copies of oxygenase genes, and genes coding for different oxygenases (Geiselbrecht et al., 1998, Romine et al., 1999; Moody et al., 2001; Tittabutr et al., 2011). Moreover some bacteria, specifically strains of *Pseudomonas* and *Rhodococcus* genera, were shown to carry genes coding for enzymes that degrade aromatics and alkanes simultaneously (Whyte et al., 1997; Andreoni et al., 2000). Identifying and characterizing the type of oxygenase enzyme involved in degradation can provide important information about kinetics of degradation as initial reactions can be overall rate limiting.

1.2.2   Hydrocarbon degraders

HC-degrading bacteria were first isolated almost 100 years ago (ZoBell, 1946). Since then, a vast diversity of prokaryotic and eukaryotic organisms has been identified as actors in HC biodegradation (Prince, 2010). Eukaryotic species, mainly terrestrial, but recently also marine fungi has been found to degrade aromatic HCs (Passarini et al., 2011). Even though, formally no *Arhcaea* has been described as HC degrader yet, some methanogenic *Archaea* have been found to be involved in utilization of alkanes under methanogenic conditions (Gray et al., 2011). Furthermore it was suggested that the absence of archaeal communities can indicate oil pollution, as they tend to disappear following oil exposure in laboratory microcosms, and recover as the HC concentrations lower. This statement however remains controversial due to contradictory observations in field experiments (Röling et al., 2004). In marine systems, phototrophic diatoms and algae has been found to participate in HC biodegradation, however, the mechanisms are not clarified yet. Besides photosynthetic eukaryotes also phototrophic bacteria were found to facilitate biodegradation of HCs (Gutierrez et al., 2012).

The major target group of interest in relation to HC biodegradation is the aerob heterotroph prokaryotic degraders as they are known to consume significant amounts

of a variety of crude oil components within a relatively short time (Head et al., 2006). HC degraders have been found in several different environments, e.g. soil (Szoboszlay et al., 2008), groundwater (Cavalca et al., 2004), salt marshes (Daane et al., 2001), mangroves (Guo et al., 2005), sea ice (Gerdes et al., 2005), Alpine soil (Margesin, 2000), oil reservoirs (Hubert and Judd, 2010), Antarctic soil (Aislabie et al., 2000) and cold HC seeps (Orcutt et al., 2010). The majority of isolated strains, covering more than 170 genera, originate from terrestrial habitats but an increasing number of marine species are discovered (Prince et al., 2010). Prokaryotic HC degraders have been found within phylum Actinobacteria, Bacteroidetes, Cyanobacteria, Deinococcus-Thermus, Firmicutes, and Proteobacteria. The majority of formally described HC degraders belongs to phylum Proteobacteria, including the relatively recently discovered obligate hydrocarbonoclastic genera, e.g. *Alkanivorax* (Yakimov et al., 1998), *Cycloclasticus* (Dyksterhouse et al., 1995), *Oleiphilus* (Golyshin et al., 2002), *Oleispira* (Yakimov et al., 2003), and *Thalassolituus* (Yakimov et al., 2004a). Members of these genera are considered to be the global players in marine crude oil biodegradation (Harayama et al., 2004; Head et al., 2006). Some of them are obligate HC degraders (Yakimov et al., 2007), using HCs almost exclusively as carbon and energy source (Head et al., 2006; McKew, et al., 2007).

Specifically in marine environments, commonly found HC degrading bacteria belong to genera: *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Nocardia*, *Arthrobacter* and other coryneforms, *Vibrio*, *Bacillus*, *Micrococcus* and *Acinetobacter* (Atlas, 1995), also *Alteromonas* (Math et al., 2012), *Polaromonas* (Jeon et al., 2006) and *Neptunomonas* (Hedlund et al., 1999) have been identified. Nowadays *Cycloclasticus* and *Alcanivorax* are believed to be the most important genera involved in crude oil biodegradation in seawater and marine sediment (Prince et al., 2010).

Cycloclasticus is a genus specialized in aerobic degradation of PAHs in a variety of marine environments (Teramoto et al., 2010). The type strain, *Cycloclasticus pugetii* (Geiselbrecht et al., 1998) carries two distinct dioxygenase genes and is able to utilize naphthalene, phenanthrene, anthracene, biphenyl, salicylate, toluene, benzoate, acetate, propionate and glutamate as sole carbon sources. *Cycloclasticus* species have been isolated from several regions and are thought to be cosmopolitan (Button et al.,

1998; Kasai et al., 2002; Maruyama et al., 2003; Teira et al., 2007). Although reported to be phylogenetically similar, no comparative information about biodegradation rates of geographically different isolates has been published yet.

Alcanivorax is known for the alkane degradation capability of its members. Similarly to *Cycloclasticus*, *Alkanivorax* species are also cosmopolitan. This ubiquity is possibly originating from the capacity of the over 250 *Alkanivorax*-like bacteria to grow on many saturated HCs, biogenic HCs (e.g. pristine and phytane), and long side-chain cyclic or aromatic compounds (Cappello and Yakimov, 2010). So far, they have been isolated only from temperate and subtropical environments. However, the presence of *Alkanivorax*-related 16S r RNA gene sequences have been detected also in cold polar areas. In general, *Alkanivorax* are considered mesophilic with optimum temperatures between 25 and 30 ℃ (Golyshin et al., 2005). Psychrotolerant or psychrophilic variants might also exist though not reported yet. Genome sequence of *Alkanivorax borkumensis* revealed the genetic basis for several mechanisms that make members of this genus efficient degraders of saturated HCs. Mechanisms include a) expression of multiple oxygenase systems (5 distinct oxygenases), b) biosurfactant production in the presence of alkanes, c) biofilm formation on the oil/water interface, d) ability to scavenge nutrients in the oligotrophic marine environment, and e) capability to produce storage lipids (Sabirova et al., 2006, 2008). Different *Alkanivorax* species have different physiological properties which might affect biodegradation kinetics and extent (Yakimov et al., 1998; Dutta and Harayama, 2001).

Though some hydrocarbonoclastic species can possess enzymes for degradation of structurally different HCs, thus able to grow on both alkanes and aromatics, no strain has been found to be able to degrade all the diverse components of crude oil alone. Compositional complexity of crude oil thus requires the concerted action of a specialized bacterial consortium (Valentine et al., 2012).

The number of hydrocarbonoclastic bacteria is usually very low in pristine environments (i.e. down to only 0.1% of the total biomass). Upon exposure to their favored substrate, they quickly become dominating (i.e. reaching up to 100% relative abundance). In general, the bloom of primary HC degraders accompanied by the

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growth of secondary degrader populations, consuming metabolic products excreted by primary degraders, facilitates the quick disappearance of pollutants. In case transformation of the HC does not provide sufficient energy for bacterial growth it may be co-metabolized (Van Hamme et al., 2003; Harayama et al., 2004; Haritash and Kaushik, 2009). Earlier it was hypothesized that there is a sequential degradation order of different HC types and homologues of different chain-length. It is now becoming clear that the false impression of sequential utilization originates from the lower degradation rates of certain compounds attributed to the lower number of bacteria degrading them (Yassine et al., 2013).

Though exact mechanisms are not clearly understood yet, HC degraders appear to possess some remarkable physiological capabilities such as, a) tolerating solvents due to active efflux transport facilitated by solvent pumps, b) possessing a large number of transport systems that facilitate uptake of nutrients from oligotrophic environments, c) high affinity for their preferred substrate, d) specialized transport systems enhancing HC uptake, e) ability to adjust their cell membrane hydrophobicity in the presence of HCs, and f) production of surfactants to increase bioavailability of HCs (Heipieper et al., 2010).

Despite the advances in molecular methods for detection and identification of microorganisms in environmental samples, it remains a challenge to identify the most active HC degraders as currently used molecular methods lack the ability to link phylogeny and function. Identifying HC degraders based on 16S rRNA gene sequence does not reveal their metabolic capacity (i.e. achievable biodegradation rate). Approaches such as stable-isotope probing (SIP) have demonstrated that there are still novel HC degraders to be found whose activity is overlooked when using popular 16S rRNA-based community analysis of exposed samples (Singleton et al., 2006; Jones et al, 2008; Xie et al., 2011).

1.2.3   Kinetics of hydrocarbon biodegradation

While identifying the major degraders in a given environment provides a qualitative insight into biodegradation, rate and extent of biodegradation activity is the quantitative information necessary for being able to assess duration and

environmental concentration of HC exposure of marine biota following a spill event. According to Zahed et al. (2011), kinetic analysis is a key factor for understanding the biodegradation process and for the development of efficient clean up strategies. The first step in estimating biodegradation rates in nature is to understand the kinetics of the process and the factors influencing this kinetics (Alexander, 1994).

Assuming that most HC degrading bacteria grow well on their preferred substrate (i.e. depletion of a compound is coupled to biomass formation), removal of HCs (substrate) is influenced by the concentration of active degraders (X; mg L^{-1}), their specific growth rate (μ; day^{-1}), and the proportion of substrate which is assimilated into biomass (Y, unit biomass formed per unit substrate) according to the following:

$$-\frac{dS}{dt} = \frac{\mu \cdot X}{Y} \qquad (1.2\text{-}1)$$

Where S is substrate concentration (mg L^{-1}) and t is time (day). Yield is assumed to be constant during biodegradation of a single compound. Growth rate however, depends on the concentration of the limiting substrate. Most growth based degradation models assume saturation kinetics as described by the Monod model (Monod, 1949):

$$\mu = \frac{\mu_{max} \cdot S}{K_S + S} \qquad (1.2\text{-}2)$$

Where μ is the observed growth rate (day^{-1}), K_S is the half saturation coefficient (the inverse is known as substrate affinity) (mg L^{-1}) and μ_{max} is the maximum growth rate (day^{-1}). During growth related degradation, biomass changes depending on the growth and death rate as:

$$\frac{dX}{dt} = \mu \cdot X - k_d \cdot X \qquad (1.2\text{-}3)$$

Where k_d is the cellular decay rate (day^{-1}), usually assumed to be constant (0.1 day^{-1}). As cell decay only influences the biomass mass balance, rate of substrate removal can be expressed according to:

$$-\frac{dS}{dt} = \frac{1}{Y} \cdot \frac{\mu_{max} \cdot S}{K_S + S} \cdot X \qquad\qquad (1.2\text{-}4)$$

Yield, affinity, and maximum growth rate are considered constants. During fast exponential growth, the decrease of biomass due to cell death is negligible and can be ignored. Determining the kinetics of substrate removal requires the integration of this equation. Depending on the relative concentrations of K_S to S, and the relative amount of X to S, several types of kinetics can be deducted (Simkins and Alexander, 1984).

Considering the case of crude oil in seawater, the initial biomass involved in degradation is usually very low, unless previous exposure enriched for HC degraders. Substrate concentration is also expected to be low and vary according to HC type. Alkanes, which are practically insoluble in water, can be assumed to be available for degradation in concentrations much lower than K_S of degrading bacteria (case I: S << K_S). On the other hand, this assumption may not stand for BTEX compounds and low molecular weight PAHs with relatively high aqueous solubility (case II: S ~ K_S).

At the extreme case of S << K_S (case I), changes in substrate concentration occur either in an exponential fashion (case a) or following a logistic model (case b). If the initial number of degraders is high, the available low substrate concentration will not support significant population growth, and the substrate depletion can be described with pseudo first order kinetics (case a). Typically, this pseudo first order kinetics is applied when assessing biodegradation rates of crude oil in seawater (Stewart et al., 1993). However, if the number of degraders is initially low, as expected in seawater, this low initial biomass begins to mineralize with little observable substrate loss but with significant increase in population size. Depletion will become gradually faster as the size of the degrading population increases (following a lag phase). However, shortly after, substrate limitation will constrain the degradation rates due to high biomass to substrate ration. This type of kinetics can be described with logistic function (case b) as it was shown for degradation of selected HCs (Aislabie et al., 2011; Mohn and Stewart, 2000).

Considering case II (S ~ K_S), biodegradation of low molecular weight PAHs, with aqueous solubility ~10 mg L^{-1}, is expected to follow the Monod model with growth according to the guidelines of Simkins and Alexander (1984). BTEX compounds with even higher aqueous solubility (~1000 mg L^{-1}) are expected to be degraded according to a logarithmic model.

It is important to note that one major assumption underlying the previously described kinetic models for biodegradation was that initial substrate concentration equals the biologically available substrate concentration. This may not be true for an event of oil spill in the sea, where HCs are becoming available for microbial degradation continuously over time, through transport from the oil phase into water. Bioavailability refers to the amount of substrate that is available for microbial uptake and conversion. Alternatively, bioavailability can be defined as the ratio of the actual biodegradation rate of an extant microbial community to its theoretical degradation capacity (Thullner et al., 2008). Bioavailability influences degradation kinetics through controlling the actual (utilizable) concentration of substrate (i.e. via mass transfer limitation from the bulk oil phase into water). In case inherent biodegradation rate is high, HC depletion kinetics is limited by the kinetics of mass transfer of HCs. Alternatively, when biodegradation proceeds with a slower rate compared to mass transfer, HC depletion kinetics is limited by the kinetics of microbial degradation process (Bosma et al., 1996; Yassine et al., 2013). Hence, differences in bioavailability of different HC types results in distinct degradation kinetics.

Overall, when choosing a kinetic model to interpret HC biodegradation results, it is important to consider the number of bacteria actively involved in the degradation and biologically available concentrations of the substrate.

1.3 Environmental factors influencing hydrocarbon biodegradation

Factors determining biodegradation kinetics are those which influence the number of degrading bacteria, concentration of available substrate (bioavailability), and growth rate of degraders.

1.3.1   Top down and bottom up control

In their natural habitats, HC degrading bacteria are organic parts of an ecological network. This network consists of the traditional food chain and the microbial loop, which is at the base of the chain (Azam et al., 1983). Within the context of the microbial loop, there are several mechanisms that control the abundance and growth rate of bacteria, and through that influencing biodegradation processes (McGenity et al., 2012). Top down control of grazing by heterotrophic nanoflagellates and viral lysis directly regulates their abundance. The effect of grazing can be negative as it reduces the number of bacteria involved in biodegradation (Kota et al., 1999). However, predation was found to increase the per-cell biodegradation of toluene and benzene (Mattison and Harayama, 2001). This might be due to that predation and viral lysis (Cochran et al., 1998) can increase the turnover of biomass thereby stimulating biodegradation through intrinsic recycling of nutrients (Head et al., 2006).

Nutrients (e.g. nitrogen and phosphorous) are essential for microbial growth and are usually the limiting factor for HC degrading bacteria in oligotrophic marine environments (Leahy and Colwell, 1990). Several studies showed that fertilization has the capacity to enhance biodegradation process. This strategy has often been used in bioremediation although with variable success (Swannell et al., 1996). Röling et al. (2002) showed that the initial degradation can be influenced by fertilizer addition, while the final extent of oil biodegradation remains unaffected. Nutrient requirements of bacteria in general, including HC degraders, are not uniform. Even within one genus the different species can exhibit significantly different nutrient needs. Therefore, nutrient availability is an important selective factor determining community composition. Interestingly, while the effect of nutrient amendment on the microbial composition of oiled microcosms was variable, (i.e. several different community compositions were observed resulting from identical nutrient treatment) biodegradation rates remained similar, indicating that there are several combinations of bacteria that perform equally well (Röling et al., 2002). Since not only HC degraders but all prokaryotes use the same nutrient pool, a competitive situation is created which might be unfavorable for HC biodegradation. Competition is a

mutually negative interaction for all parties involved presumably reducing HC biodegradation rates as a result.

There are, however, also cooperative interactions which may enhance biodegradation. An interesting mutualistic interaction is related to the surfactant production of non-HC degraders stimulating bioavailability of HCs. Extracellular polysaccharides produced by a *Rhodococcus* strain enabled *Cycloclasticus* to become dominant in nutrient amended seawater (Iwabuchi et al., 2002). Though this area is not well studied yet, such mutualistic interactions are expected to result in enhanced biodegradation rate of HCs in natural environments.

As a summary, Figure 1.3-1 illustrates the above described microbial interactions expected to occur during oil biodegradation in seawater between HC-degraders and non-HC degraders and secondary consumers, including competition for nutrient among non-HC degraders and HC-degraders, viral lysis and predation on all these bacterial populations.

1.3.2 Bioavailability of hydrocarbons

Besides availability of nutrients, often bioavailability of HCs themselves limits their biodegradation resulting from their low aqueous solubility and high $logK_{ow}$ values. As stated earlier (section 1.2.3) bioavailability influences degradation kinetics through controlling the utilizable concentration of HCs. Since microorganisms live in the water phase, HCs can be taken up from the water phase or from the oil/water interface. Dissolved compounds are therefore considered readily available for microbial uptake by passive diffusion or active transport through cell membranes, while HCs within the context of the oil NAPL are not available (Parales and Ditty, 2010). In order to overcome bioavailability limitations, HC degraders evolved special strategies such as producing biosurfactants, actively migrating to the oil/water interphase, and attaching to oil droplets and forming biofilms (Baldi et al., 1999).

Dispersion of the oil NAPL into small droplets enhances HC bioavailability through increasing contact surface between water and oil. Dispersion can be assisted by the use of chemical dispersant as a response strategy (NRC, 2005; Prince et al., 2013) or

it can be facilitated by bacteria themselves through the production of biosurfactants (Brown, 2007; Kanga et al., 1997). Several strains have been found to produce biosurfactants (Batista et al., 2006). Some are actively involved in HC biodegradation while some are not HC degraders. Biosurfactant production is thought to have a mutually positive effect on both HC degraders and generalist heterotrophic bacteria (McGenity et al., 2012).
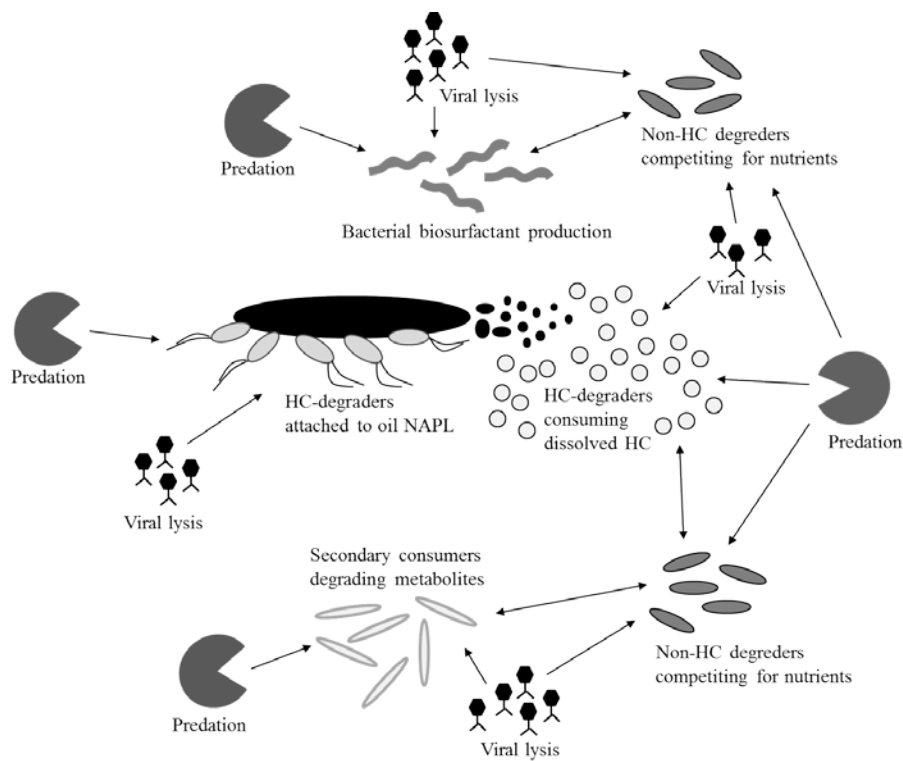
Figure 1.3-1 Major players and microbial interactions during biodegradation of oil in seawater.

Motility traits of degraders were found to influence degradation rate of single HC substrates and chemotaxis is now believed to have the potential to increase the rate of biodegradation (Law and Aitken, 2003). Naphthalene chemotaxis genes have been found in naphthalene degradation related operons indicating that bacteria are indeed

able to detect their preferred substrate and engage in active movement towards its source (Grimm and Harwood, 1999).

Once bacteria reach the vicinity of oil NAPL, they were shown to enhance bioavailability by changing hydrophobicity of their outer cell membrane in order to facilitate contact with hydrophobic substrates (Hermansson, 1999; Wick et al., 2002). Increased hydrophobicity was found to be one of the major mechanisms exhibited by a psychrotrophic *Rhodococcus* sp. strain Q15 to access alkanes at low temperature (Whyte et al., 1999). Furthermore, two *Acinetobacter* species, *Acinetobacter calcoaceticus* RAG-1 and *Acinetobacter venetianus* VE-C3 were shown to disperse hexadecane and also to adhere to droplets and form biofilm around diesel oil drops (Baldi et al., 1999). Once bacteria are attached to the oil/water interface, they maintain a constant driving force for diffusion of their preferred substrate from the bulk NAPL to water by utilizing them continuously (Harms et al., 2010a, 2010b).

1.3.3   Temperature

Temperature has a prominent role among all other environmental factors because it influences all aspects of life as a result of fundamental thermodynamic laws (Arrhenius, 1889). The temperature of oceans above the thermocline varies considerably according to location and season, ranging from below zero in the Arctic winter, up to around 30 °C in hot season of the tropical areas. However, the majority of marine habitats are constantly cold with a temperature lower than 5 °C (Morita, 1975). It is well known that the growth rate and rate of metabolism of bacteria changes according to temperature (Farrell and Rosa, 1967) and that decreasing temperature limits oil biodegradation (Zobell, 1963; Atlas and Bartha, 1972; Brakstad et al., 2009). Hence it can be expected that biodegradation of spilled oil in distinct marine environments proceeds with a different rate due to variations in ambient temperature.

An example of temperature effect related to HC degradation was reported early by Zobell et al., (1943) showing a culture of HC degrading bacteria to consume seven times more paraffin at 55 °C compared to 22 °C. It was also observed that mineralization of crude oil is significantly higher at 20 °C compared to 10 °C (Atlas,

1975). Several studies have found similar results, i.e. decreasing HC biodegradation rates at low incubation temperature (Mulkins-Phillips and Stewart, 1974; Gibbs et al., 1975; Minas and Gunkel, 1975; Gibbs and Davis, 1976; Garrett et al., 2003; Deppe et al., 2005; Brakstad and Bonaunet, 2006; Coulon et al., 2007; Venosa and Holder, 2007). However, contradicting results also appeared claiming that temperature did not have any effect on biodegradation (Delille et al., 2009; Rodríguez-Blanco et al., 2010).

The following section presents biological and physico-chemical factors involved in mechanisms responsible for the effect of temperature (specifically on petroleum HC biodegradation) and broadly applied modeling approaches developed to describe the effect of temperature on metabolic processes.

1.4 Temperature control of petroleum hydrocarbon biodegradation

The effect of temperature on crude oil biodegradation is a multifaceted problem with biological and physico-chemical factors interacting (Brakstad et al., 2009). On one hand, there is the effect of temperature on the activity of microorganisms, their adaptation level and community compositions evolved under different thermal regimes (biological factors). On the other hand, there is the effect of temperature on the oil itself (physico-chemical factors).

1.4.1 Biological factors involved in temperature control of petroleum hydrocarbon biodegradation

Metabolism and growth of bacteria is controlled by temperature primarily through its effect on enzymes. The fundamental concept behind temperature control of any metabolic activity is the temperature dependence of enzyme activity resulting from thermodynamic constrains (Struvay and Feller, 2012). Reactions carried out by enzymes are characterized with catalytic rate constants (maximum number of substrate molecules converted to product per active site, per unit of time), which depend on temperature according to the Eyring equation:

$$k_{cat} = \kappa \frac{k_B T}{h} e^{-\Delta G^{\#}/RT}$$ (1.4-1)

Where k_{cat} is the catalytic rate constant (s^{-1}), κ is the transmission coefficient generally close to 1, k_B is the Boltzmann constant ($1.3805 \cdot 10^{-23}$ J K^{-1}), h is the Planck constant ($6.6256 \cdot 10^{-34}$ J s), $\Delta G^{\#}$ is the free energy of activation (J mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}) and T is temperature (K). Hence, there is an exponential relationship between activity and temperature which results in increasing rates at higher temperatures. This is however, only true for the temperature range below optimum. It is important to note, that from Equation 1.4-1, it is the free energy of activation ($\Delta G^{\#}$) that determines catalytic activity at a given temperature.

Biodegradation is not carried out by single and pure enzymes but through the activity of populations of degrading bacteria, where each individual is involved in complex processes regulating and controlling maintenance and growth of its cellular structure. Though metabolic activity of populations is far more complex than activity of single enzymes, its temperature dependence can be described with very similar exponential relationship according to:

$$r = A_0 \cdot e^{\left(\frac{-E_a}{R \cdot T}\right)}$$ (1.4-2)

Where r is the metabolic rate (mol s^{-1}), A_0 is a constant (mol s^{-1}), E_a is the activation energy (J mol^{-1}), R is the universal gas constant (8.314 J K^{-1} mol^{-1}) and T is the temperature (K). Equation 1.4-2, known as the van't Hoff-Arrhenius equation (Reichardt and Morita, 1982; Margesin and Schinner, 1994; Robador et al., 2009), was originally developed to formalize the temperature dependence of chemical reactions (Arrhenius, 1889). Later it was adapted to biochemistry and microbiology (Hanus and Morita, 1968). From Equation 1.4-2 it is apparent that temperature response of a population is defined by the activation energy (E_a). This concept was adapted to temperature response of whole communities (Price and Sowers, 2004), and to metabolic theories (Brown et al., 2004; Kooijman, 2010). In the context of a microbial community, E_a describes the temperature response of the whole community.

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When studying temperature sensitivity of HC biodegradation with whole communities, usually temperature controlled experiments are carried out. These tests are used to determine Q_{10} values, the multipliers by which metabolic rates increase at ten degrees temperature rise. The Q_{10} value reflects temperature sensitivity of microbial communities, similarly to E_a. The relationship between the two, based on the Arrhenius interpretation of temperature effect, is as follows:

$$\frac{r_{(T+10)}}{r_T} = Q_{10} = e^{\left[\frac{E_a \cdot 10}{R \cdot T \cdot (T+10)}\right]}$$
(1.4-3)

Exposing given microbial communities of environmental samples to incubation temperatures lower than their ambient temperature will result in reduced biodegradation rates according to Equation 1.4-3. However, this does not imply that biodegradation in cold environments is necessarily slower compared to warm environments.

Adaptation of enzymes to ambient temperature allows bacteria living in cold environments to express similar metabolic activities as those living in warmer environments (Arnosti et al., 1998; Robador et al., 2009). This results from unique characteristics of psychrophilic enzymes, e.g. high specific activity at low temperatures at the expense of lower affinity (Feller, 2003; Struvay and Feller, 2012). Adaptation of bacteria to ambient temperature is not a result of only a few enzymatic adjustments, but the outcome of a concerted effect of complex physiological and genetic adjustments (Methé et al., 2005, D'Amico et al., 2006; Filler et al., 2008). These involve for example increased membrane fluidity and synthesis of antifreeze proteins and cryoprotectants. The degree of temperature adaptation of bacteria has traditionally been believed to be reflected in the temperature range for growth (Ingraham and Stokes, 1959; Mohr and Krawiec, 1980). Warm-adapted bacteria (mesophiles) were defined as those growing well from 15 to 20 °C, while cold-adapted bacteria (psychrotrophs or psychrophiles) as those growing well below 5 °C (Morita, 1975). This definition of the thermal groups has lately been debated due to differences found in optimal temperatures depending on whether maximum growth rate or maximum growth yield was selected as classification criteria (Margesin,

2009). Perhaps it is more practical to interpret differences in temperature sensitivity as a continuum in temperature adaptation as suggested by D'Amico et al. (2006).

Several studies demonstrated the capability to degrade HCs at low temperatures by psychrophilic or psychrotolerant hydrocarbon-degrading marine bacteria of high phylogenetic diversity, including members of the genera *Sphingomonas*, *Marinobacter*, *Marinomonas*, *Halomonas*, *Psychrobacter*, *Psychromonas*, *Colwellia*, *Oleispira*, *Acinetobacter*, *Shewanella*, *Pseudoalteromonas*, *Pseudomonas*, *Cytophaga*, *Agreia*, *Arthrobacer* and *Rhodococcus* (Michaud et al., 2004; Yakimov et al., 2004b; Deppe at al., 2005; Gerdes et al., 2005; Powell et al., 2005; Brakstad and Bonaunet, 2006). Studies reporting comparison of inherent biodegradation capacity of microbial communities inhabiting permanently cold and temperate seawaters are so far lacking. In case of crude oil, physico-chemical constrains from the substrate side are likely to cause limited degradation rates.

1.4.2 Physico-chemical factors involved in temperature control of petroleum hydrocarbon biodegradation

Temperature influences the physic-chemical properties of HCs (e.g. solubility, viscosity and volatility), the extent and rate of weathering processes (e.g. dissolution, spreading and evaporation) and through that affects the concentration of the biologically available fraction of oil in seawater. Solubility of HCs generally decreases with declining temperatures (Whitehouse, 1983), directly causing available aqueous substrate concentrations to be lower at low temperatures (e.g. in cold seawater like the Arctic Ocean). Moreover, dissolution rate is also reduced under cold conditions (Faksness et al., 2008). This is partly due to the increased viscosity of oil at colder temperatures limiting diffusion from the bulk oil phase into the water. Viscosity of oil changes dramatically at the pour point. Crude oils spilled at sea temperature below their pour point form solid and thick layers and patches (ITOPF, 2002). These patches can persist over a long period of time without significant degradation resulting from reduced bioavailability. Viscosity also influences the size and thickness of oil slicks, hence, indirectly affects evaporation as well (Brandvik and Faksness, 2009). Volatilization of low molecular weight alkanes and aromatics is retarded at lower temperatures. This has an adverse effect on the microbiota due to the

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toxic effects of compounds such as benzene, toluene etc. (Atlas and Bartha, 1972). Overall, low temperature does not favor biodegradation due to reduced bioavailability of potential substrates for microbial growth and elevated toxicity. Bioremediation of crude oil spills in cold marine environments is more challenging compared to bioremediating spills at warmer sites.

1.5   Modeling the fate and effect of oil in seawater

Offshore oil & gas industry and related transport activities represent a major source of anthropogenic oil pollution in the marine environment (NRC, 2003). Governmental regulations require that environmental impacts are assessed before operations are started to evaluate the possible effects of accidental oil releases (Thérivel and Minas, 2002). A key regulatory risk assessment approach, commonly applied in the petroleum industry in Norway, is simulation of discharge scenarios using numerical fate and exposure models. These simulation methods, based on worst case assumptions, have been found to be useful in environmental risk management, though they have limited capacity to simulate the marine biosphere in realistic detail (Carroll and Smit, 2011).

Comprehensive oil spill models (third generation models) consist of a set of algorithms used to simulate fate and transport of oil in three dimensions (Berry et al., 2012). Weathering processes (e.g. advection, diffusion, surface spreading, vertical mechanical dispersion, evaporation, emulsification and stranding) are defined by mechanistically or empirically determined differential equations and are solved simultaneously by numerical integration (Sebastião and Soares, 1995). An important criterion is that transport models are coupled to state of the art meteorological and oceanographic forecasts, such as it has been implemented in the OILTRANS model (Berry et al., 2012). Models are also required to be flexible so that oil characteristics can be adjusted to an actual scenario (Guo and Wang, 2009; Wang and Shen, 2010).

Numerical models are applied to estimate and compare efficiency of different clean up scenarios (Aamo et al., 1997). In this case, the fate and transport model needs to be coupled to a module that simulates selected response strategies. Setting either

economic or responsive criteria, an optimal planning of oil spill response operations can be designed with consideration of oil weathering process (Zhong and You, 2011).

1.5.1 The Oil Spill Contingency and Response model

In Norway, the Oil Spill Contingency and Response (OSCAR) model has become an industry standard for environmental impact assessments of oil discharges. The OSCAR model has been developed at SINTEF (Trondheim, Norway) offering a tool for objective analysis of alternative spill response strategies (Reed et al, 1995). The OSCAR model computes the fate of oil spills using a multi-component representation of crude oils and petroleum products. Key components of the system are: a) SINTEF's oil weathering model, b) a three-dimensional oil trajectory and chemical fate model, c) an oil spill combat model, and d) exposure models for fish and ichthyoplankton, birds, and marine mammals (Aamo et al., 1997). An oil and chemical database is integrated, which supplies chemical and toxicological parameters for the model. Biological data, including biodegradation, acute toxicity and bioaccumulation in pelagic organisms is incorporated to quantify environmental consequences of various oil spill scenarios. Both physical and biological measures of success and effectiveness are provided as outcome, hence the model is a useful tool for Net Environmental Benefit Analysis (NEBA), used for decision making during choice of combat strategies to reduce the overall impacts of surface oil spills (Reed et al., 1999a, 1999b; Brakstad and Faksness, 2000).

1.5.2 Implementation of biodegradation in fate and effect models

Biodegradation is rarely considered as an important weathering process in numerical fate models. A recent example, OILTRANS (Berry et al., 2012), does not take it into account, while a model developed by French-McCay (2009) considers degradation process as a sum of biodegradation, photooxidation, and other chemical reactions described by a first-order decay algorithm, with a specified (total) degradation rate. Though evaporation and dispersion are responsible for the disappearance of oil slicks, only biodegradation eliminates degradable fractions of oil from the water column. Transport processes result in gradually diluting the dispersed fraction, however, without biodegradation the oil would remain in the ecosystem. Importance of

microbial degradation (natural and enhanced) has been exemplified following the two worst spills of the US history (i.e. the Exxon Valdez and the BP Deepwater Horizon oil spills) showing that microbes were largely responsible for the mineralization of the spilled oil (Atlas and Hazen, 2011). Scientific understanding of the unique conditions of the spill (i.e. volume of oil spilled, the chemical nature of the oil, and the ecosystems with their specific environmental conditions), and both natural and enhanced biodegradation processes are necessary for efficient response planning.

Table 1.5-1 Pseudo first order rate coefficients (k_1) implemented in the OSCAR model to describe transformation rate of WAF, aerobic sediments and droplet oil (from Reed et al., 2000). WAF=water accommodated fraction, SO=sediment oil, DO=droplet oil.

#	Compound group	k_1 WAF (d^{-1})	k_1 SO (d^{-1})	k_1 DO (d^{-1})
1	C_1-C_4 gases	1.000	1.000	0.100
2	C_5-saturates	0.288	0.100	0.100
3	C_6-saturates	0.248	0.100	0.100
4	Benzene	0.266	0.100	0.100
5	C_7-saturates	0.267	0.100	0.100
6	C_1-benzene (toluene)	0.462	0.100	0.100
7	C_8-saturates	0.267	0.100	0.100
8	C_2-benzene (xylenes)	0.433	0.100	0.100
9	C_9-saturates	0.267	0.100	0.100
10	C_3-benzene	0.433	0.100	0.100
11	C_{10}-saturates	0.200	0.0073	0.102
12	C_4 and C_5-benzene	0.385	0.100	0.100
13	C_{11}-C_{12}	0.200	0.0077	0.0825
14	Phenols (C_0-C_4 alkylated)	0.200	0.0894	0.010
15	Naphthalenes (C_0-C_1 alkylated)	0.630	0.040	0.173
16	C_{13}-C_{14}	0.200	0.0067	0.00737
17	Unidentified C_{10}-C_{36}	0.117	0.001	0.010
18	Naphthalenes (C_2-C_3 alkylated)	0.495	0.036	0.0608
19	C_{15}-C_{16}	0.200	0.0039	0.0707
20	PAH 1 (3 rings non-alkylated)	0.462	0.0254	0.0066
21	C_{17}-C_{18}	0.200	0.0049	0.0707
22	C_{19}-C_{20}	0.200	0.004	0.0635
23	C_{21}-C_{25}	0.200	0.0041	0.0647
24	PAH 2 (3 rings-alkylated, 4-5+ rings)	0.408	0.0018	0.001
25	C_{25}+ (total)	0.200	0.0033	0.0376

Implementation of biodegradation in the OSCAR model

The OSCAR model takes biodegradation into account as a pseudo first order process, and calculates biodegradation rates based on compound group specific pseudo first order rate coefficients (k_1) of 25 compound groups, currently (Table 1.5-1).

Reference k_1 values of each compound group have been either measured in laboratory tests or derived from literature data (Reed et al., 2000). Experimentally determined reference k_1 values were obtained using seawater from temperate area (Trondheimsfjorden, Trondheim, Norway, from 70 m depth) at 13 °C incubation temperature (Brakstad and Faksness, 2000). Biodegradation rates are adjusted to local conditions by taking into account the temperature difference between the incubation temperature where reference k_1 values were determined and the ambient temperature of the area where the model is applied, according to:

$$k_1 = k_1^{ref} \cdot 10^{(0,03 \cdot (T_w - T_{ref})}$$
(1.5-1)

Where k_1 is the temperature compensated rate coefficient (day^{-1}), k_1^{ref} is the coefficient measured in laboratory tests (day^{-1}), T_w is the *in situ* water temperature (°C) and T_{ref} is the temperature where k_1^{ref} values were determined (°C) (Brakstad and Faksness, 2000). This temperature compensation approach is based on the Q_{10} concept, the constant 0.03 in Equation 1.5-1 represents the $Q_{10}=2$ rule of thumb.

1.6   Problem formulation

One fundamental problem of temperature compensation of HC biodegradation rates originates from the theoretical frame it is based on. According to van der Meer (2006), an appropriate mathematical model describing the effect of temperature on organism level is lacking. Furthermore, understanding and mathematical description of temperature dependence of bacterial growth rates in pure cultures are not agreed upon (Huang et al., 2011) and there is little data on metabolic rates of communities (Brauer et al., 2009). The theoretical frame for modeling temperature effect on biodegradation rates is based on the Arrhenius model (Equation 1.4-2). Even though

the Arrhenius equation was found to be suitable for the normal biological range of temperature (0–40 °C) (Gillooly et al., 2001), it can only describe one part of the whole temperature response curve. This implies that temperature response measured outside that range, cannot be described with a Q_{10} value. Hence, Q_{10} values should only be derived from experiments, where a wide range of temperatures have been measured and it was confirmed that Q_{10} was calculated in the temperature range where Arrhenius model is still applicable. Alternatively, new models could be applied. For example, Ratkowsky et al. (2005) and Huang (2011) suggested thermodynamic models that fitted experimental data for the whole range of observation and has been successfully used to evaluate temperature effects on soil respiration (Pietikåinen et al., 2006). As the currently implemented temperature compensation method is based on the Q_{10} approach, investigations carried out during this research project focused on that.

The other fundamental problem originates from the fact that the temperature compensation method implemented in the OSCAR model assumes that microbial communities have the same temperature response and the same biodegradation capacity at a given (reference) temperature (i.e. assume the existence of a universal Arrhenius plot of k_1 values). If a microbial community has a different temperature response (i.e. $Q_{10} \neq 2$) or exhibits different biodegradation rate at the reference temperature, the Q_{10} approach becomes futile, leading to erroneous rate predictions. These cases can be illustrated as shown in Figure 1.6-1, where fragments of theoretical Arrhenius plots of three abstract microbial communities are displayed. Temperature scale of the plot shows relative temperatures calculated from the *in situ* temperature of theoretical communities and the incubation temperatures used in biodegradation experiments (reference temperature in the OSCAR model). Using this relative temperature scale, Arrhenius curves are shifted in a way that it is easier to see whether biodegradation rate coefficients of two distinct communities are similar at their *in situ* temperature. Possible deviations from assumptions of currently implemented temperature compensation approach are illustrated with communities A, B, and C. Community A and B of Figure 1.6-1 responds to temperature change with a Q_{10} of 2, however, their k_1 values at *in situ* temperature are not the same. Community

B and C has different temperature response, however, at *in situ* temperature their k_1 values are the same.
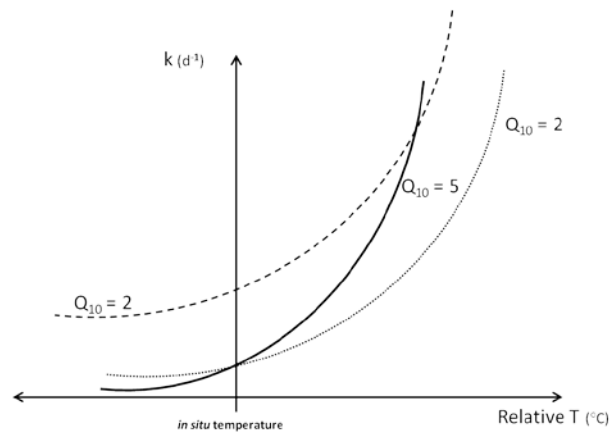
Figure 1.6-1 Theoretical Arrhenius plots of degradation rate coefficients of three microbial communities; A: dashed line, B: dotted line, and C: continuous line. T = temperature.

Considering a) the dynamic nature of diversity, abundance and *in situ* activity of marine bacterial communities (Kirchman et al., 2009), b) the versatility of HC degraders and their physiology (Prince et al., 2010), and c) the temperature adaptation strategies of bacteria inhabiting cold seawaters (Methé et al., 2005), accuracy of biodegradation rate estimations based on merely temperature compensation appears to be questionable. Regarding the OSCAR model, selection of the $Q_{10}=2$ has not been experimentally confirmed or validated, hence it is questionable how appropriate this choice has been and to what extent it can provide accurate biodegradation rate predictions. Answering these questions requires comparative testing of HC biodegradation rates in geographically and climatically different areas. Until today, there is a lack of data regarding crude oil biodegradation rates in Arctic marine environments. The little data available is difficult to compare with bidegradation rates measured with temperate seawater due to differences in experimental methodologies. Using a standardized (commonly accepted) approach for measuring biodegradation would greatly advance our understanding of this process.

Chapter 2. General methodology

This chapter first presents a brief introduction of the two major model compounds used in the experimental part of the research work then introduces the method and measurement system used for testing biodegradation in seawater and the techniques applied for microbial community composition analysis.

2.1 Model substrates: Naphthalene and Sodium-benzoate

Naphthalene, the smallest PAH, (Figure 2.1-1) was selected as a model compound for studying biodegradation of aromatic HCs in seawater. It is often used as a model compound due to its relatively high water solubility compared to PAHs with 3 or more benzene rings. Sodium-benzoate (Figure 2.1-1) was used as a positive control as suggested in the OECD 306 protocol (OECD, 1992).
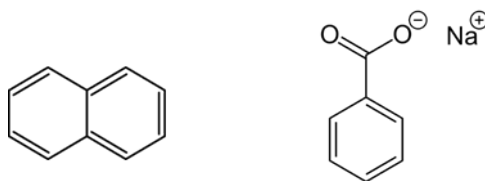
Figure 2.1-1 Chemical structure of naphthalene (left) and sodium-benzoate (right).

Naphthalene is found in abundance in crude oil, while sodium-benzoate can be found as a preservant in food and beverages. Naphthalene is produced by distillation and fractionation of petroleum. Its principal use is as intermediate in the production of phthalic anhydride, which is important in the manufacture of phthalate plasticizers, resins, dyes, and insect repellents (EPA, 1998). Worldwide production of sodium-benzoate was estimated to be around 100 kt by OECD SIDS (2001).

2.1.1 Physico-chemical properties of naphthalene and sodium-benzoate

Table 2.1-1 summarizes selected physico-chemical properties of the model and positive control substrate. Both compounds are solid at room temperature,

naphthalene appears as white crystals or crystal flakes and sodium-benzoate as white or colorless crystalline powder. Naphthalene has a characteristic aromatic odour detectable below 0.1 ppb, while sodium-benzoate is odourless.

Table 2.1-1 Physico-chemical properties of naphthalene and sodium-benzoate. mp=melting point, bp=boiling point, vp=vapor pressure, ws=water solubility, $logK_{ow}$=octanol-water partitioning coefficient (EPA, 1998, WHO, 2000; OECD SIDS, 2001; Jonsson, 2003).

Property	Naphthalene	Sodium-benzoate
molecular formula	$C_{10}H_8$	$NaC_6H_5CO_2$
molar mass (g mol^{-1})	128.17	144.11
mp (°C)	80.26	>300.0
bp (°C)	218.0	464.9
vp (mm Hg at 25 °C)	0.03	<0.00075
ws at 25 °C (mg L^{-1})	30	>500000
$logK_{ow}$	3.34	-2.269
density (g cm^3)	1.14	1.497

Naphthalene has a significantly lower aqueous solubility and higher octanol-water partition coefficient compared to sodium-benzoate, predicting lower bioavailability in aqueous environments. Water solubility of naphthalene highly depends on both salinity and temperature of water, hence in cold seawater (T=3.6 °C, 35 PSU) maximum 13 mg L^{-1} can be dissolved (compare with 30 mg L^{-1} at 25 °C in distilled water) (Gold and Rodriguez, 1988). Due to its high water solubility, sodium-benzoate is not expected to adsorb on surfaces of organic particles, while naphthalene can be, as indicated by its sorption coefficient (log K_{oc}) of 3.11 at 25 °C. Sodium-benzoate is non-bioaccumulative, while naphthalene has a bioaccumulation factor of 1.64 in mussel and 4.11 in bile of rainbow trout (OECD SIDS, 2001; Mackay et al., 2006). Regarding environmental toxicity, sodium-benzoate is considered as a low risk compound with acute toxic effects observed in fish, daphnia, algae, and bacteria above 100 mg L^{-1} concentration (OECD SIDS, 2001). No carcinogenic effects were found in Ames tests. Carcinogenic effects of naphthalene have not been confirmed (EPA, 1998). Environmental toxicity of naphthalene is significantly higher compared to sodium-benzoate based on 24 h LC$_{50}$ level of 3.798 mg L^{-1} for the estuarine copepod *Eurytemora affinis* (Ott et al., 1978). Naphthalene has a tendency for

evaporation at room temperature (low boiling point and higher vapour pressure) and crystals readily sublimate over time.

2.1.2 Aerob biodegradation of naphthalene and sodium-benzoate: oxygen demand, pathways, and bacteria involved

Biodegradation of organic material by aerob heterotroph bacteria requires a stoichiometrically determinable amount of oxygen, according to:

$$C_nH_m + \left(1 + (m-4) + (n-5)\right)O_2 + NH_3 = C_5H_7O_2N + \left(\frac{m-4}{2}\right)H_2O + (n-5)CO_2 \qquad (2.1\text{-}1)$$
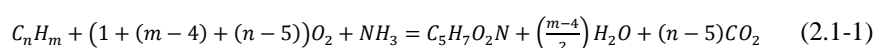
Without taking into account biomass ($C_5H_7O_2N$) formation, the amount of oxygen required for complete mineralization of organic material can be calculated as theoretical oxygen demand (ThOD). The ThOD of naphthalene is 3.0 mg oxygen per mg naphthalene, while for sodium-benzoate it is 1.67 mg oxygen per mg substrate. Oxygen requirement of biodegradation is usually lower than ThOD as a certain fraction of carbon atoms are not respired to CO_2, but assimilated into biomass. This fraction is expressed in the yield coefficient (Y, mg biomass/mg substrate), which varies among types of bacteria and types of substrate. ThOD can be considered as a maximum theoretical biological oxygen demand (ThBOD) for biodegradation of a compound. Hence, ThOD is a good indicator of the amount of oxygen required for biodegradation of organic materials. BOD is a key parameter measured in wastewater treatment systems for monitoring organic pollutants.

Catabolic pathways of naphthalene degradation have been proposed to follow common initial steps until the formation of salicylate (upper pathway reactions) (Figure 2.1-2). The majority of characterized naphthalene degraders convert salicylate into catechol, however, strains transforming salicylate into gentisate has also been found. Key enzymes, the naphthalene dioxygenase systems (NDO) are useful for oxidizing not only naphthalene but also other bi-cyclic and also tri-cyclic PAHs (Peng et al., 2008). Commonly reported genes coding for components of NDO systems are *nah* (Li et al., 2004), *nag* (Jeon et al., 2006) and *phn* (Laurie and Lloyd-Jones, 1999) genes.

Ability to utilize sodium-benzoate as carbon source necessitates the presence of significantly fewer specific enzymes compared to naphthalene. In only two steps, a benzoate dioxygenase and a benzoate *cis*-diol dehydrogenase converts sodium-benzoate into catechol, the same intermediate compound as that of one branch in the naphthalene metabolic pathway (Figure 2.1-3). From this molecule, catechol, degradation of both sodium-benzoate and naphthalene occurs through reactions of the well conserved β-ketoadipate pathway.

Great diversity of bacteria has been found to degrade naphthalene. Prince et al. (2010) listed over 175 genera as prokaryotic HC degraders, from which the following had naphthalene as typical substrate: *Clavibacter*, *Coccochloris*, *Nostoc*, *Tropibacter*, *Acidocella*, *Polaromonas*, *Rahnella*, *Neptunomonas*, and *Salinicola*. According to the UniProt database, there are several species belonging to genera *Achromobacter*, *Acinetobacter*, *Alteromonas*, *Azoarcus*, *Bacillus Burkholderia*, *Comamonas*, *Cycloclasticus*, *Marinobacter*, *Maritimibacter*, *Neptunomonas*, *Pseudoalteromonas*, *Pseudomonas*, *Raltsonia*, *Rhodococcus*, etc. (UniProt, 2013a), which carry genes coding for naphthalene dioxygenase enzymes.

From this diversity of bacteria capable of degrading naphthalene, it is most commonly *Cycloclasticus*, *Pseudomonas* and *Pseudoalteromonas* species identified as major players in naphthalene or PAH degradation in marine environments (Teramoto et al., 2010).

As sodium-benzoate has been suggested as a reference compound by OECD guidelines for testing biodegradability, presumably an even wider range of bacteria is able to utilize it as sole carbon source. According to the UniProt database, benzoate dioxygenase enzyme has been found in species in several genera, e.g. *Acinetobacter*, *Alcanivorax*, *Alteromonas*, *Athrobacter*, *Azotobacter*, *Burkholderia*, *Cycloclasticus*, *Glaciecola*, *Halomonas*, *Marinobacter*, *Oceanimonas*, *Polaromonas*, *Pseudomonas*, *Pseudoxanthomonas*, *Ralstonia*, *Rhodococcus*, *Roseobacter*, *Stenotrophomonas*, *Vibrio*, *Xanthomonas* etc. (UniProt, 2013b). Several species are able to degrade both naphthalene and sodium-benzoate, hence sodium-benzoate is an appropriate positive control for naphthalene degradation testing.
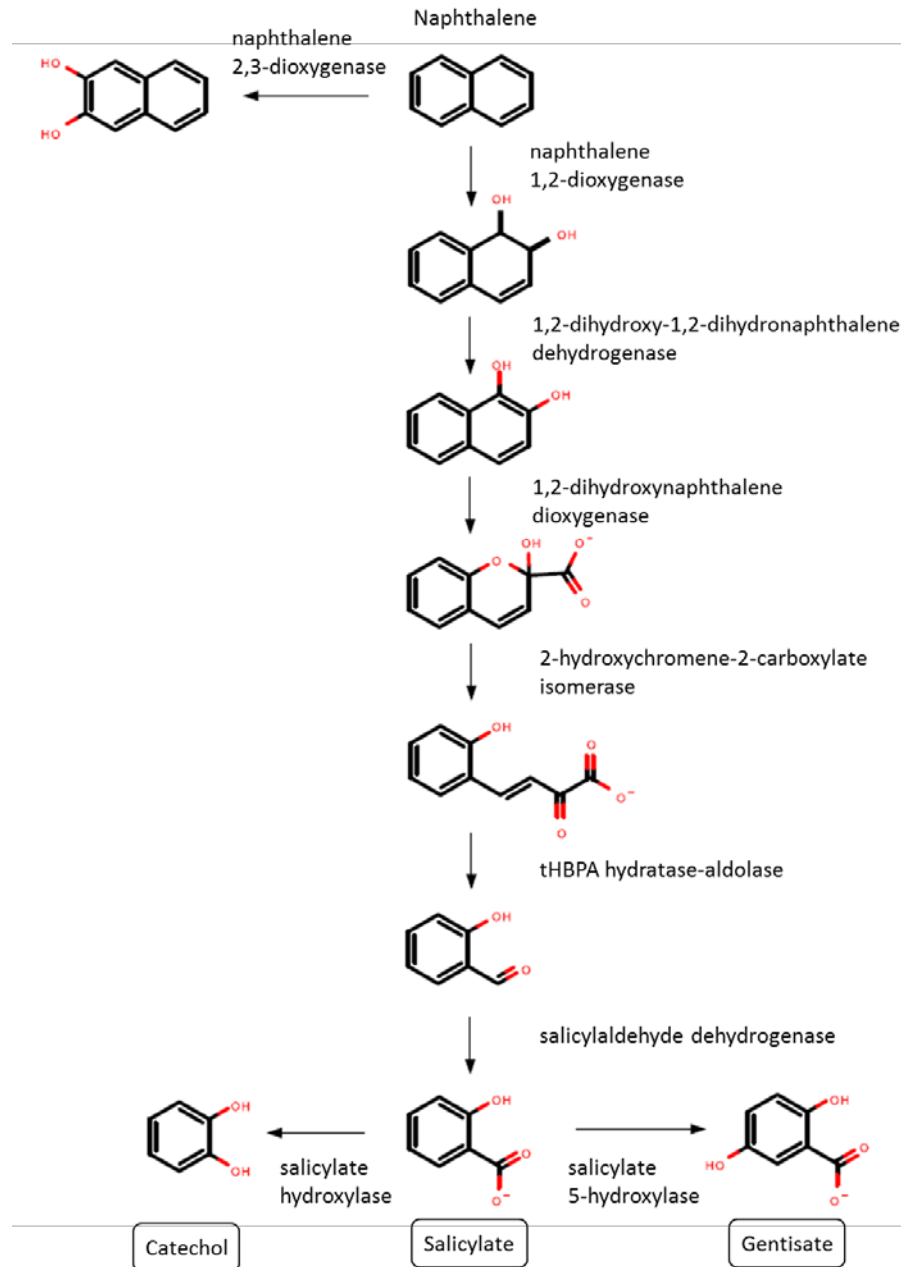
Figure 2.1-2 Naphthalene degradation pathway map based on:
http://umbbd.ethz.ch/naph/naph_map.html (Zeng and Essenberg, 2011).
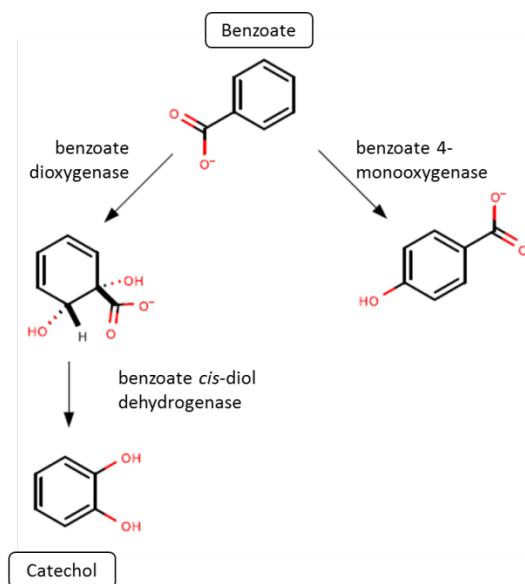
Figure 2.1-3 Benzoate degradation pathways map based on:
http://umbbd.ethz.ch/benz2/benz2_image_map.html (Feng and Ellis, 2012).

2.2 Measuring hydrocarbon biodegradation by respirometry

Crude oil biodegradation in seawater is most often measured by gas-chromatography
(GC) analysis of remaining components and bacterial counts (total and HC degrading)
(Mulkins-Phillips and Stewart, 1974; Garrett et al., 2003; Deppe et al., 2005; Brakstad
and Bonaunet, 2006; Venosa and Holder, 2007; Coulon et al., 2007; Delille et al.,
2009). Besides GC measurements, different respirometry methods have also been
used to derive quantitative information about HC degradation process (Atlas and
Bartha, 1972; Gibbs et al., 1975; Gibbs and Davis, 1976; Minas and Gunkel, 1995).
Respirometry refers to set of different techniques applied to determine the amount of
oxygen consumed or carbon-dioxide produced during biodegradation (Equation 2.1-
1). The first technique has been developed in 1924 by Otto Heinrich Warburg. His
method was based on measuring changes of pressure in a closed system. Following
Warburg's invention, several different kinds of respirometry techniques have been
developed based on manometeric, electrolytic principles or on direct measurement of
oxygen consumption (Roppola, 2009). Respirometry was suggested to be the most

suitable technique to use when biodegradation of poorly water soluble compounds (e.g. HCs) is to be measured (Hoffmann et al., 1997). Application of respirometry techniques is also recommended by standardized biodegradability testing guidelines (OECD, 1992; Reuschenbach et al., 2003). One of the latest instrumental developments in respirometry has been the OxiTop®-C system. It has been found to be suitable to assess biodegradability of different types of forestry oils in groundwater and in soil, and suggested to be a useful tool for testing biodegradation of volatile compounds (Kuokkanen et al., 2004; Kurola and Salkinoja-Salonen, 2007; Karhu et al., 2009).

2.2.1 OxiTop®-C measurement system

The OxiTop®-C is a static respirometry system that allows continuous and automated recording of oxygen consumption in a closed bottle based on manometric principles. Test flasks containing test media amended with a selected carbon source are capped with OxiTop®-C heads (Figure 2.2-1). These heads detect and record pressure change, which is related to the consumption of oxygen in the closed system. Ensured by continuous mixing, oxygen concentration in the liquid and gas phase is in equilibrium, hence depletion of oxygen from the liquid phase due to respiration can be followed by the decrease in partial pressure of oxygen in the headspace as described by Henry's law:

$$p_{O_2} = H_{O_2} \cdot c_{O_2} \qquad (2.2\text{-}1)$$

Where p_{O2} is the partial pressure of oxygen in the headspace (atm), H_{O2} is the Henry's constant for oxygen (L atm mol^{-1}) and C_{O2} is the concentration of oxygen in the liquid phase (L mol^{-1}). Produced CO_2 is captured from the headspace with sodium-hydroxide and therefore the change in measured pressure can directly be related to the change in oxygen partial pressure. The OxiTop®-C head calculates changes in partial pressure into biological oxygen demand (BOD) values, according to:

$$BOD = \frac{M(O_2)}{R \cdot T_m} \cdot \left(\frac{V_{tot} - V_l}{V_l} + \frac{\alpha \cdot T_m}{T_0} \right) \cdot \Delta p(O_2) \qquad (2.2\text{-}2)$$

Where $M(O_2)$ is the molecular weight of oxygen (32 g mol^{-1}), R is the universal gas constant (83.144 L·hPa mol^{-1} K^{-1}), T_m is the measuring temperature (K), T_0 is the reference temperature (273.15 K), V_{tot} is the bottle volume (L), V_1 is the filling volume (L), α is the Bunsen absorption coefficient (0.03103) and $\Delta p(O_2)$ is the difference in the partial pressure of oxygen (hPa). BOD data can be downloaded and viewed at any time without perturbation of the experiment.

An important step of the experimental design when using the OxiTop®-C, is the determination of an approximate theoretical oxygen consumption (ThOD) of the carbon source as described previously (section 2.1.2). As the OxiTop®-C system does not allow for the replenishment of oxygen, in contrast to dynamic respirometers (e.g. Sapromat, MicroOxymax, etc.), it is important to ensure that the volume of headspace in the test flask is sufficient, i.e. contains the amount of oxygen corresponding to less than the theoretical oxygen demand of the substrate.

Figure 2.2-1 Test flasks capped with OxiTop®-C heads on magnetic stir plate inside the temperature controlled cabinet.

Oxygen consumption data obtained with the OxiTop®-C system is ideal for developing and validating kinetic models describing biodegradation due to the frequency of measurements (one measurement every 30 minutes at maximum resolution). Moreover, assessment of biodegradation in a large number of test flasks is significantly less laborious compared to GC-approaches, increasing experimental

capacity. Therefore this method is highly suitable for comparative experiments where several replicates of each test conditions are required.

Besides the several advantages (e.g. easy set-up and operation, automated measurement and possibility to view data real time), the limitations of the OxiTop®-C system needs to be considered. A concern may be the interference of nitrogenous oxygen demand (i.e. oxygen consumption due to nitrification) with the oxygen demand of the carbon source. Oxygen is used for the oxidization of reduced forms of nitrogen (e.g. ammonia), unless prevented by an inhibitor (Reuschenbach et al., 2003). This issue has caused misinterpretation of BOD results in activated sludge tests where high numbers of nitrifiers and ammonia are present (Buswell et al., 1954). It is less likely that nitrification occurs when biodegradation is measured in seawater, as it generally does not contain high concentrations of ammonium or nitrifier microorganisms (Falkowski et al., 1998; Kirchman, 2010). Nitrifiers are autotrophic microorganisms with low growth rates (0.3–0.8 day^{-1}), duplicating approximately 10 times slower than aerob heterotrophs, hence they are expected to be easily outcompeted, especially at temperatures lower than 15 °C (Henze et al., 2006; LeBlanc, 1972). Ambient rates of ammonia and nitrite oxidation was measured as 20–747 nM d^{-1} by Capone et al. (2008), 21.6 nmol L^{-1} d^{-1} in the Arabian Sea open ocean environment (Newell et al., 2011), and 0.319 and 0.478 µmol L^{-1} d^{-1} in a British Fjord (Grundle and Juniper, 2011). Hence, oxygen consumption related to nitrification can be considered negligible when seawater inocula are tested in case excess ammonium source is not added to the test medium.

Application of biodegradation rates measured with the OxiTop®-C system under field conditions is problematic mainly due to nutrient addition. Addition of nutrients biases measured degradation rates as discussed earlier (section 1.3.1). Nevertheless, this method is a very useful tool to study the effect of different environmental and experimental factors on biodegradation kinetics by comparative tests.

2.3 Microbial community composition analysis

Discovering the diversity, abundance and identity of bacteria involved in metabolic processes in nature is a key aspect of microbial ecology. Traditionally, culture based enrichment and isolation techniques have been widely and routinely used to assess the diversity of environmental samples, and to identify the major players responding to perturbations. However, it was found that culture dependent methods often fail to answer these central questions, mainly due to the disparity between cultivable organisms and the organisms actually present in the environment (Amann et al., 1995; Van Hamme et al., 2003). Laboratory enrichments select for a narrow part of the whole diversity (Ward et al., 1990), therefore cultured microorganisms represent only a small fraction of natural microbial communities (von Wintzingerode, et al., 1997). Nevertheless, culture-dependent methods are essential tools to determine physiology and metabolic activity of microorganisms (Vartoukian et al., 2010).

The discovery that similarities of 16S rRNA gene sequences can be used to classify microorganisms and the invention of the polymerase chain reaction (PCR) by Kary B. Mullis in 1983 allowed the rapid advance of culture independent molecular microbial methods (Woese et al., 1984; Pace et al., 1986; Bartlett and Stirling, 2003). Extraction of whole community DNA, PCR amplification of target sequences followed by cloning and sequencing became a widely used method to identify microorganisms *in situ* (Britschgi and Giovannoni, 1991; Schmidt et al., 1991). Several other methods, e.g. denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and ribosomal intergenic spacer analysis (RISA) have also been established (Muyzer et al., 1993; Muyzer, 1999). All these genetic fingerprinting techniques allowed fast screening of temporal changes of community composition and comparison of microbial communities in distinct samples (Head et al., 1998).

2.3.1 Denaturing Gradient Gel Electrophoresis

DGGE was originally developed to detect point mutations, including single nucleotide polymorphisms (SNPs) and was then applied in environmental microbiology in the 90's (Muyzer et al., 1993; Muyzer, 1999). DGGE is capable of separating sequences

differing only in one nucleotide, making it ideal for separation of complex mixtures of 16S rRNA fragments (Macnaughton et al., 1999; Ogino et al., 2001; Brakstad et al., 2004; Brakstad and Bonaunet, 2006; Castle et al., 2006; Coulon et al., 2007).

Sample preparation prior to DGGE analysis requires extraction of the whole community DNA followed by PCR amplification of target sequences (Figure 2.3-1). The most commonly used templates for community composition assessment are variable regions of the 16S rRNA gene. Amplicons are loaded onto a polyacrylamide gel that contains a gradient of denaturing agents (urea and formamide). DGGE technique takes advantage of the fact that the denaturing point of a DNA molecule depends on its sequence and that migration of denatured DNA inside a gel is almost completely retarded. Hence, amplicons with different nucleic acid sequence migrate to distinct distances on the gel. Each sequence is assumed to represent a member of the bacterial community, hence the bands visualized after gel staining denote the community members. The number of bands is assumed to accurately reflect the diversity of microbes in the sample while the relative intensity of bands is thought to reflect the relative abundance of particular organisms represented by the band. The DGGE apparatus used during this research work is shown in Figure 2.3-2.
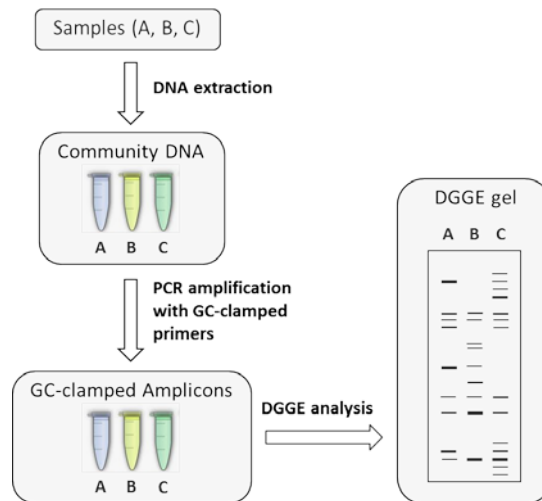
Figure 2.3-1 Work-flow of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) analysis.

DGGE has the advantage of easy interpretation of results based on band composition, which can be used for similarity analysis (Zhang and Fang, 2000). Similarity matrix of a set of samples is calculated from relative intensities of unique bands, and hierarchical clustering is applied to visualize the relative similarity of each sample. As mentioned before, this technique became increasingly popular among environmental microbiologists and microbial ecologists due to the promise of high sensitivity compared to the relatively easy methodology and data analysis despite the serious limitations and challenges that have been discovered later on.

The underlying hypothesis of DGGE analysis is that DNA from all community members is extracted with equal efficiency and PCR amplification also occurs non-specifically. Hence, the original diversity and relative abundance of different sequences are not supposed to alter during PCR. Methodological difficulties of DGGE include: i) difficulty of achieving reproducible and efficient extraction of DNA from environmental samples containing complex and diverse microbial communities, ii) issues related to PCR biases, iii) the co-migration of DNA from different species in the same band, and iv) the formation of multiple bands from single species (de Araujo and Schneider, 2008). PCR primer choice influences diversity estimates, while bacterial community profile reflected by the DGGE band pattern is highly influenced by different DNA extraction methods (Luo et al., 2007). Moreover, DGGE apparatus also influences the observed band composition profile. Thus the use of one and the same DGGE apparatus throughout comparative experiments is recommended, especially if monitoring of microbial community structures requires multiple gel-to-gel analysis (Ascher et al., 2010).

Quantitative analysis based on band intensities can be biased as it is not always only DNA from one single bacterial strain in one single band (Sekiguchi et al., 2001). Moreover, heterogeneity in the copy number of SSU rRNA in different bacteria also biases intensity based comparison. Identification of community members by excision and sequencing of unique bands can be difficult if the band contains a mixture of sequences. On the other hand, multiple bands for a single organism could greatly confound interpretation of DGGE profiles (de Araujo and Schneider, 2008).
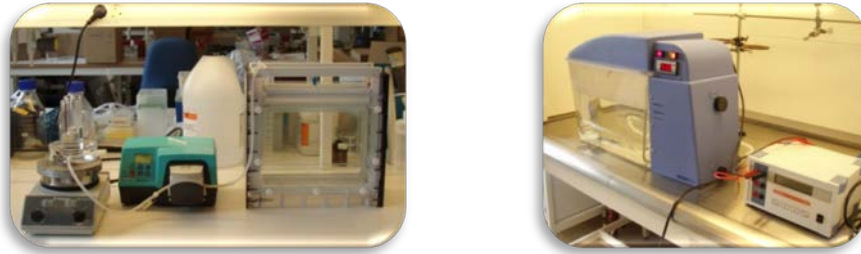
Figure 2.3-2 Equipment used for gel casting (left) and the Ingeny4U denaturing gradient gel electrophoresis (DGGE) system (right).

DGGE therefore represents a semi-quantitative method, which is best applied as a comparative and screening tool for monitoring changes during the course of biodegradation. It is preferably to be applied in combination with other molecular and culture-dependent techniques.

2.3.2 Pyrosequencing

With the development of molecular microbial ecology tools, sequencing became routinely used to identify microorganisms from environmental samples. However, traditional Sanger sequencing required isolation of individual sequences from the mixtures of whole community DNA, for example by cloning (one clone-one sequence), which is time consuming and labour intensive (Sanger et al., 1977). Though cloning and sequencing provided improved coverage of diversity compared to culture-dependent methods, the number of clones sequenced from an environmental sample could not possibly account for the true diversity (Venter et al., 2004). Pyrosequencing technology bypasses the necessity for cloning, as a mixture of target sequences from whole community DNA can be amplified in one PCR reaction and directly subjected to analysis, resulting in reduced labour and time requirements for sample preparation (Ronaghi, 2001; Ahmadian et al., 2006). 454 pyrosequencing allows the generation of short reads relatively rapidly and inexpensively, with accuracy, and permits cost-effective exploration of microbial communities from various environments (Sogin et al., 2006; Huse et al., 2008; Galand et al., 2009; Vaz-

Moreira et al., 2011). Pyrosequencing allows parallel sample analysis using barcoded primers, and yields high number of sequences, (>200 000 reads per run) currently of approximately 800 bp (February, 2013). As a consequence, sequencing of standard taxonomic markers (e.g hypervariable regions of the small subunit of 16S ribosomal RNA (16S rRNA) has become a fast and efficient method for studying environmental microbial communities.

Several studies have used pyrosequencing to track changes of diversity and community composition over time (Brown et al., 2009; Lanzén et al., 2011; Nacke et al., 2011; dos Santos et al., 2011). In comparison to DGGE, 454 pyrosequencing method was shown to have a higher capacity to explore the bacterial richness and to detect cultured organisms, being also less laborious (Vaz-Moreira et al., 2011).

Major challenges regarding pyrosequencing analysis of microbial communities come from the data analysis side. Analysing and interpreting high-throughput sequencing data requires sophisticated mathematical tools. These include algorithms that detect and remove PCR biases, chimeras and sequencing error, and through that prevent inflated estimation of microbial diversity (Quince et al., 2008). Furthermore, a huge amount of 16S rDNA sequence data has been collected and is continuously organized in databases such as SILVA (Pruesse et al., 2007), Ribosomal Database Project (RDP; Cole et al., 2009) and Greengenes (DeSantis et al., 2006). Data processing and storage are becoming critical issues as sequencing technology is nowadays improving faster compared to computer power (Logares et al., 2012).

Two major aims of community analysis are the quantification of diversity and relative abundance of taxa, and phylogenetic classification of community members. Measuring microbial diversity is not straightforward since delineating species in microbes has proven to be a very challenging task (e.g. Doolittle and Zhaxybayeva, 2009). Furthermore, distinguishing truly novel diversity from 'noise' has also been problematic (Gihring et al., 2012). The pragmatic solution to the species concept problem has been to group sequences into operational taxonomic units (OTU) using a 97% rDNA sequence similarity threshold (Logares et al., 2012).

A reliable and flexible system for taxonomic classification, i.e. Classification Resources for Environmental Sequence Tags (CREST), was recently developed aiding the utilization of large 16S rRNA databases. CREST provides a set of tools for generating and utilizing custom taxonomies and reference datasets for classification of environmental sequences (Lanzén et al., 2012). However, due to the short-read lengths currently available on pyrosequencing platforms detailed phylogenetic characterization has not been possible below genus level. Generally, the percentage of classified sequences drops significantly below the order level.

2.3.3 Methodological limitations common to molecular methods

A common drawback of molecular ecology methods originate from the efficiency of the DNA extraction and the occurrence and frequency of PCR biases, e.g. inhibition, differential amplification of target sequences, and formation of PCR artefacts (von Wintzingerode et al., 1997). PCR amplification of 16S rDNA cannot be used directly to infer microbial abundance due to the bias in PCR amplification of different templates (Chandler et al., 1997). Bacterial diversity estimates are further challenged by the lack of a robust species concept. Even when several methods are applied in combination, determining true diversity remains challenging.

Objectives

Based on the problems presented in Chapter 1 (section 1.6 on page 33-36) regarding temperature compensation of HC biodegradation rates using the Q_{10} approach, and considerations presented in Chapter 2 regarding measurement methodology and microbial community analysis, the following objectives were formulated.

Aims of the research work were:

   I.   to assess whether temperature response of crude oil biodegradation follows the commonly used $Q_{10}=2$ rule of thumb

  II.   to assess the limitations of predicting biodegradation rates based on temperature compensation

 III.   to adapt a static respirometry method for studying hydrocarbon biodegradation in seawater

 IV.   to evaluate the sensitivity of the static respirometry method toward different sampling approaches (filtered vs. non-filtered seawater), sample storage (aging of seawater), and the initial number of bacteria

  V.   to examine the effect of the geographical origin of seawater on inherent biodegradation rate and temperature response

 VI.   to investigate bacterial community composition of different seawater types associated with hydrocarbon degradation using PCR-DGGE and pyrosequencing

The following three chapters describe the research work carried out in order to address these goals. Chapter 3 presents the preliminary study carried out addressing aims I. and II, Chapter 4 addresses aims III. and IV., and in Chapter 5, experimental work directed to address objective V. and VI. is presented.

Chapter 3. Preliminary survey

Title of the manuscript

Estimation of hydrocarbon biodegradation rates in marine environments: A critical review of the Q_{10} approach

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3.1 Summary

Oil & gas industry is moving exploration and production activities into frontier regions like the Arctic and deep water environments. Governmental regulations require that environmental impacts are assessed before operations are started to evaluate the possible effects of accidental oil releases. Environmental impact assessments of oil discharges are often performed by numerical fate and exposure models, like the Oil Spill Contingency and Response (OSCAR) model, which has become an industry standard in Norway. Herein the effect of temperature on hydrocarbon biodegradation in marine environments is reviewed, and the temperature compensation approach of this process implemented in the OSCAR model is investigated, based on published data and novel obtained results. The OSCAR model uses a Q_{10} approach for temperature compensation of biodegradation. Overall, biodegradation rate predictions calculated by temperature compensation are found to be ambiguous, and choosing one universal Q_{10} value is not feasible. The high variation in Q_{10} values is in fact attributed to indirect effects of temperature (e.g. influence on bioavailability, active bacteria number). Implementing mechanistic models in risk assessment tools to describe biodegradation kinetics may represent a better alternative compared to the currently applied temperature compensation approach.

3.2 Introduction

Offshore petroleum resources in temperate and shallow water regions are being depleted, and the oil and gas industry is moving exploration and production activities into frontier regions, like the Arctic and the deep sea environments. Before opening up for oil production international and national regulations require industrial developers to conduct assessments of environmental impacts to evaluate the possible effects of accidental and operational oil releases (Thérivel and Minas, 2002). A key methodological approach is simulation of discharge scenarios using numerical fate models (Reed et al., 1995; Sebastião and Soares, 1995; French-McCay, 2011; Zhong and You, 2011).

In the North Atlantic regions, these models have earned a central role in environmental risk management and contingency planning (Daling and Strøm, 1999; Ly et al., 2004). They are used for evaluation of fate and estimation of the effect of crude oil in marine environments by simulating physico-chemical and biological weathering processes (Reed et al., 1999a). Models have been designed to be generic, i.e. applicable in different regions, while reflecting local environmental conditions. Marine meteorology data (e.g. wind, wave, currents etc.) can be obtained from online databases and used as input for these fate models, ensuring that physico-chemical processes (e.g. evaporation, dispersion, dissolution etc.) are adapted to local conditions (Guo and Wang, 2009; Hackett et al., 2009; Liu et al., 2011; Berry et al., 2012). However, information (databases or on-site measurements) about biodegradation in different regions is not available.

The Oil Spill Contingency and Response (OSCAR) model is a three-dimensional dynamic tool, and is currently the most widely used model in the North Atlantic region. This model includes a number of parameters like description of site-specific physical environments and physical-chemical fate processes. The OSCAR model also includes a description of biodegradation of 25 pseudo-oil component groups. Biodegradation rates for water-soluble fractions, dispersed oil in the water column, and for sedimented oil are used to calculate overall biodegradation (Reed et al., 2000). Due to the lack of locally relevant data, biodegradation rates are adjusted to local conditions by temperature compensation (Mark Reed, SINTEF, personal communication). Accurate estimation of biodegradation rates is important since this is the only process that completely eliminates oil compounds from the environment (Zobell et al., 1943; Atlas, 1981; Head et al., 2006; Brakstad et al., 2009). In fact, oil fate estimations of the OSCAR model were found to be very sensitive to changes in biodegradation rates (Brakstad et al., 2003).

Herein, the effect of temperature on HC biodegradation in the marine environment is reviewed, and the temperature compensation approach of this process implemented in the OSCAR model is investigated. The existing approach is evaluated based on discussion of published data and novel results obtained in a laboratory experiment.

3.2.1 Temperature effect on hydrocarbon biodegradation

Temperature is a major factor influencing all aspects of life, also the metabolic activity of microorganisms (Price and Sowers, 2004). The influence of temperature on biodegradation of HCs has been reported over the last 70 years (Zobell, et al., 1943; Zobell, 1946, 1963; Atlas and Bartha, 1972; Atlas, 1981; Brakstad, 2008). It has been extensively studied through temperature controlled experiments where seawater samples are exposed to HCs at several incubation temperatures, biodegradation rates are determined and the effect of temperature is expressed as Q_{10} values (Mulkins-Phillips and Stewart, 1974; Gibbs et al., 1975; Gibbs and Davis, 1976; Minas and Gunkel, 1995; Garrett et al., 2003; Coulon et al., 2007; Venosa and Holder, 2007; Delille et al., 2009). By definition, these values are the multipliers by which rates of enzymatic reactions increase at a 10 °C temperature rise. Crude oil biodegradation is most often interpreted as a pseudo first order process according to:

$$\frac{dS}{dt} = -k_1 \cdot S \qquad (3.2\text{-}1)$$

Where S is the substrate concentration (mg L^{-1}), t is time (day), and k_1 is the pseudo first order rate coefficient (day^{-1}). The k_1 values determined from biodegradation measurements are then used for Q_{10} calculations as:

$$Q_{10} = \frac{k_1^{T+10}}{k_1^T} \qquad (3.2\text{-}2)$$

Where k_1^T is the pseudo first order degradation rate coefficient at temperature T (°C), and k_1^{T+10} is the rate coefficient at 10 degrees higher temperature. The Q_{10} approach implies using Equation 3.2-2 to calculate k_1 value at a certain temperature based on k_1 measured at a different temperature. Following the work of Gibbs et al. (1975), a Q_{10} of 2.7 became a generally accepted value for temperature compensation of biodegradation rates (Stewart et al., 1993). However, a Q_{10} of 2.0 has later been used for temperature compensation of HC biodegradation rates measured in groundwater (Van Stempvoort and Biggar, 2008). It appears that a Q_{10} range of 2-3 is nowadays

the most widely accepted as a rule of thumb for calculating biodegradation rates from one temperature to another (NRC, 2003; Brakstad et al., 2009).

3.2.2 Temperature compensation of hydrocarbon biodegradation

The OSCAR model simulates biodegradation for each of 25 oil pseudo-component groups, separated on the bases of boiling point characteristics, by using a database of first-order biodegradation rate coefficients (Reed et al., 2000). It is well known that HC biodegradation depends on several environmental factors other than temperature, that vary considerably among marine environments, e.g. nutrient concentration and type of oil degraders (Leahy and Colwell, 1990). The effect of these factors has not been mathematically formulated and implemented in numerical models. However, the relationship between temperature and biodegradation rate has been established (Equation 3.2-2). Location specific biodegradation rate coefficients are calculated by the OSCAR model from the database of reference k_1 values based on the temperature difference between the incubation temperature where reference k_1 was measured and the ambient temperature of the environment where the OSCAR model is applied, according to:

$$k_1 = k_1^{ref} \cdot 10^{(0,03 \cdot (T_w - T_{ref})}$$
(3.2-3)

Where k_1 is the temperature compensated rate coefficient, k_1^{ref} is the coefficient measured in laboratory tests, T_w is the ambient water temperature and T_{ref} is the incubation temperature where the biodegradation rates were originally determined (Brakstad and Faksness, 2000). This temperature compensation approach is derived from the Q_{10} concept, and the constant 0.03 in Equation 3.2-3 represents the $Q_{10}=2$ rule of thumb. The OSCAR model is applied in several different areas using the same set of k_1^{ref} values and the same Q_{10}. Using the same reference rate coefficients implies that biodegradation capacity of all marine HC degrading microbial communities is assumed to be the same, while using the same Q_{10} implies that temperature responses are also assumed to be uniform. However, prokaryotic communities of the oceans are diverse, dynamically changing and adapted to local conditions (Hewson et al., 2006; Martiny et al., 2006; Fuhrman et al., 2008;

Kirchman, 2010; Baltar et al., 2010; Ruiz-González et al., 2012), hence expecting uniform biodegradation capacity and temperature response is questionable. Degradation efficiency of two different Antarctic HC degrading isolates has been found to differ at the tested two incubation temperatures during the first two weeks of the experiment (Michaud et al., 2004). Degradation profile and capacity of cosmopolitan HC degrader, *Cycloclasticus* was shown to vary with geographical origin (Geiselbrecht et al., 1998). A key bottle-neck in predicting HC degradation rates in marine environments is the lack of quantitative information on inherent biodegradation capacity (rate) of geographically and climatically distinct marine microbial communities. Additionally, warm and cold adapted bacteria have been shown to exhibit distinct temperature sensitivity, yet displaying similar metabolic activity at ambient temperatures of their environment (Arnosti et al., 1998; Robador et al., 2009). Hence, both assumptions underlying the Q_{10} approach are debatable. This review is focusing on investigating the latter, i.e. the uniform temperature response.

3.3   Literature survey

3.3.1   Literature survey: Materials and Methods

A dataset of Q_{10} values was constructed from literature by collecting published oil biodegradation rates and calculating Q_{10} values. Data were selected from biodegradation studies carried out at various incubation temperatures with mixed culture inocula or natural microbial communities from seawater, and sufficient quantitative data was reported for the calculation of degradation rates. The focus of this literature assessment was to gather information about temperature response of naturally occurring microbial communities in seawater when exposed to crude oil (in one case diesel oil). Therefore, temperature manipulation experiments with pure cultures were excluded. Also, experiments performed in other environmental media, such as soil, were not considered. Reported oil concentrations were converted to a consistent unit (gC m^{-3}) using the following conversion factors: 0.85 gC goil^{-1} and 0.7 gC gBOD^{-1} (biological oxygen demand) (Stewart et al., 1993). Independently of the type of crude oil, a density of 0.8 g mL^{-1} was used, unless specific values were reported (ITOPF, 2002). All biodegradation rates were calculated assuming pseudo

first order kinetics (Stewart et al., 1993). From data reported by Brakstad and Bonaunet (2006), the degradation rate of hexadecane instead of crude oil was calculated due to lack of data. The Q_{10} values were determined either as the ratio of degradation rates measured at incubation temperatures at ten degrees difference, or from Arrhenius plots (Brauer et al., 2009; Robador et al., 2009). In the latter case, the logarithm of biodegradation rates were plotted against 1000/temperature (K) and E_a was determined from the slope of the Arrhenius curves. E_a was then converted into Q_{10} according to:

$$Q_{10} = e^{\left[\left(\frac{E_a}{R \cdot T \cdot (T+10)}\right)\right] \cdot 10}$$

(3.3-1)

3.3.2 Literature survey: Dataset

A total of 32 Q_{10} values were calculated (Table 3.3-1). The values ranged from 1.1 to 16.2 with an average of 2.8. The frequency distribution of Q_{10} ranges across the reviewed studies showed that the highest occurrence of obtained Q_{10} values was around 2.5 (Figure 3.3-1). Q_{10} values lower than 2.0 were determined in 47% of the cases while Q_{10} values higher than 2.0 were determined in 53% of the cases. The reviewed studies varied considerably in several parameters, e.g. location, pollution history of the sampling sites, type of inoculum, type of oil, season, and measurement method used. For this reason statistical analysis was not performed. However, the following hypotheses were formulated: a) crude oil type does modify observed temperature response as shown by Atlas (1975), b) incubation temperature range can be an influencing parameter as Q_{10} values appeared to be higher at low temperatures (Atlas and Bartha, 1972; Gibbs and Davis, 1976; Deppe et al., 2005). Moreover, the effect of season on temperature response was either not observed (Minas and Gunkel, 1995) or inconclusive (Atlas and Bartha, 1972). Measurement method also influenced the Q_{10}, as for example gravimetric and respirometric analysis of the same biodegradation experiment resulted in different values (Atlas and Bartha, 1972).

In general, literature concerning temperature response of crude oil biodegradation in seawater is scarce. A new experimental approach using static respirometry was therefore tested to establish a procedure that could be standardized and applied to

Table 3.3-1 Details of the surveyed studies: type of oil used, concentration of the oil (C_0), inoculum, season, measurement method, temperature where Q_{10} was calculated ($T(Q_{10})$), Q_{10} value and reference. Letter code for methods: G-gravimetry, R-respirometry, GC-gas chromatography, B-bacterial number and for season: W-winter, Su-summer, A-autumn, Sp-spring.

Oil type	C_0 (gC/m³)	Inoculum	Season	Method	$T(Q_{10})$ (°C)	Q_{10}	Reference
Bunker C crude	850	enrichment culture #1	Winter	G	5	2.8	Mulkins-Phillips and Stewart, 1974
		enrichment culture #2			5	3.4	
		enrichment culture #3			5	2.7	
Sweden crude	6800	fresh seawater	Autumn	R	5	>2.7	Atlas and Bartha, 1972
				R	10	3.8	
				G	5	5.7	
				G	10	1.5	
			Winter	R	5	3.3	
				R	10	3.2	
				G	5	2.3	
				G	10	1.2	
Kuwait crude	340	fresh seawater	N/A	R	4	2.7	Gibbs, 1974
Solvent mix	8500	fresh seawater	N/A	G	10	1.9	Atlas, 1975
Sweden crude						4.5	
South Louisiana						1.5	
Lagomedio						1.7	
Prudhoe Bay						1.3	
Mirando Humble						1.6	
Kuwait residues	4590	beach gravel and fresh seawater	N/A	R	6	3.3	Gibbs and Davis, 1976
					11	2.1	
Bunker C crude	17	fresh seawater	Summer	R	4	2.4	Minas and Gunkel, 1995
			Winter		4	2.4	
weathered Alaska North slope	8500	enrichment culture	N/A	GC	6	1.3	Garrett et al., 2003
				G	6	1.5	
crude oil from North Sea field	680	enrichment culture	N/A	B	5	4.4	Deppe et al., 2005
					15	1.2	
Statfjord crude	0.786	acclimated seawater	Winter	GC	0	16.2	Brakstad and Bonaunet, 2006
Prudhoe Bay crude	19	enrichment culture	N/A	GC alkanes	5	1.8	Venosa et al., 2007
				GC PAHs	5	1.9	
weathered Forties crude	765	fresh estuary water	Winter	GC	4	1.9	Coulon et al., 2007
crude oil	680	fresh seawater	Spring	GC	4	1.1	Delille et al., 2009
diesel oil					4	1.3	
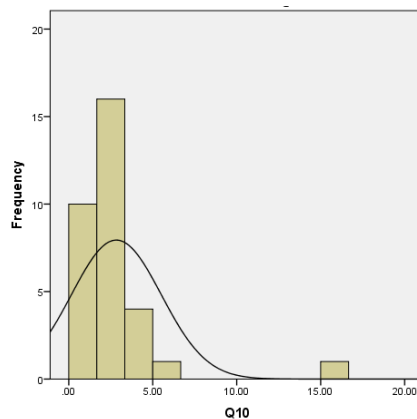
Figure 3.3-1 Frequency distribution of determined Q_{10} ranges. Solid line shows the normal distribution curve (mean=2.8, S.D.=2.7, n=32).

create comparable datasets over different conditions (e.g. using different crude oil types, temperature ranges, and seawater from different regions or different seasons).

3.4 Experimental data

3.4.1 Experimental data: Materials and Methods

The OxiTop®-C static respirometry measurement system was used to study the effect of temperature on crude oil (Tyrihans; light, paraffinic crude oil, density of 0.825 g cm^{-3}) biodegradation in seawater. The measuring principle and application of the OxiTop®-C system has been previously described (Kuokkanen et al., 2004). Water samples were collected from Byfjorden (North: 58° 57' 48" East: 5° 43' 8", Randaberg, Norway, 80 m depth) without filtration (March, 2012, water temperature: 7.7 °C). Prior to distribution into test flasks (500 ml), seawater samples were aerated for 5 min with sterile-filtered air. Flasks were then filled with seawater (375 ml) and inorganic nutrients, vitamins and amino acids were added to ensure non-limiting conditions (see Chapter 4). Incubation temperatures were 0.5, 5, 10, and 15 °C. Nine flasks were prepared for each incubation temperature: 5 with crude oil (100 mg L^{-1}), 2 with sodium-benzoate (30 mg L^{-1}) (positive control), 1 without additional carbon (blank) and 1 with oil (100 mg L^{-1}) and sodium-azide (1 g L^{-1}) to diminish bacterial activity (negative control). Finally, a carbon-dioxide trap was fixed in each flask. Bottles were then capped with OxiTop®-C heads and placed in temperature controlled incubator cabinets (first exposure). Magnetic stirring ensured mixing. As soon as oxygen consumption reached 40-50 mg L^{-1} (10-20 mg L^{-1} in the case of the 0.5 °C test), test flasks were opened and spiked with crude oil (second exposure). At the same time positive controls were spiked with sodium-benzoate (Na-benzoate). The same concentrations of each substrate were added as during the first exposure. Maximum oxygen consumption rate (r_{max}) was determined using a linear approximation at the most active phase of degradation. Half-lives and k_1 were calculated for Na-benzoate. Q_{10} values were determined from the ratios of rates (k_1 and r_{max}) at ten degrees temperature difference.

3.4.2 Experimental data: Results

Prior to the addition of Na-benzoate, lag times and crude oil degradation were similar and increased at declining temperature (Figure 3.4-1).
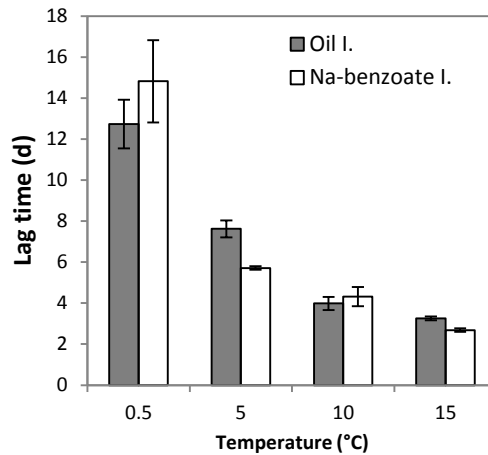
Figure 3.4-1 Lag times prior to sodium-benzoate (SB) and crude oil degradation at four incubation temperatures (mean ± S.D.).

Maximum oxygen consumption rates and k_1 values increased at elevated incubation temperatures, except the r_{max} of Na-benzoate, which showed a maximum at 10 °C during the first exposure (Figure 3.4-2).
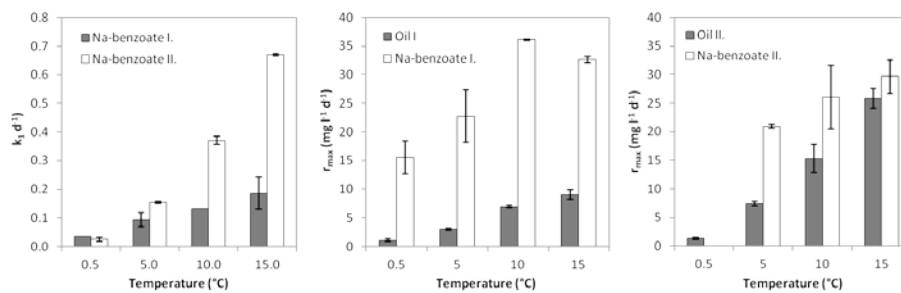
Figure 3.4-2 Pseudo first order rate coefficients (left), and maximum oxygen consumption rates of sodium-benzoate (middle) and crude oil (right). Legend codes: SB-sodium-benzoate, I.-first exposure, II.-second exposure (mean ±S.E.).

Temperature responses of the two substrates were different, Q_{10} values were in all cases higher for oil compared to Na-benzoate (Table 3.4-1). It is worth noting that Q_{10} was systematically higher at 0.5 °C compared to 5 °C. In general, Q_{10} values were higher during the second exposure than during the first one, except the value calculated from r_{max} of Na-benzoate at 5 °C, which remained the same during both exposures.

Table 3.4-1 Q_{10} values of oil and sodium-benzoate (SB) degradation calculated from pseudo first order rate coefficients (Q_{10} (k_1)) and maximum oxygen consumption rates (Q_{10} (r_{max})) during the first (I.) and second (II.) exposures at two temperatures.

Substrate	T_{Q10} (°C)	Q_{10} (k_1)		Q_{10} (r_{max})	
		I.	II.	I.	II.
Oil	0.5	-	-	9.9	25.6
Oil	5.0	-	-	2.6	3.5
SB	0.5	3.7	14.8	2.3	21.8
SB	5.0	2.1	4.5	1.4	1.4

3.5 Variability of Q_{10} values

A key question that constrains accuracy of temperature compensation based biodegradation rate predictions is how temperature influences crude oil biodegradation. As introduced earlier, the Q_{10} approach is accepted describing this relationship, and a Q_{10} of 2 is implemented in the OSCAR model. However, the literature data gathered herein does not corroborate the choice of Q_{10}=2. The Q_{10} values determined during the reported laboratory experiment are also not in agreement with this rule of thumb. Establishing a range of Q_{10} values is anyway controversial, in fact, the proposed universal temperature dependence theory (UTD), which suggests that E_a ranges between 57.9 and 67.5 kJ mol^{-1}, and consequently Q_{10} values between 2.3 and 2.7, is still under debate (Brown et al., 2004; Clarke, 2004). Hence, proposing another universal Q_{10} value, or range of values, would be ambitious. Instead, possible reasons underlying the variability in Q_{10} values obtained from temperature controlled experiments must be understood.

3.5.1 Effect of bioavailability on the Q_{10} value

It has been observed that temperature affects the physico-chemical state of oil (Margesin and Schinner, 1999). Toxicity and bioavailability of oil is determined by its composition, ambient temperature and also other factors (e.g. presence of dispersants) (Atlas, 1975). Some oils are more available for microbial degradation at low temperatures than others, as exemplified by low pour points (Brakstad and Bonaunet, 2006). The appearance and bioavailability of oils at different temperatures varies significantly, resulting from different composition and pour points of different oil types (ITOPF, 2002). Bioavailability influences degradation rate and its temperature dependence will also be reflected in the measured Q_{10} values. Therefore temperature response of different crude oils has to be described by different Q_{10} values. This effect of temperature on bioavailability was observed in the reported laboratory experiment. Biodegradation of Na-benzoate at low temperature was not limited due to physico-chemical restrictions as it was completely dissolved (i.e. available for microbial uptake) at the beginning of the exposure at all temperatures. On the other hand, crude oil had decreased solubility and increased viscosity at low temperatures, limiting microbial uptake and utilization (Harms et al., 2010b). The influence of low solubility of hydrophobic compounds on their biodegradation rates has recently been reported by Yassine et al. (2013). It appears that the effect of temperature on physico-chemical properties of oil caused substrate limitation at cold temperatures during the reported experiments. Initial biomass was not the limiting factor responsible for reduced biodegradation of oil at low temperatures, as lag times preceding Na-benzoate and oil degradation were similar at all temperatures, indicating that the number of bacteria consuming the tested compounds was on the same order of magnitude. Summarizing, temperature dependence of bioavailability implies that properties of the substrate influence the Q_{10} value. Consequently, the temperature compensation using the same Q_{10} value for different oils is not realistic.

3.5.2 Effect of temperature on the Q_{10} value

It has been previously reported that the Q_{10} value varies with incubation temperature (Li and Dickie, 1987; Ratkowsky et al., 1983; Chablain et al., 1997; Pietikäinen et al.,

2005; Sand-Jensen et al., 2007; Tarpgaard et al., 2006). In fact, Q_{10} values were higher at low temperatures in several of the reviewed studies and also in the reported laboratory experiment. Herein, three hypotheses are suggested to explain this effect. First, there is an inherent temperature dependence of the Q_{10} value which predicts that Q_{10} will increase at low temperatures (Equation 3.3-1). However, the difference between Q_{10} values determined at 0.5 and 5 °C in the reported laboratory experiment cannot be explained by this inherent relationship. Similarly, the difference between Q_{10} values calculated at 5 °C and 10 °C from Atlas and Bartha (1972) is greater than expected from the inherent increase of Q_{10}. On the other hand, these differences could be explained by deviation from the Arrhenius model below 5 °C observed by several authors (Ratkowsky et al., 1983; Pietikäinen et al., 2005; Sand-Jensen et al., 2007). Second, in the case of crude oil biodegradation, the physico-chemical changes that oil undergoes towards its pour point could cause the increase in the Q_{10} value due to limited substrate bioavailability. However, a similar increase in Q_{10} value was observed for Na-benzoate in the reported laboratory experiment. Hence, substrate limitation is not likely to be the main reason for temperature influencing Q_{10} value. A third hypothesis could be that the initial number of bacteria participating in the degradation is affected by temperature. Besides biodegradation activity and substrate concentration, biodegradation rate also depends on the number of active bacteria possessing the degrading enzymes (Valentine et al., 2012; Yassine et al., 2013). According to the Monod growth model, substrate consumption rates (i.e. HC degradation rate) can be expressed as:

$$\frac{dS}{dt} = \frac{1}{Y} \cdot \frac{\mu_{max} \cdot S}{K_s + S} \cdot X \qquad (3.5-1)$$

Where Y is the yield coefficient, μ_{max} is the maximum growth rate (day^{-1}), K_s is the half-saturation coefficient (mg L^{-1}), S is the substrate concentration (mg L^{-1}) and X is the biomass concentration (mg L^{-1}). Temperate sea waters are thought to be dominated by mesophilic bacteria, which have a minimum growth temperature of around 5 °C, and also inhabited by cold-adapted bacteria (psychrophilic and psychrotolerant), which grow below 5 °C (Morita, 1975). At the incubation

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temperature of 0.5 °C, cold-adapted species would be expected to grow and become dominant in temperate sea water (Brakstad and Bonaunet, 2006). If these cold-adapted species are in low abundance, then biomass limitation could be the reason for Q_{10} values being higher than expected below 5 °C. This can be more clearly seen if one expresses the k_1 values from Equation 3.5-1, taking into account the assumptions that the first order interpretation of the Monod model requires, i.e. substrate concentration is much lower than the half-saturation coefficient ($S \ll K_s$) and biomass is constant ($dX/dt = 0$):

$$k_1 = \frac{1}{Y} \cdot \frac{\mu_{max}}{K_s} \cdot X \qquad (3.5\text{-}2)$$

Biomass (X) clearly influences the pseudo first order rate coefficient. In fact, number of active bacteria was shown to influence degradation rate of alkanes (Yassine et al., 2013). Though pseudo first order interpretation of crude oil biodegradation assumes that biomass does not increase while HCs are consumed, this is generally not the case. Number of degrading bacteria increases several orders of magnitude during HC biodegradation (Valentine et al., 2012). Hence, the assumption underlying the pseudo first order interpretation of crude oil biodegradation is erroneous. More importantly, when Q_{10} values are calculated from k_1, the influence of biomass on biodegradation rate is taken into account causing observed Q_{10} values to vary. In other words, what appears to be the effect of temperature is in reality the result of the influence of biomass.

3.6 Conclusions

As an overall result of the present study, biodegradation rate predictions calculated by temperature compensation are ambiguous. Choosing a Q_{10} value based on the herein reviewed studies was neither feasible nor anticipated. In fact, experimentally determined Q_{10} values were found to reflect not only the direct effect of temperature on enzymatic activity, but also other indirect effects. The high variation in the observed Q_{10} values can be attributed to indirect effects of temperature such as the influence on bioavailability and number of active bacteria. Intrinsic temperature

sensitivity of oil biodegradation (i.e. sensitivity of the metabolic processes involved in biodegradation) has to be determined after removing the contribution of these additional factors. Assuming that $Q_{10}=2$ describes this intrinsic temperature sensitivity universally, adequate degradation rate predictions still depend on the existence of a common inherent biodegradation capacity, characteristic to all marine microbial communities at the same temperature. Implementing mechanistic models in risk assessment tools to describe biodegradation kinetics might be applicable in cases where biomass heterogeneity and aqueous phase diffusion do not limit degradation. A coupled physical-metabolic model established in this manner predicting HC biodegradation rates based on exponential growth of bacteria could represent a better alternative compared to estimating biodegradation rates based on temperature compensation.

Chapter 4. Static respirometry

The work presented in this chapter is in preparation for submission.

Proposed title:

Adaptation and evaluation of static respirometry method for testing biodegradation in marine samples

4.1 Summary

Accurate biodegradation rates are essential input data for numerical models assessing fate and effect of pollutants in the marine environment. Lag time and half-life values derived from standard tests are also useful for predicting environmental fate. A static respirometry method using the OxiTop®-C system has been applied to investigate the influence of sample handling (e.g. filtration and storage) and initial bacterial number on sodium-benzoate and naphthalene degradation in seawater. Dilution of the seawater influenced all determined parameters (lag time, maximum oxygen consumption rate and pseudo first order degradation rate), while the parameter most often reported for HC and crude oil biodegradation (i.e. pseudo first order rate coefficient) was not affected by filtration and aging of the seawater. Overall, the OxiTop®-C system was found to be a suitable tool for kinetic analysis and comparative assessment, especially due to the high resolution of oxygen consumption data it provides.

4.2 Introduction

Complete removal of hydrocarbons (HCs) present in the marine environment occurs via biodegradation (Atlas, 1981). Accurate information about the kinetics of this process and better understanding of how environmental factors are influencing its kinetics is important (Leahy and Colwell, 1990). Petroleum HC biodegradation rates are usually determined from microcosm scale laboratory experiments applying gas chromatography analysis of remaining HCs, counting total and/or HC degrading bacteria, or respirometry (Atlas and Bartha, 1972; Mulkins-Phillips and Stewart, 1974; Gibbs et al., 1975; Gibbs and Davis, 1976; Minas and Gunkel, 1995; Garrett et al., 2003; Deppe et al., 2005; Brakstad and Bonaunet, 2006; Venosa and Holder, 2007; Coulon et al., 2007; Delille et al., 2009). Pseudo first order rate coefficients (k_1) are often reported as a quantitative measure of biodegradation. Moreover, k_1 values are implemented in the most commonly used fate model (i.e. the OSCAR model) to describe biodegradation of HC groups.

It has been suggested that biodegradation of HCs with low solubility is best measured by respirometry (Reuschenbach et al., 2003). Quantifying consumed oxygen or produced carbon-dioxide provides information about microbial activity directly. OECD guidelines for biodegradability testing of chemicals also advise this type of experimental approach (OECD, 1992). The closed bottle test described in OECD306 protocol for biodegradability testing in seawater relies on determining consumed oxygen using an oxygen probe. Filtration of seawater to remove coarse particles and aging of seawater to remove dissolved organic carbon is recommended by the guidelines prior to experiments. Determination of bacterial numbers can optionally be carried out, however, not necessary for approved test. Major results are reported as % biodegradability after 28 days of exposure, lag time and half-life. Influence of pre-treatments of seawater samples used as inoculum (e.g. filtration and aging) has not been well studied, although they are important parameters to investigate.

Filtration of seawater is not unusual in biodegradation studies (Brakstad and Bonaunet, 2006). Regarding this pre-treatment, presumably the following two major aspects need to be considered. One is connected to the removal of grazers larger than the filter pore size, while the other is related to the removal of particulate organic matter (POM). Bacterial communities in marine environments are part of the microbial loop which serves the basis for conventional planktonic food chain of zooplankton and fish (Azam et al., 1983; Azam, 1998). The importance of microbial interactions within the microbial community and between the community and other members of the microbial loop has also been recognized as an important factor affecting biodegradation in seawater (McGenity, 2012). Bottom up control of the bacterial populations (e.g. nutrient availability) taking part in biodegradation has been well studied, while the effect of sideways interactions (e.g. competition or mutualism) and top down control mechanisms (e.g. grazing or viral lysis) is not well studied. Bacteria live in seawater either attached to particles (e.g. to different types of POM) or freely in the water column. The role of POM has been relatively well studied in pelagic and deep-water environment (Kirchman, 2010). POM has a particularly important role in deep-water environments providing microhabitats for bacterial communities (DeLong et al., 2006). Though in pelagic waters, POM attached bacteria

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accounts for less than 10% of total bacterial abundance, their contribution to bacterial production can reach 50% in nutrient rich areas (Crump et al., 1998). POM can be converted into dissolved organic matter (DOM) by hydrolytic activity of extracellular enzymes of bacteria, consequently providing carbon source for bacterial growth (Hoppe et al., 2002; Simon et al., 2002).

Storage or aging of seawater sample is also a relevant issue for studying HC biodegradation. Seawater samples taken offshore need to be transported to an on shore laboratory prior to testing, in case biodegradation experiments cannot be carried out on board. Hence, storage time necessarily precedes experimental start. General guidelines for seawater storage suggest keeping the sample in a closed bottle (completely filled with seawater) in darkness at reduced temperature (Goya et al., 1963; Crompton, 2006). About 70 years ago, it was believed that processes taking place in stored seawater without additional carbon supply are a) release of ammonia, phosphate and carbon-dioxide, b) inter-conversion of ammonia, nitrate and nitrite, c) utilization of oxygen, d) attachment and growth of bacteria on the wall of sampling bottle, e) decay, and f) loss of biomass due to predation by grazers and viral lysis (Harvey, 1941). All these processes can potentially alter biodegradation capacity of a seawater sample. Especially loss of biomass, as initial number of bacteria participating in a particular degradation process is known to affect both the kinetics and rate of the degradation (Alexander, 1994).

The aim of this study was to evaluate the effect of experimental factors, such as seawater filtering, aging, and the initial number of bacteria, on measured oxygen consumption using sodium-benzoate (an easy biodegradable substrate) and naphthalene (a model compound for PAHs). A respirometry technique, the OxiTop®–C system, has been applied in the experiments which were designed based on OECD306 principles. The OxiTop®-C system has been previously used to measure biodegradation of several different types of organic material including fuel oil and forestry oils in soil and groundwater (Kuokkanen et al., 2004). It has been found to be a suitable tool to assess biodegradability of low solubility and volatile materials (Karhu et al., 2009; Reuschenbach et al., 2003). Moreover, studying the effect of experimental variables such as nutrient addition was also successfully performed

(Roppola, 2009). Use of the OxiTop®-C system is not widespread in marine biodegradation studies, though it is a promising tool for kinetic studies.

4.3   Materials and methods

4.3.1   Experimental setup

Biodegradation of naphthalene crystals and sodium-benzoate was measured by manometric respirometry using the OxiTop®-C system. Seawater was used as inoculum for microcosm scale biodegradation experiments. Two sets of tests were prepared (Table 4.3-1). In the first set, seawater samples were immediately used after sampling, while in the second set, seawater was stored for 10 days at 8 °C in darkness before experiment start.

Table 4.3-1 Experimental set up of the two sets of tests. V_{sw} is the volume of seawater in each test flask, V_{tot} is the total volume. F=filtered, NF=non-filtered, 10x=10 times diluted, 100x=100 times diluted.

Set	Test	Storage time (day)	V_{sw} (ml)	V_{tot} (ml)
1	F	0	448	450
	NF	0	448	450
	NF 10x	0	44.8	450
	NF 100x	0	4.48	450
2	NF aged	10	448	450

4.3.2   Sampling

Seawater samples were collected via a fixed pipeline system from Byfjorden (North: 58° 57' 48" East: 5° 43' 8", Randaberg, Norway, 80 m depth, 8 °C) in January, 2013. Filtered seawater samples were collected using an in line filter (Millipore, 10 µm). Samples were transported to the laboratory where the first set of experiments was started immediately. One batch of non-filtered seawater was left for aging as indicated in the previous section.

4.3.3 Preparation of the OxiTop flasks

Prior to distribution into test flasks (500 mL), seawater samples were aerated for 10 min with sterile-filtered air. OxiTop®-C flasks were then filled with seawater (375 mL). Dilution test flasks were prepared mixing the non-filtered seawater with autoclaved seawater in 1:10 (10x diluted) and 1:100 (100x diluted) ratios. Inorganic nutrients (16.2 mg L^{-1} K_2HPO_4, 0.8 mg L^{-1} KH_2PO_4, 42.0 mg L^{-1} $NaNO_3$, 0.05 mg L^{-1} $FeCl_3$, 2.5 mg L^{-1} $CaCl_2$ and 1.5 mg L^{-1} $MgSO_4$) and trace minerals according to Balch (1979) were added. Amino acids (10µl L^{-1} RPMI 1640 amino acids solution 50x, Sigma) and vitamins (10 µL L^{-1} of a stock solution with 20 mg L^{-1} myoinositol, 0.1 mg L^{-1} thiamine-hydrochloride, 0.1 mg L^{-1} pyridoxine-hydrochloride, 1.0 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} glycine, 0.01 mg L^{-1} biotin and 0.1 mg L^{-1} folic acid) (modified from Balch, 1979) were also added.

Flasks were kept in temperature controlled cabinet at 15 °C overnight for equilibration. Each set of tests consisted of 14 flasks: 6 contained naphthalene (33 mg L^{-1}) as the sole carbon source, 6 received sodium-benzoate (25 mg L^{-1}), 1 had no additional carbon (blank) and 1 was prepared with naphthalene (33 mg L^{-1}) and sodium-azide (1 g L^{-1}) to diminish bacterial activity (negative control). Naphthalene was added as crystals, while sodium-benzoate was added from a stock solution. Finally, a carbon-dioxide trap was fixed in each flask containing 2 pellets of sodium-hydroxide. Bottles were then capped with OxiTop®-C heads and placed back in the temperature controlled cabinets. Magnetic stirring ensured continuous mixing. Data collection was started immediately.

4.3.4 Biodegradation data analysis

Oxygen consumption (OC) values were used to determine lag times (t_{lag}), maximum oxygen consumption rates (r_{max}) and pseudo first order rate coefficients (k_1). The t_{lag} values were determined following logarithmic transformation of the OC curves. The beginning of the linear phase of log-transformed curves was defined as t_{lag}. The r_{max} values were determined from the derivate of the OC curves. Half-life values for sodium-benzoate were calculated according to the method described in the OECD guidelines for testing biodegradability in seawater (OECD, 1992). For naphthalene,

$t_{1/2}$ was defined as the time where 50% of the final OC was reached (i.e. OC on day 28). The $t_{1/2}$ values were then converted into pseudo first order rate coefficients (k_1), according to the following equation:

$$k_1 = \frac{ln2}{t_{1/2}} \qquad (4.3\text{-}1)$$

After checking the normality, one-way ANOVA analysis was used to test the null hypothesis; H_0: all sample handling conditions are equal implying that the means of t_{lag}, r_{max} and k_1 values are the same at $\alpha = 0.05$. Based on the degrees of freedom between and within samples, a limit of 3.6823 for F value was selected from an F-table and the three sample handling conditions were concluded significantly different in case $F > 3.6823$.

4.4 Results

Averaged OC curves for sodium-benzoate and naphthalene are shown in Figure 4.4-1 and 4.4-2, respectively. All sodium-benzoate curves depicted a similar sigmoid shape, consisting of a lag period with no observable respiration, followed by exponential increase in oxygen consumption until a plateau has been reached. Endogenous respiration phase was observed (data not shown). Naphthalene degradation resulted in clearly different curves compared to the ones observed with sodium-benzoate. Lag periods were detected, followed by a logarithmic shape reaching a plateau as substrate was depleted.

Average t_{lag}, r_{max}, and k_1 values are summarized in Figure 4.4-3 and 4.4-4. Lag times appeared to be similar for filtered, non-filtered and aged seawater tests with both substrates. The r_{max} values of sodium-benzoate were highest in non-filtered seawater, whereas r_{max} was highest in the filtered seawater in case of naphthalene. Pseudo first order rate coefficients of sodium-benzoate were very similar for filtered, non-filtered and aged seawater samples similarly to k_1 values for naphthalene.
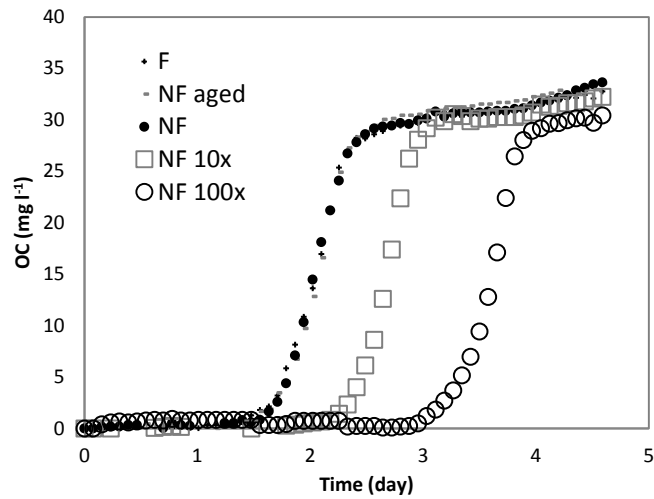
Figure 4.4-1 Average oxygen consumption (OC) of sodium-benzoate degradation in seawater (n=6). F=filtered, NF aged=non-filtered aged, NF=non-filtered, NF 10x=non-filtered 10 times diluted, and NF 100x=non-filtered 100 times diluted.
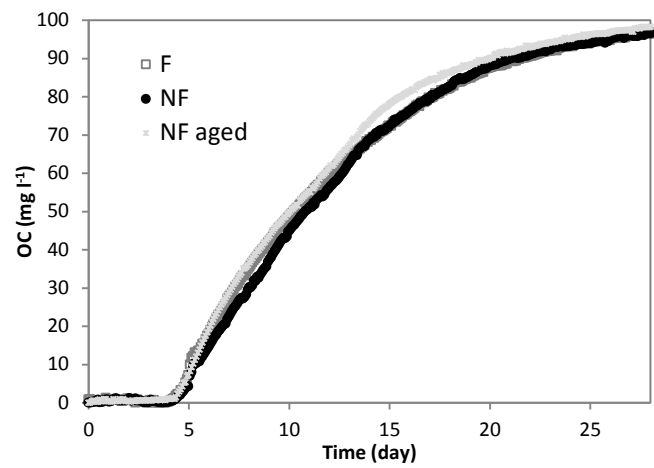
Figure 4.4-2 Average oxygen consumption (OC) of naphthalene degradation in seawater (n=6). F=filtered, NF=non-filtered, and NF aged=non-filtered aged.
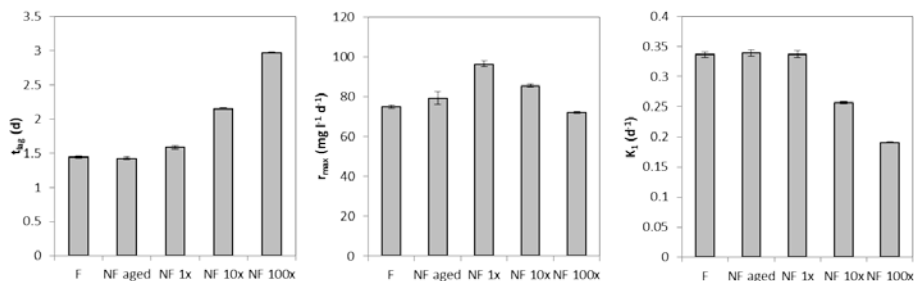
Figure 4.4-3 Lag times (t_{lag}), maximum oxygen consumption rates (r_{max}) and pseudo first order rate coefficients (k_1) of sodium-benzoate degradation in filtered (F), non-filtered aged (NF aged), non-filtered non-diluted (NF 1x), non-filtered 10 times diluted (NF 10x), and non-filtered 100x diluted (NF 100x) seawater (mean ± sd).
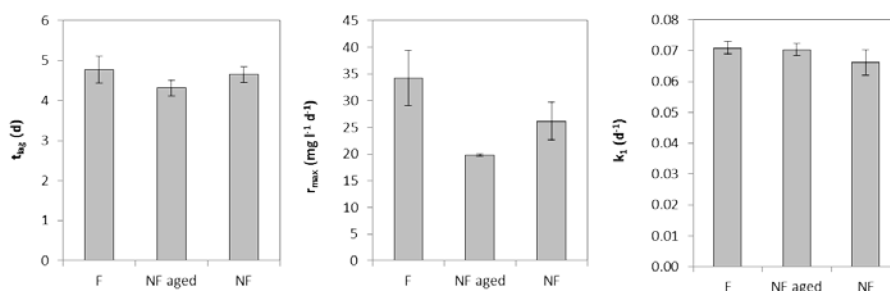
Figure 4.4-4 Lag times (t_{lag}), maximum oxygen consumption rates (r_{max}) and pseudo first order rate coefficients (k_1) of naphthalene degradation in filtered (F), non-filtered (NF) and non-filtered aged (NF aged) seawater (mean ± sd).

Dilution of the seawater influenced all derived parameters as observed with sodium-benzoate (Figure 4.4-3). Lag times increased, while r_{max} and k_1 values decreased with increasing dilution factor. A 10 fold dilution of seawater resulted in an increase in t_{lag} of 0.57 day, while a 100 fold dilution resulted in an increase of 0.82 days (compared to the undiluted seawater). In case of naphthalene, degradation was observed in 2 of the 6 replicate flasks of the 10 times diluted seawater, with lag times of approximately 7 days (data not shown). No degradation was observed in any of the flasks containing 100 times diluted seawater, within 28 days of incubation (data not shown).

Filtration and aging did not have significant effect on sodium-benzoate and naphthalene biodegradation according to k_1 values. F values were 1.34 and 0.016 ($p < 0.05$) for naphthalene and sodium-benzoate, respectively. The opposite was indicated by r_{max} values, where F values were 5.87 and 7.36 ($p < 0.05$) for naphthalene and sodium-benzoate, respectively. Comparing t_{lag} values, means were not significantly different in case of naphthalene ($F=1.33$, $p < 0.05$) while they were in case of sodium-benzoate ($F=4.18$, $p < 0.05$).

4.5 Discussion

Degradation kinetics of the two substrates was different. This can be explained by the facts that the number of naphthalene degrading bacteria was probably orders of magnitude below the number of heterotrophic bacteria capable of utilizing sodium-benzoate, and naphthalene concentration in the water phase was probably significantly lower throughout the experiment as crystals were slowly dissolving. Sodium-benzoate degradation kinetics has been previously observed to follow the Monod model with growth (Loh and Chua, 2002), similarly to the results reported herein. Depending on experimental factors, naphthalene biodegradation can also follow Monod model (MacRae and Hall 1998; Ahn et al., 1998; Jajuee et al., 2006), or modified Gompertz model (Lu et al., 2011). Herein, however, naphthalene degradation appeared to follow first order kinetics, as expected when substrate concentration is too low to support net bacterial growth (i.e. at very high biomass to carbon source ratio) (Alexander, 1994; Yuan et al., 2001; Desai et al., 2008; Maletic et al., 2009).

Dilution of seawater decreased the number of bacteria involved in degradation, which clearly affected the time point where respiration became observable (t_{lag}) in case of sodium-benzoate. Assuming that lag time prior to both sodium-benzoate and naphthalene degradation is mostly affected by the initial number of bacteria (i.e. adaptation to both substrates is assumed to take equally long), number of naphthalene degraders in the seawater was several orders of magnitude less compared to number of sodium-benzoate consumers, initially. Further dilution (100 times) of the seawater

decreased the abundance of naphthalene degraders to a level, where no observable degradation could occur within 28 days.

Filtration of seawater resulted in the removal of grazers and particles greater than 10 µm in diameter. Removal of grazers of this size range should not directly affect the predation pressure on bacteria, as the size range of heterotrophic nanoflagellates preying on them is usually between 2 and 5 µm (Kirchman, 2010). Indirect effects can be originating from the removal of the predators feeding on these nanoflagellates. This phenomenon would increase predation pressure on bacteria due to uncontrolled nanoflagellate growth, resulting in reduced biodegradation rates due to reduced biomass. The other aspect related to filtration, is the removal of bacteria attached to POM. Poeton et al. (1998) found in fact, that 50% of biomass involved in phenanthrene degradation was attached to particles. Consequently, biodegradation rate coefficients in filtered seawater were expected to be lower compared to non-filtered seawater. Hence, it was surprising, that observed k_1 values were not affected by filtration.

During aging, bacteria carry out maintenance metabolism, and regeneration of dissolved organic material (DOM) and nutrients occurs via decay or predation and viral lysis. Loss of bacterial diversity and abundance is a possible outcome of sample storage, hence biodegradation rate coefficients were expected to be lower in aged seawater compared to fresh. Brakstad and Bonaunet (2006) found that microbial community composition changed during storage and acclimation of seawater. In that study, the influence of these changes on biodegradation rate was not investigated. It was however suggested, that reduction of bacterial diversity may not influence broader functioning of the whole microbial community (i.e. respiration of easy biodegradable organic carbon such as sodium-benzoate), but it is believed to affect well-defined narrow niche functions (i.e. degradation of naphthalene). During the experiment reported herein, no difference between the effect of aging on the two substrates was observed.

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4.6 Conclusions

Static respirometry using the OxiTop®-C system provides high resolution oxygen consumption data. Hence, it is a suitable tool for kinetic model development, validation and comparative analysis. Lag time, a parameter often reported in OECD test results, was not influenced by seawater pre-treatment (i.e. filtration and aging), however it was clearly affected by the number of bacteria present in seawater. The parameter most often reported for HC and crude oil biodegradation (i.e. k_1) was not affected by filtration and aging of seawater. However, dilution of the seawater inoculum clearly influenced the k_1 value. This implies that the effect of biomass concentration is in fact reflected in this kinetic "constant" assumed to be independent of it. Development of mechanistic models for the analysis and interpretation of biodegradation test results is essential for understanding fine scale differences between the effects of experimental conditions and other factors that possibly influence observed degradation kinetics.

Chapter 5. Comparative study

The work presented in this chapter has been accepted for publication in *Biodegradation*, DOI: 10.1007/s10532-013-9644-3.

Title

Naphthalene biodegradation in temperate and arctic marine microcosms

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5.1 Summary

Naphthalene, the smallest polycyclic aromatic hydrocarbon (PAH), is found in abundance in crude oil, its major source in marine environments. PAH removal occurs via biodegradation, a key process determining their fate in the sea. Adequate estimation of PAH biodegradation rates is essential for environmental risk assessment and response planning using numerical models such as the Oil Spill Contingency and Response (OSCAR) model. Using naphthalene as a model compound, biodegradation rate, temperature response and bacterial community composition of seawaters from two climatically different areas (North Sea and Arctic Ocean) were studied and compared. Naphthalene degradation was followed by measuring oxygen consumption in closed bottles using the OxiTop® system. Microbial communities of untreated and naphthalene exposed samples were analysed by polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and pyrosequencing. Three times higher naphthalene degradation rate coefficients were observed in arctic seawater samples compared to temperate, at all incubation temperatures. Rate coefficients at *in situ* temperatures were however, similar (0.048 d^{-1} for temperate and 0.068 d^{-1} for arctic). Naphthalene biodegradation rates decreased with similar Q_{10} ratios (3.3 and 3.5) in both seawaters. Using the temperature compensation method implemented in the OSCAR model, $Q_{10} = 2$, biodegradation in arctic seawater was underestimated when calculated from the measured temperate k_1 value, showing that temperature difference alone could not predict biodegradation rates adequately. Temperate and arctic untreated seawater communities were different as revealed by pyrosequencing. Geographic origin of seawater affected the community composition of exposed samples.

5.2 Introduction

Naphthalene is a two-ring polycyclic aromatic hydrocarbon (PAH) often used as a model compound when studying the environmental fate of PAHs (Bauer and Capone, 1985; Heitkamp et al., 1987; Geiselbrecht et al., 1998; Castle et al., 2006). PAHs are known to be toxic, mutagenic and potentially carcinogenic chemicals representing

substantial environmental risk (Moore et al., 1989). PAHs enter the marine environment from oil seeps, as a result of accidental discharges of crude oil and from operational releases of produced water from offshore installations (Cerniglia, 1992; Latimer et al., 2003; Pampanin and Sydnes, 2013). In order to evaluate the environmental impact of hydrocarbon (HC) releases and to plan response strategies, risk assessment models have been implemented to predict the fate of oil in the sea (Reed et al., 1999a). Temporal change in concentrations of HCs present in oil is predicted based on physiochemical and biological weathering processes. Processes, such as evaporation and photo-oxidation, can reduce the amount and/or alter the properties of HCs in seawater. However, only biodegradation can truly eliminate them from the environment through mineralization (Atlas, 1981; Zobell et al., 1943). Hence, models need to provide an adequate estimation of HC biodegradation rates. The Oil Spill Contingency and Response (OSCAR) model, commonly used in the Norwegian sector, estimates the biodegradation process using first order rate coefficients assigned to chemically similar compound groups (Brakstad and Faksness, 2000; Reed et al., 2000). The Q_{10} approach is used to adjust biodegradation rate coefficients for the temperature of the area of interest. (Mark Reed, personal communication). The Q_{10} value, the factor by which metabolic rates increase for a 10 °C temperature increase, has been used to describe the effect of temperature on metabolic rates (Winkler et al., 1996; Robador et al., 2009; Jenkins and Adams, 2011). It is well known that the rate of oil biodegradation decreases at lower incubation temperatures similarly to the temperature dependence of other metabolic processes (Zobell, 1946; Atlas, 1975; Gibbs et al., 1975; Brakstad, 2008). The Q_{10} approach implies that biodegradation rates in two different environments can be predicted based on the difference in their *in situ* temperatures. A Q_{10} value between 2 and 4 is accepted as a rule of thumb (Feller and Gerday, 2003; Brakstad, 2008; Brakstad et al., 2009) for comparing studies performed at different temperatures (Stewart et al., 1993; Van Stempvoort and Biggar, 2008). Specifically, the OSCAR model adopts a Q_{10} value of 2, which predicts biodegradation rates to halve with a ten degree temperature reduction.

Factors other than temperature have been shown to influence the rate of biodegradation (e.g. nutrients, dispersants and bacterial community composition) (Atlas, 1981; Leahy and Colwell, 1990; Siron et al., 1995). In general, less is known about the influence of the microbial community actually involved in the process. However, a dynamic model, based on the locally present HC degraders and their growth characteristics, has been shown to well describe the observed biodegradation rate after the Deepwater Horizon blowout (Valentine et al., 2012). Still, the specific *in situ* metabolic activity of most of the HC degraders needs to be studied in more detail.

The aims of the present study were to compare naphthalene biodegradation rate, temperature response and bacterial community composition of seawater samples collected in two different geographic areas: North Sea (temperate) and Arctic Ocean. The following questions were addressed: (1) Do naphthalene biodegradation rates differ in temperate and arctic seawater? (2) Do temperature responses of temperate and arctic seawater microbial communities differ (in terms of Q_{10} approach)? (3) Do untreated temperate and arctic seawater microbial communities differ? (4) Does the geographic origin of seawater effect the microbial community composition of naphthalene exposed samples? The possibility to predict biodegradation rates in a new area of interest (e.g. the Arctic) using the Q_{10} approach is also discussed.

5.3 Materials and Methods

5.3.1 Experimental set up

Biodegradation of naphthalene in temperate and arctic seawater samples was followed by measuring oxygen consumption in closed bottles using the OxiTop® system. The measuring principle of the OxiTop® heads has been previously described (Kuokkanen et al., 2004). Two sets of experiments were carried out at four different incubation temperatures (0.5, 4, 8, 15 °C). Incubation times were 48 and 28 days for the temperate and arctic experiments, respectively. These lengths of the tests were determined by the degradation process; each experiment was terminated after biodegradation reached the endogenous respiration phase. One test flask was sacrificed and sampled for gas chromatographic (GC) analysis at the beginning of

each experiment. Samples for DNA extraction were planned to be taken in the most active phase of the degradation for microbial community analysis using polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) and pyrosequencing. For each sampling point and each temperature one flask was selected. DNA samples were collected on day 6 for 15 °C, day 22 for 8 and 4 °C, and day 37 for 0.5 °C for temperate experiment. For the arctic experiment, samples for DNA extraction were taken on day 1 for 15 °C and day 14 for 8, 4, and 0.5 °C.

5.3.2   Sampling

Seawater samples were collected and used directly as inoculum for microcosm scale (1 L) biodegradation experiments. The first experiment was carried out with seawater from Byfjorden, Norway (North: 58° 57' 48" East: 5° 43' 8", from 80 m depth, 7.0 °C), collected via a fixed pipeline system, and filtered through 10 µm in February 2009. Seawater was transported to the laboratory immediately and the experiment started the following day. The second experiment was performed using seawater batch sampled southwest of Sjuøyane, Spitsbergen, Norway (North: 80° 34' 039" East: 19° 25' 795", from 60 m depth, 1.4 °C), collected using Niskin bottles, without filtration in August 2009 during the COPOL cruise with R/V Lance and shipped to the laboratory 3 days after sampling. Precautions were taken to reduce the exposure of seawater to light and heat. The seawater was stored for an extra week before the experiment started due to laboratory constraints. The effect of sample handling differences (filtration and aging) was tested in our laboratory (see Chapter 4). These supplemental tests confirmed that lag times and pseudo first order rate coefficients were not influenced by differences in sample filtration and aging.

5.3.3   Preparation of OxiTop® flasks

Prior to distribution into flasks (1 L), seawater samples were aerated for 5 min, with sterile-filtered air. OxiTop® flasks were then filled with seawater and inorganic nutrients were added (16.2 mg L^{-1} K$_2$HPO$_4$, 0.8 mg L^{-1} KH$_2$PO$_4$, 42.0 mg L^{-1} NaNO$_3$, 0.05 mg L^{-1} FeCl$_3$, 2.5 mg L^{-1} CaCl$_2$ and 1.5 mg L^{-1} MgSO$_4$). Trace minerals were added according to Balch (1979). Amino acids (10µl L^{-1} RPMI 1640 amino acids solution 50x, Sigma) and vitamins (10 µL L^{-1} of a stock solution with 20 mg L^{-1}

myoinositol, 0.1 mg L^{-1} thiamine-hydrochloride, 0.1 mg L^{-1} pyridoxine-hydrochloride, 1.0 mg L^{-1} nicotinic acid, 0.5 mg L^{-1} glycine, 0.01 mg L^{-1} biotin and 0.1 mg L^{-1} folic acid) (modified from Balch, 1979) were also added.

For each experiment, 10 flasks were prepared for each incubation temperature: 6 contained naphthalene (10 mg L^{-1}) as the sole carbon source, 2 received sodium-benzoate (10 mg L^{-1}) as positive controls, 1 had no additional carbon (blank) and 1 was prepared with naphthalene (10 mg L^{-1}) and sodium-azide (1 g L^{-1}) to diminish bacterial activity (negative control). Flasks containing naphthalene were prepared as follows: seawater (800 mL) was transferred into a 1 L flask and 200 mL naphthalene stock solution (50 mg L^{-1} prepared in heated distilled water) together with sea salts was added to adjust salinity to 35 psu. Approximately 100 mL of headspace remained above the liquid phase. Finally, a carbon-dioxide trap was fixed in each flask containing 2 pellets of sodium-hydroxide. Bottles were then capped with OxiTop® heads and placed in temperature controlled incubator cabinets. Magnetic stirring ensured mixing. Data collection was started on the following day (this was taken into account when lag times and half-life values were calculated).

5.3.4   Gas chromatography analysis

An Agilent 6890N GC with Flame Ionization Detector (FID) and Supelco Equity 1 fused silica capillary column (10 m x 200 µm diameter, 1.2 µm film thickness) was used for naphthalene analyses in seawater samples. Carrier gas was nitrogen (N$_2$) with a flow rate of 0.7 mL/min. The inlet was set to splitless mode and the inlet temperature was kept at 260 °C. The oven temperature program was 0.2 min at 60 °C initial temperature, followed by an increase of 70 °C/min until the final temperature of 240 °C. The run time was 4 min. Samples (2.0 mL) were taken from test flasks, preserved with 200 µL 1 M HCl and stored below 10 °C until further processing. Prior to injection, vials were heated and incubated at 65 °C for 5 min under vigorous stirring. Finally a subsample of 500 µL from the headspace was automatically injected onto the column. Quantification of naphthalene concentration was based on external standard curve. Samples were measured in three replicates.

5.3.5   Biodegradation data analysis

Oxygen consumption values were used to determine lag times and half-life ($t_{1/2}$) values. Half-life values were calculated according to the method described in the OECD guidelines for testing biodegradability in seawater (OECD, 1992) and converted into pseudo first order rate coefficients (k_1) using.

$$k_1 = \frac{ln2}{t_{1/2}}$$

(5.3-1)

After checking the normality, two-sample t-test analysis ($p < 0.05$) was used to compare lag time and k_1 values between the four incubation temperatures, and between arctic and temperate samples using XLSTAT. The k_1 values were then used to prepare Arrhenius plots to determine activation energy values (E_a). The slope of the Arrhenius curve multiplied by the universal gas constant (8.314 kJ mol^{-1}) is the actual E_a value which was then finally converted into Q_{10} according to:

$$Q_{10} = e^{\left[\left(\frac{E_a}{R \cdot T \cdot (T+10)}\right)\right] \cdot 10}$$

(5.3-2)

5.3.6   Community composition analysis based on PCR-DGGE

The nucleic acid extraction procedure is described in detail in Brakstad et al (2008). Universal primers 341F (5'-CCTACGGGAGGCAGCAG-3') and SD907-r (5'-CCCCGTCAATTCCTTTGAGTT-3') with GC-clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGG-3') (Brakstad et al. 2008) targeting the V3-V4 hypervariable regions of 16S rRNA gene were used. Each reaction was carried out in a total volume of 50 µL. The PCR master mix contained the buffer provided (5 Prime), 0.1 mM of each dNTP's, 10 pM of each primer, 1 µL of community DNA and 0.25 µL of Taq polymerase (5U/µL, 5 Prime). The PCR program began with an initial activation at 94 °C for 2 min and was followed by 25 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 40 sec and elongation at 72 °C for 1 min. A final elongation step at 72 °C for 7 min was included. Products (5 µL of each) were checked on a 2 % agarose gel and relative amounts of DNA were estimated using Image Lab™ software (BioRad, version 2.0.1)

in order to have the same amount of DNA in each well. The DGGE gel contained 6 % acrylamide and the denaturing gradient was 20–70 %. DGGE analysis was performed using an Ingeny4U system (in 17 L TAE running buffer, at 60 °C, for 18 h, at 90 V). Gels were stained in GelRed (VWR) solution for 30 min. Images were taken in a BioRad GelDoc XR imaging system. Relative front and relative intensity of each band was determined using the Image Lab™ software. A matrix was then constructed and used to calculate similarities (Bray-Curtis distance measure) and to construct an UPGMA tree using JExpress2012.

5.3.7   Pyrosequencing

The V3-V4 region of the 16S rRNA gene was amplified by PCR using fusion primers containing the Roche 454 pyrosequencing adaptors and unique multiplex identifier sequences. The same universal forward, 341F (5'-CCTACGGGAGGCAGCAG-3') and reverse, SD907-r (5'-CCCCGTCAATTCCTTTGAGTT-3') primers were used as for PCR-DGGE analysis (Brakstad et al., 2008). Fusion primers were designed according to Roche 454 guidelines for unidirectional sequencing with Lib-L chemistry. All DNA samples were amplified in 50 µL PCR reactions (4 tubes per sample). Each reaction contained 10 µL reaction buffer (BioRad), 1 mM $MgCl_2$, 0.4 mM d'NTPs, 0.5 µM of each primer, 1U of iProof high fidelity DNA polymerase (BioRad), 1 µL of each DNA sample and molecular grade water. PCR conditions were: activation step at 98 °C for 2 min, denaturation at 98 °C for 30 sec, annealing at 57 °C for 30 sec and elongation at 72 °C for 1 min. A final elongation at 72 °C for 7 min was performed. PCR cycle numbers (26-30) were optimized previously to give a similar yield for each sample. All reaction products from the same sample were then pooled and concentrated to a volume of 50 µL using MinElute kit (Quiagen). Samples were run on agarose gel and DNA was extracted from the gel with MinElute Gel Extraction kit (Quiagen). All samples were pooled in equimolar amounts and submitted for pyrosequencing to the DNA Sequencing Facility at the University of Cambridge (Department of Biochemistry). The reaction was carried out on a Roche GS-FLX sequencer using Titanium chemistry.

5.3.8 Pyrosequencing data analysis

Filtering and noise removal of the raw amplicon sequences was carried out using AmpliconNoise (v1.25) (Quince et al., 2011), in order to crop barcode- and primer sequences, remove noise and chimeric sequences. All sequences shorter than 250 bp were also removed from the dataset. The resulting unique sequences were clustered into operational taxonomic units (OTUs) using maximum linkage clustering, based on pairwise distances generated using the Needleman-Wunsch algorithm with a 3 % distance cutoff (Quince et al., 2011). AmpliconNoise and the Diversity Estimates package (Quince et al., 2008) were used to calculate Bayesian parametric diversity estimates, Chao1 estimates of minimum diversity (Chao, 1987), rarified number of OTUs, and Shannon indices (Shannon and Weaver, 1949). Parametric diversity estimates were calculated based on the inverse Gaussian model, which showed the best fit to the data. OTUs were taxonomically classified using CREST with the SilvaMod 106 database (Lanzén et al., 2012).

5.3.9 Comminity composition analysis based on pyrosequencing

Statistical analysis of the pyrosequencing results was carried out using the R software (v2.14.2) (R Development Core Team, 2011), including the HCLUST function for hierarchical clustering. Community compositions were compared using two approaches. All OTUs and corresponding relative abundances were included in hierarchical clustering; distance measure was Bray-Curtis and clustering was performed according to the average linkage method. Principal Component Analysis (PCA) was also carried out with the most abundant taxa (> 5%) classified on genus level using XLSTAT (Pearson).

5.4 Results

5.4.1 Experimental design

In the temperate experiment, the initial naphthalene concentration was below the designated concentration (10 mg L^{-1}); the mean initial concentrations were 2, 6.6, 6.2 and 6.2 mg L^{-1} at 0.5, 4, 8 and 15 °C respectively. In the arctic experiment, the initial

naphthalene concentrations were above the designated concentration (with only one exception). The mean initial concentrations were 14.4, 12.0, 14.2 and 3.9 mg L^{-1} at 0.5, 4, 8, and 15 °C, respectively.

5.4.2 Biodegradation data analysis

Lag time values showed that naphthalene degradation started significantly earlier in the arctic compared to the temperate samples (Figure 5.4-1). GC analysis confirmed naphthalene degradation (data not shown). The effect of temperature was also observed as increasing lag periods at lower incubation temperatures. In Figure 5.4-2, the calculated pseudo first order rate coefficients of naphthalene (k_1) are reported. An exponential increase in the naphthalene k_1 values was observed for both experiments (temperate and arctic) at increasing incubation temperatures. Moreover, naphthalene degradation rate coefficients were approximately three times higher in arctic samples compared to temperate ones. For sodium-benzoate degradation (positive control), k_1 values also showed an increasing trend at higher incubation temperatures, however, they were similar for both temperate and arctic samples at all temperature (Figure 5.4-3). It is worth noting that the degradation rate of naphthalene was higher than the degradation rate of sodium-benzoate at all incubation temperatures in the arctic experiment, whereas, the opposite was observed in the temperate experiment.
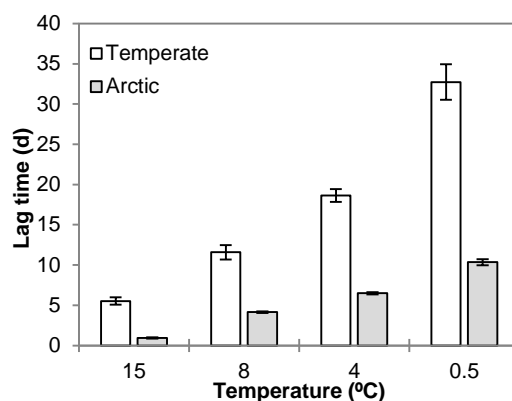
Figure 5.4-1 Lag times of naphthalene biodegradation (d = day) in temperate and arctic experiments (mean±s.d, n = 6).

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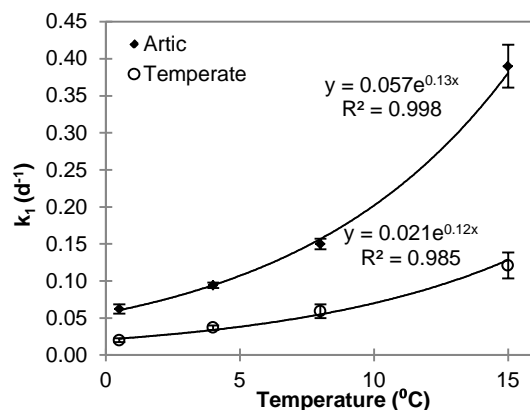
Figure 5.4-2 Pseudo first order rate coefficients (k_1) of naphthalene biodegradation in temperate and arctic experiments (mean±s.d., n = 6).
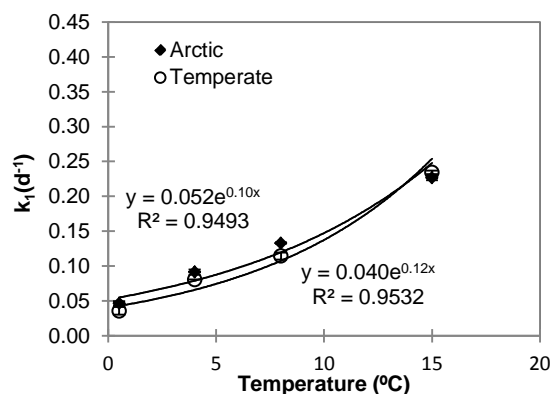
Figure 5.4-3 Pseudo first order rate coefficients (k_1) of sodium-benzoate biodegradation (positive control) in temperate and arctic experiments (mean±s.d., n = 6).

Activation energy (E_a) values in the temperature range of 0.5-15 °C for naphthalene biodegradation were determined as 83.2 (Q_{10} = 3.3) and 79.8 (Q_{10} = 3.5) kJ mol^{-1} for temperate and arctic samples respectively. The k_1 values at *in situ* temperatures were determined as 0.048 d^{-1} for temperate and 0.068 d^{-1} for arctic seawater using exponential curves in Fig. 2. The arctic k_1 value was calculated to be 0.047 d^{-1} based on the measured temperate k^1 using the Q_{10} = 2 approach, in order to simulate the temperature compensation method implemented in the OSCAR model. Using the

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measured $Q_{10} = 3.5$ instead of 2.0, the arctic k_1 value was estimated to 0.024 d^{-1}. For the positive controls, two lines fitted the Arrhenius curves best, and therefore two different E_a values were determined for the degradation process. Between 4 and 15 °C, E_a values of 64.7 ($Q_{10} = 2.7$) and 54.3 ($Q_{10} = 2.3$) kJ mol^{-1} were calculated for temperate and arctic experiments respectively. Between 0.5 and 4 °C, E_a values were 147.2 ($Q_{10} = 9.8$) and 120.9 ($Q_{10} = 6.5$) kJ mol^{-1} for temperate and arctic experiments.

5.4.3   Microbial community composition

PCR-DGGE and pyrosequencing were performed to study the bacterial diversity and community composition. Sample descriptions are reported in Table 5.4-1. Samples were coded as follows: T represents temperate, A arctic, U untreated and the number is the incubation temperature.

Table 5.4-1 Summary of sample details; location, description regarding the phase of degradation when DNA was extracted, incubation temperature (T), sampling day and naphthalene degradation half-life ($t_{1/2}$ (d = day)). Sample code: T = temperate, A = arctic, U = untreated, 0.5 = 0.5 °C, 4 = 4 °C, 8 = 8 °C, 15 = 15 °C.

Experiment	Sample code	Description	T (°C)	Sampling day	$t_{1/2}$ (d)
Temperate	TU	Seawater prior to experiment	-	0	-
	T0.5	Most active phase	0.5	37	36
	T4	Short afterdegradation	4	22	19
	T8	Long afterdegradation	8	22	12
	T15	Most active phase	15	6	6
Arctic	AU	Seawater prior to experiment	-	0	-
	A0.5	Short afterdegradation	0.5	14	11
	A4	Long afterdegradation	4	14	7
	A8	Long afterdegradation	8	14	5
	A15	Just before most active phase	15	1	2

5.4.4   Comparing community structure based on PCR-DGGE

Differences in prokaryotic community compositions of temperate and arctic samples were revealed by the DGGE analysis (Figure 5.4-4).
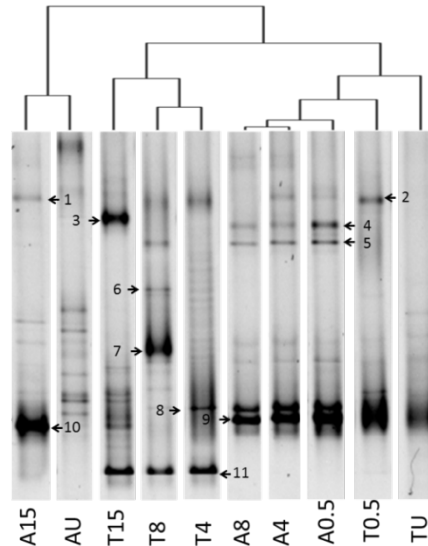
Figure 5.4-4 PCR-DGGE analysis of bacterial 16S rRNA genes extracted from naphthalene exposed samples during the temperate and arctic experiments. UPGMA tree based on the relative intensities of the 43 identified bands (Bray-Curtis distance measure). Sample code: T = temperate, A = arctic, U = untreated, 0.5 = 0.5 °C, 4 = 4 °C, 8 = 8 °C, 15 = 15 °C. Interesting bands are numbered.

Exposure of seawater to naphthalene at different incubation temperatures clearly affected banding patterns. According to the UPGMA tree, samples AU and A15 were distinct from all others. All naphthalene exposed arctic samples diverged from the AU sample, except A15. The most dominant band in A15 (band 10) did not appear in any other naphthalene exposed arctic samples, however a weaker band in A15 (number 1) also appeared in samples A0.5 and A4. The three arctic exposed samples (A0.5, A4 and A8), which clustered tightly, had several common bands (numbers 4, 5, 8, and 9). Temperate exposed samples showed similar banding patterns and shared one predominant band (number 11) except for T0.5. Samples T4, T8 and T15 clustered together on the UPGMA tree, confirming the similarity of their band composition. Interestingly, a dominant band of T4 (number 8) also appeared in samples A0.5, A4 and A8. It needs to be considered, that difference in sampling time could have influenced structure of microbial community as microbial community changes during

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the whole biodegradation process. The similarity between A15 and AU might be due to the early sampling of A15 compared to other arctic samples.

5.4.4.1 Comparison of community structure and diversity based on pyrosequencing

PCR-DGGE results were also used to select samples for pyrosequencing analysis. The pyrosequencing output files were submitted to the NCBI Sequence Read Archive (accession number: SRA061588; BioProject number: PRJNA179559). The pyrosequencing run resulted in a total of 113 164 reads (reads ranged between 1 074 and 22 289). After filtering and noise removal, 2 157 unique sequences remained in the dataset, which were then clustered into 1 669 unique OTUs. OTU richness, assessed by parametric diversity estimates, was between 4 and 13 times greater than the total number of OTUs determined from the dataset, showing that the sequencing effort was not exhaustive (data not shown).

Table 5.4-2 Non-parametric diversity estimates: total OTUs from maximum linkage clustering at 3% distance using all clean reads; Chao1 estimate of minimum diversity; rarified number OTUs (the number of OTUs at minimum sampling depth, 2 259 reads as in AU); and Shannon index. Sample code: T = temperate, A = arctic, U = untreated, 0.5 = 0.5 °C, 4 = 4 °C, 8 = 8 °C, 15 = 15 °C.

Sample	Total OTUs	Chao1	Rarified OTUs	Shannon
TU	719	1450	278	3.19
T0.5	132	261	60	1.18
T4	81	140	26	1.03
T8	142	332	49	1.67
T15	116	236	72	1.87
AU	229	587	229	2.39
A0.5	89	239	59	2.04
A4	62	114	59	1.68
A8	57	137	53	1.25
A15	42	108	N/A	2.41

Bacterial diversity was highest in samples TU, AU, and A15 as shown by Shannon indices (Table 5.4-2). Rarefied number of OTUs and Shannon index were higher in

sample TU compared to AU showing greater diversity in the untreated temperate seawater. The numbers of rarefied OTUs and Shannon indices decreased during the biodegradation for both experiments except for A15. The lowest diversity was estimated in sample T4, sampled only 3 days after the half-life time at 4 °C.

The overall tree structure was different, however, some consistent patterns were also observed between hierarchical clustering of all OTUs (Figure 5.4-5) and the UPGMA tree based on PCR-DGGE results (Figure 5.4-4). For example, sample T4, T8 and T15 grouped together according to both analyses. Sample TU and AU formed a distinct group based on pyrosequencing data analysis, in contrast to the DGGE results where they clustered together with exposed samples. According to OTU composition, samples T0.5 and A15 showed more similarity to the rest of arctic exposed samples. Finally, the A0.5, A4 and A8 sample cluster was also observed, in agreement with DGGE analysis.
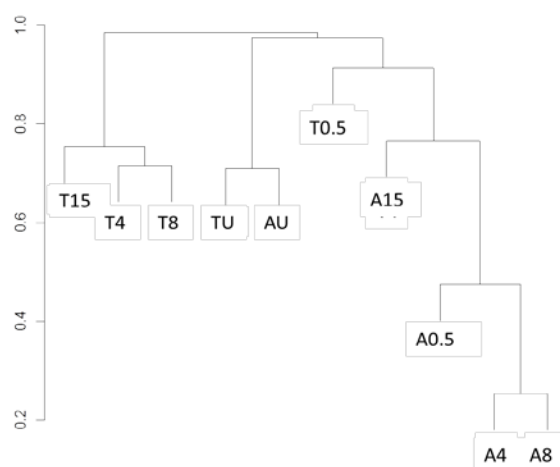
Figure 5.4-5 Hierarchical clustering of the ten samples based on relative abundance of all OTUs using Bray-Curtis distance measure and average linkage clustering. Sample code: T = temperate, A = arctic, U = untreated, 0.5 = 0.5 °C, 4 = 4 °C, 8 = 8 °C, 15 = 15 °C.

5.4.4.2 *Composition of initial seawater samples*

Relative taxon abundances revealed differences in the composition of samples TU and AU already at class level. More than 90 % of OTUs were classified at family level

(with two exceptions). Five abundant classes were observed. *Alphaproteobacteria* (37 % in TU, 70 % in AU), *Gammaproteobacteria* (42 % in TU, 17 % in AU), *Deltaproteobacteria* (3.6 % in TU and 0.4 % in AU). *Flavobacteria* (phylum *Bacteroidetes*) (5 % in TU and AU) and *Cytophagia* (0.16 % in TU, 4.4 % in AU). *Alphaproteobacteria* were dominated by *SAR11 clade* (32 % in TU and 64 % in AU) and *Gammaproteobacteria* by *Alteromonadales* (35 % in TU and 3 % in AU) and *Oceanospirillales* (6 % in TU and 11 % in AU). Within the *Alteromonadales* order, the genus *Colwellia* dominated the TU sample (34 %), while it was represented with only 1.4 % abundance in sample AU. OTUs classified as members of the *SAR11* clade were not classifiable at genus level, therefore the genus level composition of the most dominant family within *Alphaproteobacteria* was not revealed. Higher relative abundances of the genus *Balneatrix* and *Marinoscillum* were found in sample AU compared to TU. Whereas the *NS5 Marine group* of the family *Flavobacteriaceae* was represented by similar abundances in both samples (2 %).

5.4.4.3 *Correlating taxon composition with experimental factors*

UPGMA tree and taxon composition of all 10 samples at family rank is shown in Figure 5.4-6. Hierarchical clustering of all samples based on relative abundance of classified families showed that some taxa were characteristic of temperate and others of arctic exposed samples. *Vibrionaceae* and *Piscirickettsiaceae* were more abundant in samples T4, T8 and T15, whereas *Helicobacteraceae*, *Moritellaceae* and *Pseudoalteromonadaceae* were more abundant in samples A0.5, A4 and A8. *Flavobacteriaceae*, *Colwelliaceae* and *Oceanospirillaceae* also appeared to be important taxa involved in degradation. It is interesting to note that the structure of the UPGMA tree was not in agreement with the UPGMA tree constructed based on relative abundances of all OTUs, and it did not show the same structure as the tree determined from DGGE banding patterns. The genus level composition of all 8 mentioned families was determined and the most abundant genera were selected for further evaluation (Table 5.4-3). Comparison of the 10 microbial communities was carried out using PCA based on relative abundances of the ten most abundant genera (Figure 5.4-7). The first two factors of the PCA explained 60 % of the total variance.
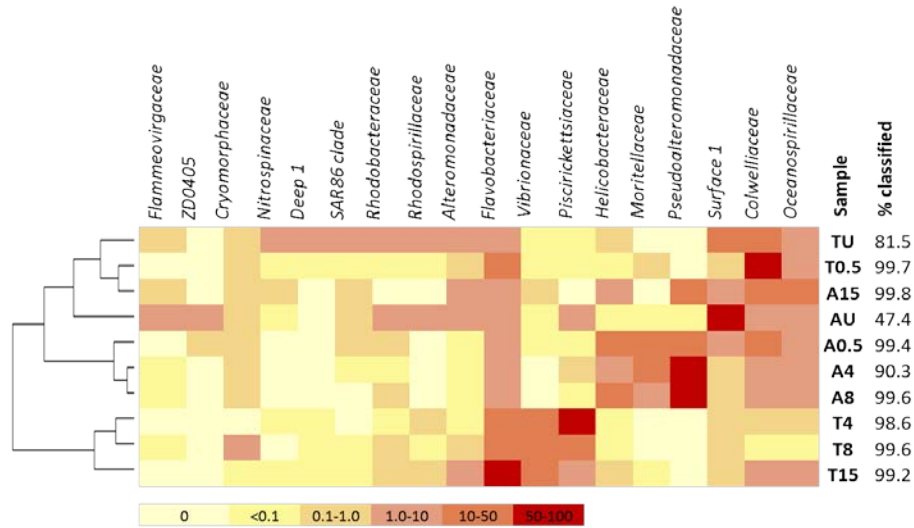
Figure 5.4-6 Heat map and hierarchical clustering (Bray-Curtis, UPGMA) of all samples based on relative abundances of the ten most abundant families (> 1 %).Classification percentage is shown for family rank (% classified). The colour code for the heat map is shown below the map,numbers indicate relative abundance range coloured. Sample code: T = temperate, A = arctic, U = untreated, 0.5 = 0.5 °C, 4 = 4 °C, 8 = 8 °C, 15 = 15 °C.

Table 5.4-3 Relative abundance of the ten most abundant genera in all ten samples. Sample code: T = temperate, A = arctic, U = untreated, 0.5 = 0.5 °C, 4 = 4 °C, 8 = 8 °C, 15 = 15 °C.

Genus	Relative abundance (%)									
	TU	T0.5	T4	T8	T15	AU	A0.5	A4	A8	A15
Amphritea	0.00	0.00	0.07	0.00	0.11	0.00	0.02	0.00	0.04	12.9
Arcobacter	0.22	0.07	0.03	0.01	0.35	0.09	18.7	7.21	13.5	2.45
Colwellia	33.0	76.4	0.30	0.03	8.93	1.15	13.32	1.99	7.61	49.8
Cycloclasticus	0.04	0.15	70.8	10.5	1.61	0.00	0.00	0.12	0.04	0.00
Maribacter	0.00	0.45	0.43	6.58	0.00	0.00	0.00	0.00	0.00	0.00
Moritella	0.00	0.19	0.00	0.00	0.02	0.04	22.31	13.28	2.16	0.00
Oleispira	0.00	0.95	0.00	0.00	0.02	0.49	0.43	0.35	0.31	3.27
Photobacterium	0.01	0.05	10.7	16.8	20.5	0.09	0.02	0.00	0.00	0.16
Polaribacter	0.12	10.6	12.8	6.61	51.0	0.09	4.72	7.01	1.04	3.76
Pseudoalteromonas	0.00	0.00	0.00	0.00	0.00	0.04	33.6	64.9	71.12	11.95

The two untreated samples (TU and AU) clustered together close to the middle of the PCA plot. All exposed samples diverged from the untreated ones into three distinct groups. As also observed in DGGE analysis and hierarchical clustering of all OTUs, samples A0.5, A4 and A8 appeared to be similar and T4, T8 and T15 grouped also together. The samples exposed to temperatures furthest from their respective *in situ* temperature (A15 and T0.5) grouped apart from all the other samples. This separation was mainly due to the high relative abundance of *Oleispira*, *Amphritea* and *Colwellia*. PCA also showed the genera that most contributed to clustering patterns. *Moritella*, *Arcobacter* and *Pseudoalteromonas* were characteristic of arctic samples, while *Polaribacter*, *Cycloclasticus*, *Maribacter* and *Photobacterium* were characteristic of temperate samples.
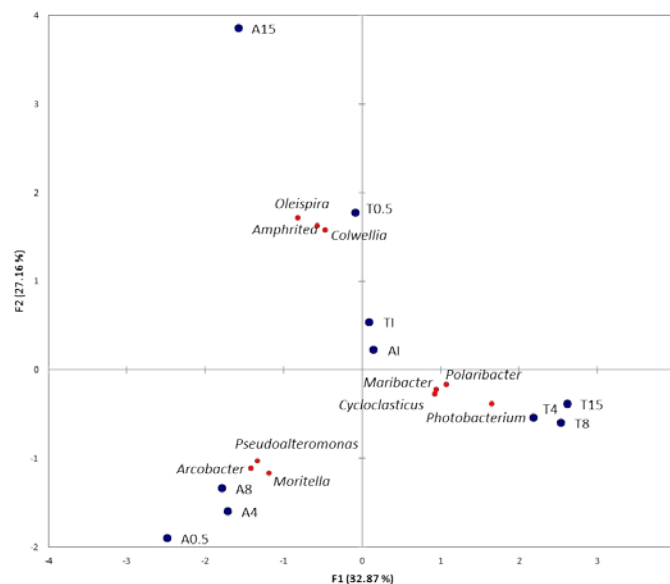
Figure 5.4-7 Principal component analysis of the ten most abundant OTUs (> 5 % relative abundance) classified at genus level. Sample code: T = temperate, A = arctic, U = untreated, 0.5 = 0.5 °C, 4 = 4 °C, 8 = 8 °C, 15 = 15 °C.

5.5 Discussion

In this study, oxygen consumption measurements were used to compare naphthalene biodegradation rates in seawater from two geographically and climatically different

marine environments (temperate and arctic). The selected methodology, OxiTop® measuring system, has been previously applied in only a few studies examining biological decomposition (Karhu et al., 2009). The selection of this method was based on the fact that it is convenient when dealing with highly volatile and poorly water soluble compounds (e.g. entirely automated measurement method carried out in a closed system) (Kuokkanen et al., 2004; Karhu et al., 2009).

Performing quantitative studies with naphthalene as the sole carbon source is challenging due to its low solubility in water, which also strongly depends on temperature (Gold and Rodriguez, 1988). Using a carrier solvent such as methanol or methylene chloride, as usually done (Ahn et al., 1998; Geiselbrecht et al,. 1998), was avoided in this study due to possible interference with oxygen consumption measurement. Unexpectedly, the concentration of naphthalene at the beginning of the experiment was not uniform for all test flasks. It is possible that the method for preparation of the stock solution was affected by some factors (e.g. non-dissolved crystals, adsorption to glassware, evaporation). In any case, differences in initial naphthalene concentrations have been observed not to influence the parameters selected for this study (i.e. lag time and k_1) (Bauer and Capone, 1985; Stewart et al., 1993).

5.5.1 Biodegradation rates

Biodegradation of HCs at low temperature (close to 0 °C) has been previously demonstrated in seawater from cold and permanently-cold regions (Minas et al., 1995; Michaud et al., 2004; Deppe et al., 2005; Brakstad and Bonaunet, 2006; Brakstad et al., 2008; Delille et al., 2009). In the present study, naphthalene degrading ability of temperate and arctic seawater samples was confirmed in the selected temperature range (0.5-15 °C).

Pseudo first order rate coefficients (k_1) of naphthalene degradation found in this study were higher than previously reported values for polluted estuary water (Castle et al., 2006) and pristine and contaminated fresh water (Heitkamp et al., 1987). Nutrients were not added to the test media in these two studies. This lack of nutrient addition could explain the lower rate coefficients observed by Castle et al. (2006) and

Heitkamp et al. (1987), as nutrient concentration is known to influence biodegradation rate of HCs (Delille et al., 2009). The aim of adding mineral salts and nutrients in this study was to ensure non-limiting conditions for biodegradation in different types of seawater samples to be able to compare their intrinsic degradation capability. The observed values were however, also higher than rate coefficients measured in a non-polluted Norwegian fjord, where nutrients were added (Brakstad and Bonaunet, 2006). Naphthalene degradation rate coefficients reported herein are therefore indicative of potential biodegradation capacity under natural conditions.

Naphthalene degradation k_1 values were higher than the k_1 of sodium-benzoate degradation in the arctic experiment. This phenomena is unusual considering that sodium-benzoate is an easy biodegradable compound, used as a positive control in biodegradability tests (OECD, 1992; Courtes et al., 1995). Moreover, these two compounds share the same metabolic pathway, with sodium-benzoate requiring less enzymatic reactions (Zeng and Essenberg, 2011; Feng and Ellis, 2012). Higher k_1 values of sodium-benzoate compared to naphthalene in arctic seawater cannot be explained up to our knowledge based on the parameters measured during the experiments.

In the present study, three times higher naphthalene degradation rate coefficients were found in arctic samples compared to temperate ones at all incubation temperatures. Higher relative activity observed in arctic samples suggests that arctic naphthalene degraders were well adapted to cold conditions, and capable of achieving similar k_1 values at *in situ* temperatures as temperate degraders. Microorganisms adapted to lower temperatures are physiologically different from those found in warmer climates (Methé et al., 2005). Psychrophilic sulphate reducing bacteria for example showed higher relative activity at low temperature in marine sediments compared to mesophilic counterparts (Arnosti et al., 1998; Knoblauch et al., 1999). However, no significant difference was observed in k_1 values of sodium-benzoate degradation between arctic and temperate seawater samples. Overall, these results indicate that biodegradation capacity in arctic seawater is not intrinsically lower compared to temperate seawater.

5.5.2   Temperature effect on biodegradation

Temperature strongly influenced biodegradation rates of naphthalene, as expected. The relationship between temperature and k_1 values was exponential as predicted by the Arrhenius model (Arrhenius, 1889). Metabolic rates in general change with temperature and this correlation has been expressed as universal activation energy (E_a) values. Various authors have proposed fixed values for implementation into ecological models (e.g. Metabolic Theory of Ecology). E_a values in this study were higher than the one suggested by Gillooly et al. (2001) and Brauer et al. (2009) and lower than the one from Price and Sowers (2004). This suggests that establishing a single value for all metabolic rates is not feasible. In comparison to other naphthalene biodegradation studies, the obtained E_a values were similar to those determined from naphthalene mineralization in intertidal sediments (Bauer and Capone, 1985) and in seawater (Brakstad and Bonaunet, 2006).

Regarding HC biodegradation, temperature dependence is usually described with a $Q_{10} = 2$ rule of thumb (Brakstad, 2008). In the present study, Q_{10} values were higher than the rule of thumb, confirming that this rule should be considered more as a range of values than a fixed number.

Applying the temperature compensation method implemented in the OSCAR model, $Q_{10} = 2$, to the present results, k_1 in arctic seawater was underestimated by 30 % when calculated from the measured temperate k_1 value. Replacing the adopted $Q_{10} = 2$ with the actual measured $Q_{10} = 3.5$ did not improve the estimation; in fact k_1 was underestimated even more (65 %). This demonstrates that temperature difference of these two environments alone did not predict biodegradation rates.

The temperature response was different for naphthalene and for sodium-benzoate, indicating that Q_{10} depends on the compound being degraded. This can have further implications in fate models, such as OSCAR, where the same Q_{10} value is used for all compound groups (Mark Reed, personal communication). Moreover, the temperature response of sodium-benzoate could only be described with two different Q_{10} values, one for low (0.5-4 °C) and one for high temperatures (4-15 °C). This is in agreement

with previous studies, where two linear fits were also required to describe the temperature response (Chablain et al., 1997).

Bacteria adapted to different ambient temperatures are expected to show distinct temperature responses due to molecular mechanisms underlying the short-term temperature response (D'Amico et al., 2006), e.g. mesophilic organisms have often been found to be more sensitive to temperature decreases compared to psychrophiles (Arnosti et al., 1998; Knoblauch et al., 1999; Robador et al., 2009). In the present study, temperate and arctic samples had similar Q_{10} values. This could be due to the selected temperature range not being broad enough to highlight differences.

5.5.3   Comparison of community compositions

Exposure to PAHs and crude oil has been shown to alter microbial community composition due to the selective pressure of the HCs being the sole source of carbon and energy (Brakstad et al., 2004; Castle et al., 2006). In this study, the DGGE banding patterns showed that initially dominating species disappeared and new ones emerged as a result of naphthalene addition.

The hierarchical clustering tree based on OTU composition showed that in general communities clustered together according to the origin of the seawater. There was only one exception: the 0.5 °C temperate sample grouped together with the arctic samples. Incubation of temperate seawater at 0.5 °C may have induced the growth of bacteria usually found in arctic seawater, showing that an extremely low incubation temperature can alter the community composition markedly (Brakstad and Bonaunet, 2006).

Although, both DGGE and pyrosequencing provided similar information, the latter revealed the fine scale differences among microbial communities. The use of the DGGE technique is therefore more suitable for screening purposes.

5.5.4   Taxon composition

Prokaryotic community composition in geographically different marine environments differs, and changes dynamically during seasons (Kirchman, 2010). In this study, the untreated prokaryotic communities of the arctic and temperate seawater samples were

in fact different, as revealed by DGGE and pyrosequencing analyses. Domination of *Alpha-* over *Gammaproteobacteria* was observed in the arctic seawater, in agreement with previous findings from Bano and Hollibaugh (2002). Moreover a gradient in species diversity increasing from the Poles towards the Equator is known to occur (Fuhrman et al., 2008). In this study, higher OTU richness was found in the temperate seawater samples compared to the arctic ones, as expected. As generally recognised, members of the *Roseobacter* clade are commonly found in high abundance (up to 25 %) in the sea surface of various geographical areas (Kirchman, 2010). However, in the North Sea coast near the sampling site of this study, lower relative abundance of the *Roseobacter* was found (Giebel et al., 2011). In the present study, *Roseobacter* were represented with less than 1% relative abundance in all untreated samples. The other major group of *Alphaproteobacteria* known to be widely distributed, the *SAR11* clade, were found in high abundance in both the untreated arctic and temperate seawater (Kirchman, 2010). Greater relative abundance of members of the genus *Colwellia* was found in temperate seawater compared to arctic. Most reported members of this genus are strictly psychrophilic and mainly isolated from cold habitats (Methé et al., 2005; Lauro et al., 2011). Nevertheless, seasonal dynamics of bacterial communities in temperate areas may explain the increased abundance of cold-adapted species, such as *Colwellia*, during the cold season (Fuhrman et al., 2006; Gilbert et al., 2012).

Community composition of naphthalene exposed seawater samples was characterised at the genus level. Few of the genera which became dominant during the degradation process were detected in the untreated samples by pyrosequencing. Other genera detected during the exposure were not identified in the untreated samples with the sequencing effort of this study. Possibly a deeper sequencing would be able to describe the starting conditions in more detail.

Two of the ten most abundant genera, *Cycloclasticus* and *Oleispira*, are known obligate HC degraders (Yakimov et al., 2007). Four other genera (*Colwellia, Maribacter, Pseudoalteromonas* and *Arcobacter*) have been identified as oil degraders in similar studies (Yakimov et al., 2004b; Brakstad and Bonaunet, 2006; McKew et al., 2007; Brakstad et al., 2008; Niepceron et al., 2009; Røberg et al.,

2011). The last four genera (*Amphritea*, *Photobacterium, Moritella* and *Polaribacter*) have not been reported before as dominant HC degraders in the marine environment.

5.6   Conclusions

Three times higher naphthalene degradation rate coefficients were found in the arctic seawater compared to temperate at each tested incubation temperature, confirming that psychrophilic bacteria can have higher relative activity compared to their mesophilic counterparts in marine microcosms. Moreover, temperate and arctic communities degraded naphthalene with similar k_1 at *in situ* temperatures calculated by using the obtained Q_{10} values. Due to protocol choices, these results are indicative of the potential biodegradation capacity of the two seawater samples. The obtained results suggest that biodegradation capacity in cold seawater is not necessarily inherently lower compared to temperate seawater.

Naphthalene biodegradation rates decreased with the same Q_{10} ratio in both temperate and arctic seawater samples. Applying the $Q_{10} = 2$ approach to the present results would have led to an underestimation of biological degradation in arctic samples, showing that the temperature difference of these two environments alone did not predict biodegradation rates.

The untreated temperate and arctic seawater communities were different as revealed by pyrosequencing.

The geographic origin of seawater affected the community composition of naphthalene exposed samples. *Polaribacter*, *Cycloclasticus*, *Maribacter* and *Photobacterium* genera were characteristic of naphthalene exposed temperate seawater, while *Moritella*, *Arcobacter* and *Pseudoalteromonas* were characteristic of naphthalene exposed arctic samples.

Chapter 6. Overall conclusions

Research work described in Chapter 3, 4, and 5 has been engaged in addressing the goals presented earlier (page 52). This section summarizes major conclusions regarding each main objective.

I. Crude oil biodegradation rate data obtained from the literature survey showed high variability of Q_{10} values (1.1-16.2) and did not support a choice of 2.0 for general temperature compensation. Hence, temperature response of crude oil biodegradation appeared not to follow the commonly used $Q_{10}=2$ rule of thumb and biodegradation rate predictions calculated by Q_{10} based temperature compensation are concluded to be ambiguous.

II. Choosing one universal Q_{10} value was neither feasible nor anticipated. Experimentally determined Q_{10} values were found to reflect indirect effects of temperature, such as the influence on HC bioavailability and number of active degraders. Hence, intrinsic temperature sensitivity of HC biodegradation can only be measured accurately if the interference of these factors is eliminated.

III. Static respirometry, using the OxiTop®-C system was found to be a suitable tool for comparative analysis of the influence of different sample handling conditions prior to biodegradation testing. Moreover, this method is suggested to be used for kinetic model development and validation, especially due to the high resolution of measurement data obtained and experimental capacity.

IV. The parameter most often reported for HC and crude oil biodegradation (i.e. pseudo first order rate coefficient, k_1) was not influenced by filtration and aging of seawater samples, while it was clearly affected by dilution. Therefore the OxiTop®-C system was found to be most sensitive toward the initial number of bacteria when used for comparing k_1 values.

V. Bacteria present in seawater from the Arctic Ocean were able to utilize naphthalene with a three times higher rate coefficient compared to bacteria from the North Sea. Hence, this study showed that bacteria inhabiting permanently cold environments can have higher relative activity at low temperatures compared to their mesophilic counterparts inhabiting temperate waters. Naphthalene biodegradation rate coefficients decreased at low temperature with similar Q_{10} ratio in both temperate and arctic seawater samples. This indicates that temperature sensitivity of the two geographically and climatically different seawater microbial communities was similar. When calculated at *in situ* temperatures, pseudo first order rate coefficients of temperate and arctic communities were comparable, suggesting that biodegradation capacity in cold marine environments may be similar to temperate areas. Hence, biodegradation can presumably be a significant weathering process in permanently cold seawater.

VI. Initial bacterial communities of temperate and arctic seawater samples were different, as revealed by pyrosequencing analysis, and geographic origin of seawater appeared to affect the community composition following naphthalene exposure. Differences in dominant bacteria, however, did not influence the observed biodegradation rate coefficients at *in situ* temperatures. This implies that inferring different biodegradation potential to different seawater types based on dissimilarity in their initial bacterial community composition is questionable.

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Chapter 7. Future research

Numerical models, applied to assess fate and effect of crude oil in seawater, currently take biodegradation into account according to a simplified model. Future studies need to focus on developing mechanistic models that can replace this approach. Mechanistic models would also be beneficial for the analysis and interpretation of biodegradation test results. A common framework of data analysis could enhance our understanding of the mechanisms underlying effects of different factors influencing observed biodegradation kinetics (e.g. nutrient amendment, bacterial diversity and abundance, dispersants and temperature).

Bioavailability of crude oil is possibly one of the most important characteristic that affects biodegradation rates, especially under cold conditions. There are several research gaps related to this issue, covering areas such as the mechanisms microorganisms access biodegradable compounds of oil spilled in seawater, toxicity or inhibition effects of dissolved oil components, and the influence of oil composition on these. In relation to bioavailability, fundamental understanding of transport mechanisms of hydrocarbons through cell membranes and cell walls are currently still unclear and represent an area for further research.

Available biodegradation rate data is difficult to compare due to the several experimental variables that differ between studies. One important factor is the measurement method. Static respirometry is a promising technique which can possibly be standardized and used to obtain comparable sets of data. Exploring methodological advantages and limitations is necessary.

This study showed that temperature compensation based biodegradation rate predictions are ambiguous. Implementing mechanistic models could circumvent this issue, making it unnecessary to determine a "better" Q_{10} value as rule of thumb. Achieving that, however, requires knowledge of inherent biodegradation capacity and growth rate of populations from different marine microbial communities. Certainly more research is required in this particular area. Though a vast diversity of HC degraders has been isolated, quantitative and comparative analyses of the oil

biodegradation rates of degraders from geographically and climatically different regions are today lacking. Future studies should aim to obtain these types of data.

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