THE HYDROCARBON BIODEGRADATION POTENTIAL OF FAROE-SHETLAND CHANNEL BACTERIOPLANKTON

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ABSTRACT

The Faroe-Shetland Channel (FSC) is an important gateway for dynamic water exchange between the North Atlantic Ocean and the Nordic Seas. In recent years it has also become a frontier for deep-water oil exploration and petroleum production, which has raised the risk of oil pollution to local ecosystems and adjacent waterways. In order to better understand the factors that influence the biodegradation of spilled petroleum, a prerequisite has been recognized to elucidate the complex dynamics of microbial communities and their relationships to their ecosystem. This research project was a pioneering attempt to investigate the FSC's microbial community composition, its response and potential to degrade crude oil hydrocarbons under the prevailing regional temperature conditions. Three strategies were used to investigate this. Firstly, high throughput sequencing and 16S rRNA gene-based community profiling techniques were utilized to explore the spatiotemporal patterns of the FSC bacterioplankton. Monitoring proceeded over a period of 2 years and interrogated the multiple water masses flowing through the region producing 2 contrasting water cores: Atlantic (surface) and Nordic (subsurface). Results revealed microbial profiles more distinguishable based on water cores (rather than individual water masses) and seasonal variability patterns within each core. Secondly, the response of the microbial communities to crude oil was investigated in laboratory-based microcosms. Microbial communities from all water masses exhibited hydrocarbon biodegradation activity at average FSC temperatures (4°C), albeit with markedly delayed and potentially slower response in comparison to those exposed to moderate control temperatures (20°C). A collection of bacterial isolates, comprising of 230 FSC strains with putative hydrocarbonoclastic activity was created, which included psychrotolerant members belonging to the genera *Marinobacter, Pseudoalteromonas, Cycloclasticus, Halomonas, Thalassolituus* and *Glaciecola.* Lastly, a sophisticated molecular technique called DNA-based stable isotope probing (DNA-SIP) was used to directly target and identify hydrocarbon-degrading taxa that may not be easily amenable to cultivation. Using DNA-SIP, hydrocarbonoclastic FSC strains affiliated with the genera *Phaeobacter* and *Lentibacter* were identified, along with strains affiliated with known hydrocarbon-degraders from the genera *Thalassolituus, Alcanivorax, Oleispira, Glaciecola, Marinobacter* and *Cycloclasticus*. Correlating the findings from all three experiments, revealed that ~41% of the baseline FSC microbial community constituted bacteria affiliated to genera with hydrocarbon-degrading capacities. Their response to the

presence of hydrocarbons, however, appeared to be largely influenced by temperature. This work is the first to establish a microbial baseline for the FSC and to investigate the microbial repose to crude oil in the water column of the region. Results are expected to contribute to the development of biotechnologies and oil-spill mitigation strategies tailored for the FSC region in the event of an oil spill.

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DEDICATION

This thesis is dedicated to my grandmother Radka Yanakieva, who has always been my inspiration, motivation and strength.

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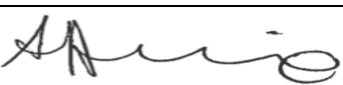| Name*:*	Angelina Angelova		
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GLOSSARY

AWC	Atlantic Water Core
CRO	Crude oil
CRO-EE	Crude oil Exposure Experiment
CsCl	Caesium Chloride
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
DNA-SIP	DNA-based Stable Isotope Probing
EPS	Extracellular polymeric substances
FSC	Faroe-Shetland Channel
GHCB	Generalist hydrocarbonoclastic bacteria
HC	Hydrocarbon
HCB	Hydrocarbonoclastic bacteria
HEX	Hexadecane
MEIW	Modified East Icelandic Water
MNAW	Modified North Atlantic Water
NAW	North Atlantic Water
NGS	Next Generation Sequencing
NSAIW	Norwegian Sea Arctic Intermediate Water
NSDW	Norwegian Sea Deep Water
NWC	Nordic Water Core
OHCB	Obligate hydrocarbonoclastic bacteria
OTU	Operational Taxonomic Unit
PAH	Polyaromatic hydrocarbons
PCR	Polymerase Chain Reaction
PHE	Phenanthrene
qPCR	Quantitative Polymerase Chain Reaction
rRNA	Ribosomal ribonucleic acid
SIP	Stable Isotope Probing
SW	Seawater

CHAPTER 1: INTRODUCTION

1.1 Oceanography of the Faroe-Shetland Channel

The Faroe-Shetland Channel (FSC) is a rift basin of depth between 100 to 1600 m, situated to the north-west of Scotland between the Scottish Continental Shelf and the Faroe plateau at 0–6°W, 60–63°N (Narayanaswamy *et al.*, 2010). The Channel is an important component of the 'great ocean conveyer belt' as it is one of three gateways of water exchange between the North Atlantic and the Nordic seas (Norwegian and Greenland Seas): The Faroe-Shetland Channel, The Island-Faroe Ridge and the Denmark Straight; as part of the 'great ocean conveyer belt' (Berx, 2012). It is generally accepted that the FSC has 5 uniquely defined water masses – two different Atlantic water masses flowing on top of three deep-sea Nordic water masses (Hansen and Østerhus, 2000). At the surface, the North Atlantic Water mass (NAW) dominates the Scottish side of the FSC basin (southeast) and is associated with the salt and temperature maximum in the region (Hansen and Østerhus, 2000; Berx, 2012). The Faroese slope side (northwest) supports the other surface water mass coming from the North Atlantic – the Modified North Atlantic Water mass (MNAW), that is similar (only slightly lower) in salinity and temperature to the NAW. MNAW passes on the northern side of the Faroe Islands before turning back to enter the FSC through the north (Hansen and Østerhus, 2000; Berx, 2012). These two surface water masses (<600 m) have different routes and directions around the FSC, but are both comprised of North Atlantic water, and therefore comprise the Atlantic Water core (AWC) of the region. Below 600 m, between the Scottish slope and Faroe plateau, flow the contrasting water masses of the FSC, which originate from the Nordic seas and are characterized by their comparatively lower salinity and temperature properties. These water masses are the Modified East Icelandic Water (MEIW), Norwegian Sea Artic Intermediate Water (NSAIW) and the Norwegian Sea Deep Water (NSDW) (Hansen and Østerhus, 2000; Berx, 2012). Collectively those three water masses comprise Nordic water core (NWC) of the region. Due to the contrast in water mass characteristics, a dynamic mixing zone is generated at depths ~ 350 - 650m where the two major water cores meet. The mixing zone is distinguished by rapid changes in temperature and salinity (Narayanaswamy *et al.*, 2010; Berx, 2012). All masses are distinguished by their characteristic path through the channel, as well as by their temperature and salinity profiles – refer to Table 1.1 and Figure 1.1. The FSC is among the most consistently studied regions in the ocean, with systematic hydrographic and oceanographic observations proceeding for

more than a century (Figure 1.2) (Berx, 2012). The long term oceanographic monitoring of the FSC has revealed high seasonal and inter-annual variability, which also contribute to the dynamics and complexity of the region (Berx, 2012). The unusual temperature gradient, produces a heterogeneous habitat supporting a diverse array of benthic marine life (Jones *et al.*, 2007; Narayanaswamy *et al.*, 2010).
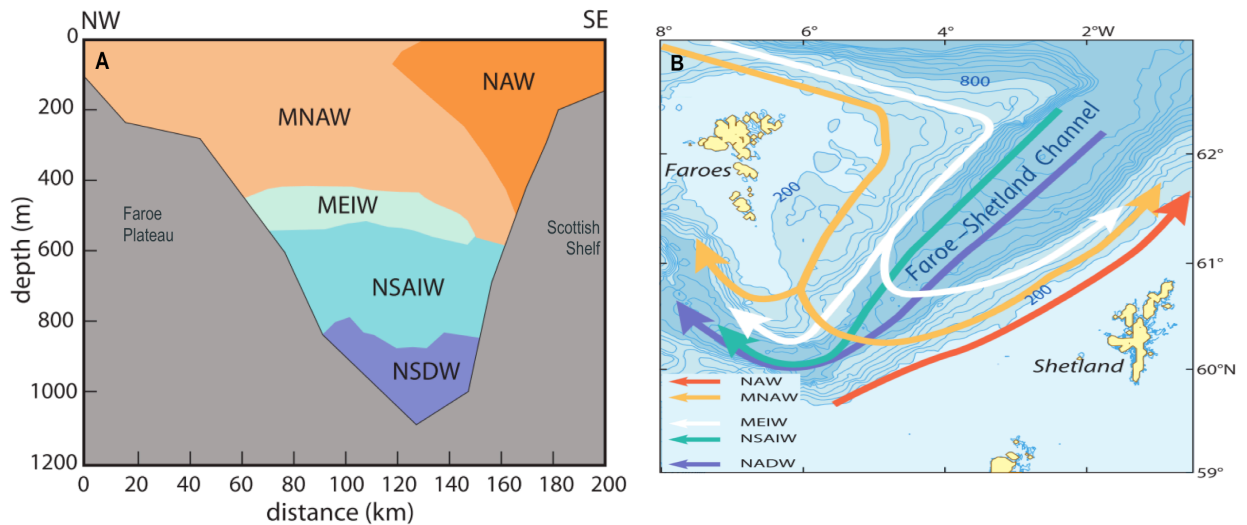
Figure 1.1. Schematic representations of the FSC and associated water masses. A. FSC water column vertical transect. Orange palette of colours represents the water masses belonging to the Atlantic Water Core (AWC). Blue palette of colours – the Nordic Water Core (NWC). B. Areal representation of the water mass pathways through the FSC. Figures adapted from Berx (2012)**.**

Table 1.1. Water masses in the FSC and their defining properties (adapted from Berx, 2012)

Water core	Water Mass Name (Acronym)	Salinity Range	T°C Range	Depth Range
Atlantic Water Core (AWC)	North Atlantic Water (NAW)	35.35 – 35.45	> 9.5°C	0-300m Shetland Shelf
	Modified North Atlantic Water (MNAW)	35.1 – 35.3	7.0 – 8.5°C	0-500m Faroe Plateau
Nordic Water Core (NWC)	Modified East Icelandic Water (MEIW)	34.7-34.90	1.0 – 3.0°C	400-600m Faroe Plateau
	Norwegian Sea Arctic Intermediate Water (NSAIW)	34.87 – 34.90	-0.5 – 0.5°C	600-900m
	Norwegian Sea Deep Water (NSDW)	34.91	< -0.5°C	900-1200m
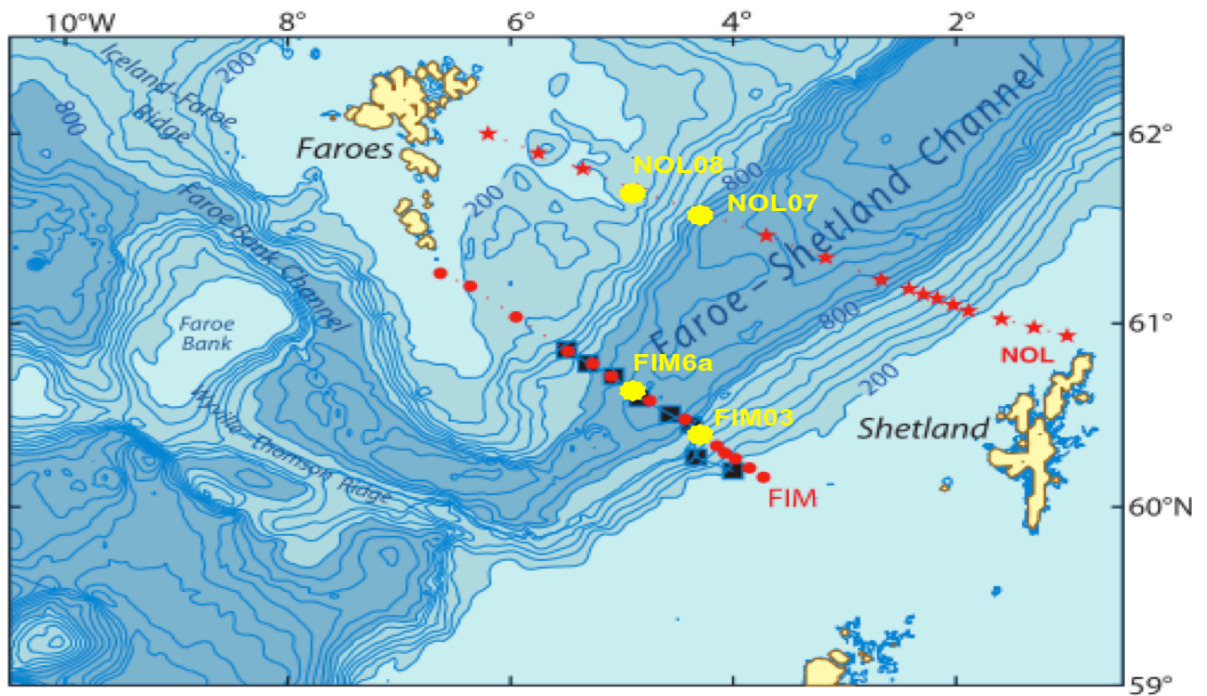
Figure 1.2. A bathymetry map of the FSC with marked transient lines and stations undergoing systematic monitoring. Red dots indicate the Fair Isle-Munken line (FIM) and red stars indicate the Nolso-Flugga line (NOL). Mooring locaitons are marked in black squares. Yellow stars distinguish the sampling locations for the current study. Figure adapted from Berx (2012)

1.2 Crude oil in the marine environment

1.2.1 Natural oil seeps

Although oil leaking into the environment has the reputation of being a disastrous human-mediated event, liquid and gaseous hydrocarbons have been entering the environment for millennia due to natural geological processes (Schmidt-Etkin, 2011). When oil is discharged into the environment, the release is usually sporadic and diffuse, yet occasionally larger releases may cause noticeable environmental impact (Schmidt-Etkin, 2011; Joye *et al.*, 2014). It is estimated that about 47% of all crude oil entering the marine environment worldwide is from natural oil seeps (equates ~600,000 tons annually (GESAMP, 2006)), whereas 53% come from anthropogenic sources (Kvenvolden and Cooper, 2003). However, such assessments have been argued to be based not on actual measurements, but on the estimation of Wilson *et al.* from 1974 conducted primarily on seepage rates from California

and western Canada (Schmidt-Etkin, 2011; Wilson *et al.*, 1974). Nonetheless, these estimates are still widely used today (Schmidt-Etkin, 2011).

Natural seepages of crude oil (a.k.a. cold seeps) appear most commonly along continental margins, especially around the Gulf of Mexico, the Atlantic Ocean, West Africa and the Mediterranean Sea (Tyler *et al.*, 2002; German *et al.*, 2011). These regions are defined by high tectonic activity where the thickness of sedimentary rocks exceeds a certain minimum (GESAMP, 2006). The UK Continental Shelf, however, is not recognized as one such region. Although a few cold seeps have been identified by the petroleum industry along the UK Continental Shelf, they are still considered very rare in this margin (Judd *et al.*, 1997). Despite the presence of numerous hydrocarbon accumulations in the FSC, none of them appear to naturally leak from under the extensive and thick layers of basaltic rock covering them (Austin *et al.*, 2014; German *et al.*, 2011).

1.2.2 Anthropogenic oil sources

Oil from human activities enters the marine environment through oil spills, intentional or accidental discharges from oil tankers, oil refineries, production platforms as well as terrestrial sources such as sewage treatment plants and urban runoff (Blackburn *et al.*, 2014). Since petrol prospecting commenced back in the 1800s, it has flourished through terrestrial, marine (coastal and offshore) and Arctic environments. With it has emerged a significant increase in the risk of accidental oil spills into the environment. Some historic marine disasters include the *Tsesis* oil spill of 1977 in the Baltic Sea, the *Ixtoc-I* of 1979 in the Gulf of Mexico, the *Piper Alpha* of 1988 in the North Sea, the *Exxon Valdez* of 1989 in Alaska, the Gulf War oil spill of 1991 in the Arabian Gulf, and the *Prestige* oil spill of 2002 in Spain. The most recent major spill was the *Deepwater Horizon* disaster that occurred on April 20, 2010, in the Gulf of Mexico (Pallardy, 2017). It was due to a massive build up of gas from the Macondo wellhead which eventually resulted in an explosion on the rig (Pallardy, 2017). It was recorded as not only one of the largest accidental offshore spills in recorded history, but has been distinguished for producing an unprecedented, massive and long-lasting oil plume at a depth of ~1000-1300 m (Camilli *et al.*, 2010; Deepwater Horizon Natural Resource Damage Assessment Trustees, 2016; Joye *et al.*, 2014). It generated a focused discharge of an estimated 5 million barrels (ca. 700,000 tonnes) of crude oil (Crone and

Tolstoy, 2010; Joye *et al.*, 2014) in addition to 500,000 tonnes of natural gas (Joye *et al.*, 2014) into the Gulf of Mexico. An estimated one quarter of the spilled oil was collected or burned by contingency efforts, whereas much of the rest of the spilled oil partitioned within the water column, sediment, shoreline and the atmosphere (Deepwater Horizon Natural Resource Damage Assessment Trustees, 2016). It proved to be a major ecological disaster, significantly affecting coastal and marine ecosystems, as well as the economies that depend on them (Beyer *et al.*, 2016; McCrea-Strub *et al.*, 2011). Since 2010, the catastrophe has been well documented by the media and a vast array of research has been performed in order to increase our understanding in oil prospecting safety as well as the economical and ecological affects of anthropogenic oil spills.

The UK Continental Shelf is the largest remaining exploration region for the development of the oil and gas industries (Austin *et al.*, 2014). Since the 1990s, the Faroe Shetland Channel (as a part of the UK Continental Shelf, West of Shetland area) has become a frontier for deepwater oil and gas exploration to the oil and gas industry (Smallwood and Kirk, 2005). Numerous gas and oil fields have been discovered in rapid succession over the past 30 years including Clair, Victory, Laxfornd, Strathmore, Solan, Foinaven, Schiehallion, Suilven, Cambo, Rosebank (main), Tormore, Lancaster, Glenlivet, Tornado, Edradour (Figure 1.3; Austin et al., 2014). Yet the region is still considered 'underdeveloped' as only three of the fields are in production (Foinaven, Schiehallion and Clair), primarily because they are in shallower and more easily accessible waters (down to 600 m), which translates to having more easily attainable value (Austin *et al.*, 2014). Due to technological advancements since the turn of the century, multiple more fields are currently being developed (e.g. the Lagan-Tormore field) (Austin *et al.*, 2014).
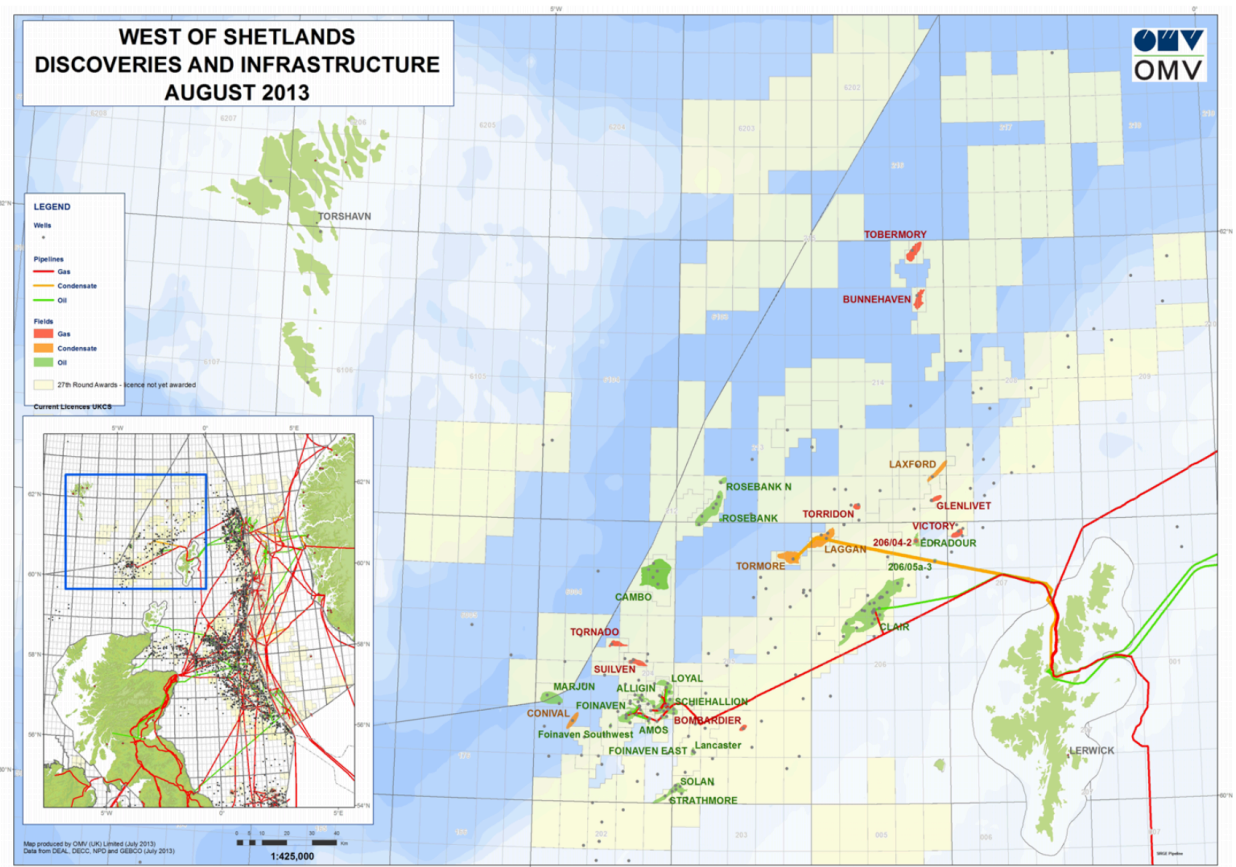
Figure 1.3. West of Shetland infrastructure map showing sites of significant gas and oil discoveries (Austin *et al.*, 2014)

1.3 Composition of crude oil

Crude oil is a complex mixture of hydrocarbons, categorized into four main chemical groups (a.k.a. SARA fractions; Figure 1.4) 1) saturates (aliphatic hydrocarbons which can vary based on degree of saturation and branching); 2) aromatics (mono- and poly- aromatics); 3) resins (terpenes and terpenoids); and 4) asphaltenes (high-molecular-weight polar compounds highly variable in structure) (NRC, 2003; Demirbas and Taylan, 2016).
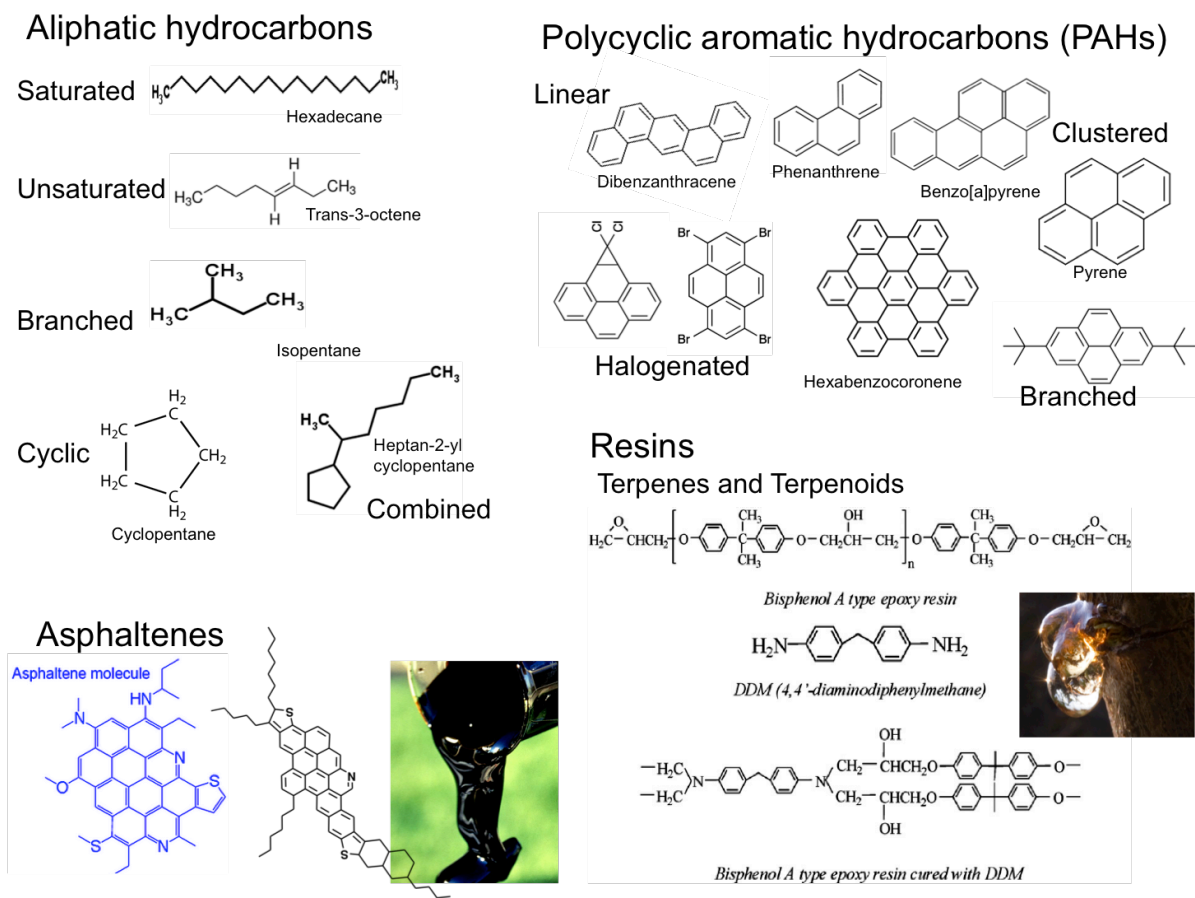
Figure 1.4. SARA fractions, example chemical components within each fraction, their chemical structure and variations

1.3.1 Aliphatics

Aliphatic hydrocarbons (a.k.a. paraffins) are straight chain, branched or cyclic in structure and can be saturated or unsaturated based on the presence of π bonds (double or triple bonding) between carbons, as long as the bonding does not form aromatic ring structures (McNaught and Wilkinson, 2014). Aliphatic hydrocarbons can range from 15 to 60% of the crude oil composition by weight (Hyne, 2012), and can vary significantly in molecular weight, shape and saturation level (NRC, 2003). The lower molecular-weight aliphatic hydrocarbons (e.g. propane, butane, pentane) are highly volatile and constitute the major components of natural gas (NRC, 2003). Medium to high molecular-weight paraffins are liquid compounds exhibiting various degrees of hydrophobicity, depending on their level of saturation and branching (NRC, 2003). Despite their physical behaviour, most of these crude oil components are sufficiently bioavailable and are usually the first ones to succumb to microbial attack (NRC, 2003).

1.3.2 Aromatics

Aromatics (arenes) contain one to multiple benzenoid rings within their structure and can account for 3 to 30% of all hydrocarbons in crude oil (Hyne, 2012). Due to resonance/mesomeric stabilization, the aromatic hydrocarbons tend to be more stable than the conjugation of double bonds alone (Wilkes and Schwarzbauer, 2010). This can make them very chemically stable, hence persistent in the environment (NRC, 2003). Aromatic hydrocarbons may exhibit branching and saturation variations the side chains of their benzene rings (substituents) (Hyne, 2012). Monoaromatic hydrocarbons are low molecular-weight compounds that may be volatile and may confer the crude oil with a fruity smell (Hyne, 2012). Typical examples are benzene, toluene, ethylbenzene and xylene, which are highly volatile monoaromatic hydrocarbons with low molecular weight, collectively known as the BTEX compounds (NRC, 2003). Polyaromatic hydrocarbons (PAHs) contain multiple fused aromatic rings arranged in linear, angular or clustered form (Kerr *et al.*, 1999). The fused rings increase their degree of aromaticity (Hyne, 2012) which increases their molecular stability (Hyne, 2012). Due to their high solubility in lipids, PAHs are readily absorbed into fatty tissue of animals where they exhibit toxic, mutagenic and/or carcinogenic effects (Samanta *et al.*, 2002).

1.3.3 Resins

Resins are found between 1 to 30% in crude oil (e.g. pyridines, quinolines, carbazols, sulfoxides, amides); these are polar multinuclear heteroatomic molecules containing fused aromatic rings (like PAHs), branched paraffins and cyclic and polar substituents (Demirbas and Taylan, 2016; Fingas, 2011). Resin molecules can also contain alternative chemical elements such as metals, sulphur, nitrogen and oxygen (Demirbas and Taylan, 2016; Fingas, 2011). The distinguishing characteristic of resins (differentiating them from asphaltenes) is that they are more soluble in mid-sized alkanes (C4-C7 chain alkanes) than larger or smaller alkanes (Demirbas and Taylan, 2016; Liao *et al.*, 2009). Like asphaltenes (see below), resins have high aromatic structure, however their aromaticity is reduced in comparison to PAHs (Demirbas and Taylan, 2016), probably due to the destabilizing effect of the large polar substituents. Resins are largely responsible for oil adhesive behaviour (Fingas, 2011) and are considered notoriously resistant to microbial alterations (Liao *et al.*, 2009).

1.3.4 Asphaltenes

Asphaltenes are the fraction of crude oil polar components with highest molecular weights, most complex and variable molecular structure (Demirbas and Taylan, 2016; NRC, 2003; Fingas, 2011). Again, the molecular structure consists of multiple aromatic nuclei, fused, condensed or connected with very large and diverse substituents (Liao *et al.*, 2009). They are distinguished from resins by their large molecular size and insolubility in midsize alkane solvents (e.g. *n*-heptane) (Demirbas and Taylan, 2016; Liao *et al.*, 2009; Fingas, 2011). Asphaltenes have the lowest commercial value of the crude oil constituents, and therefore are used as the main component in asphalt (Fingas, 2011). Asphaltenes have only remnant presence in crude oils, which on average is about 6% (Hyne, 2012).

Resins and asphaltenes are considered the most environmentally persistent components of crude oil, composing a large part of crude oil remnants such as tarballs (unresolved complex mixture), which are exceptionally resistant to microbial alterations (Liao *et al.*, 2009; NRC, 2003; Fingas, 2011). This is probably due to their complex molecular structure making them bulky, amphoteric and amphipathic substrates, with very low bio-availability. However, some low rates of biodegradation have been reported to occur on the resin or asphaltenes fraction of crude oils (Liao *et al.*, 2009).

1.3.5 General properties of crude oils

Crude oil is classified as 'light' or 'heavy' based on the ratio of these four chemical groups (the SARA fractions) (Fan *et al.*, 2002). Light oils, which are more fluid and transparent (e.g. gasoline), tend to be more valuable as they are composed predominately of saturated (55-90%) and aromatic (10-36%) hydrocarbons (Hyne, 2012; Fingas, 2011). Resins and asphaltenes are largely responsible for the dark reddish-brown or black colouration that is associated with crude oil (Demirbas and Taylan, 2016). Therefore, heavy oils, which are commonly richer in resins (up to 25%) and asphaltenes (up to 20%), are darker and more viscous, and contain considerably higher amounts of metals and sulphur (Hyne, 2012; Fingas, 2011). Heavy oils form from light oils after a period of weathering whereby abiotic and biotic influences can reduce the saturated and aromatic fractions, resulting in higher levels of resins and asphaltenes in the oil (Head *et al.*, 2006; Liao *et al.*, 2009). Oil classification into 'light' or 'heavy' is based on their degree of viscosity relative to water, and

is defined as the API gravity index (Fingas, 2011). Light oils, which are commercially more valuable as they are high in aliphatic and aromatic hydrocarbons, generally have low viscosity as denoted by high API gravity values above 35°. Heavy oils are easily recognized by their relatively high viscosity and descriptive low API gravity values of below 25°. Intermediate oils range from API 25-35° (Hollebone, 2011; Fingas, 2011). In the FSC, the most productive oil fields are Foinaven, Schiehallion and Clair, which are estimated to contain 250-500 million barrels of intermediate oil with API gravity of 24-28° (Scotchman *et al.*, 1998).

1.4 Fate of crude oil upon its entry into the marine environment

Once crude oil enters the marine environment, any number of environmental factors will ensue (physical, chemical, biological), which produce alterations in its physicochemical fingerprint, behaviour, fate and environmental impact (Hollebone, 2015; Tarr *et al.*, 2016; Brakstad *et al.*, 2010; Fingas, 2011). Factors that can contribute to the weathering of oil include temperature, salinity, pressure (at depth), wave/mixing energy, redox potential (of the environment), presence/absence of oxygen, nutrients and light. The type and amount of released oil, including the ratio and characteristics of its SARA fractions (e.g. molecular weight, polarity, structural complexity) will also influence the weathering process (Tarr *et al.*, 2016; Fingas, 2011; Bej *et al.*, 2010; Brakstad *et al.*, 2010). Weathering is also influenced by transformational (photooxidation and microbial degradation) and migratory/transference processes (evaporation, dissolution, sedimentation, aggregation, dispersion) (Hollebone, 2015; Tarr *et al.*, 2016; Karant *et al.*, 1999; Head *et al.*, 2006; Fingas, 2011). The extent and progression of weathering and removal of oil can also be affected (or facilitated) by human interventions, such as burning, the application of dispersants or fertilizers (Tarr *et al.*, 2016).

Depending on which of the above factors and processes are at work, they can have varying effects on the composition of crude oil and the rate and extend to which it weathers (Brakstad *et al.*, 2010; Fingas, 2011). For example, the fraction of low-molecular-weight and volatile compounds (paraffins/*n*-alkanes and mono-aromatics) are more easily susceptible to evaporation (Colwell, 1977; Fingas, 2011) and could potentially partition into the atmosphere within hours (Tarr *et al.*, 2016). Compounds with active groups, hydrophilic properties or

unbranched carbon chains would undergo rapid degradation (within days) that is largely driven by microbial processes (McFarlin *et al.*, 2014; Hamme *et al.*, 2003; Okoh, 2006; Atlas, 1995; Dubinsky *et al.*, 2013). Aromatic compounds (e.g. PAHs) are most susceptible to photooxidation, which can proceed over months (Dutta and Harayama, 2000; Bacosa *et al.*, 2015), depending in part on their molecular weight, the prevailing light and oxygen conditions, as well as structural complexity of the compounds (Tarr *et al.*, 2016; Dubinsky *et al.*, 2013; Bacosa *et al.*, 2015).

Oil components that undergo transformation by one or more weathering process can subsequently become susceptible to a different set of weathering processes. For example, aromatic compounds that have undergone photooxidation usually transform into polar compounds, which are then more easily susceptible to bacterial or chemical oxidation (Dutta and Harayama, 2000; Bacosa *et al.*, 2015). Furthermore, soluble alkanes and smaller aromatic compounds are continuously leached (liquid-liquid extraction) from oil droplets (migratory effect) into the seawater (Tarr *et al.*, 2016) where they then become more biologically available for biodegradation by microorganisms, absorption, adsorption and/or uptake by higher organisms and inert materials (e.g. plastics). Over time the less dense, lower molecular weight, soluble and bioavailable hydrocarbons become removed from the environment, leaving behind the more recalcitrant and higher molecular weight fraction of oil components that may eventually aggregate to form tarballs, surface residue balls and mats (Tarr *et al.*, 2016; Leahy and Colwell, 1990). These 'heavier' oil residues include highly branched aliphatics and aromatics, as well as asphaltenes and resins (Brakstad *et al.*, 2010). With sorption to settling particles in the water column (e.g. marine snow, faecal pellets or sediment), the heavy oil residues bioaccumulate and persist in the environment for prolonged periods of time, sink, surface, emulsify or undergo long-range transportation within the marine environment (Brakstad *et al.*, 2010; Leahy and Colwell, 1990; Mason *et al.*, 2014).

In a study of Main *et al.* (2016), oceanographic general circulation modelling and a particle tracking algorithm was used to assess the temporal variability of oil-plume distribution from a randomly selected FSC deep-sea oil well (namely Lagavulin) after a hypothetical blowout at various potential depths. Although not a forecast, results from the model indicated that the transport of the plume, the distance it would travel and its persistence would be significantly affected by oil injection depth. An oil spill produced in near-surface Atlantic core waters

was theorized to transfer northward towards the Arctic Ocean, specifically into Norwegian and Barent Seas. Conversely, an oil plume released at near-seabed depths was anticipated to be carried by the Nordic core towards Icelandic and Greenlandic waters, potentially reaching as far as Labrador Sea and Newfoundland (Main *et al.*, 2016). Over a longer time period, an oil plume released in shallower waters (≤200m depth) of the FSC was shown to be transported the furthest, and the deep-water plumes (≥600m depth) were shown to persist the longest (Main *et al.*, 2016). This study demonstrated that any major oil spill in the FSC would be uncontainable to the vicinity of the spill site, but would likely be to spread over long distances (as a subsurface plume), and cause potential adverse global impact.

1.5 Microbial degradation of hydrocarbons in the ocean

Microbial degradation is one of the most effective processes in the removal of petrochemical pollutants that enter the marine environment (Colwell, 1977; Dutta and Harayama, 2000; Das and Chandran, 2011; Heipieper and Martínez, 2010). Considering the wide variety of compounds and factors that contribute to the degradation and ultimate fate of oil hydrocarbons in the marine environment, the microbial processes involved tend to be complex and proceed at different rates for the different types of crude oil constituents. Generally, under aerobic conditions this rate variation creates an illusion of sequential degradation, which appear to follow the following biodegradation pattern: *n*-alkanes (fastest degradation rates) > branched alkanes > branched alkenes > monoaromatics > cyclic alkanes > PAHs >> resins > asphaltenes (slowest degradation rates) (Wang and Fingas, 2003; Huesemann, 1995; Gros *et al.*, 2014; Perry, 1977; Das and Chandran, 2011; Head *et al.*, 2006; Dubinsky *et al.*, 2013).

1.5.1 Factors influencing microbial degradation

Three types of factors appear to contribute a significant role to the biodegradation rates of crude oil: environmental, biological and chemical (molecular) factors. Environmental factors include temperature, pressure, presence of oxygen and nutrients (nitrates NO_3^-, phosphates PO_4^{3-}, iron Fe) (Atlas and Hazen, 2011). Significant chemical factors (referred to as molecular factors in the text for specificity) are molecular size of the hydrocarbons, their solubility/hydrophobicity and structural complexity (Atlas and Hazen, 2011; Head *et al.*, 2006). Biological factors include viral lysis and predator grazing (protozoans, zooplankton,

etc.) (Head *et al.*, 2006; Beaudoin *et al.*, 2014). Because of their ultimate effects and better-understood role on crude oil biodegradation, only environmental factors will be discussed in the upcoming section. The distinct hydrodynamic regime and environmental conditions of the FSC, predicate for unique communities of hydrocarbon-degrading bacteria in terms of phylogeny, metabolic and/or functional potential.

1.5.1.1 Temperature

Microbial degradation of crude oil constituents relies significantly on their bioavailability as well as the extent of weathering (Bej *et al.*, 2010). Considering that temperature substantially affects some of the migratory processes during weathering (evaporation, emulsification, dissolution, dispersion), microbial degradation will also be equivalently affected. Some crude oil components are less likely to evaporate or become dissolved into the water phase at lower temperatures, resulting in a different crude oil mixture to be subjected to biodegradation (Brakstad *et al.*, 2010). Therefore, microbial degradation at low temperatures will proceed at different rates (usually slower) than at tropical regions. Furthermore, low temperature tends to negatively affect cellular growth, division and metabolic rates of bacteria (Gibbs *et al.*, 1975; Colwell, 1977). Hence, microbial degradation would be carried out at different rates by different microbial assemblages, depending on the temperature tolerance of the members in the community. Despite the typically inhibitory effects of lower temperatures, microbial degradation has been shown to occur in cold environments, often carried out by psychrophilic and psychrotolerant bacteria (Atlas, 1975; Bej *et al.*, 2010; Colwell, 1977).

1.5.1.2 Nutrients

Studies have shown that the availability of inorganic nutrients (commonly NH_4^+, PO_4^{3-}, SO_4^{2-} and Fe^{3+}), significantly enhances the biodegradation of petroleum hydrocarbons, even at lower temperatures (Beyer *et al.*, 2016). The biodegradation of hydrocarbons requires these nutrients for cell growth and division, consequently leading to the depletion of these macro- and micro- nutrients in affected environments (Head *et al.*, 2003; Das and Chandran, 2011). Bioremediation strategies often involve the addition of these limiting nutrients (referred to as fertilizers) in order to promote microbial growth and hence biodegradation of the hydrocarbons (Acosta-Gonzalez *et al.*, 2013; Timmis, 2010; Yakimov *et al.*, 2006; Fingas, 2011). These nutrients, however, are highly water soluble and rarely remain contained within the location of their highest demand (Obbard *et al.*, 2004). Organic nutrients, such as

readily-oxidizable fatty acids, cationic surfactants and urea have also been used as nutritional supplements during fertilization in order to increase the bioavailability of the crude oil constituents (Pelletier *et al.*, 2004; Obbard *et al.*, 2004). However, these do not appear to be part of the basic requirements of hydrocarbon degrading bacteria (Pelletier *et al.*, 2004).

1.5.1.3 Oxygen

Hydrocarbons are a major source of electrons (acting as electron donors/reductants), therefore high amounts of electron acceptors are required for their oxidation (Head *et al.*, 2003; Acosta-Gonzalez *et al.*, 2013; Edwards *et al.*, 2011). Since oxygen is one of the strongest electron acceptors (oxidants), its utilization is one of the most efficient and widespread microbial strategies for hydrocarbon degradation (Brakstad *et al.*, 2010; McNaught and Wilkinson, 2014). Oxygen is also required by most organisms for another major biological function - respiration (Das and Chandran, 2011). Despite fluid dynamics and oxygen dissolution in the water column, oil spills often result in oxygen-limiting conditions in the marine environment (especially in sediment), which stems from the production of increased oxygen demand and its decreased dissolution potential within the oil plume (Colwell, 1977; Bej *et al.*, 2010; Acosta-Gonzalez *et al.*, 2013; Head *et al.*, 2003; Yakimov *et al.*, 2007; Fingas, 2011; Atlas, 1981; Beyer *et al.*, 2016). In the absence of oxygen, the hydrocarbon load can be degraded by microorganisms capable of anaerobic respiration, which is based on another electron acceptor such as nitrogen, iron, sulphur, carbonate or manganese (Das and Chandran, 2011; Acosta-Gonzalez *et al.*, 2013; Nealson and Saffarini, 1994; Braker *et al.*, 2001). Normally, however, a diverse microbial community is necessary in order to utilize the variety of electron acceptors present in the surrounding environment. Since elements used as electron acceptors also vary in electron affinity (strength), a diverse community of bacteria may produce a vertical (or sequential) zonation in the water column (or sediment), depending on the availability of the next best electron acceptor (Acosta-Gonzalez *et al.*, 2013). Therefore, upon oxygen depletion, generally follow the sequential utilization of nitrogen (reducing nitrates NO_3^- to NO_2), manganese (reducing MnO_2 to MnO, $MnCO_3$, $Mn(SO_3)_2$ or even MnS), iron (Fe^{3+}), sulphur (reducing sulphate (SO_4^{2-}) to sulphide (S^{2-} such as FeS or FeS_2 (pyrite))) and lastly carbonate (CO_3^{2-}) (Acosta-Gonzalez *et al.*, 2013; Nealson and Saffarini, 1994; Braker *et al.*, 2001). In the absence of any of these electron acceptors, some members of the bacterial community may resort to another respiration strategy in which methane is produced (methanogenesis)

(Acosta-Gonzalez *et al.*, 2013). Although some bacteria are capable of switching from one metabolic strategy to another, and bacterial communities are capable of utilizing a variety of electron acceptors, anaerobic degradation of petroleum hydrocarbons generally occurs at almost negligible rate in comparison to its aerobic counterpart (Atlas and Hazen, 2011; Atlas, 1981; Acosta-Gonzalez *et al.*, 2013). This is why active biodegradation tends to be measured as biological oxygen demand (Minas and Gunkel, 1995; Atlas, 1981)

1.5.2 Strategies used by microbes for hydrocarbon degradation

Many different species of bacteria (and other microorganisms) have been shown to degrade hydrocarbons, to possess an assortment of metabolic capabilities for it and to be promoted by different temperatures, oxygen levels and other factors or conditions. When hydrocarbon molecules become solubilized (bioavailable), they are subjected to attack by hydrocarbon-degrading bacteria via direct contact (Ron and Rosenberg, 2014). However, the majority of hydrocarbons are quite insoluble (<5 mg/L) and hence are not easily accessible by microorganisms. Bacteria have evolved strategies to resolve this, which involve production of biosurfactants (to disperse and/or solubilize the hydrocarbons) and adhesion of the cells to oil droplets (biofilm formation) (Das and Chandran, 2011; Ron and Rosenberg, 2014). Once the substrate is accessed the most common bacterial strategy for its utilization is enzymatic oxidation (Ron and Rosenberg, 2014).

1.5.2.1 Biosurfactant production

In order to gain direct access to insoluble crude oil hydrocarbons, bacteria initiate an attack on the hydrocarbon droplets. This is done by synthesis of a wide range of surface-acting compounds (SACs) (Banat *et al.*, 2010). Molecularly, SACs are made up of 3 parts: a hydrophilic moiety (acid or polar peptide), a chain of one to multiple saccharide units (mono-, di-, or poly- saccharides), and a hydrophobic moiety (unsaturated or saturated hydrocarbon or fatty acid) (Banat *et al.*, 2010). SACs are classified as biosurfactants or emulsifiers based on their molecular weight, physicochemical properties and mode of action (Banat *et al.*, 2010). Biosurfactants (e.g. glycolipids) are generally lower molecular weight molecules which act to reduce surface tension of oil droplet interface, reduce viscosity and aid to increase the surface area of the droplet (Karant *et al.*, 1999; Ron and Rosenberg, 2010). Bioemulsifiers (e.g. liposan; RAG-1 emulsan; alasan) are usually higher molecular weight SACs, composed of polysaccharides, proteins, lipopolysaccharides, lipoproteins or a

complex mixture of these biopolymers (Ron and Rosenberg, 2010). Bioemulsifiers are more effective at tightly binding to oily surfaces, preventing their coalescence and therefore stabilizing emulsions (dispersed oil in water), rather than at reducing surface tension (Banat *et al.*, 2010; Ron and Rosenberg, 2010). SAC production in bacteria is strain specific, meaning that different species and strains specialize in the emulsification of different types of hydrocarbons, all while possibly inhibiting mineralization from other strains (Banat *et al.*, 2010; Ron and Rosenberg, 2010). Microbially-produced SACs, however, can also have different molecular-based efficiencies on different hydrocarbons (e.g. some SACs can be more efficient on PAH+aliphatic mixtures but not on pure aliphatics; other SACs can be more efficient on hydrocarbons with specific molecular weight) (Banat *et al.*, 2010; Ron and Rosenberg, 2010). Type and amount of SACs produced is also affected by nutrients (esp. N), oxygen, trace elements or primary metabolites, agitation, pH and temperature (Karant *et al.*, 1999). SACs may also play an inhibitory or stimulative role in adhesion to hydrophobic surfaces (depending on the nature of the substrate), affecting biofilm formation (Banat *et al.*, 2010; Franzetti *et al.*, 2008). Anaerobic biosurfactant production is also possible, although rates of production and fictional efficiency of surfactants appear lower than those produced in the presence of oxygen (Karant *et al.*, 1999).

1.5.2.2 Biofilm formation/Substrate adhesion

Biofilm formation is one of the most effective strategies that bacteria use for gaining access to low solubility hydrocarbon substrates. As bacteria require direct contact with hydrocarbon molecules in order to degrade them, they form biofilms on the oil-water interface – the oil droplet surface serving as both a substrate and substratum of attachment (Grimaud, 2010). Biofilms are constructed from extracellular polymeric substances (EPS), such as lipopolysaccharides and capsular polysaccharides and are highly organized multi-layered structures with complex architecture essential to their nutritive functions (Whyte *et al.*, 1999; Grimaud, 2010). The bacterial communities embedded in these extracellular matrixes require cell differentiation and collective behaviour (quorum sensing) in order to coordinate the initiation and development of the biofilm (Grimaud, 2010; Dang *et al.*, 2008). Because of phylotype-specific efficiencies in adhesion, EPS formation and substrate degradation, biofilm-forming communities are often multi-species assemblages (Grimaud, 2010; Singh *et al.*, 2006). Therefore, crude oil degradation can be viewed as a cooperative biodegradation network (Joye *et al.*, 2014).

1.5.2.3 Enzymatic oxidation

Once bacteria gain access to their preferred hydrocarbons, they initiate enzymatic oxidation of the hydrocarbon substrate(s). This often occurs extracellularly. Most aliphatic substrates will be converted to 1-alkanols by membrane-bound oxidoreductase enzymes called alkane mono-oxygenases (Wyatt, 1984). Once the substrate is oxygenated, it is introduced into the cell where it is fragmented by alkane hydroxylase and dehydrogenase systems or other peripheral degradation pathways (Das and Chandran, 2011; Schneiker *et al.*, 2006). Intermediates are "fed" into the TCA cycle of the cell (Das and Chandran, 2011) which stimulates respiration and biosynthesis of cell components. Oxidation of aromatic hydrocarbons occurs similarly, except that two oxygen molecules are incorporated onto one of the aromatic hydrocarbon ring (Luz *et al.*, 2010). Aromatic hydrocarbon degradation is mediated by a complex system of multi-component enzymes called Aromatic Hydroxylating Di-oxygenases (ARHDs), which are also involved in electron respiration throughout the cell (Das and Chandran, 2011; Luz *et al.*, 2010). The incorporation of oxygen into the molecule disturbs the mesomerism (resonance) and symmetry of the aromatic system, and therefore also its stability (Das and Chandran, 2011). This contributes to the solubility of the compound and its transfer through the cell membrane where further degradation is continued. These alkane and aromatic oxygenative systems are the predominant mechanisms for hydrocarbon utilization in bacteria and eukaryotes (Baelum *et al.*, 2012; Das and Chandran, 2011; Luz *et al.*, 2010). Their prevalence is indicative of the efficiency of this strategy, as well as the significance of oxygen for the hydrocarbon biodegradation process (Atlas, 1981; Brakstad *et al.*, 2010). As discussed in Chapter1, Section 1.4.1.3, aliphatic and aromatic oxidation, can also be performed under anaerobic conditions, but using other less energy efficient (though apparently sufficient) electron acceptors (Luz *et al.*, 2010).

Although there are two major types of hydrocarbon oxygenative systems, they are each composed of numerous oxygenative enzymes (as well as hydrolases and dehydrogenases) that can be quite complex in structure (Luz *et al.*, 2010). The enzymes are highly specialized for a particular hydrocarbon, and an individual organism is unlikely to possess the genes coding for them all (Ron and Rosenberg, 2014). Therefore, a consortium of hydrocarbon-degrading bacteria encoding, amongst them, a wide spectrum of enzymes and pathways for degrading the multitude of different hydrocarbons under the prevailing environmental

conditions, is required in order for much of the biodegradable fraction of hydrocarbons in crude oil to be biodegraded (Ron and Rosenberg, 2014).

1.5.3 Hydrocarbon degrading bacteria in the marine water column

With the exception of the domain Archaea, hydrocarbon-degrading organisms have been identified across all phylogenic lineages (Prince, 2010; Prince *et al.*, 2010; C. E. Cerniglia and Sutherland, 2010) and from marine environments of varying temperature and oxygen conditions (Bej *et al.*, 2010; Timmis, 2010). The marine water column is generally considered an aerobic environment, although anoxic conditions can be produced in oil plumes as evidenced during the *Deepwater Horizon* oil spill (Kessler *et al.*, 2011). Hydrocarbon degrading bacteria (a.k.a. hydrocarbonoclastic bacteria; HCB) can be categorised into two groups: "obligate" or "generalist", based on the stringency of their diet (Chronopoulou *et al.*, 2015; Yakimov *et al.*, 2007) which is determined via laboratory-based growth/substrate tests (Yakimov *et al.*, 2007; Kostka *et al.*, 2011). For clarity, this nomenclature will be used from hereon in this text. Generalist hydrocarbonoclastic bacteria (GHCB; e.g. *Pseudoalteromonas, Halomonas*) are capable of utilizing a variety of substrates (e.g. sugars), but also exhibit hydrocarbonoclastic traits, utilizing a wide range of hydrocarbons as long as those are available in solution (Chronopoulou *et al.*, 2015; Gerdes *et al.*, 2005). Because of their substrate versatility, GHCB are commonly detected in high relative abundance ($\geq 5\%$) in uncontaminated marine waters (Chronopoulou *et al.*, 2015). Obligate hydrocarbonoclastic bacteria (OHCB; e.g. *Alcanivorax, Cycloclasticus*) utilise hydrocarbons almost exclusively as a sole source of carbon and energy and can be found in pristine environments, but at almost undetectable levels (Yakimov *et al.*, 2007). In addition they commonly exhibit strain-specificity to different hydrocarbons (e.g. straight-chain alkanes, branched alkanes or monoaromatic hydrocarbons) (Chronopoulou *et al.*, 2015; Gerdes *et al.*, 2005).

Considering the levels of crude oil that enter the marine environment every year from seeps and anthropogenic sources, it is of no surprise that OHCB primarily inhabit marine environments (Yakimov *et al.*, 2007). In the vast area of the ocean where hydrocarbon levels are almost undetectable, the abundance of these organisms is typically negligible (<0.1% of the total bacterial community) (Yakimov *et al.*, 2007; Head *et al.*, 2006). There is a

relatively limited number of aerobic marine hydrocarbon degrading bacteria particularly distinguished for playing a major role in the removal of hydrocarbons, although a variety of other bacteria have also been identified (Prince *et al.*, 2010). Interestingly, despite a few exceptions, most hydrocarbon degraders belong to the class *Gammaproteobacteria* (Chronopoulou *et al.*, 2015; Yakimov *et al.*, 2007). Hydrocarbon degraders recognized for their significant role in hydrocarbon biodegradation in marine environments are listed in Table 1.2 alongside their preferred temperature and hydrocarbon substrate(s).

OHCB are opportunistic heterotrophs, although they also possess features that are found typical to oligotrophic bacteria: outer cellular membrane enriched with wide range of transport, oligo- and nutrient- capture systems optimized for scavenging nutrients in nutrient-depleted environments (Yakimov *et al.*, 2007). This duality could be due to the severe imbalance in carbon source relative to nutrient and oxygen sources produced by the extensive microbial activity during an oil spill. OHCB commonly exhibit chemotaxis to hydrocarbon substrates, and high initial hydrocarbon utilisation rates (Grimaud, 2010; Das and Chandran, 2011). However, high degradation rates may quickly lead to a depletion in nitrogen, phosphorus and oxygen in their surrounding environment despite potential replenishment from water mixing (Yang *et al.*, 2014; Chronopoulou *et al.*, 2015; Das and Chandran, 2011; Valentine *et al.*, 2010). An additional cellular distinction of OHCB is that they lack carbohydrate transferring systems on their cell membranes, or intact carbohydrate catabolic pathways that are common to other microorganism for capturing and processing simple sugars (Schneiker *et al.*, 2006). Such cellular properties explain OHCB's behaviour and strict dietary preferences for hydrocarbons. OHCB and GHCB are capable of transferring genes specific for the degradation of certain substrate (organized in gene cassettes) between strains or other organisms via horizontal gene transfer (Hedlund and Staley, 2006; Bej *et al.*, 2010; Cappello *et al.*, 2007; Luz *et al.*, 2010; Yakimov *et al.*, 2007; Head *et al.*, 2006). This trait complicates the link between their rRNA-based taxonomic identification and absolute metabolic capacity (Berry *et al.*, 2017; Yakimov *et al.*, 2007).

1.5.4 Community dynamics in crude oil impacted marine systems
In an uncontaminated marine environment, a certain level of microbial diversity and community stability over time is generally detected (Head *et al.*, 2006; Fuhrman *et al.*, 2015;

Fuhrman *et al.*, 2006). The introduction of crude oil in seawater, however, leads to a disruption of community stability and decrease in diversity through an emergence of blooms of normally low abundance OHCB (Head *et al.*, 2006). The composition of OHCB blooms can be highly variable, although, researchers have revealed almost consistent patterns: a succession of blooms from certain types of OHCB over the time scale of a spill (Head *et al.*, 2006; Yakimov *et al.*, 2007). Studies indicate that alkane degraders, such as *Alcanivorax*, are the first to bloom and dominate, where they begin to utilise the easily degradable alkane fraction of the oil (Yakimov *et al.*, 2007; Yang *et al.*, 2014). As the alkane fraction decreases, the community of alkane degraders proportionally also decreases in abundance, while a wave of aromatic degraders subsequently blooms (e.g. *Cycloclasticus*) (Yakimov *et al.*, 2007; Head *et al.*, 2006; Yang *et al.*, 2014). Studies on the time scale of the successive blooms, the rates of degradation and exact community structure show noticeably inconsistent results, probably due to the recalcitrant effects of the various prevailing physicochemical factors (temperature, oxygen, nutrients, crude oil characteristics, etc.) (Yakimov *et al.*, 2007; Joye *et al.*, 2014; Hazen *et al.*, 2010; Camilli *et al.*, 2010). Despite the variable community composition of HCB that may result from oil spills at different spill sites, the degradation process generally leads to an analogous degradation end-point (Head *et al.*, 2006). This indicates that there is a persistent level of HCB in the marine water column that has the capacity to effectively degrade hydrocarbons. At the point, when the majority of the biodegradable fraction of the spilled crude oil is degraded, biodegradation rates subside and the microbiota in the afflicted sites begin restoring to their natural diversity and microbial composition (Yang *et al.*, 2014; Kessler *et al.*, 2011). However, studies have shown an increased presence (i.e. above pre-spill abundance levels) of OHCB and GHCB during the post-spill phase, even after 1 year since a spill had been considered remediated (Yang *et al.*, 2014; Kessler *et al.*, 2011).

Table 1.2. Genera of aerobic marine hydrocarbonoclastic bacteria that are found in marine environments and commonly play a significant role in the biodegradation of hydrocarbons

Genus	Temperature preference	Preferred hydrocarbons	References
Obligate Hydrocarbonoclastic Bacteria (OHCB)			
Alcanivorax	Broad range	Broad range	(Chronopoulou *et al.*, 2015; Yakimov *et al.*, 2007; Timmis, 2010)
Cycloclasticus	Mesophilic	PAH only	(Chronopoulou *et al.*, 2015; Staley, 2010)
Marinobacter	Broad range	Broad range	(Yakimov *et al.*, 2007; Duran, 2010; Kostka *et al.*, 2011)
Oleibacter	Mesophilic	Alkanes	(Chronopoulou *et al.*, 2015; Teramoto *et al.*, 2011)
Oleiphilus	Mesophilic	Alkanes	(Golyshin *et al.*, 2003; Cappello *et al.*, 2007)
Oleispira	Psychrophilic	Alkanes	(Yakimov *et al.*, 2007; Golyshin *et al.*, 2010)
Thalassolituus	Broad range	Broad range T°C dependent	(Chronopoulou *et al.*, 2015; Yakimov *et al.*, 2004)
Generalist Hydrocarbonoclastic Bacteria (GHCB)			
Alteromonas	Mesophilic	PAH	(Chronopoulou *et al.*, 2015; Gutierrez *et al.*, 2013)
Colwellia	Psychrophilic	Broad range	(Joye *et al.*, 2014; Baelum *et al.*, 2012; Yang *et al.*, 2014; Gutierrez *et al.*, 2013)
Glaciecola	Psychrophilic	Methanol	(Chronopoulou *et al.*, 2015)
Halomonas	Broad range	Broad range	(Chronopoulou *et al.*, 2015; Yang *et al.*, 2014)
Neptunomonas	Aromatics	Naphthalene	(Yakimov *et al.*, 2005; Head *et al.*, 2006)
Pseudoalteromonas	Broad range	Broad range	(Chronopoulou *et al.*, 2015; Timmis, 2010)
Pseudomonas	Psychrotolerant	Aromatics	(Chronopoulou *et al.*, 2015; Yakimov *et al.*, 2007)
Psychromonas	Psychrophilic	N/A	(Yakimov *et al.*, 2007; Brakstad and Bonaunet, 2006; Agogué *et al.*, 2011)
Shewanella	Psychrophizophilic	Aliphatic	(Yakimov *et al.*, 2007; Gerdes *et al.*, 2005)
Vibrio	Broad range	Broad range	(Yakimov *et al.*, 2007; Hedlund and Staley, 2001)
Xanthomonas	Mesophilic	Broad range	(Timmis, 2010)

1.6 Current state of knowledge on the microbiota of the FSC

Although the hydrocarbonoclastic microbiota of the FSC has, thus far never been explored, studies of the North Atlantic and Nordic seas indicate that common OHCB in the cold FSC region are likely to be *Alcanivorax*, *Cycloclasticus, Marinobacter* and *Thalassolituus* (Yakimov *et al.*, 2007). Generalist hydrocarbon degraders found in the North Sea are likely similar inhabitants of the FSC, as both regions share some similarities in environmental

conditions. Likely HCB to be found in the FSC could be *Pseudoalteromonas*, *Roseovarius*, *Pseudomonas*, *Alteromonas*, *Glaciecola* and *Halomonas* species (Chronopoulou *et al.*, 2015). Psychrotolerant and psychrophilic bacteria, previously reported to degrade hydrocarbons from the Arctic and the Antarctic environments, might also be found to comprise the FSC oil-degrading community, and which may include *Psychromonas*, *Oleispira*, *Shewanella* and *Colwellia* (Brakstad and Bonaunet, 2006; Brakstad *et al.*, 2010).

Despite a century of effort gone into intensely studying the hydrography and oceanography of the FSC (Turrell *et al.*, 1996), no information currently exists on the microbial communities of this region, and nor how they would respond to a mass influx of crude oil, especially under the prevailing conditions (temperature, salinity etc.). The complex hydrodynamics and sub-Arctic conditions in the FSC are likely to play a significantly role in the transport, weathering and biodegradation of an oil plume in the event of a large accidental discharge of crude oil into the region. Considering the variety of currents that traverse through the FSC, an oil spill may also have a detrimental environmental impact on the ecosystems of the North Atlantic and the Nordic Sea waters, as indeed also to the vast coastlines that shape the landscape of these major waterways. The oil-degrading microbial communities in the FSC, like in any other water body, would be expected play a crucial role in the biodegradation and ultimate removal of any petrochemical input into the system. It is imperative, therefore, to understand the diversity of these organisms in the FSC, their seasonal dynamics, and what factors influence their activity in degrading oil hydrocarbons in the event of an oil spill.

1.6.1 Project overview and general design

The overarching aim of this study was to gain a fundamental understanding of the bacterioplanktonic communities in the FSC and to investigate the abundance and diversity of hydrocarbon-degrading bacteria, which has not been done before for this region of the NE Atlantic. In this study the hydrocarbon biodegradation potential of the FSC bacterioplankton was investigated and its response to crude oil under conditions of ambient FSC temperature (4°C) and salinities at different depths. Another goal of this study was to investigate for the presence and activity of psychrotolerant and psychrophilic hydrocarbon-degrading bacteria, and to potentially discover novel HCB taxa in the FSC waters. This study represents the first

creation of a microbial baseline for the water column of the FSC, as well as an investigation into the presence and abundance of hydrocarbon-degrading bacteria, their potential to degrade hydrocarbons and their response upon exposure to crude oil.

1.6.2 Project Objectives

1. To explore the inherent microbial community composition and diversity in the major water masses of the Faroe Shetland Channel (FSC), as well as create a spatiotemporal baseline dataset for these communities within the water column (0 to 1,000 m depth) over a 2-year period. This microbial baseline would represent a 'pre-spill' reference of significant value in the event that a spill were to occur in this region.

2. To explore the response of indigenous microbial communities in the FSC (from within each water mass) to typical FSC crude oil (Schiehallion crude oil, abbreviated as CRO), under controlled laboratory conditions at moderate control (20°C) and average FSC (4°C) temperatures. This would provide estimation of the time frame required for microbial response to crude oil in each water mass at both temperatures.

3. To obtain and identify FSC strain isolates potentially capable of degrading FSC crude oil (CRO), model hydrocarbons such as hexadecane (HEX) or phenanthrene (PHE). The isolation of these organisms would allow for their further interrogation for hydrocarbon-degrading abilities, using cultivation-based methods.

4. To follow the CRO-exposed microbial community dynamics and changes in diversity at average FSC temperatures (4°C). This study would elucidate the capacity of FSC microbiota to degrade hydrocarbons under these temperature conditions, reveal potential HCB likely to be active in the FSC, as well as provide an idea about the timeframe under which CRO response may occur.

5. To identify and prove the metabolic functionality of hydrocarbon-degrading bacteria from surface and subsurface FSC waters using DNA-based Stable Isotope Probing (DNA-SIP). The technique allows for these organisms to be identified without the need

for their isolation, which is particularly important for bacteria not easily amenable to cultivation in the laboratory.

1.6.3 Strategy and general methodology for accomplishing goals of research

Chapter 2: Baseline Microbial Community experiments

Identify stations and depths most closely representing each annually stable water mass in the FSC. Determine water sample collection volumes sufficient for bacterial DNA extractions and 16S rRNA gene profiling via Illumina MiSeq sequencing platform. Perform cluster-based microbial community analysis to explore the community structure and diversity for each water mass as well as the dynamics of these communities over a period of 2 years. Utilize bioinformatics tools such as USEARCH, BLAST, Ribosomal Database Project and R Studio to accomplish the proposed goal.

Chapter 3: Crude Oil Enrichment Experiments (CRO-EE)

For this study, one FSC monitoring station of strategic location was selected, with the following characteristics: proximity to oil production platform, maximum monitoring depth, optimal coverage of water-column characteristics and potentially of water masses. Determine minimum sample volumes necessary microcosm setup and sufficient for DNA extractions after exposure. Produce culture-based microcosms utilizing Schiehallion crude oil and incubate them under moderate control (20°C) and average FSC temperature conditions (4°C) to confirm and compare microbial response to crude oil. Perform 16S rRNA gene profiling of microcosm microbial communities using Illumina MiSeq platform in order to follow community dynamics occurring at 4°C over a set time period. Attempt isolations of FSC bacteria with putative hydrocarbonoclastic activity, based on their growth on hydrocarbon sources such as alkane (HEX), aromatic (PHE) or crude oil (CRO) as a sole source of carbon. Produce full 16S rRNA gene sequences and determine phylogenic identity of isolates.

Chapter 4: DNA-based Stable Isotope Probing (SIP) Experiments

Utilize DNA-based Stable Isotope Probing (DNA-SIP) technique for exploring the alkane and aromatic degrading consortia of FSC surface and subsurface communities. Confirm

presence and activity of FSC strains associated with known hydrocarbon degraders and potentially reveal presence of novel hydrocarbonoclastic strains.

Chapter 5: Discussion

Discuss results through an *in silico* interrogation of the newly obtained FSC baseline microbial community dataset (from Chapter 2) for the presence and abundance of known, expected and newly revealed hydrocarbon degraders discovered in the CRO-EE microcosms, isolate libraries and DNA-SIP experiments (Chapters 1, 3 and 4). Define the abundance of the hydrocarbon-active microbial consortium of the FSC.

1.6.4 Hypothesis

Despite regular hydrographic and oceanographic monitoring of the FSC, an investigation of the planktonic microbiology, not to mention also sediment, of this region has hitherto not been performed. Expected is a water-mass specificity of each microbial community, based on the findings of Agogue *et al.* (2011) which investigated the planktonic microbiota of the North Atlantic (Agogué *et al.*, 2011). Also low *in situ* levels of hydrocarbonoclastic bacteria is expected as per lack of major quantities of crude oil in the water column (Head *et al.*, 2006; Yakimov *et al.*, 2007). It is hypothesized that despite likely presence of psychrophilic and psychrotolerant hydrocarbon degraders, observed hydrocarbon degradation response will be slower at average FSC temperature conditions ($\sim 4°C$), than at moderate temperatures (20°C chosen as control).

The ultimate goal of this project was to produce the first pre-spill baseline of a microbial community, to investigate community structure composition and dynamics, as well as to explore microbial hydrocarbon degradation response under average FSC temperature conditions. This information is critical to aid the development of microbially-mediated contingency efforts and mitigation techniques for crude oil spill bioremediation in the FSC and adjacent marine environments. This study could also provide materials for other biotechnological research such as bioprospecting and developing novel bio-surface-acting compounds.

CHAPTER 2: SPATIOTEMPORAL DYNAMICS OF PLANKTONIC BACTERIAL COMMUNITIES IN THE FAROE-SHETLAND CHANNEL

2.1 Introduction

With oil prospecting and extraction likely to expand into deeper waters of the FSC, the threat of oil pollution in this region is undeniably increasing. Numerous studies have shown that microorganisms play a major role in oil hydrocarbon remediation at contaminated sites in the environment (Head *et al.*, 2006; Yang *et al.*, 2014; Yakimov *et al.*, 2007; Chronopoulou *et al.*, 2015). Due to experience gained from the response efforts to past oil spills (e.g. *Exxon Valdez*, *Deepwater Horizon*), a prerequisite has been recognized to elucidate the diversity, dynamics and structure of the bacterioplankton of a region as well as its potential for responding to an injection of crude oil, prior to any major accident (Yang *et al.*, 2014; Atlas and Hazen, 2011). Lack of knowledge and understanding into the indigenous microbial diversity structure and dynamics of the Gulf of Mexico for example, left some questions unanswerable during the *Deepwater Horizon* remediation response, concerning microbial behaviour post plume and the level of recovery of the ecosystem (Yang *et al.*, 2014). In the FSC, despite extensive hydrographic exploration over the past 100 years (Berx, 2012), basic research and understanding into the region's microbial communities and their dynamics is still lacking.

As discussed in Section 1.1, the FSC can be defined as a region of dynamic mixing of waters with contrasting temperature and salinity characteristics. To simplify this complexity, the region's confluence of different waters is distinguished into two bodies of water: the Atlantic Water Core (AWC), which contains two water masses originating from the North Atlantic ocean (NAW and MNAW) and the Nordic Water Core (NWC), which contains three water masses (MEIW, NSAIW and NSDW). Despite presence of three NWC water masses in the FSC, only two (NSAIW and NSDW) are relatively stable throughout the year (Hansen and Østerhus, 2000; Read and Pollard, 1992). The MEIW mass, in particular has been found to exhibit the highest seasonal variability (strengthening and weakening based on season) due to the influx of fresh, cold glacier water from the polar regions and mixing with other water masses (Hansen and Østerhus, 2000; Read and Pollard, 1992). Therefore, in order to

thoroughly understand the microbial communities of the FSC, microbial community profiles would need to be obtained not only for each water mass, but also across the different seasons of the year.

Several challenges should be taken into consideration when performing PCR-based community profiling of natural environments, such as seawater. The development of Next Generation Sequencing (NGS) technologies in recent years has significantly increased the opportunities for studying entire microbial communities in environmental samples. Whilst these technologies bypass the problematic culturing steps, they still heavily rely on the polymerase chain reaction (PCR) amplification. The flaw in PCR-dependent community profiling techniques, however, emerges from PCR-produced bias and sequence artefact formations (sequence errors, chimeras, heteroduplexity), especially in complex environmental multi-bacterial samples such as from marine environments (Acinas *et al.*, 2005; Acinas *et al.*, 2004). Although the problem is acknowledged, so far NGS is still the best available technique for studying the diversity and structure of microbial communities in environmental samples. Furthermore, most NGS platforms require a unique sample-specific sequence (barcode) and platform-specific adapter to be added to the DNA locus of interest. This is usually done during the PCR amplification step under the assumption that the amendments on the locus-specific primers do not interfere with the template and its amplification (Berry *et al.*, 2011). Recent research, however, has concluded that the barcode variation on the PCR primers induces the problematic PCR bias and causes replicate inconsistencies (Berry *et al.*, 2011). Therefore, Berry *et al*. (2011) proposed a 2-step PCR approach in which the environmental DNA samples are initially amplified with PCR primers amended with only sequence-consistent platform-specific adaptors. After the desired amplicon yield is achieved, a short second PCR step (~8 cycles) is performed that adds the variable barcodes to the amplicons prior to library construction (Berry *et al.*, 2011). This approach has shown to significantly reduce PCR bias and replicate variability within samples (Berry *et al.*, 2011) and therefore is a lucrative approach for environmental microbial profiling, and was hence used in this study for analysing the bacterial communities in the FSC.

Whilst the microbiology of the FSC has thus far not been explored, it was anticipated that its microbial profile would be similar to that of adjacent regions, such as other regions of the

northeast Atlantic, the Norwegian Sea, and the water bodies of the Arctic and Icelandic regions. From all the water bodies that flank the FSC, the prokaryotic communities in the North Sea have been studied the most (Giebel *et al.*, 2011; Chronopoulou *et al.*, 2015; Hahnke *et al.*, 2013; Teeling *et al.*, 2012; Fuhrman *et al.*, 2015; Eilers *et al.*, 2000). Although North Sea outflow water does not appear to flow through the FSC (Hansen and Østerhus, 2000) and the North Sea landscape of oil and gas industrial activity is predominant and significantly more advanced than in the FSC, the microbiology of these two regions can be presumed as comparable, due to the similarity of climate conditions in the regions as well as their proximity. The primary water sources for the FSC include surface water from the North Atlantic and deep water from primarily the Norwegian Sea, which is sometimes mixed in with variable fractions of Arctic and Icelandic water (Berx, 2012). Agoguè *et al.* (2012) reported a high abundance (55-68%) of *Alphaproteobacteria* in north-eastern Atlantic surface waters, and dominated by SAR11 with maximum abundance of ~37% of the total bacterial community (Agogué *et al.*, 2011). A similar community dominance of these bacterial groups was also found to the east of the FSC in North Sea waters (Giebel *et al.*, 2011). Generally, the SAR11 clade (*Pelagibacterales*) is considered the most ubiquitous bacteria in the marine environment, preferring warmer pelagic and oligotrophic waters, however also containing members with cold and deep-water adaptations (Giovannoni, 2016; Giebel *et al.*, 2011). *Gammaproteobacteria* averaged at about 20% abundance in the North Atlantic water masses, dominated by the groups *Alteromonadales*, *Oceanospirillales* and *Pseudomonadales*, which increased in dominance with depth (Agogué *et al.*, 2011). The deeper water masses of the eastern North Atlantic (adjacent to the FSC), which include waters originating from the Arctic and Nordic regions, showed higher abundance of psychrophilic clades such as SAR324 (~7%) and SAR406 (~2%) (Agogué *et al.*, 2011). Both groups are common members of the deeper ocean layers and were also both detected in the water column of the Norwegian Sea, currents of which flow through the deep layers of the FSC (Jensen *et al.*, 2012; Hansen and Østerhus, 2000; Berx, 2012). Therefore it is expected that these groups of bacteria might also have noticeable presence in the water column of the FSC.

Common means for exploring the diversity within a community or between communities in microbial ecology studies utilise the so-called alpha and beta diversity community analyses. Alpha diversity is usually a means of interrogating the total diversity of a community as well as the extent of community coverage obtained during sampling (Lozupone and Knight,

2008). To quantify the total number of species in a community, and possibly also compare diversity of species across different communities, the Shannon diversity index is commonly used (Lozupone and Knight, 2008). Contrarily, in order to measure and present the extent of community coverage obtained during sampling, accumulation plots (a.k.a. rarefaction curves) are used (Lozupone and Knight, 2008; Gotelli and Colwell, 2010). Microbial communities in the environment, however, are highly diverse, and despite recent developments in NGS technologies that have yielded unprecedented levels of sequencing depth, it is still quite uncommon to find rarefaction curves that reach saturation levels (Chao *et al.*, 2014; Gotelli and Colwell, 2010). Therefore, environmental samples are generally considered sufficiently explored if their accumulation curves show the "levelling off" behaviour characteristic for plateau approach (Gotelli and Colwell, 2010; Lozupone and Knight, 2008). On the other hand, beta diversity inquires into the change in community composition depending on certain factors of interest and evaluates the extend to which two or more communities differ (Lozupone and Knight, 2008). Among the approaches used, the main ones are multivariate analyses, such as Principle Component Analysis (PCA) and non-metric multidimensional scaling (nMDS) (Ramette, 2007). Both are used to visualize the similarity (or dissimilarity) between various communities or environmental factors, although nMDS is more commonly used in microbial ecology as it is not affected by null values (e.g. lack of certain taxonomic units in some samples) and produces more simplified plots which still fully represent the relationships between communities (Paliy and Shankar, 2016). In this study, both alpha and beta diversity were used to analyse the microbial communities of the different water masses in the FSC.

Based on the study by Agogué *et al.*, (2011), who showed a watermass-specificity of North Atlantic microbial communities, the hypothesis posed in the present study was that the FSC microbial communities might also exhibit a watermass-based specificity. To investigate this, water column samples from the FSC, representing each different water mass, were collected during the warmer and colder seasons of the year for a period of 2 years and analysed by the NGS approach Illumina MiSeq sequencing. The alpha and beta diversity of the communities across the water masses was also assessed. The aim of this study was to provide, for the first time, information on the microbiology for the water column of the FSC and construct a reference baseline vertical microbial profile for this region. The results are anticipated to be

especially valuable if the need occurs for assessing the changes and response of the indigenous microbial communities to a major anthropogenic perturbation, such as an oil spill.

2.2 Materials & Methods

2.2.1 Sample Collection

Sampling of the different water masses was performed for spring (April 24th to May 9th) and fall (September 23rd to October 8th) seasons in two consecutive years - 2014 and 2015. From the routinely monitored FSC sampling stations of MRV *Scotia* and under the advice and guidance of on-board experienced oceanographers from Marine Scotland Science (Dr Bee Berx and Dr George Slesser), suitable locations and depths targeting each water mass were selected prior to collection. Sample collection was performed based on the sampling procedures of MRV *Scotia* and with the guidance and aid from trained scientific personnel on-board MRV *Scotia*. Targeted water characteristics specific for each water mass, along with the names of the most suitable stations, geographic coordinates of the stations and depth of collection are presented in **Table** 2**.**1.

Table 2.1. Geographical coordinates, depth and station names of sampling locations for microbial profiling of each targeted FSC water mass (and its expected characteristics) during research cruises in 2014 and 2015**.**

Water mass targeted	Targeted Temperature Characteristics	Targeted Salinity Characteristics	Station name	Depth of collection	Geographical Coordinates
NAW	> 9.5°C	35.35-35.45	FIM03	150m	60° 20.25' N, 04° 09.00' W
MNAW	7.0 - 8.5°C	35.1-35.3	NOL08	175m	61° 42.00' N, 04° 51.00' W
NSAIW	-0.5 - 0.5°C	34.87-34.90	NOL07	700m	61° 35.00' N, 04° 15.00' W
NSDW	< -0.5°C	34.91	FIM6a	1100m	61° 38.00' N, 04° 54.00' W

Samples from the distinct water masses were collected in 3 L volumes using 10 L CTD (conductivity, temperature, depth) rosette bottles. The volume was then separated in triplicate 1 L water volumes and immediately filtered through individual sterile 0.22 μm pore size, 47 mm diameter gridded Mixed Cellulose Esters (MCE) filters (Millipore Sigma). This resulted in 3 filters (triplicates) per water sample collected per depth. Filters were handled with alcohol-sterilized tweezers and placed inside sterile 50 mL Falcon[TM] centrifuge tubes (Eppendorf). Tubes were carefully labelled and stored at -20°C for subsequent DNA extraction at a later date.

2.2.2 DNA extractions

Under aseptic conditions, filters were taken out of their storage tubes and cut into three equal pieces. One third of the filter was placed inside 1.5-mL centrifuge tubes (Eppendorf) and lowered into liquid nitrogen for 15 seconds. The frozen filters were crushed with sterilized round-end tweezers and then treated with 50 μL TER buffer (TE+RNaseA buffer) according to the protocol of Tillett and Neilan (2000). The method was selected for its efficiency in cell disruption without enzymatic or mechanical treatment, but a chemical cell lysis with potassium xanthogenate buffer (Tillett and Neilan, 2000). The method has been optimized to produce contaminant and inhibitor-free DNA and RNA material from bacterial and archaeal microorganisms (Tillett and Neilan, 2000) and has been regularly proven successful in nucleic acid isolations from marine environmental samples (Dombrowski *et al.*, 2016; Gutierrez *et al.*, 2013; Mishamandani *et al.*, 2014). For this study, the protocol of Tillett and Neilan (2000) was amended only at the DNA precipitation step (extended to 30 min and performed at -20°C), which allowed for more efficient DNA precipitation (Green and Sambrook, 2016). During DNA extractions, along with every set of marine samples, a set of negative controls was also added (using sterile filters or molecular grade water instead of cell culture) in order to assure the sterility of the method and used reagents. Briefly, lysis potassium xanthogenate buffer of volume 750 μL (a.k.a. XS buffer) was added to the filters and incubated at 75°C for 30 min. The tubes were then mixed by vortexing and incubated on ice for 30 min. Filter fragments and cell debris were pelleted by centrifugation (17,000 ×*g*; 10 min) and the resultant supernatant carefully transferred (without any debris carry-over) into sterile 1.5-mL centrifuge tubes containing an equal volume of 100% molecular-grade isopropanol. Tubes were gently mixed and placed at -20°C for at least 30 min. The DNA was then pelleted at 4°C (17,000 ×*g*; 30 min). The supernatant fractions were discarded and the DNA pellets washed twice with 750 mL of 70% molecular-grade ethanol (17,000 ×*g*; 10 min). The ethanol fractions from the final wash step were discarded and the DNA pellets left to air dry in a laminar flow cabinet under sterile conditions. DNA pellets were then resuspended in 50 μL of 1 mM TE buffer. To confirm DNA was extracted and assess its quality, a sub-sample (ca. 2-3 μL) of each extracted DNA fraction was run by gel electrophoresis at 90 V for 30 min through 1% agarose gels made with 1× TAE. Gels were stained with ethidium bromide (EtBr) and imaged with SynGene gel imaging system InGenius3.

2.2.3 Barcoded-amplicon Illumina MiSeq Sequencing

PCR amplifications of the V3-V4 16S rRNA gene fragment were performed on all DNA extracts. For PCR, 1 μL DNA (~1-3 ng/μL) was added to 25 μL PCR reaction that was composed of the following: 5 μL 5× MyTaq buffer (1× final; BioLine), 1.5 μL Primer mix (0.25 μM final; 4 μM each primer), 0.5 μL DMSO (3% final), 16.8 μL H_2O and 0.2 μL 10 U MyTaq (1 U final; BioLine). Negative controls were also prepared for each set of PCR reactions and treated similarly using molecular grade water instead of template DNA. Negative controls were used to determine the sterility of the PCR reaction. Primer sequences of choice were provided by the University of Liverpool's Centre for Genomics Research (CGR) sequencing facility. The primers included a universal bacterial primer of forward or reverse direction (341F and 785R) amended with an Illumina Nextera XT adapter as described in Table 2.2. The two-step PCR approach was performed as previously described by Berry *et al*. (2012), which aimed at reducing PCR-based bias in amplification reactions designated for sequencing using a NGS approach (Berry *et al.*, 2011). Replicate samples were processed separately. Target-specific primer pair coverage distribution was tested with TestPrime 1.0 tool against SILVA RefNR database (https://goo.gl/A1PmGF) and includes 85.9% Bacterial, 0.6% Archaeal and 0% Eukaryotic coverage.

Table 2.2. Primer information for MiSeq library amplifications

Primer Name	Target-Specific Primer Sequence 5'-3'	Linker sequence	MiSeq Adapter Sequence 5'-3'	T_M °C	Start position	Orig. Org.
341F[*]	CCTACGGGNGGCWGCAG	AG	TCGTCGGCAGCGTCAGT-GTGTATAAGAGACAG	55	341	*E. coli*
785R[*]	GGACTACHVGGGTATCTAATCC	CA	GTCTCGTGGGCTCGGAG-ATGTGTATAAGAGACAG	55	785	*E. coli*

*(Full sequences as follows 341F = 5' T CGT CGG CAG CGT CAG ATG TGT ATA AGA GAC **AG** CCT ACG GGN GGC WGC AG 3'; and 785R = 5' G TCT CGT GGG CTC GGA GAT GTG TAT AAG AGA **CA** GGA CTA CHV GGG TAT CTA ATC C 3')

For the first round of PCR, conditions were as follows: initial denaturation step 95°C for 3 min, followed by 32 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 30 s. Amplifications were completed with a 5 min extension step at 72°C. Expected amplification product was of size 444 bp. PCR products were quantified using a Nanodrop 3300 spectrometer (ThermoScientific) and also checked by gel electrophoreses (90 V for 30 min.) on a 1% 1× TAE agarose gel and staining with EtBr. All PCR products that produced one band at the expected size on agarose gels were enzymatically cleaned of polymerase and residual primers

by treating 20 µL of each product with 0.5 µL *Exonuclease I* (10 U final; ThermoFisher), 1 µL FastAP Thermo-sensitive *Alkaline Phosphatase* (1 U final; ThermoFisher) and 3.5µL H_2O for 30 min at 37°C. Reactions were terminated by a 15 min enzyme denaturation step at 85°C. Based on gel electrophoresis, the best two PCR products (from triplicates of each sample) were distributed into a 96-well plate. The plate was tightly sealed with MicroAmp® strip caps and then shipped overnight on ice to the University of Liverpool (UL) Centre for Genomic Research (CGR) for Illumina MiSeq sequencing. Upon arrival at the CGR, the PCR products underwent a second round of PCR (8 cycles) using the same forward and reverse primers, except with barcoded sequences added to the 5' end of the Illumina Nextera XT adapter. Final amplicons were quality controlled using Qubit and Bioanalyzer (ThermoFisher Scientific), concentrations were normalized and pooled and run on a 2× 250 paired-end run of the Illumina MiSeq platform using v2 reagents (Illumina).

2.2.4 MiSeq sequencing data processing

De-multiplexed and primer trimmed data files were provided from UL-CGR in ".fastq" (fasta+quality) format for each sample. Forward and reverse sequences with overlap of >10 bp were merged using PANDAseq assembler (Bartram *et al.*, 2011), and then qualified using PRINSEQ software (Schmieder and Edwards, 2011). Ends of reads with quality score <35 were trimmed and reads with total quality score <30 and/or shorter than the mean length within the sample were discarded (~15-20% of reads) using PRINSEQ software. All good quality reads were of size 390-460 bp length, depending on sample quality. QIIME (Caporaso *et al.*, 2010) was used to edit read headers and add more meaningful unique labels for each read. Chimera check and screening for homopolymers and ambiguous base sequences was performed using MOTHUR (Schloss *et al.*, 2009).

2.2.5 16S rRNA gene profiling

The sequence analysis tool USEARCH (version 9.0; http://www.drive5.com) was used to perform 16S rRNA gene fragment profiling of the MiSeq data within each library sample, based on the manufacturer's recommendations. The analysis pipeline involved extracting each unique read from the large sequence dataset ("fastx_uniques" option), clustering all unique reads with >97% similarity to generate operational taxonomic units (OTUs) and selecting a representative read from each OTU ("cluster_otus" option). The original sequence dataset was then compared to each representative read and upon match, assigned to the corresponding OTU ("usearch_global" option). The number of sequences in each OTU

was then enumerated, producing absolute abundances for each OTU in each sample ("otutabout" flag). OTUs were assigned taxonomies to the deepest possible taxonomic rank, using the UTAX algorithm of USEARCH ("utax" option) and the latest RDP database (Release 11.5; date 30/09/2016) (Wang *et al.*, 2007). Absolute abundances for each OTU were converted into relative abundances (normalisation) when the counts for each OTU was divided by the cumulative number of species for each site and multiplied by 100%. This was performed using RStudio software (www.rstudio.com). Relative abundances OTUs per sample were summed to highest taxonomic rank of interest (e.g. the relative abundance of all OTUs with taxonomic rank "family" and classification Candidatus_Pelagibacter were summated to represent the "order SAR11"), in order to reduce accumulation of OTUs with redundant taxonomic classification (e.g. Candidatus_Pelagibacter) and low relative abundance (<0.5%). Thus accurate representations of each taxonomic rank and taxonomic classification were produced for each duplicate. The OTU with highest relative abundance within each taxonomic classification was selected to represent the lineage at taxonomic rank "order". The average relative abundance and standard deviation across samples was calculated based on the abundance for each taxonomic ranking of interest (e.g. specific order or genus). Community profile visualization graph was produced based on profiles averaged between duplicates, since most duplicated samples (~65%) produced relatively consistent results. Representative OTUs for each taxonomic classification were used to produce a phylogenic tree of the FSC microbial community. This was done using maximum-likelihood phylogenetic tree software tool FastTree (Price *et al.*, 2010), utilizing its default parameters. The tree was beautified with iTol web-based tool (Letunic and Bork, 2007).

2.2.6 Alpha and beta diversity analysis

The OTUs of each site was examined for diversity as well as sampling coverage. RStudio (packages vegan and iNext and ggPlots2) was utilized to produce rarefaction curves for each site (Oksanen *et al.*, 2017; Hsieh *et al.*, 2016; Wickham and Chang, 2016). Species richness was interpolated and extrapolated (depending on sample) to 100,000 individual reads per OTU using Hill numbers (a.k.a. effective number of species) (Chao *et al.*, 2014) and the iNext RStudio package tools (Hsieh *et al.*, 2016). Diversity and diversity variance for each water mass was determined using Shannon index and using the same tools. Sample diversities between water masses and water cores was compared using Welch's t-test using Shannon diversity indices. Beta diversity community composition plot for the FSC microbial

communities were produced using RStudio and vegan packages utilizing nonmetric Multidimensional Scaling (nMDS) tool (RStudioTeam, 2016; Oksanen *et al.*, 2017) through collapsing and simplifying information from multiple factors and improving visualization and result interpretation. To compare community composition between samples, a pair-wise Bray-Curtis similarity matrix (Bray and Curtis, 1957) was produced with Primer 7 (Clarke and Gorley, 2015) (when needed dissimilarity matrix was also produced via R Studio). Permutational multivariate analysis of variance (PERMANOVA) tests (Anderson, 2001) with 999 permutations were performed with Primer v7 in order to show community composition variation and its significance between water cores and water masses. Multivariate dispersion (PERMDISP) tests (Anderson and Walsh, 2013) were performed in order to confirm the homogeneity of community dispersion (and its significance) between samples (also graphically represented in the nMDS plots). Significance of results was based high F values and level of significance based on low *p* values, with *p* value cut-off being <0.05 (5%) for a comparison to be considered a significantly different.

2.2.7 Seasonal dynamics analysis

Based on results from previous sections, seasonal variability was examined per water core rather than water mass. This was performed by plotting (RStudio) the Shannon Diversity Index of each water core according to season. Additionally, community profile graphs for each water core were presented by averaging the abundance of each OTU at "order level" within water core across season-founded sampling dates. The significance of seasonal variability was also determined using season-based pair-wise PERMANOVA test for each water core, performed in PRIMER v7.

2.3 Results

2.3.1 Sample collection, DNA processing and MiSeq sequencing results

Sample collection resulted in a collection of 60 filters from 20 sites, sorted in triplicates and containing FSC bacterial communities from 4 water masses for Spring 2014 and 2015 and Fall 2014 and 2015. Samples from December 2015 cruise from the same locations were also courteously provided from *MRV Scotia* scientific crew, however such were not obtained from December 2014. DNA extractions and PCR amplifications were successful producing ~50 ng/μL PCR product from each sample. Of the three replicates from each sample, two were

selected and shipped for sequencing. A total of 11,037,306 bi-directionally sequenced reads were returned after MiSeq sequencing. After pre-processing (merging, trimming, short-read, and low quality filtering) ~4,739,293 good quality sequences were produced.

2.3.2 Bacterial community profiling of the FSC

The resultant good quality V3-V4 16S rRNA gene fragment sequences were clustered, at 97% identity into 1,933 OTUs. The microbial profiles for each site, season and year are shown in Figure 2.1. The identified taxonomic groups cumulatively comprised 99.5-100% of the total microbial community (Table 2.3). *Alphaproteobacteria* dominated the AWC with average abundance as high as 51%, represented primarily by the orders SAR11 and *Rhodobacterales*. The group SAR11 of the *Alphaproteobacteria* showed distinctly higher representation in the AWC (~23%) than by any other order, along with the order *Rhodobacterales* (~16%), which was noticeably dominated by the genus *Lentibacter* (12.7%). Members of the class *Gammaproteobacteria* represented the second most abundant group in the AWC microbial profiles (~21% relative abundance) and were dominated by members of the order *Methylococcales* (almost 8%). *Deltaproteobacteria* comprised ~5% of the total community and were mostly represented by the order SAR324 (~4%). Contrarily, in the NWC, the most abundant class of bacteria were *Gammaproteobacteria* at 32% average relative abundance. Despite continual dominance of the order *Methylococcales* within this water core (~9%) the order *Alteromonadales* was also noticeably increased in presence in the FSC deep-sea waters (from ~1.5% to ~14%). The genera *Pseudoalteromonas* and *Alteromonas* were the major contributors in abundance to the order *Alteromonadales* (10.7% and 3.6% respectively) within the NWC. Next in abundance were the *Alphaproteobacteria* at 29% and the *Deltaproteobacteria* at 15% of relative abundance. Both classes were again dominated by the orders SAR11, *Rhodobacterales* and SAR324 (7.5%, 17% and 14.5% respectively). A phylogenic analysis of all representative OTUs was also performed in order to confirm their taxonomic identities and reveal their relationships to GenBank type strains (Figure 2.2).

o:Other <0.5% · o:Rhodobacterales · o:SAR11 · o:SAR324 · o:Alteromonadales · o:Methylococcales · o:Acidimicrobidae · o:Oceanospirillales · o:Flavobacteriales · o:Rhodospirillales · o:Nitrospinales · o:SAR406 · o:SAR86 · o:Chromatiales · o:Rhizobiales · o:Kordiimonadales · o:Sphingomonadales · o:Planctomycetales · o:Thiotrichales · o:Chloroplast

Relative Abundance — MNAW, NAW, NSAIW, NSDW; Spring 2014, Spring 2015, Fall 2014, Fall 2015, Winter 2015

Figure 2.1. 16S rRNA gene-based microbial profiles of FSC communities over a variety of seasons in the water masses MNAW and NAW of the Atlantic Water Core (AWC) and NSAIW and NSDW of the Nordic Water Core (NWC). Relative abundance was calculated based on taxonomic level "order" and a cut-off value for minimum relative abundance of 0.5% was used.

Table 2.3. Prominent bacterial taxa (average abundance >0.5%) of the microbial groups found in each FSC water core. Each taxon is represented by an OTU (if applicable), its average abundance within each water core and its standard deviation across the 10 duplicated samples (n=20) collected from each water core over the course of the study.

Prominent bacterial taxa	Rep OTU	AWC abundance (St.Dev; n=20)	NWC abundance (St.Dev; n=20)
d:Bacteria			
p:Actinobacteria	OTU_9	1.99 (0.55)	5.00 (2.01)
p:Bacteroidetes	OTU_34	4.86 (7.71)	3.15 (1.80)
p:Cyanobacteria/Chloroplast	OTU_70	2.21 (1.71)	0.16 (0.16)
p:Marinimicrobia	OTU_8	1.88 (0.48)	3.35 (1.25)
p:Nitrospinae	OTU_24	2.97 (1.25)	1.39 (0.67)
p:Proteobacteria	OTU_7	77.87 (6.25)	76.69 (8.19
p:Other		5.35 (1.26)	5.79 (0.24)
p:Verrucomicrobia	OTU_506	0.89 (0.37)	1.09 (0.49)
% of total community		**98.01**	**96.63**
p: Proteobacteria			
c:Alphaproteobacteria	OTU_7	50.81 (12.09)	28.96 (10.95)
c:Deltaproteobacteria	OTU_3	4.66 (2.16)	15.07 (5.49)
c:Gammaproteobacteria	OTU_2	21.09 (8.79)	31.93 (14.40)
c:Other proteobacteria		1.31 (0.14)	0.72 (0.23)
% of total community		**77.87**	**76.69**
o: Rhodobacterales (of Alphaproteobacteria)			
g:Lentibacter	OTU_1	12.68 (11.26)	15.02 (12.83)
g: other		3.60 (0.58)	1.76 (0.41)
% of total community		**16.28**	**16.78**

Prominent bacterial taxa	Rep OTU	AWC abundance (St.Dev; n=20)	NWC abundance (St.Dev; n=20)
c:Alphaproteobacteria			
o:Rhodobacterales	OTU_1	16.28 (10.60)	16.78 (12.24)
o:SAR11	OTU_7	23.33 (9.28)	7.53 (2.62)
o:Other		11.20 (1.35)	4.65 (0.42)
c:Deltaproteobacteria			
o:SAR324	OTU_3	4.19 (2.00)	14.48 (5.28)
o:other		0.47 (0.34)	0.59 (0.45)
c:Gammaproteobacteria			
o:Alteromonadales		1.51 (1.23)	14.33 (15.49)
o:Methylococcales	OTU_4	7.60 (5.92)	8.68 (3.13)
o:Oceanospirillales	OTU_20	2.22 (1.00)	1.75 (1.78)
o:SAR86	OTU_25	2.20 (1.79)	0.07 (0.06)
o:Other		7.55 (0.40)	7.10 (0.31)
c:Other proteobacteria			
o:other		1.31 (0.03)	0.72 (0.08)
% of total community		**77.87**	**76.69**
o: Alteromonadales (of Gammaproteobacteria)			
g:Alteromonas	OTU_5	0.14 (0.21)	3.59 (9.79)
g:Pseudoalteromonas	OTU_2	1.37 (0.27)	10.73 (12.34)
g:other		0.00 (0.50)	0.00 (0.41)
% of total community		**1.51**	**14.33**
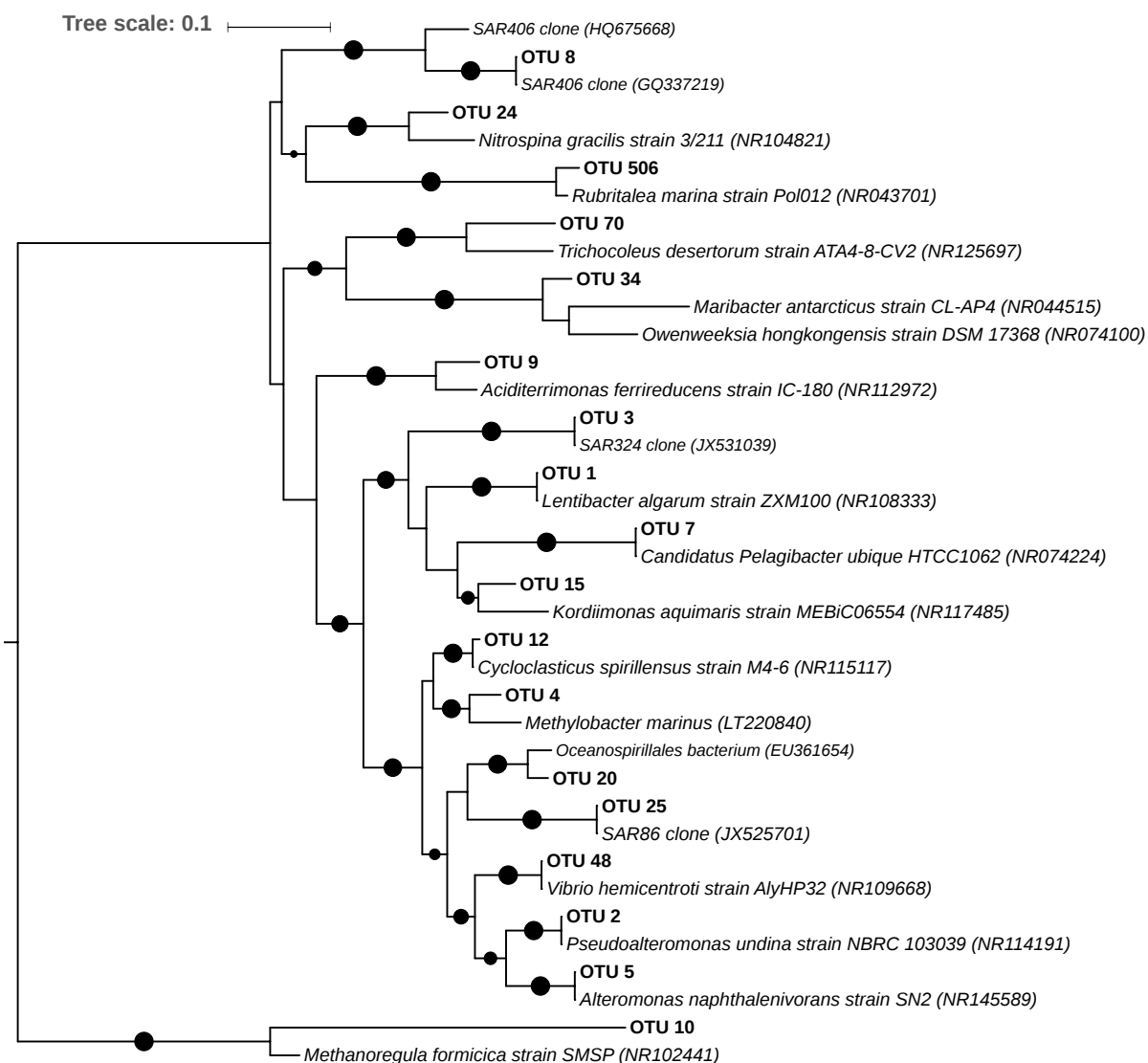
Figure 2.2. Neighbour-joining tree based on 16S rRNA gene V3-V4 fragment sequences, showing the phylogenic associations of the representative OTUs across all FSC samples to known type strains. Accession numbers starting with "NR" represent closely-related established type strains from NCBI GenBank, but in the absence of such, non-type strains were also included. Black dot and their sizes correspond to bootstrapping values of 70% and above. Archaeal sequences of representative OTU10 and *Methanoregula formicica* were used to root the tree. The scale bar indicates the number of substitutions per site.

2.3.3 Alpha and Beta Diversity

Alpha diversity, presented in Figure 2.3 as rarefaction curves, indicates whether examined sites were sufficiently sampled and whether amount of sequences produced were sufficient to represent the majority (significant faction) of the taxa that were identified in the water

column of the FSC by MiSeq sequencing. The rarefaction curves each approached an asymptote, thus indicating that sampling coverage was sufficient for the examined environmental marine locations. Results also indicated that samples NSAIW from the Fall of 2014 had the highest species diversity, whereas sample NAW from the Spring of 2015 had the lowest diversity from all samples. Sample MNAW from Spring of 2014 showed the lowest number of individual reads (only ~45,700 individual reads) and species diversity for 100,000 individual reads had to be extrapolated.
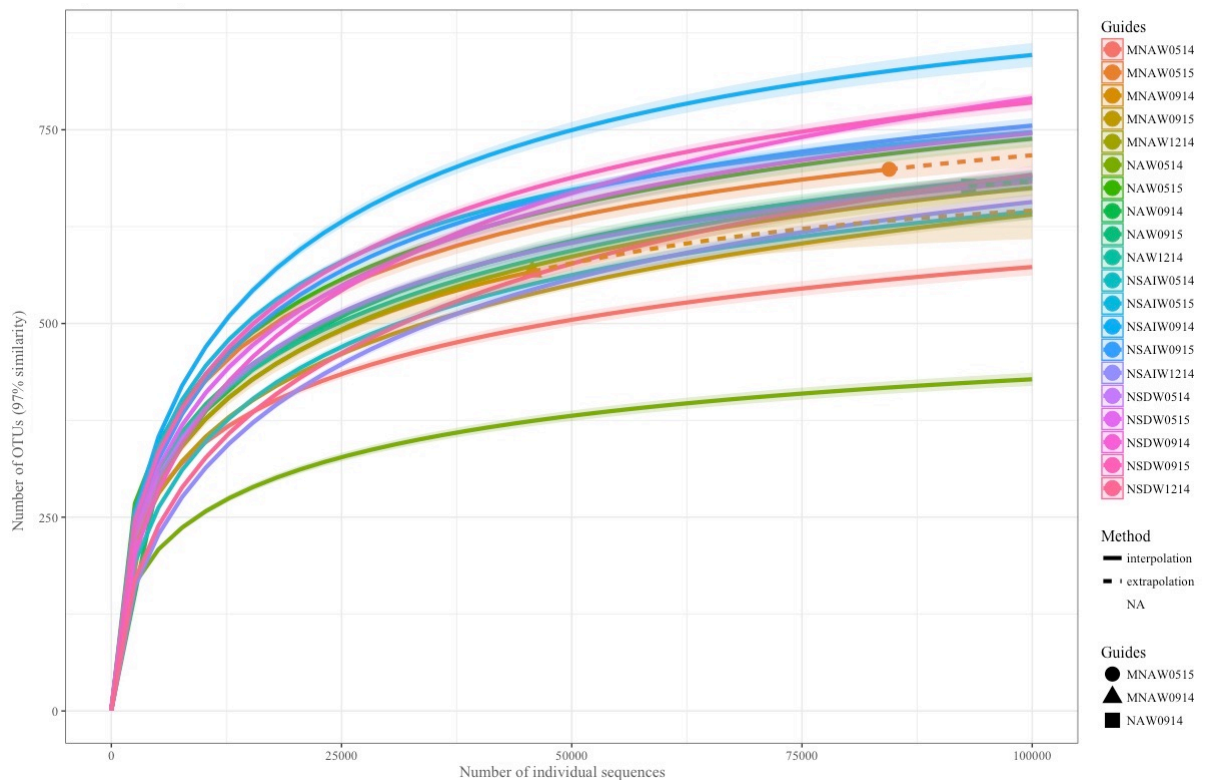
Figure 2.3. Rarefaction curves of FSC sampled sites, interpolated and extrapolated (as needed) up to 100,000 individual sequences.

Shannon Index was used to determine the microbial diversity variance of each water mass (Figure 2.4) and compare diversities based on water masses and cores. Results indicate that the AWC carries microbial diversity higher than that of the NWC, with an average Shannon index of 3.9 (st.dev=0.37) for the Atlantic water masses and an average of 3.6 (st.dev =0.67) for the Nordic water masses across the 2-year timeframe. Species richness comparison between water masses and water cores (Welch's t-test) indicated that there was no significant difference in communities of water masses belonging to the same water core (*p* values ~0.85

(> 0.05)), however a significant species richness difference was found in communities if they are grouped in water cores and compared based on water core variation (*p* value < 0.005 (below 0.05 threshold)).
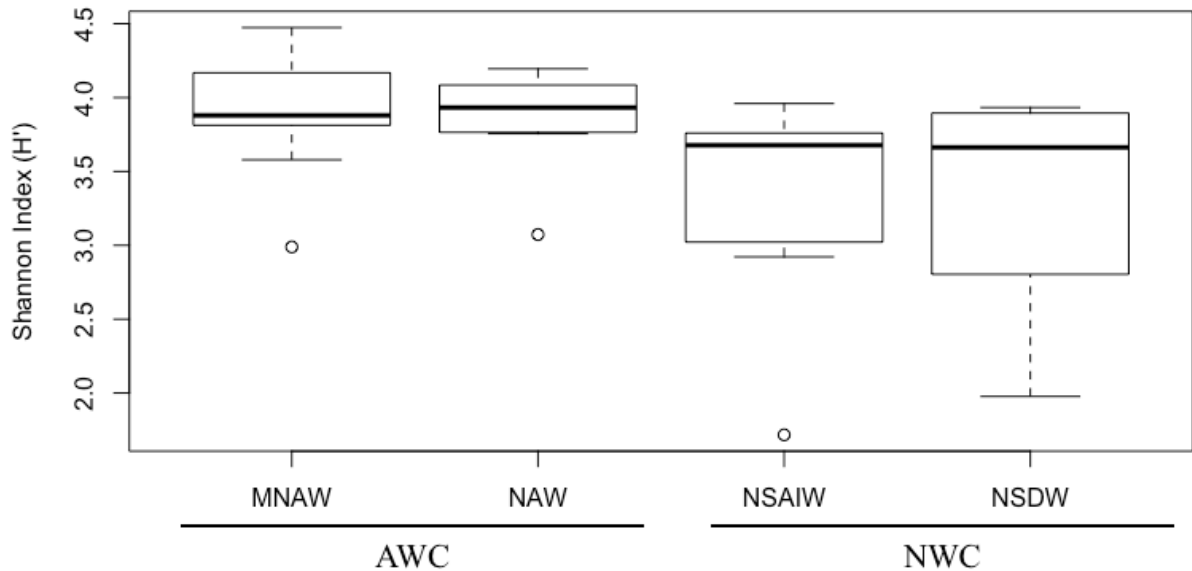
Figure 2.4. A boxplot representation of the Shannon Diversity indices for each water mass and water core, including mean values (bold lines), range values (rectangles), standard deviation (whiskers) and outliers (circles). Abbreviations: NAW: North Atlantic Water, MNAW: Modified North Atlantic Water, NSAIW: Norwegian Sea Arctic Intermediate Water; NSDW: Norwegian Sea Deep Water; AWC: Atlantic Water Core, NWC: Nordic Water Core.

In addition to changes in diversity, communities were further compared based on composition of present organisms via nMDS plot indicating whether the water masses belonging to the same water core contain microbial communities more closely related to each other than those belonging to water masses from different water cores. The community distribution plot across FSC water temperature is presented in Figure 2.5. The plot in addition to the PERMANOVA test show that both NWC and AWC have distinct microbial communities, which are more closely related to each other based on water-core (*p* value = 0.001 (< 0.05)) than on water mass (*p* values > 0.2 (> 0.05)). Additionally the PERMANOVA tests indicate that a major portion of the total variability of community compositions (36.7%) can be accounted for by their difference in Water Core (Water Core specificity). Dispersion tests (PERMDISP) based on water core, showed small pseudo-F

values (0.73) and large *p* values (0.67 (>0.05)), which indicated that the water core differences of the samples within the nMDS plots were truly based on clustering, not just a happenstance from their dispersion. This confirmed the distinguished groupings of the samples (emphasised with ellipses) based on water core not water mass.
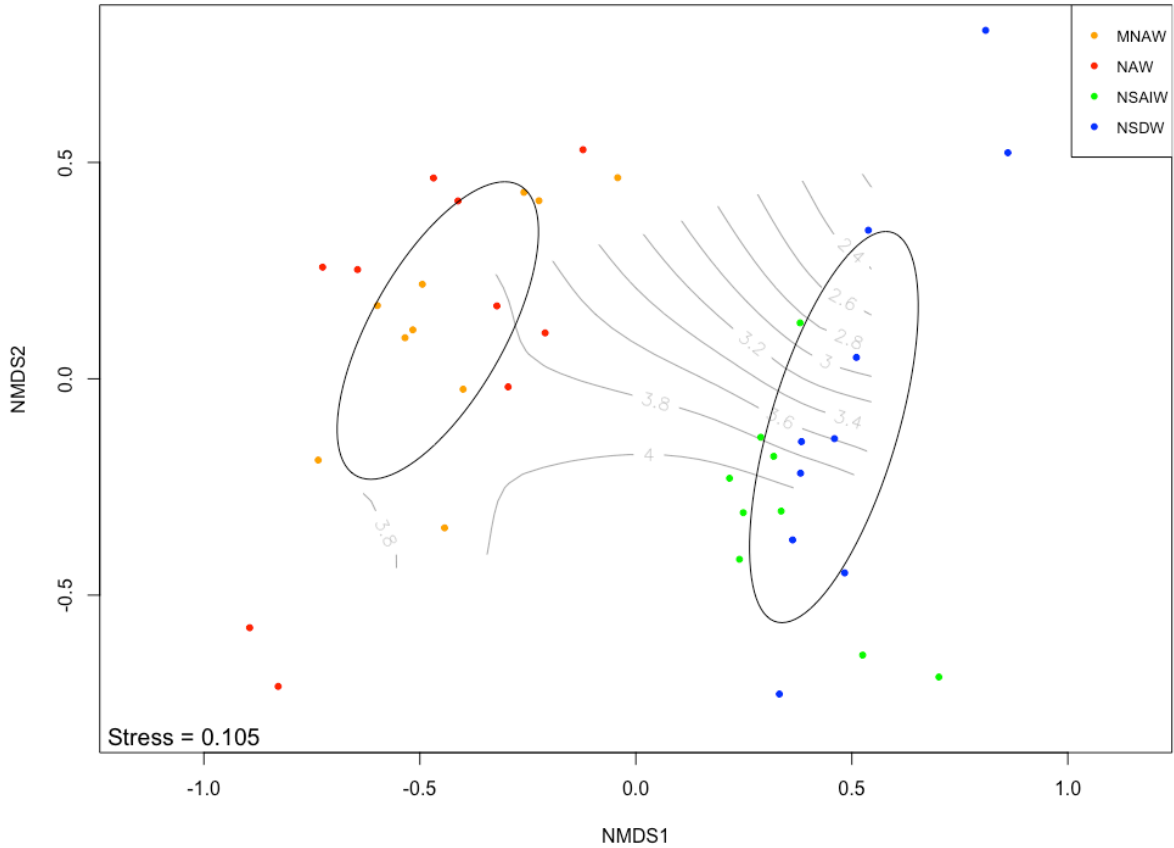
Figure 2.5. Non-metric multidimensional scaling ordination diagram of community composition variations in FSC water masses and a gradient of their Shannon diversity indices (contour lines). The ordination was based on the Bray-Curtis similarity matrix of the relative abundance data obtained from MiSeq 16S rRNA gene fragment amplicon sequencing. Dots indicate samples. Ellipses highlight sample groupings based on water core.

2.3.4 Seasonal changes in FSC microbial community

As shown in Figure 2.4, the microbial communities of the AWC showed considerably higher species richness than those of the NWC (Section 2.3.3). The seasonality interrogation via the Shannon diversity indices, however, allow for viewing the diversity variation within each water core based on season. Figure 2.6 shows increased diversity during the spring seasons and relatively even levels between the fall and winter seasons in the AWC. Significant

variation in diversity of the communities (Welch's t-test) within the AWC was not found (p values > 0.1), however, significant variation in the composition of the communities (PERMANOVA) was found between the Fall, Spring and Winter communities (p values < 0.05). Conversely, the NWC had relatively stable levels of diversity throughout the spring and fall seasons of 2014 and 2015 (p values > 0.8 based on Welch's t test), but a significant decrease in species richness was observed in the winter of 2015 (p values <0.05) (Figure 2.6). Similarly, significance in variation of community composition (PERMANOVA tests) was not observed between the spring and fall seasons (p value > 0.2), however a significant variation was observed for the winter season (p value = 0.001). Results indicate that the winter season of the NWC accounts for the majority of community changes. PERMDISP test where season was used as a factor explored whether seasonality was a major contributor to sample dispersion within the nMDS plot (Figure 2.5). Results showed a large pseudo-F value (5.05) and a small p value (0.024 (<0.05 threshold)) indicating that the nMDS plot accurately represented the seasonal variation of both water cores and that this community variation was accounted for by the dispersion of the samples throughout each sample cluster on the nMDS plot.

The described analysis results from microbial community seasonality are also reflected in the shift of dominant microbial taxa during the different seasons in the two water cores, which can be viewed in the microbial profiles (Figure 2.7). As the microbial profiles are classified only to "order" level, a more detailed breakdown of community taxonomic composition is shown in Table 2.4. Genus *Lentibacter belonging to the order Rhodobacterales* showed distinctly higher abundance in the AWC during the fall and winter seasons (19-22%), while the order SAR11 clade showed highest abundance in AWC during the spring (25%). In the NWC, the genus *Lentibacter* showed prevailing abundance in the NWC during the winter season (~37%). The order *Alteromonadales* also showed noticeable seasonal variation within the NWC, with the genus *Alteromonas* increasing from <0.5% to 8.5% abundance during the spring seasons and the genus *Pseudoalteromonas* increasing to levels as high as 26% during winter in the NWC microbial community (Figure 2.7).
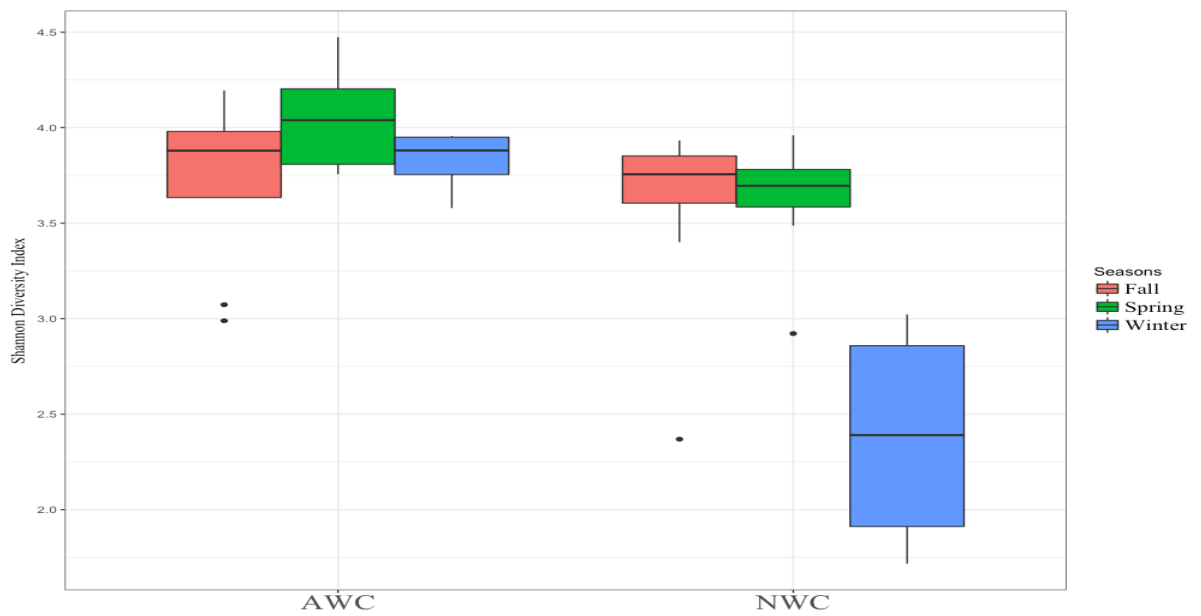
Figure 2.6. Differences in Shannon diversity indices from the microbial communities of each water core and season. Whiskers represent standard deviation and black dots indicate data outliers.
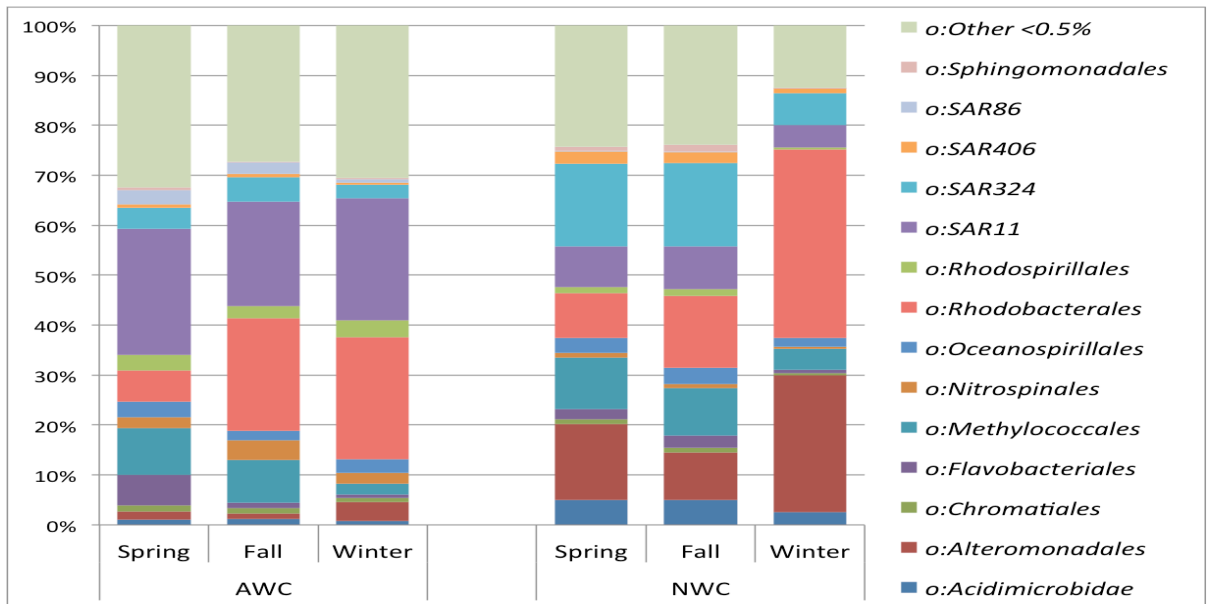
Figure 2.7. Microbial community profile for each water core during spring, fall and winter seasons of years 2014 and 2015.

Table 2.4. Average relative abundance (and standard deviation, n=4 or 8) of each taxonomic classification (most abundant order (>0.1%) and prominent genera) over spring, fall and winter seasons of years 2014 and 2015 for each water core.

Average Relative Abundance For Each Water Core per Season							
Water Core	AWC				NWC		
Taxa	Spring	Fall	Winter		Spring	Fall	Winter
o:Acidimicrobidales	1.09 (0.00)	1.16 (0.01)	0.84 (0.00)		4.9 (1.59)	4.96 (2.20)	2.55 (0.25)
o:Alteromonadales (residuals)	0.08 (0.02)	0.13 (0.61)	0.09 (0.00)		0.11 (0.05)	0.13 (0.02)	0.05 (0.00)
g:Alteromonas	0.25 (0.31)	0.06 (0.06)	0.08 (0.07)		8.49 (15.28)	0.36 (0.65)	0.27 (0.04)
g:Colwellia	0.05 (0.04)	0.13 (0.15)	0.07 (0.02)		1.13 (1.87)	0.55 (0.42)	0.21 (0.08)
g:Marinobacter	0.06 (0.06)	0.02 (0.01)	0.02 (0.02)		0.09 (0.07)	0.07 (0.08)	0.00 (0.00)
g:Pseudoalteromonas	1.07 (0.41)	0.63 (0.48)	3.45 (1.49)		5.18 (6.08)	8.29 (14.15)	26.73 (0.64)
o:Chloroplast	1.77 (1.73)	0.22 (0.20)	0.91 (0.19)		0.05 (0.05)	0.08 (0.03)	0.02 (0.01)
o:Flavobacteriales	6.01 (8.98)	1.08 (0.38)	0.57 (0.06)		1.73 (0.92)	2.46 (1.52)	0.61 (0.05)
o:Kordiimonadales	2.21 (0.99)	0.50 (0.21)	1.13 (0.19)		0.01 (0.00)	0.02 (0.02)	0.00 (0.00)
o:Methylococcales	9.42 (7.89)	8.43 (4.29)	2.26 (0.30)		10.23 (0.74)	9.42 (3.31)	4.11 (0.66)
o:Nitrospinales	2.10 (0.03)	4.02 (0.06)	2.15 (0.20)		0.95 (0.18)	0.88 (0.33)	0.45 (0.11)
o:Oceanospirillales (resisduals)	2.84 (0.24)	1.69 (0.08)	2.11 (0.14)		2.32 (0.10)	1.68 (0.09)	0.81 (0.01)
g:Alcanivorax	0.26 (0.02)	0.06 (0.06)	0.02 (0.02)		0.50 (0.59)	0.07 (0.07)	0.01 (0.01)
g:Halomonas	0.05 (0.03)	0.09 (0.15)	0.09 (0.00)		0.15 (0.09)	1.47 (2.89)	0.17 (0.03)
g:Oleispira	0.05 (0.04)	0.07 (0.02)	0.40 (0.30)		0.09 (0.07)	0.05 (0.03)	0.79 (0.63)
o:Rhodobacterales (residuals)	3.42 (0.51)	3.02 (0.27)	2.31 (0.07)		1.38 (0.56)	0.52 (0.48)	0.18 (0.02)
g:Lentibacter	1.83 (1.12)	18.99 (9.85)	21.79 (7.09)		6.30 (7.55)	12.81 (3.16)	36.89 (1.23)
g:Sulfitobacter	0.66 (0.63)	0.10 (0.16)	0.33 (0.11)		0.82 (0.57)	0.33 (0.43)	0.39 (0.16)
o:Rhodospirillales	3.13 (1.57)	2.45 (0.60)	3.36 (0.68)		1.24 (0.32)	1.30 (0.48)	0.45 (0.06)
o:SAR11	25.24 (13.75)	20.92 (7.22)	24.35 (3.43)		8.11 (2.10)	8.52 (2.73)	4.39 (1.11)
o:SAR324	4.24 (2.99)	4.88 (0.90)	2.72 (0.79)		16.50 (2.56)	16.54 (4.54)	6.30 (1.58)
o:SAR406	0.58 (0.52)	0.63 (0.24)	0.37 (0.19)		2.48 (0.51)	2.24 (0.67)	0.96 (0.15)
o:SAR86	2.87 (2.63)	2.27 (0.77)	0.72 (0.01)		0.06 (0.05)	0.10 (0.07)	0.03 (0.02)
o:Verrucomicrobiales	0.43 (0.38)	0.47 (0.06)	0.50 (0.18)		0.44 (0.21)	0.67 (0.42)	0.15 (0.03)
Other + low abundance (<0.1%)	28.39 (0.64)	25.64 (0.07)	25.22 (0.10)		23.06 (0.76)	21.41 (0.18)	10.68 (0.03)
Total Abundance	98.01	97.66	95.86		96.32	94.93	97.20

2.4 Discussion

2.4.1 Sampling logistics of the FSC water masses

As mentioned in Chapter 1, the FSC has been hydrographically monitored for more than a century. This involved the employment of particular route and sampling logistics, kept consistent over decades and which did not accommodate for optimized interrogation of the microbial communities throughout the FSC water column. In order to comply to the already established sampling programme and protocols of MRV *Scotia*, the sampling logistics of the present study required targeting of each water mass through pre-selection of locations and depths based on accumulated hydrographic knowledge of the region (**Table** 2.1 and Figure 1.2). This required the annual stability and general predictability of each water mass, especially through the routinely monitored locations and during the period of the sampling

cruises. As discussed in the introduction section of this chapter (Section 2.1), the MEIW mass exhibits seasonal variability, and presence within the dynamic mixing zone of the FSC (within 400-600m depth) (Berx, 2012; Read and Pollard, 1992). Due to the variability of this water mass and the sampling logistics of MRV *Scotia*, a consistent location and depth for this water mass could not be reliably selected. Therefore this water mass was excluded from this FSC microbial community interrogation.

2.4.2 Vertical distribution of community profiles

Before commencing with any analysis of the microbial profiles, the results from the extent of community coverage analyses (rarefaction curves) need to be considered. The rarefaction curves presented in Figure 2.3 exhibited a plateauing behaviour typical for sampling (Lozupone and Knight, 2008; Gotelli and Colwell, 2010), which has captured the major portion of the absolute microbial diversity existing within the studied sites. The results from this analysis allowed for the reliable comparability of the community profiles and subsequent analyses. As the majority of the diversity within a site was represented in the sequences of this study, comparative analyses of the structure, diversity, composition and dynamics of the communities from different depths and locations could be more reliably produced.

The FSC microbial community profiling was performed per water mass under the assumption that the microbial assemblages would have unique structure and diversity specific for each water mass, similarly to the North Atlantic water masses reported in the study of Agogué *et al.* (2011). The community profiles of both Atlantic water masses (comprising the AWC), however, were found to be relatively similar (Figure 2.1 and Table 2.3), with high representation by members of the class *Alphaproteobacteria*, especially the SAR11 clade and the genus *Lentibacter*, seconded by the class *Gammaproteobacteria* with dominant representation by the order *Methylococcales*. Since the waters from both AWC masses originate from the North Atlantic body, it was not surprising that both microbial profiles would be concurrent with the North Atlantic surface water profiles reported by Agogue *et al*. (2011) (discussed in Section 2.1). The two Norwegian Sea water masses forming the NWC also showed major similarities in their microbial profiles (Figure 2.1 and Table 2.3) with *Alpha-* and *Gamma- proteobacteria* being relatively equally dominant, followed by predominance of *Deltaproteobacteria* represented by SAR324. In comparison, the Norwegian sea deep-water microplanktonic investigation of Jensen *et al*. (2012), also

suggested of very similar profiles: almost shared dominance of *Alpha-* and *Gamma-proteobacteria*, high presence of SAR324, SAR406 and *Bacteroidetes* (Jensen *et al.*, 2012). Considering the role of the FSC as a dynamic gateway for water exchange between the Norwegian Seas and the North Atlantic, these microbial profiles confirm the expected transference effect between two environments. Based on this findings, it was determined that the microbial profile results of this study disagreed with the initial hypothesis that FSC microbial profiles would be water mass-specific. The similarities in microbial profiles between water masses belonging to the same water core, as well as the differences between the microbial profiles belonging to different water cores, suggested of a water core (and origin-based) specificity, more than of a water mass specificity. Aside from origin-based specificity, these results could also be explained by the temperature contrast of each water core. Considering that both water masses within the AWC display considerably higher temperatures (7°C - ~ 10°C) and salinities (>35.1), contrasting those of the NWC (–0.5°C - 3°C and <34.1 respectively) (**Table** 2**.**1), it appears likely that that both origin and water characteristics are the driving factors of the vertical water column microbial variability within the FSC.

The alpha and beta diversity analyses confirmed the water core-based specificity observations suspected from the microbial profiles. The Welch's test performed on the Shannon diversity indices confirmed the observation that microbial communities belonging to different water masses within the same water core are generally indistinguishable in their diversity (*p* value > 0.05) and that community diversity contained within AWC (in both NAW and MNAW masses) was significantly different (*p* value < 0.05) from community diversity within NWC (NSAIW and NSDW) (Figure 2.4). Shannon diversity indices of the NWC[1] (comprised primarily of Norwegian Sea water) were concurrent (and very similar (~3.3)) to those obtained by Jensen *et al.* in 2012, in a study exploring the microplankton communities of 300-370 m Norwegian Sea waters (Jensen *et al.*, 2012), suggesting that the region's diversity was accurately measured in this study. While Shannon diversity indices measured the species richness of each communities and allowed for their comparison, the beta diversity measurements such as the nMDS plot and the PERMANOVA tests allowed for deeper comparison between communities, taking into account community composition in

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[1] Shannon indices for bacterioplankton communities in the North Atlantic Ocean obtained through high-throughput sequencing was not found so AWC diversity comparison was not possible.

addition to richness. The beta diversity tests showed the core-based specificity, by highlighting the distance in community compositions between the two water cores. The PERMDISP test (dispersion test) factored in the water mass variance between communities. Its results confirmed that the two water cores formed two genuinely distinct communities. The water core specificity observed in this study is concurrent with observations of declining total organic carbon concentrations (by approx. 16%) and bacterioplankton production with increase in depth for the FSC region, especially >600 m (hence the NWC reigns) (Kramer *et al.*, 2005). The vertical variation in organic carbon, primary production, temperature and salinity could therefore be major driving factors in community composition differences between the AWC and NWC. As each water core appear to have its own physico-chemical "identity", it is likely to reflect in the microbial community profiles of the FSC.

2.4.3 Seasonal variability of FSC microbial communities

Seasonality appeared to play a major role in the differences observed between the two water core communities. The AWC communities appeared to respond favourably to spring conditions, with slight decrease in diversity during the fall or winter season (Figure 2.6). This could be due to seasonal temperature variability, found within the AWC (Hátún *et al.*, 2005). In addition to the expected change in temperature with season, multiple studies have reported on seasonal variability of nutrient, oxygen, carbon dioxide, bacterioplankton production and organic carbon levels of the North Atlantic surface waters, which is also likely transferable and reflecting onto the community composition of AWC within the FSC (Peng *et al.*, 1987; Lazarevich *et al.*, 2004; Laane *et al.*, 1996). Although seasonality variation of nutrient levels specifically for the FSC have thus far not been reported, independently-collected oceanographic data from the FSC have suggested of potential nutrient seasonal variability in the Atlantic waters of the FSC (personal communication, Bee Berx).

While the AWC communities appeared to respond favourably to spring conditions more than to fall or winter season (indicated by increased diversity during Spring), the NWC communities appeared to respond sharply to the winter season in their community's diversity (Figure 2.6). The communities within the deep-sea FSC waters (the NWC) appeared unaffected by the change of Spring or Fall seasons, yet significantly affected by winter

conditions (Figure 2.6 and Figure 2.7). Changes in community diversity within the NWC during the winter season could again be explained by an adverse affect of low temperatures and decreased nutrient supply to the deep-sea layers during the winter season. However, consideration needs to be taken of the lack of winter season duplication from this study (lack of Winter 2014 samples, relying only on Winter 2015 samples), allowing for the possibility that the observed winter-related NWC community shift was an uncharacteristic one time occurrence. A complex interaction of multiple environmental factors such as inflow of specific nutrient or recent weather event leading to water column disruption could have resulted in blooms in specific genera (e.g. *Lentibacter* or *Pseudoalteromonas*), which could have produced the observed species diversity decline. A study by Turrell *et al.* (1999) reported a larger scale inter-annual (in terms of decades) rather than intra-annual variability (within a year) of the physico-chemical composition in the deep-sea FSC waters, suggesting that the observed winter community variation may not be a true reflection of the intra-annual community seasonality. A more systematic and long-term approach to determining seasonality of the communities of both the Atlantic and Nordic Water Cores could be beneficial to authenticate and qualify the observed seasonal variations of all FSC microbial communities.

2.4.4 Bacteria of the FSC and their spatiotemporal dynamics

Although this study represented a relatively short (2-year) spatiotemporal analysis of the microbial community dynamics in the FSC, the data suggested specific microbial signatures associated to certain seasons and water cores. Figure 2.7 and Table 2.4 showed that bacterial taxa, such as *Bacteroidetes*, SAR11 and *Cyanobacteria*, were enriched mainly during the spring in the AWC compared to in any other season or water cores. These taxa may have a preference for warmer waters, and thus their abundance may be temperature dependent, although nutrient concentration variability during the seasons should not be discounted.

The SAR11 clade, the most ubiquitous bacteria in the marine environment, is known for preferring warm oligotrophic pelagic marine waters (Giovannoni, 2016) as also observed also in this study (~25% average abundance in the spring-time communities of the AWC) Table 2.4. Ecotypes of the SAR11 clade, such as HTCC1062, were likely to contribute to the microbial seasonality observed in the AWC of the FSC waters (Giovannoni, 2016; Peng *et*

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al., 1987; Lazarevich *et al.*, 2004). The clade, however also contains psychropiezophilic ecotypes (Giovannoni, 2016) which would also explain the 8% abundance of these organisms in the deep layers of the FSC. The seasonal variation found in the phylum of *Cyanobacteria* was also not surprising (~2% in the spring-time surface communities compared to <1% presence in other seasons and depths), especially in the northern latitudes where light intensity and exposure is significantly variable over the different periods of the year (Michelou *et al.*, 2007). With respect to the phylum *Bacteroidetes*, which are known for specialization in CO_2 fixation and degradation of higher molecular weight compounds (Fernandez-Gomez *et al.*, 2013), their enrichment during the spring season (9% compared to <4% in other seasons and depths) may have been related to the higher inflow of nutrients and total organic carbon concentration during this period of the year (Peng *et al.*, 1987; Lazarevich *et al.*, 2004). The presence and consistent levels of *Bacteroidetes* (~3-4%) in the NWC, affirms the existence of psychropiezophilic members within NWC.

Other taxa, such as the genera *Lentibacter* and *Pseudoalteromonas*, and the group SAR324 (represented by OTUs 1, 2 and 3 respectively in this study), appeared to exhibit a preference for colder conditions, proliferating in either the colder seasons of the year or in the deeper colder waters in the FSC. Their elevated abundance under such conditions was suggestive of a psychrotolerant/psychrophilic adaptation, or even psychropiezophilic behaviour. The presence of the genus *Lentibacter* was found highly variable for the AWC with an average of 1.83% in the spring seasons and a sharp increase to ~19% and even ~22% during the fall and winter seasons. Although high abundance of the genus was also found in the NWC (6% to ~37%), the highest average abundance of the genus appeared to be in the AWC. This appears consistent with its typical associations with higher organisms such as phytoplankton, which would primarily be found in the surface water layers (Pujalte *et al.*, 2014). The genus *Lentibacter*, which is associated with the order *Rhodobacterales*, is recognized for its high environmental adaptability, broad metabolic capacity, associations with more complex organisms, potential for copiotrophic behaviour (thriving in nutrient and carbon rich environments such as chemically polluted ecosystems) and biofilm production (Pujalte *et al.*, 2014; Dang *et al.*, 2008; Gilbert *et al.*, 2012; Li *et al.*, 2012). Based on these characteristics, strains within the *Lentibacter* genus could potentially hold functional potential for hydrocarbon degradation throughout the FSC water column.

Among the most abundant bacteria within the NWC of the FSC, were also the genera *Pseudoalteromonas* and *Alteromonas* of the order *Alteromonadales*. The order *Alteromonadales* increased in abundance from an average of 1.5% in the AWC to ~14% in the NWC especially during the spring season. Notably the genus *Pseudoalteromonas* was the most dominant genus of the *Alteromonadales* order with a relative abundance of ~11%. The genus was also abundantly found in isolate libraries from the North Seas (Eilers *et al.*, 2000), and has been reported to become enriched in these waters when exposed to crude oil (Chronopoulou *et al.*, 2015). Additionally, both *Alteromonas* and *Pseudoalteromonas* have been described as psychrotolerant, (Médigue *et al.*, 2005; Giudice *et al.*, 2010), capable of producing biologically active extracellular agents (e.g. exopolymers), and of degrading a variety of alkane and polycyclic aromatic hydrocarbons (PAHs) (Hedlund and Staley, 2006; Chronopoulou *et al.*, 2015; Holmstrom and Kjelleberg, 1999).

When inspecting the microbial profiles of the NWC, an abundance of methanotrophic groups such as SAR324 (~14% average abundance) and *Methylococcales* (~9%) was noticeable (Table 2.4). Both groups are especially well adjusted to a methane utilization lifestyle and are commonly found in deep-water cold marine environments near methane sources such as hydrothermal vents or in associated plumes (Brown and Donachie, 2007; Bowman, 2014). Similarly to the NWC of the FSC, elevated levels of the SAR324 clade (10-15%) have also been reported in the Norwegian Sea (Jensen *et al.*, 2012). Notably, based on their genetic composition, the SAR324 clade have also been postulated to exhibit aromatic hydrocarbon degradation abilities (Cao *et al.*, 2016), although this remains unsubstantiated by empirical means. The elevated levels of methanotrophs in the FSC was strongly indicative of the presence of dissolved methane in the FSC water column as both groups are usually found in lower levels in marine environments if no methane is present (Biers *et al.*, 2009). Interestingly, however, there are no known hydrothermal vents in the FSC (as discussed in Chapter 1, Section 1.2.1). The presence of a few cold seeps in the Norwegian Sea along the coast of Norway as well as in the North Sea, however, have been reported (German *et al.*, 2011). It is unclear whether these distant cold seeps could be sources of these distinctly elevated methanotrophic assortment found in the FSC, although their transference with the NSAIW and NSDW masses is not impossible. Another likely source of methane into the FSC is the presence of a so-called whale-fall site near the FSC (German *et al.*, 2011). Whale carcasses are a major source of organic carbon which can sustain entire chemosynthetic

ecosystems and lead to the long-term production of methane (German *et al.*, 2011; Smith and Baco, 2003). Lastly, it is possible that the on-going crude oil prospecting and extraction processes in the region discussed in Chapter 1, Section 1.2.2, produce small amounts of methane leakage, which could support these methanotrophs.

In summary, this study produced an important microbial baseline for a developing, high pollution-risk region such as the FSC. It revealed seasonal dynamics of the microbial communities as well as their distinguishability based on water core rather than water mass. In order to extend the understanding of this ecosystem (e.g. any decadal microbial variation, MEIW communities, anthropogenic impact), a more systematic investigation into the microbiology of the distinct water masses would be required. Considering also the increasing risk of oil pollution in the FSC, a routine microbial monitoring of the FSC water masses is advised.

CHAPTER 3: INVESTIGATING THE RESPONSE OF FSC MICROBIOTA TO CRUDE OIL

3.1 Introduction

As discussed in Section 1.1, the FSC is a region of dynamic mixing of the Nordic and Atlantic waters. The intense hydrographic mixing, the increasing exploration and extraction of oil, have raised awareness of the potential threat for a major spill in the region. Additionally, the implications from the recent oil spill disaster in the Gulf of Mexico (discussed in Section 1.2.2) has revealed the need for understanding the response of the region's microbiota to crude oil in order to help develop optimized bioremediation strategies to combat future disasters of this nature. In this study, the response of FSC microbiota to crude oil was investigated in laboratory-based microcosms under average *in situ* FSC and moderate (control) temperature conditions. The changes in the microbial cultures within the microcosms during crude oil exposure were monitored and compared to non-exposed microcosms. The microbial profiles of communities exposed to crude oil at 4°C was compared to those of baseline community profiles. In addition, attempts were carried out for the isolation of FSC strains with potential for biodegradation of various model hydrocarbons as well as crude oil. Thus, this study was designed to be the first attempt at assessing the hydrocarbonoclastic activity of FSC bacterioplankton.

One location in the FSC, known as station FIM6a, was selected for sampling of the water column to conduct this investigation. The station FIM6a has been monitored by oceanographers for decades and was deemed characteristic of the FSC water column providing suitable coverage of surface and subsurface waters from above the mixing zone (both within the AWC), as well as subsurface and near-seabed (deep-sea) waters from below the mixing zone (from within the NWC) (Bee Berx; personal communication). The study implemented a crude oil exposure experiment (CRO-EE) performed twice: under a temperature of 4°C (CRO-EE/4°C), chosen for its representation of the average FSC water column temperatures, and under a moderate temperature of 20°C (CRO-EE/20°C), chosen as control temperature. Based on the findings of previous studies that have investigated the effects of temperature on the microbial degradation of crude oils (Atlas, 1975; Gibbs *et al.*, 1975; ZoBell, 1969; Mulkins-Phillips and Stewart, 1974; Ludzack and Kinkead, 1956;

Brakstad and Bonaunet, 2006), it was anticipated that low temperature (such as 4°C) would have an adverse effect on any microbial response to provided crude oil in the CRO-EE. It was therefore suspected that the microbial communities (from any depth) would respond more rapidly at 20°C than at 4°C. Unfortunately, due to logistical limitations at the time of experiment design, a detailed chemical analysis of the oil layer or cell density measurements were not possible. The response, however, was expected to be observable as changes in the aqueous and oily layers of the exposed cultures, which could be detected as a comparison to unexposed or sterile-exposed cultures (negative controls). Therefore the changes in cultures turbidity, colour, crude oil layer thickness and consistency were monitored visually and gauged comparatively to negative controls, as well as across temperatures of exposure (4°C and corresponding 20°C (control) experiments). The crude oil exposed cultures were also used for isolating and identification of FSC strains, with clearly observable or suspected (putative) hydrocarbonoclastic activity. The cultivatable strains were grown on solid synthetic seawater agar media with crude oil (CRO), phenanthrene (PHE) or hexadecane (HEX) as a sole carbon source, in order to assess their degradative capacity. Taxonomic identification for isolated strains was performed by Sanger sequencing of their near full-length 16S rRNA genes.

Based on recent studies of microbial dynamics within an oil spill (discussed in Section 1.5.4), it was suspected that the microbial communities of the FSC might exhibit a shift in species richness because of the crude oil exposure, from a highly diverse baseline community to one considerably less diverse, but predominated by a limited number of hydrocarbon utilizing bacteria (Joye *et al.*, 2014; Head *et al.*, 2006; Dubinsky *et al.*, 2013). In order to examine the response of the bacterial community in the water samples to crude oil under average FSC temperature, DNA was extracted and analysed by Illumina MiSeq sequencing. It was anticipated that the microbial assemblages cultivated with exposure to crude oil at 4°C may contain oil-degrading psychrophilic and psychrotolerant bacteria such as the *Oleispira*, *Colwellia* and *Psychromonas* (Yakimov *et al.*, 2007).

In an attempt to increase resolution into the internal diversity of the most enriched operational taxonomic units (OTUs), a technique called oligotyping was employed. Currently this is a novel computational method for recognizing minute, consistent differences between closely related sequences, such as those clustered together at 97% similarity (Eren *et*

al., 2013). Classical approaches in identifying operational taxonomic units (OTUs) are limited to clustering of similar sequences at a maximum threshold of 96-97% (about genus level), in order to curtail the effects of randomly produced sequencing errors (Huse *et al.*, 2008). The oligotyping approach, however, screens for such errors and considers only consistent discrepancies between sequences (Eren *et al.*, 2013). When applied to 16S rRNA genes for taxonomic identification, this method has been hypothesized to improve distinguishability between organisms at a finer scale (e.g. species and strains) (Eren *et al.*, 2013).

3.2 Materials & Methods

3.2.1 Sample collection

Samples for the crude oil exposure experiment (CRO-EE) were collected during routine oceanographic monitoring cruises of *MRV Scotia* to the FSC, during the fall of 2014 (September 24th – October 8th, 2014) and spring of 2015 (April 24th – May 8th, 2015). The location for sampling that was selected was station FIM6a at latitude 60° 38.00'N and longitude 04° 54.00'W. Water samples (250 mL each) were collected within the water column at depths of 5 m (AWC; surface water), 400 m (AWC above mixing zone water), 700 m (NWC below mixing zone water) and 1000 m (NWC deep-sea water). It should be noted that as part of the baseline microbial community study described in Chapter 2, 1 L samples from each water mass in the FSC were also collected during the same sampling cruises. The microbial profiles of these samples (performed and described throughout Chapter 2 (results in Section 2.3.2)) were also used in this study as supplementary controls representing FSC baseline communities. To complement this study, a small surface-layer sediment sample (~5 g) collected during the fall of 2014 cruise from station FIM6a (depth ~1100 m), was also provided as a courtesy of Dr Rob Ferguson (Aberdeen University). The sediment sample was used only for attempted isolation of putative hydrocarbonoclastic FSC strains. All samples were stored at 4°C immediately upon collection until aseptic laboratory conditions were available for further processing (less than 2 weeks).

3.2.2 Preparation of CRO-EE microcosm incubations

In order to setup the CRO-EE microcosms, the entire sample volume of 250 mL seawater from each depth was amended with 0.2% w/v nitrogen and phosphorous according to the

protocol of Yakimov et al., (2003). The samples were divided in 18× 10 mL volumes and aliquotted in acid-washed and sterilized 15 mL glass culture tubes. Three of the 10 mL cultures were immediately filtered through 0.22 μm pore size MCE filter (Millipore Sigma) to collect microbial cells for the baseline microbial community assessment (T0W-Baseline) from each depth. Of the other 10 mL cultures, 12 were amended with 0.10 μL (0.1% v/v) Scheihallion crude oil as a sole carbon-source (used as model oil throughout the experiment and provided as courtesy from British Petroleum), based on the method of Yakimov *et al.* 2003. These were setup in triplicates according to exposure duration as follows: T1W+CRO for crude oil exposure of 1 week, T2W+CRO for exposure of 2 weeks, T4W+CRO for 4 week exposure and T6W+CRO 6 weeks exposure to crude oil. The last set of 10 mL seawater cultures were designated as negative controls and incubated for 6 weeks without the addition of carbon source (SW-noCRO-Ctls treatments). Next, a set of triplicate cultures was designated as sterile controls and prepared using 10 mL sterile ONR7a medium (Dyksterhouse *et al.,* 1995) amended with 10 μL of the Sheihallion crude oil (T6W-CRO-Ctls treatments). For clarification, treatment names and sample distribution is shown in Table 3.1.

Table 3.1. Crude oil exposure experiments (CRO-EE) sample setup details for each depth. All treatments were comprised of FIM6a seawater with the exception of the ONR+CRO-Ctls, which were sterile controls performed with ONR7a media instead of seawater. All treatments were performed in triplicate for each water depth: 5 m, 400 m, 700 m and 1000 m.

Treatment name	Water sample volume	CRO added	Exposure time
T0W-Baseline	10 mL	none	0 weeks
T1W+CRO	10 mL	10 μL	1 week
T2W+CRO	10 mL	10 μL	2 weeks
T4W+CRO	10 mL	10 μL	4 weeks
T6W+CRO	10 mL	10 μL	6 weeks
SW-noCRO-Ctls	10 mL	None	6 weeks
ONR+CRO-Ctls	10 mL	10 μL	6 weeks

All cultures were maintained upright in the dark with gentle horizontal shaking (~150 rpm). Cultures prepared from water samples collected in the fall of 2014 were incubated at 20°C (CRO-EE/20°C), whereas cultures prepared from water samples collected in spring 2015 were incubated at 4°C (CRO-EE/4°C) until the termination of these incubations at 1, 2, 4 or 6 weeks. At the designated end time, samples were visually studied for changes in culture

turbidity, colour, as well as crude oil layer changes (presence and extent of droplet formation (emulsification), mucous-like substance on the water-oil interface (biofilm formation) or for any noticeable reduction in thickness of crude oil layer). Changes in these parameters were evaluated based on comparison to the sterile (ONR+CRO-Ctls) and negative control (SW-noCRO-Ctls) treatments. For the sediment sample, a small fraction of sediment (~1 g) was amended with crude oil, nitrogen and phosphorous (w/v) as described above and incubated as the CRO-EE/20°C for up to 6 weeks. The sediment samples were not considered part of the crude oil exposure experiment and were only used for isolating of potential oil-degrading strains.

3.2.3 Enrichment and isolation of hydrocarbon-degrading bacteria

At set exposure time (0 (a.k.a. baseline), 1, 2, 4, or 6 weeks), the designated samples were streaked onto ONR7a agar plates (Dyksterhouse *et al.*, 1995). Each agar plate was supplemented with crude oil (CRO), *n*-hexadecane (HEX) or phenanthrene (PHE) (99%, Fisher Scientific) as the sole carbon-source as follows: to prepare the CRO agar plates, a thin and even layer of 50 μL sterilized (Sterivex-GP 0.22 μm, polyethersulfone filters, Millipore) crude oil was spread onto ONR7a plates prior to streaking; to prepare the HEX and PHE agar plates, 1% solutions of HEX or PHE (dissolved in acetone) were made, and these were sprayed onto agar plates after streaking; this produced a thin film of the hydrocarbon on the agar surface. A non-streaked blank agar plate was similarly prepared with each carbon source in order to act as controls to assure the sterility of the method. The acetone was allowed to evaporate (ca. 5-10 sec) prior to incubating the plates in the dark at 20°C. The remaining volume the liquid water samples containing crude oil were harvested for microbial mass by centrifugation for 20 min at 17,000 ×*g* at 4°C. Harvested biomass was used for DNA extractions, 16S rRNA gene amplifications and sequencing. A small volume of the sediment sample was also streaked onto ONR7a agar plates, amended with each carbon source individually and treated as the rest of the plates.

Bacterial colonies that grew on the agar plates were examined on a weekly basis for the formation of halos on the supplied carbon source layer around present colonies (this included examination of the un-streaked control plates). Colonies with halos were selected individually with sterile toothpicks and streaked onto fresh plates supplemented with the same carbon source. Sub-cultured plates were incubated under the same conditions (in the

dark at 20°C) until growth was again observed and formation of halo was confirmed. Sub-culturing was repeated until the all the colonies in a plate appeared well separated and consistent in colour, halo formation and size (assumed pure cultures). Once pure cultures were obtained, they were grown out in liquid cultures by transferring a colony of each isolated strain (with sterile toothpicks) into 3 mL of sterile ONR7a liquid medium amended with ~0.1% w/v of HEX, PHE or CRO (v/v), based on the procedure of Gutierrez *et al.* (2013) and Yakimov *et al.* (2003). The cultures were incubated with gentle shaking (~150 rpm) in the dark at 20°C for 7-14 days, or until the cultures were observed to become sufficiently turbid. Cell biomass from each culture was collected for DNA extraction and for the cryopreservation of the isolated strains. Each isolate was labelled with the 3-letter code depictive of the substrate used for isolation as well as an identifying number. Metadata for each isolate (source water, duration of the exposure, *etc.*) were recorded. For cryopreservation, 1.4 mL of cell suspension (for each strain) was mixed with 0.6 mL of autoclaved glycerol and the glycerol stocks immediately stored in a -80°C freezer. DNA extractions were performed on cell pellets based on the protocol of Tillet and Neilan (2000), as described in Section 2.2.2.

3.2.4 PCR amplifications and sequencing of 16S rRNA genes

3.2.4.1 Partial 16S rRNA gene amplification and MiSeq sequencing
PCR amplifications of V3-V4 fragment of the 16S rRNA gene (444 bp) was performed on all DNA samples (and replicates) extracted from the CRO-EE incubated at 4°C. PCR amplification of the fragment regions, as well as the procedure for their MiSeq sequencing, data processing, microbial profiling and analysis are described in Sections 2.2.3 – 2.2.6. MiSeq sequencing was performed on only 2 out of the 3 experimental replicates per sample, depending PCR product quality and amplicon size assessed via gel electrophoresis (described below).

3.2.4.2 Near-full length 16S rRNA gene amplification and Sanger sequencing
PCR amplification of the near-full length 16S rRNA gene (1465 bp) were performed on all DNA extracted from isolates. For rRNA gene amplifications ~1 μL of DNA was used into a 25 μL PCR reactions containing 5 μL 5× MyTaq buffer (1× final; BioLine), 1.5 μL Primer

mix (0.25 μM final; 4 μM each primer), 0.5 μL DMSO (3% final), 16.8 μL H$_2$O and 0.2 μL 10 U MyTaq (1 U final; BioLine). Primers used were standard *E. coli* universal bacterial primers 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3'), synthesized by IDT (https://www.idtdna.com/). PCR conditions were as follows: Initial denaturation step 95°C for 5min, followed by 35 cycles of 95°C for 15 s, 55°C for 15 s, 72°C for 30 s. Amplifications were completed with a 7 min extension step at 72°C. Expected amplification product was of size 1465 bp. PCR product was qualified and quantified via 30min/90V gel electrophoreses step through 1% 1× TAE agarose gel using a Lambda DNA *Hind*III maker (Fisher Scientific). Gels were stained with EtBr (FisherScientific). Successful one-band PCR products from whole gene or gene fragment 16S rRNA gene amplifications were enzymatically cleaned of polymerase and primer remnants in an enzymatic reaction described in Section 2.2.3.

Sanger sequencing of the 1462 bp 16S rRNA gene fragments obtained from isolates was performed by GeneWiz, UK. At the sequencing facility, PCR amplicons underwent a PCR reaction using just one primer (27F), a mix of nucleotides (NTPs) as well as fluorescently-labelled chain terminating deoxynucleotides (ddNTPs). The products produced were therefore various lengths depending on at what base a ddNTP were incorporated. After incorporation the ddNTPs produce a fluorescent signal, which hold unique colour depending on its' nucleic acid. Therefore each ssDNA read produced carried a specific colour. The PCR products underwent capillary electrophoresis in order to separate based on size. The colour of each fragment is detected and correlated to the base number at which it was incorporated. Hence a chromatogram was produced for each forward and each reverse read fragment.

Forward reads (27F) of the Sanger sequence data were used to determine library composition. Reads were trimmed of low quality ends and manually curated for sequencing artefacts (e.g. dye blobs and base-miscalls). High quality forward reads were analysed by the BLAST against the NCBI GenBank 16S ribosomal RNA sequences (bacterial and archaeal) database. Bacterial composition of libraries was determined based on taxonomic identities. Representative sequences for each taxon were elected for sequencing with 1492R primer (reverse direction). After reverse-direction reads were sequenced, the forward and reverse reads were trimmed, conjoint/merged and manually curated using Consed/Phred/Phrap

(Gordon and Green, 2013). High quality sequences (>35 quality) representing the near full length 16S rRNA gene sequences (~1465 bp) were exported as .fasta files. Gene sequences were again analysed by BLAST in order to confirm taxonomy. Additionally taxonomic assignments were confirmed using alignments to closest-related type strains from GenBank and producing a maximum-likelihood phylogenetic, as described in Section 2.2.5. High quality sequences were then submitted to GenBank.

3.2.5 Oligotyping

Oligotyping was performed on MiSeq sequencing data obtained from the CRO-EE incubations at 4°C; this was performed with the help of the Maren Lab Oligotyping tool (https://goo.gl/PWu84c) (Eren *et al.*, 2013). For this, initially all the reads from the MiSeq analysis for the taxa of interest were selected and extracted from the preprocessed dataset using UNIX-based commands (grep, sed and cat), as well as cdbfasta tools released by The Institute for Genomic Research TIGR (Lee *et al.*, 2005). Using MOTHUR (Schloss *et al.*, 2009), selected reads were screened for homopolymers larger than 8 ambiguous bases and discarded. Reads were also subsampled using the Maren Lab oligotyping software (command "o-subsample-fasta-file"), so all samples could be equally represented. Alignment of all subsampled reads was performed via the multiple sequence aligner tool MUSCLE (Edgar, 2004). Entropy analysis and oligotyping was performed using the Oligotyping software (Eren *et al.*, 2013). Oligotyping was repeated for the most abundant OTUs as per the developer's provided pipeline until the highest possible purity score was achieved. Optimal parameters for "number of entropy components (–c) and "minimum substantive abundance" (–M) were adjusted based on the results from the repetitive oligotyping until maximum purity scores were achieved.

3.3 Results

3.3.1 Sample collection

Table 3.2 shows the characteristics of the water samples at each depth from location FIM6a during the spring of 2015 in comparison to the characteristics of the concurrently sampled water masses of the FSC. Despite collection from different locations, there were close similarities in temperature and salinity characteristics between the FIM6a water column at

depths 5 m and 700 m and the water masses NAW and NSAIW, respectively. Location FIM6a at 1000 m was selected to represent the NSDW mass for microbial profiling described in previous chapter. Water collected from depth of 400 m (FIM6a-400 m) showed some discrepancies in these characteristics relative to the closest related water mass (MNAW), which were most likely due to water mixing within the dynamic mixing zone (~350-600 m). This comparative examination between the FIM6a water column and the reciprocal FSC watermass characteristics indicated that the communities of the T0W-Baseline treatments might be considerably similar to the baseline profiles obtained for each water mass discussed in Chapter 2 (Section 2.3.2 and Figure 2.1). Therefore, the supplemental baseline controls collected from each FSC water mass as described in Chapter 2, Section 2.2.1, were also used as FIM6a baseline controls.

Table 3.2. Characteristics of water collected from 5 m, 400 m, 700 m and 1000m at FSC station FIM6a in in April –May of 2015 the FSC compared to characteristics of sampled FSC water masses during a the same time period.

Collection date	Sample name	Station name	Depth (m)	Represented Water Mass	Water Core	Location	Temp (°C)	Salinity
Spring 2015	FIM6a-5m	FIM6a	5m	NAW	AWC	61° 38.00' N, 04° 54.00' W	8.5	35.33
Spring 2015	NAW	FIM3a	150m	NAW	AWC	60° 20.25' N, 04° 09.00' W	8.7	35.34
Spring 2015	FIM6a-400m	FIM6a	400m	MNAW mix	AWC	61° 38.00' N, 04° 54.00' W	5.4	35.07
Spring 2015	MNAW	NOL08	175m	MNAW	AWC	61° 42.00' N, 04° 51.00' W	7.5	35.24
Spring 2015	FIM6a-700m	FIM6a	700m	NSAIW	NWC	61° 38.00' N, 04° 54.00' W	-0.25	34.91
Spring 2015	NSAIW	NOL07	700m	NSAIW	NWC	61° 35.00' N, 04° 15.00' W	-0.01	34.91
Spring 2015	FIM6a-1000m	FIM6a	1000m	NSDW	NWC	61° 38.00' N, 04° 54.00' W	-0.56	34.91
Spring 2015	NSDW	FIM6a	1000m	NSDW	NWC	61° 38.00' N, 04° 54.00' W	-0.53	34.91

3.3.2 CRO-EE microcosms

At the commencement of all oil exposures (time 0), the aqueous phases of all microcosms appeared clear with a smooth brownish-red crude oil layer floating at the surface. Within the first week of exposure, a noticeable increase in turbidity was observed in the aqueous phase of the cultures incubated at 20°C (CRO-EE/20°C), along with a change in colour and appearance of the oil. The aqueous phases appeared significantly more turbid compared to the sterile controls that had also been treated with crude oil. Additionally, the oil appeared to have become somewhat emulsified as observed by the formation of oil droplets, while sterile

controls (ONR+CRO-Ctls) maintained the smooth texture of their oil. By week 4 of the CRO-EE/20°C, the emulsified oil droplets appeared to be coated with mucous semi-transparent substance, presumed as biofilm (Table 3.3). At week 6, all microcosms of AWC water (from 5 m and 400 m depths) that had been exposed to crude oil and incubated at 20°C (CRO-EE/20°C) showed complete lack of crude oil layer. Conversely, the crude oil layer of all the sterile controls (*i.e.* ONR+CRO-Ctls treatments) appeared unchanged by week 6, suggesting any changes observed in the live cultures were due primarily to microbial activity. Additionally, the corresponding negative controls (SW-noCRO-Ctls) incubated at 20°C with crude oil for up to 6 weeks, did not exhibits any signs of turbidity or colour changes, suggesting that microbial activity in the live cultures was a response to the crude oil. Microcosms that were set up with the NWC water (from 700 m and 1000 m depths) exposed to crude oil and incubated at 20°C (part of CRO-EE/20°C) also exhibited noticeable changes with respect to turbidity, colour, emulsification and biofilm formation on oil droplets. However, these samples still exhibited presence of emulsified and biofilm-coated crude oil layer, albeit at noticeably reduced oil volume (Table 3.3).

In contrast, microcosms that were set up in the exact same way with the crude oil, but incubated at 4°C (CRO-EE/4°C) did not begin to exhibit changes in turbidity until the second week of exposure to crude oil. By week 6, an increase to the turbidity of the aqueous phases was observed, however it was not accompanied by noticeable changes to the cultures colour. Furthermore, only limited changes to the characteristics of the crude oil layer were observed (in comparison to the corresponding samples incubated at 20°C), thus suggesting that the crude oil may not have been as actively biodegraded (or at least to no visually noticeable extent). Sterile and negative control treatments (*i.e.* SW-noCRO-Ctls and ONR+CRO-Ctls) from the CRO-EE/4°C, showed no signs of change for any of the tracked characteristics over the course of these 6-week incubations. Details of the described visual observations that were monitored during these crude oil enrichment experiments conducted at 4°C and 20°C are shown in Table 3.3.

Table 3.3. Visual changes observed in the crude oil exposure experiment (CRO-EE) incubations conducted under 20°C (A) or 4°C (B). The microcosm observations were consistent among triplicates within samples from the AWC (5 m or 400 m), and from the NWC (700 m or 1000 m).

A.

Water Core	Treatment Name	Exposure time	Culture changes		Crude oil layer changes		
			Turbidity	Color	Emulsification	Biofilm	Volume
AWC	T1W+CRO	1 week	Moderate	Slight Yellow	Some	Some	Unchanged
	T2W+CRO	2 weeks	Moderate	Slight Yellow	Moderate	Moderate	Reduced
	T4W+CRO	4 weeks	High	Yellow	Moderate	High	Reduced
	T6W+CRO	6 weeks	High	Yellow	Dissolved	-	Almost gone
	ONR+CRO-Ctls	6 weeks	None	None	None	None	Unchanged
	SW-noCRO-Ctls	6 weeks	None	None			
NWC	T1W+CRO	1 week	Low	None	None	None	Unchanged
	T2W+CRO	2 weeks	Moderate	Whitish	Moderate	None	Unchanged
	T4W+CRO	4 weeks	Moderate	Slight Yellow	Moderate	Low	Unchanged
	T6W+CRO	6 weeks	Moderate	Slight Yellow	High	Moderate	Reduced
	ONR+CRO-Ctls	6 weeks	None	None	None	None	Unchanged
	SW-noCRO-Ctls	6 weeks	None	None			

B.

Water Core	Treatment Name	Exposure time	Culture changes		Crude oil layer changes		
			Turbidity	Color	Emulsification	Biofilm	Volume
AWC	T1W+CRO	1 week	None	None	None	None	Unchanged
	T2W+CRO	2 weeks	Low	None	Minimal	None	Unchanged
	T4W+CRO	4 weeks	Low	None	Minimal	None	Unchanged
	T6W+CRO	6 weeks	Moderate	None	Some	None	Unchanged
	ONR+CRO-Ctls	6 weeks	None	None	None	None	Unchanged
	SW-noCRO-Ctls	6 weeks	None	None			
NWC	T1W+CRO	1 week	None	None	None	None	Unchanged
	T2W+CRO	2 weeks	Low	None	Some	Minimal	Unchanged
	T4W+CRO	4 weeks	Moderate	None	Some	Minimal	Unchanged
	T6W+CRO	6 weeks	Moderate	None	Some	Some	Unchanged
	ONR+CRO-Ctls	6 weeks	None	None	None	None	Unchanged
	SW-noCRO-Ctls	6 weeks	None	None			

After 6 weeks of incubation, microcosms not exhibiting signs of turbidity, not producing visible cell pellet, detectable amounts of DNA or PCR products, included all sterile ONR+CRO-Ctls control treatments and all negative SW-noCRO-Ctls control treatments (as expected). This result confirmed that turbidity, culture colour changes, or crude oil changes were a sign of bacterial response and growth on the provided crude oil. Samples from treatment T1W+CRO incubated at 4°C (from CRO-EE/4°C) also did not produce turbidity, visible cell pellet, DNA, or PCR product – in comparison, the corresponding T1W+CRO samples incubated at 20°C had produced ample cell pellet, DNA and PCR product. Another treatment that did not produce sufficient amounts of cell material or DNA for PCR product in neither of its samples, was the baseline control treatments (T0W-Baseline). This was presumed to be a complication from the prolonged exposure of these small volumes of ocean

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seawater (10 mL) to laboratory conditions without the provision of a carbon or nutrient source. As a consequence, the microbial profiles from FSC water masses obtained during the spring of year 2015 were used as surrogate controls, to match each lost T0W-Baseline sample according to water characteristics discussed in Section 3.3.1 and Table 3.2. Cell pellets, DNA and PCR products were obtained from treatments T2W+CRO, T4W+CRO and T6W+CRO incubated at 4°C as well as treatments T1W+CRO, T2W+CRO, T4W+CRO and T6W+CRO incubated at 20°C (Table 3.3).

3.3.3 Identification of hydrocarbon-degrading bacterial isolates

In this experiment, isolates were grouped into libraries (and named accordingly), based on carbon source of isolation (HEX, CRO or PHE) as well as the CRO-EE incubation conditions from which they were obtained (CRO-EE/20°C or CRO-EE/4°C). Two bacterial libraries were generated, one comprising 91 bacterial strains from CRO-EE/20°C microcosms and the other comprising 136 strains from CRO-EE/4°C microcosms (Table 3.4). The distribution of the number of isolates in each library based on carbon source is presented in Table 3.4.

Table 3.4. Number of isolates obtained from each CRO-EE, grouped based on libraries and carbon sources. Abbreviations: CRO: crude oil, PHE: phenanthrene, HEX: *n*-hexadecane. CRO-EE/20°C: Library produced from crude oil exposure microcosms incubated at 20°C; CRO-EE/4°C: Library produced from crude oil exposure microcosms incubated at 4°C.

Carbon source	Isolate library name	
	CRO-EE/20°C	CRO-EE/4°C
CRO	35	78
PHE	8	6
HEX	48	52
Total:	91	136

Of the 227 isolates, 68 were selected for sequencing based on distinguishable colony morphology, water mass of origin, incubation temperature (4°C or 20°C), and/or growth on different hydrocarbon substrates (CO, PHE, HEX). The 68 isolates were initially sequenced with only the forward primer (27F) (as described in Section 3.2.4) in order to provide an initial determination of their taxonomic identities. Ten genera were identified across the 68 sequenced strains: *Sulfitobacter* and *Phaeobacter* of the order *Rhodobacterales* and in the class *Alphaproteobacteria*; as well as *Alcanivorax*, *Cycloclasticus*, *Halomonas*,

Marinobacter, *Pseudomonas***,** *Thalassolituus*, *Glaciecola* and *Pseudoalteromonas* of the class
Gammaproteobacteria.

Based on the distribution of cultivated strains within the isolate libraries (Figure 3.1), it was observed that some isolates were more readily isolated under specific cultivation temperature or carbon source conditions. For example, isolated strains of the genera *Sulfitobacter,* *Halomonas* and *Glaciecola* were obtained only from the CRO-EE/20°C microcosms and only with PHE as carbon source during cultivation (grouped inside CRO-EE/20°C library). Isolates from the genera *Phaeobacter*, *Thalassolituus* and *Pseudomonas* were only achieved from CRO-EE/4°C microcosms with either CRO or HEX as the sole carbon source (CRO-EE/4°C library). FSC strains of the genus *Alcanivorax* were more easily cultivatable from CRO-EE/20°C microcosms when provided with CRO or HEX as carbon source, although a few strains were also found in HEX libraries from the CRO-EE/4°C microcosms. Strains of the genus *Cycloclasticus* were isolated only when provided PHE as sole carbon source and only from the CRO-EE/4°C microcosms. Cultivatable strains from the genera *Marinobacter* and *Pseudoalteromonas* were detected in both libraries, however *Pseudoalteromonas* strains were most commonly obtained from the hexadecane carbon source.
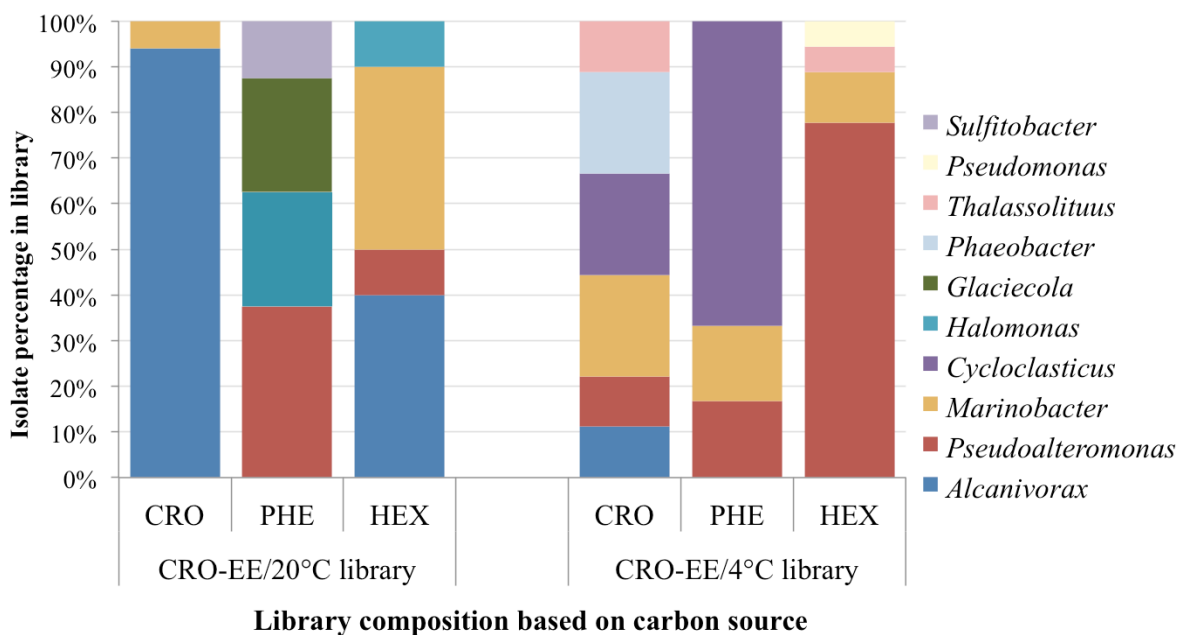
Figure 3.1. Distribution of cultivated FSC strains based on carbon source within each isolate library. Abbreviations: CRO-EE/20°C library: Isolate library produced from crude oil exposure experiment microcosms incubated under 20°C. CRO-EE/4°C library: Isolate

library produced from crude oil exposure experiment microcosms incubated under 4°C; CRO: crude oil; PHE: Phenanthrene; HEX: *n*-hexadecane.

Of the 68 isolated strains (thus far only sequenced in forward direction), 27 were elected as taxonomy representatives and sent for reverse primer sequencing (1492R). After merging the forward and reverse reads, near-full length 16S rRNA gene sequences were obtained from 26 out of the 27 representatives (one sequencing reaction failed to produce high quality data in the reverse sequencing direction). These isolates are listed in Table 3.5 together with metadata related to their origin.

Phylogenetic analysis was also performed for these representatives in order to visualize their phylogenic distance to known bacterial strains. The phylogenic tree produced is presented in Figure 3.2. Phylogenic tree confirmed the identity of isolates PHE2 and CRO81 to belong to the class *Alphaproteobacteria*, more specifically species *Phaeobacter arcticus* (isolated on CRO carbon source from CRO-EE/4°C) and *Sulfitobacter litoralis* (isolated on a PHE carbon source from CRO-EE/20°C). The tree also confirmed the identities of that all other isolates as belonging to various hydrocarbonoclastic species within the class *Gammaproteobacteria*, including isolates PHE9 and PHE13 as belonging to the species *Glaciecola mesophila* (isolated on PHE carbon source from CRO-EE/20C).

Table 3.5. Representative isolates and associated metadata from CRO-EE libraries of FSC cultivated strains.

Isolate name	Isolate library[1]	Carbon source[2]	Accession Number[3]	Water Depth[4]	Closest related type strain[5]	ID%[6]
c:*Alphaproteobacteria*, o:*Rhodobacterales*, f:*Rhodobacteraceae*						
CRO81	CRO-EE/4°C	CRO	KY508340	400m	*Phaeobacter arcticus*	99%
PHE2	CRO-EE/20°C	PHE	KY508328	SEDIM	*Sulfitobacter litoralis*	99%
c:*Gammaproteobacteria*, o:*Oceanospirillales*, f:*Alcanivoracaceae*						
CRO10	CRO-EE/20°C	CRO	KY508336	400m	*Alcanivorax borkumensis*	100%
CRO1	CRO-EE/20°C	CRO	KY508335	5m	*Alcanivorax borkumensis*	99%
HEX5	CRO-EE/20°C	HEX	KY508324	SEDIM	*Alcanivorax borkumensis*	99%
HEX4	CRO-EE/20°C	HEX	KY508323	400m	*Alcanivorax jadensis*	99%
HEX2	CRO-EE/20°C	HEX	KY508322	5m	*Alcanivorax jadensis*	99%
c:*Gammaproteobacteria*, o:*Oceanospirillales*, f:*Oceanospirillaceae*						
HEX65	CRO-EE/4°C	HEX	KY508321	5m	*Thalassolituus oleivorans*	99%
c:*Gammaproteobacteria*, o:*Oceanospirillales*, f:*Halomonadaceae*						
HEX16	CRO-EE/20°C	HEX	KY508326	700m	*Halomonas alkaliphila*	100%
PHE6	CRO-EE/20°C	PHE	KY508330	5m	*Halomonas alkaliphila*	99%
c:*Gammaproteobacteria*, o:*Alteromonadales*, f:*Alteromonadaceae*						
PHE13	CRO-EE/20°C	PHE	KY508332	SEDIM	*Glaciecola mesophila*	99%
PHE9	CRO-EE/20°C	PHE	KY508331	SEDIM	*Glaciecola mesophila*	99%
CRO52	CRO-EE/20°C	CRO	KY508338	400m	*Marinobacter algicola*	99%
HEX12	CRO-EE/20°C	HEX	KY508325	700m	*Marinobacter algicola*	99%
HEX19	CRO-EE/20°C	HEX	KY508327	700m	*Marinobacter algicola*	99%
HEX61	CRO-EE/4°C	HEX	KY508320	5m	*Marinobacter antarcticus*	98%
CRO68	CRO-EE/4°C	CRO	KY508339	700m	*Marinobacter excellens*	99%
c:*Gammaproteobacteria*, o:*Alteromonadales*, f:*Pseudoalteromonadaceae*						
HEX49	CRO-EE/4°C	HEX	KY508316	400m	*Pseudoalteromonas arctica*	99%
HEX50	CRO-EE/4°C	HEX	KY508317	400m	*Pseudoalteromonas arctica*	99%
HEX59	CRO-EE/4°C	HEX	KY508319	5m	*Pseudoalteromonas arctica*	99%
CRO30	CRO-EE/4°C	CRO	KY508337	5m	*Pseudoalteromonas elyakovii*	99%
PHE3	CRO-EE/20°C	PHE	KY508329	SEDIM	*Pseudoalteromonas elyakovii*	99%
HEX43	CRO-EE/4°C	HEX	KY508315	1000m	*Pseudoalteromonas nigrifaciens*	99%
c:*Gammaproteobacteria*, o:*Pseudomonadaales*, f:*Pseudomonadaceae*						
HEX57	CRO-EE/4°C	HEX	KY508318	5m	*Pseudomonas zhaodongensis*	99%
c:*Gammaproteobacteria*, o:*Thiotrichales*, f:*Piscirickettsiaceae*						
PHE111	CRO-EE/4°C	PHE	KY508334	1000m	*Cycloclasticus spirillensus*	100%
PHE91	CRO-EE/4°C	PHE	KY508333	700m	*Cycloclasticus spirillensus*	99%

[1]Isolate library produced from crude oil exposure experiment incubated at 20°C (CRO-EE/20°C) or 4°C (CRO-EE/4°C)
[2]Carbon source from which isolate was obtained
[3]Accession number obtained from NCBI GenBank
[4]Water depth of the original water sample
[5]Closest related type strain based NCBI GenBank database
[6]Percentage ID to closest related type strain

Tree scale: 0.1

Sulfitobacter marinus strain SW-265
PHE2 1310bp
Sulfitobacter litoralis strain Iso 3
CRO81 1263bp
Phaeobacter leonis strain 306
Phaeobacter arcticus strain 20188
HEX4 1361bp
HEX2 1361bp
Alcanivorax jadensis strain T9
HEX5 1369bp
Alcanivorax borkumensis strain SK2
CRO10 1372bp
CRO1 1371bp
PHE91 1352bp
Cycloclasticus pugetii strain PS-1
PHE111 1347bp
Cycloclasticus spirillensus strain M4-6
Pseudomonas chloritidismutans strain AW-1
HEX57 1371bp
Pseudomonas zhaodongensis strain NEAU-ST5-21
CRO68 1375bp
Marinobacter excellens strain KMM 3809
HEX61 1375bp
Marinobacter antarcticus strain ZS2-30
Marinobacter algicola strain DG893
CRO52 1379bp
HEX12 1376bp
HEX19 1382bp
Halomonas venusta strain DSM 4743
Halomonas alkaliphila strain 18bAG
HEX16 1362bp
PHE6 1377bp
Thalassolituus marinus strain IMCC1826
HEX65 1372bp
Thalassolituus oleivorans strain MIL-1
Glaciecola chathamensis strain S18K6
Glaciecola mesophila strain KMM 241
PHE9 1349bp
PHE13 1340bp
Pseudoalteromonas arctica strain A 37-1-2
HEX49 1370bp
HEX50 1370bp
HEX59 1375bp
PHE3 1382bp
Pseudoalteromonas elyakovii strain KMM 162
CRO30 1363bp
Pseudoalteromonas nigrifaciens strain NBRC 103036
HEX43 1360bp
Flavobacterium saccharophilum strain NBRC 15944
Flavobacterium flevense strain DSM 1076
Flavobacterium antarcticum strain AT1026

Figure 3.2. Phylogenic affiliations of the representative isolates to known type strains. The isolated strains are indicated with isolate name in bold face. Type strain names are spelled out and italicized. Metadata about each isolate and its % identity to the closest type strain can be found in Table 3.5. Tree was constructed by maximum-likelihood neighbour-joining method. Black circles and their size correspond to bootstrap confidence values from 70–100%. The tree was out-grouped and rooted with 16S rRNA gene sequences of the genus *Flavobacterium*. Scale bar represents number of nucleotide substitutions per site.

3.3.4 Analysis of MiSeq-sequences from microcosms incubated at 4°C

3.3.4.1 Sequencing depth and diversity

MiSeq sequencing, was performed on 2 out of the 3 replicate samples from treatments T2W+CRO, T4W+CRO and T6W+CRO incubated at 4°C. Microbial profiles from unexposed baseline communities obtained in Section 2.3 were used as T0W-Baseline controls. Sample sequencing depth analysis and Shannon diversity indices revealed contrasting differences in species diversity and richness between the baseline FSC microbial communities (T0W) and those exposed to CRO. The rarefaction curve analysis was indicative of significant species loss in the CRO-exposed cultures (T2W to T6W – all depths) compared baseline communities T0W (p values = 9.9E-12) (Figure 3.3). Baseline samples also displayed a rapid increase in OTU numbers with increase in individual sequences, while CRO-exposed samples quickly reached OTU levels of saturation (Figure 3.3). These results indicated that all the available microbial diversity in the crude oil-exposed microcosms (T2W, T4W and T6W) was captured by the MiSeq sequencing, and allowed for a reliable downstream comparative analysis of the baseline and exposed communities.
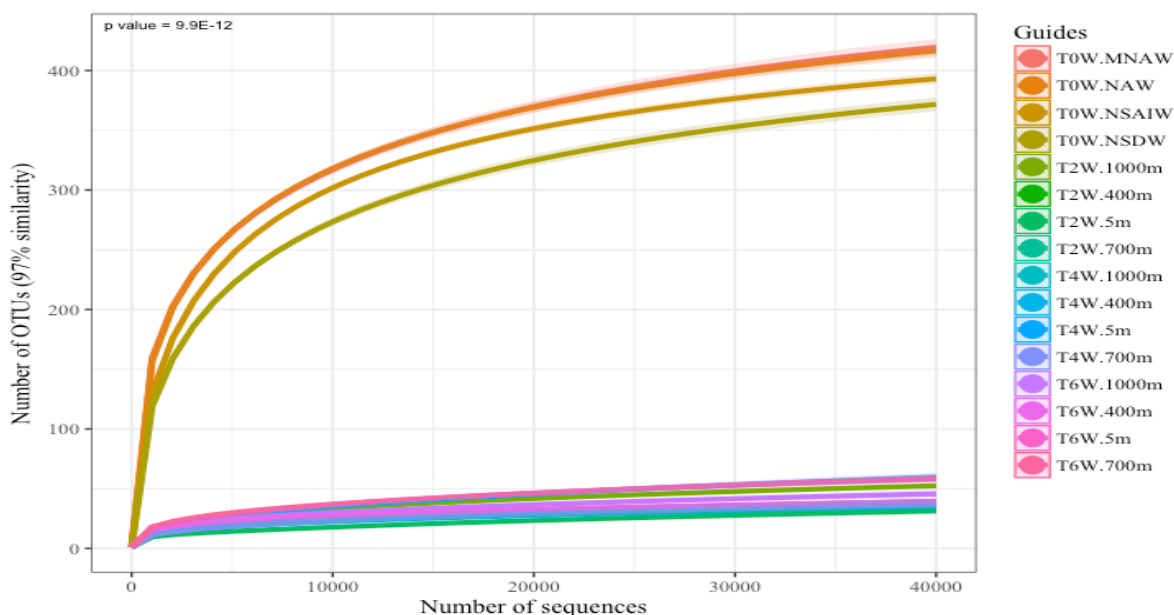
Figure 3.3. Rarefaction analysis indicating the alpha diversity of bacterial communities in baseline (T0W) *vs* communities exposed to crude oil (T2W+CRO, T4W+CRO, T6W+CRO) at 4°C.

The Shannon diversity indices analysis was a means of presenting the maximum possible level of species richness for each type of community. The Shannon diversity analysis for the crude oil enrichment experiment showed that the baseline communities in the FSC exhibited significantly higher total diversity than the oil-exposed incubations (Figure 3.4) (*p* value << 0.001 based on Welch's t-test). It also indicated that baseline communities of the AWC and had slightly higher total diversity than those of the NWC (~3.9 for AWC *vs* ~3.5 for NWC), yet after exposure to crude oil this diversity diminished to generally similar levels (~1.4 for both NWC and AWC samples). This result suggested a similar response of the two water-core based microbial communities.
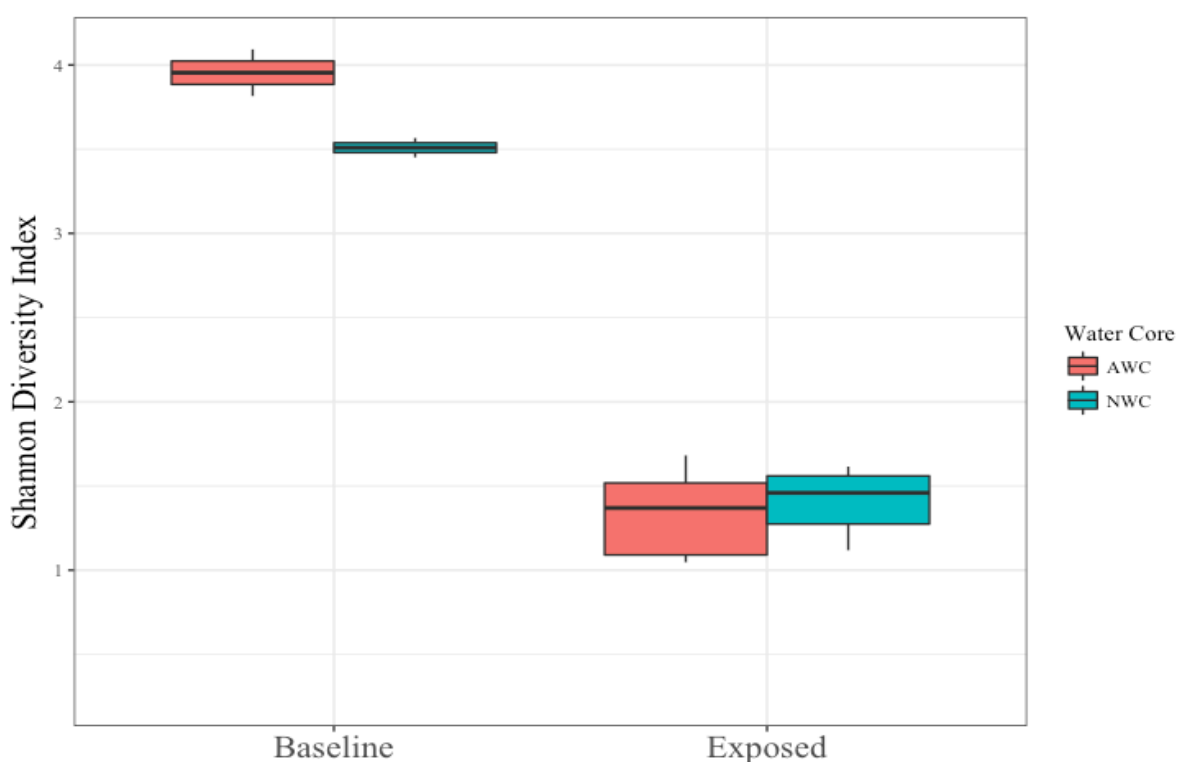
Figure 3.4. Shannon diversity analysis for the crude oil exposed samples *vs* baseline samples. Atlantic Water Core (AWC) is represented by 5 m and 400 m samples, while NWC is represented by 700 m and 1000 m samples.
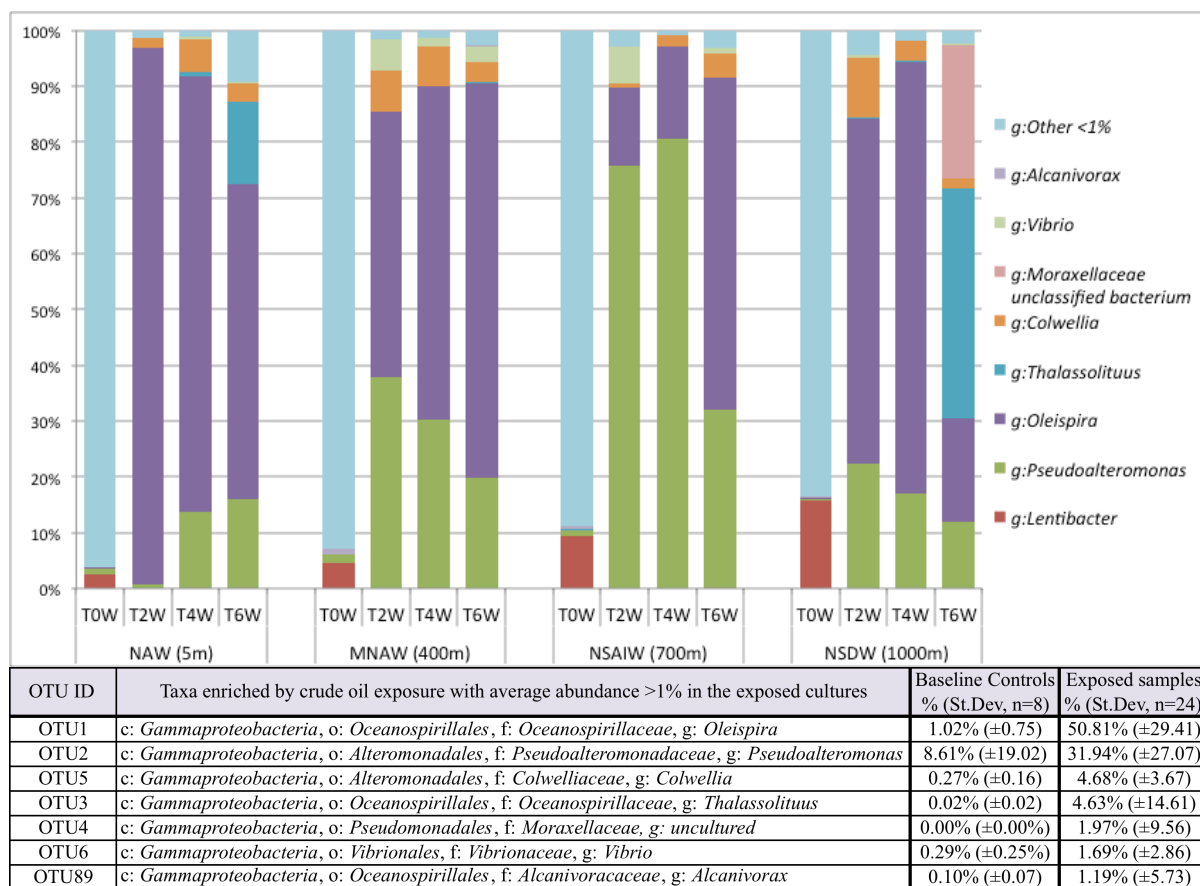
3.3.4.2 Bacterial community composition of the FSC and its response to crude oil

MiSeq sequencing was only performed on crude oil exposed cultures at 4°C as it represented average temperature conditions in the FSC. After merging and read processing, a total of 3,648,259 sequences of length 400 - 465 bp were obtained. Since biomass could not be

obtained from the control samples T0W-Baseline (original controls) or SW-noCRO-Ctls, the baseline microbial profiles from the closest water mass were used as surrogate T0W-Baseline controls throughout the subsequent analyses (Refer to Figure 2.1 and Table 3.2). As shown in Figure 3.5, the surrogate baseline community profiles of the FSC water column (denoted T0W) were distinguishable by high species richness that included the groups SAR11, SAR324, *Methylococcales*, *Rhodobacterales* (*i.e. Lentibacter*). In contrast, the community profiles that formed after oil exposure (T2W, T4W and T6W) displayed significantly lower diversity and were dominated by members belonging to the genera *Oleispira* and *Pseudoalteromonas*. For comparison, the genus *Oleispira* was found to be present at a relatively low abundance of < 0.5% in all baseline samples (T0W), whereas in all CRO-exposed communities (T2W, T4W and T6W from any depth) it significantly increased in abundance to an average 50% dominance (*p* value = 7.87E-5). The genus *Pseudoalteromonas* was found at ~1.25% relative abundance in all baseline communities, and proliferated to an average abundance of ~32% after crude oil exposure (*p* value = 0.001).

Between weeks 2 and week 6 of crude oil exposure, only relatively minor changes in community richness or composition were observed (*p* value = 0.71 >> 0.05). Generally, the genus *Oleispira* decreased in abundance between week 2 and week 6 in the 5 m communities, yet increased in abundance in 400 m and 700 m samples between the same weeks of exposure. In the 1000 m samples, the *Oleispira* OTU increased in abundance between week 2 and 4 of exposure and then sharply decreased in week 6. *Oleispira* appeared to predominate in abundance in waters of the same depth, with the exception of those from 700 m depth (NSAIW), where *Pseudoalteromonas* was found to predominate in abundance. *Pseudoalteromonas* primarily dominated the 700 m samples at 2 weeks and 4 weeks of exposure, but decreased in abundance by week 6, possibly becoming overprinted by *Oleispira*. Secondary contributors to the overall abundance were members belonging to the genera *Colwellia*, *Thalassolituus*, *Vibrio*, *Alcanivorax* and family *Moraxeliaceae* individually exhibiting <5% average abundance across all exposed microcosms. In the FSC deep-sea samples at 1000 m, the genus *Thalassolituus* and family *Moraxeliaceae* uncultured bacterium emerged with significant dominance after 6 weeks of exposure to crude oil compared to all other samples. *Thalassolituus* had generally increased in relative abundance in the 5 m depth

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water samples from almost non-detectable levels at in the baseline controls to about 40% abundance in exposed communities by week 6.

OTU ID	Taxa enriched by crude oil exposure with average abundance >1% in the exposed cultures	Baseline Controls % (St.Dev, n=8)	Exposed samples % (St.Dev, n=24)
OTU1	c: *Gammaproteobacteria*, o: *Oceanospirillales*, f: *Oceanospirillaceae*, g: *Oleispira*	1.02% (±0.75)	50.81% (±29.41)
OTU2	c: *Gammaproteobacteria*, o: *Alteromonadales*, f: *Pseudoalteromonadaceae*, g: *Pseudoalteromonas*	8.61% (±19.02)	31.94% (±27.07)
OTU5	c: *Gammaproteobacteria*, o: *Alteromonadales*, f: *Colwelliaceae*, g: *Colwellia*	0.27% (±0.16)	4.68% (±3.67)
OTU3	c: *Gammaproteobacteria*, o: *Oceanospirillales*, f: *Oceanospirillaceae*, g: *Thalassolituus*	0.02% (±0.02)	4.63% (±14.61)
OTU4	c: *Gammaproteobacteria*, o: *Pseudomonadales*, f: *Moraxellaceae, g: uncultured*	0.00% (±0.00%)	1.97% (±9.56)
OTU6	c: *Gammaproteobacteria*, o: *Vibrionales*, f: *Vibrionaceae*, g: *Vibrio*	0.29% (±0.25%)	1.69% (±2.86)
OTU89	c: *Gammaproteobacteria*, o: *Oceanospirillales*, f: *Alcanivoracaceae*, g: *Alcanivorax*	0.10% (±0.07)	1.19% (±5.73)

Figure 3.5. Microbial community profiles before (T0W) and after exposure to crude oil for 2, 4 and 6 weeks (T2W, T4W and T6W respectively) at 4°C. Complementary table represents the numerical representation of the profiles along with OTU ID, taxonomic lineage of each OTU with average abundance >1% after exposure and numerical value for average abundance (with standard deviation). All community profiles were based on experimental duplicates.

A phylogenic tree of the representative OTUs from the CRO-exposed incubations at 4°C is presented in Figure 3.6. The tree demonstrated the relationship between the identified OTUs with known type strains.
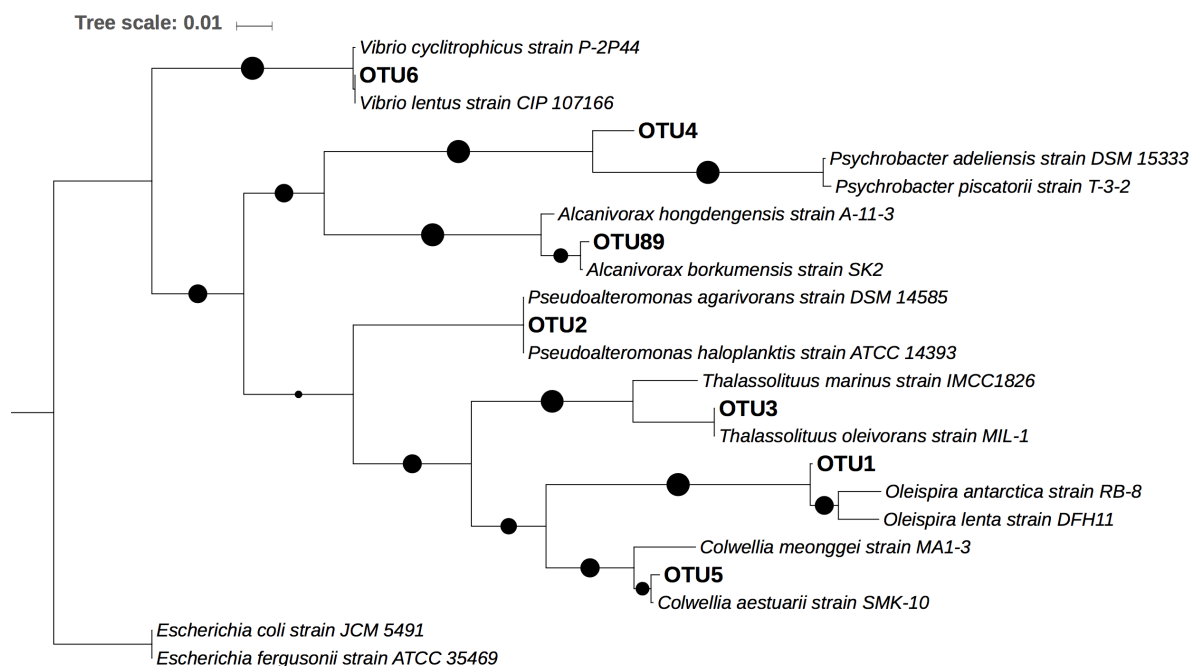
Figure 3.6. Phylogenic tree of representative OTUs found in CRO-EE/4°CC microcosms presented to closest type strains. OTUs highlighted in bold face were enriched during exposure to crude oil at 4°C. Type strain names are spelled out and italicized. The tree was rooted with sequences from the order *Flavobaceriales* (*Bacteroidetes*). Scale bar represents substitution rate (nucleotide substitutions per site). Tree was constructed by neighbour-joining method. Size of black dots corresponds to bootstrap confidence value of 70% - 100% based on 1,000 bootstrap replications.

3.3.5 Oligotyping

Oligotyping was applied in this study as a tool to resolve closely related taxa for each of the two most dominant OTUs (*Oleispira* and *Pseudoalteromonas*) in the crude oil exposed microcosms. Figure 3.7 shows the microdiversity within the genus *Oleispira* in FSC microbial communities that were not exposed to crude oil (T0W) compared to those exposed to oil over the duration of these 6-week incubations. Maximum average purity score of 92% was obtained using parameters "minimum substantive abundance" (designated with parameter –M) of 400 and "number of entropy components" (–c) of 4 (base locations: 21, 100, 112, 118), which identified 5 oligotypes. Oligotype analysis results showed an increase in the oligotype GCAG and GCTG in the NAW (from 5 m depth) water samples. No change in oligotypes was found in MNAW (from 400 m depth) water samples with exposure to crude oil. A pattern of diversity changes was not observed at 700 m depth samples, except in

oligotype GTAA, which showed noticeable increase after crude oil exposure in the 1000 m depth samples. None of the *Oleispira* oligotypes showed 100% sequence identity to a known species or strain of this genus within GenBank.
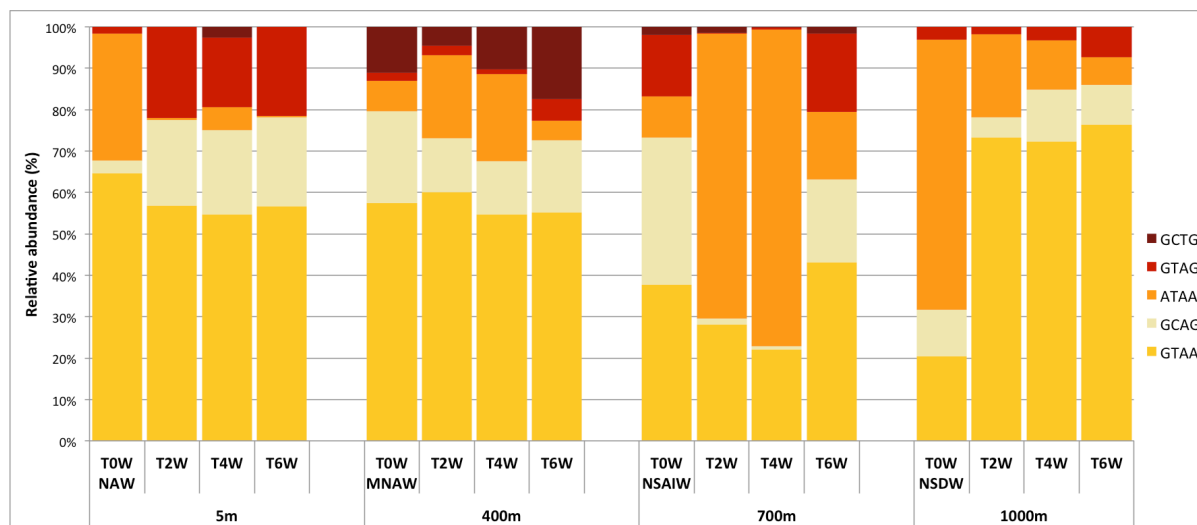
Figure 3.7. Microdiversity within the genus *Oleispira* based on oligotyping analysis of FSC *Oleispira* strains prior and after crude oil exposure for up to 6 weeks.

For microdiversity analysis within the genus *Pseudoalteromonas*, 10 high-entropy components (-c flag, base locations: 24, 87, 101, 103, 104, 114, 116, 117, 133, 427) and 500 substantive abundance threshold (-M) were selected to produce 4 oligotypes of purity score 92%. Micro-variance within the sequences showed significant increase in the CCATATCTGG oligotype in incubations that were exposed to crude oil, as well as increase in abundance of other present oligotypes. Oligotypes CCATATCTGA and CCATATCTGG showed 100% sequence identity to, respectively, *P. undina* strain NBRC 103039 and *P. haloplanktis* strain ATCC. Oligotype CCGCGCTTGG showed enrichment in crude oil exposed *Pseudoalteromonas* fractions (T2W-T6W) only in the MNAW (from 400 m depth) communities. These results, represented in more detail in figure 3.8, indicate a change in *Pseudoalteromonas* microdiversity during exposure to crude oil.
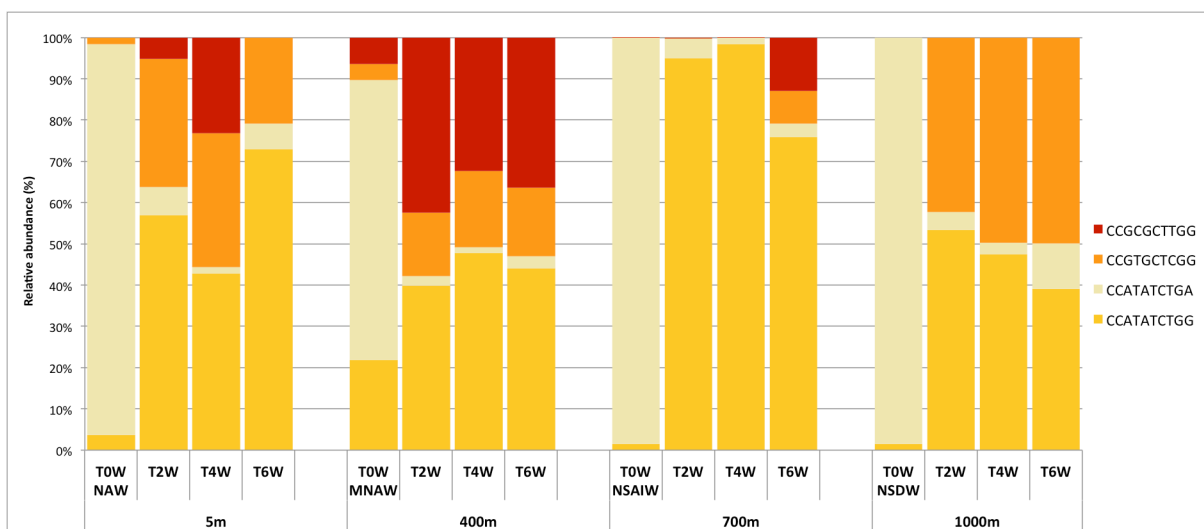
Figure 3.8. Microdiversity within the genus *Pseudoalteromonas* based on oligotyping analysis of FSC *Pseudoalteromonas* strains prior and after crude oil exposure for up to 6 weeks.

3.4 Discussion

3.4.1 Temperature effect on FSC microbial community response to crude oil

This study showed the ability of FSC microbial communities to respond to crude oil under the average FSC (~4°C) conditions of the FSC, as well as to moderate temperatures (20°C), and revealed the temporal response under these differential temperature conditions. Although the biodegradation of the crude oil was not directly measured in this study, there were marked changes that occurred in the microcosm cultures (*i.e.* turbidity, colour) as well as to the crude oil (*i.e.* emulsification, biofilm, volume) that were indicative of microbial activity. Additionally, the controls for these experiments (the sterile ONR+CRO-Ctls[2] and the negative SW+noCRO-Ctls) suggested that the observed changes in the exposed live cultures were not predominated by physico-chemical factors (e.g. photooxidation or other weathering processes), but largely microbial degradation of the CRO. Furthermore, sequencing of the exposed cultures (at 4°C) confirmed the presence and dominance of hydrocarbon degrading bacteria, usually observed in a microbial response to crude oil (Yakimov *et al.*, 2005; Head *et al.*, 2006; Brakstad *et al.*, 2015; Wang *et al.*, 2014). The

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[2]Although being a close representation of seawater, ONR-based sterile controls may not completely exclude the possibility of seawater-specific physicochemical effects to have played some role on the changes in exposed cultures. Yet, considering the significant bacterial enrichment of HCB in the CRO-exposed cultures, it appeared likely that seawater-specific physicochemical effects did not play a major role in the changes of the cultures.

observations of the cultures also showed that the onset of biodegradation in the 4°C
microcosms was noticeably delayed and possibly proceeded slower (less turbidity observed
over the 6 weeks period) than the observed at 20°C (high turbidity observed over the 6 weeks
period, Table 3.3). The effects of temperature on the bacterial response to crude oil have
been thoroughly studied in recent years and have shown that although hydrocarbon
biodegradation is possible under lower temperature conditions, lower temperature greatly
inhibits bacterial activities such as metabolism, growth rate and cell division (Giudice *et al.*,
2010; Wilkins *et al.*, 2013; Brakstad and Bonaunet, 2006; Mason *et al.*, 2012; Brakstad *et al.*,
2010; Atlas, 1975; Bej *et al.*, 2010; Timmis, 2010; Gibbs *et al.*, 1975; Colwell, 1977) (also
refer to Section 1.5.1.1). This study also adds to the plethora of data and studies affirming
delayed biodegradation response to crude oil due to reduced temperature.

Although the response of the community was expected, the observed timeframe suggested
that a potential oil spill in the FSC might take significantly longer time to bio-remediate than
if similar oil spill occurred under moderate temperatures (Gulf of Mexico). The results from
the culture observations (Table 3.3) also suggest that in the event of an oil spill in the FSC,
oil may be degraded more rapidly if injected in the warmer AWC (0-600 m depth), than if it
were released in the colder NWC environment (> 600 m). Such results corroborate with the
conclusions of Main *et al.* (2016) who revealed that spilled oil below 600 m depth in the FSC
would persist significantly longer in the environment (Main *et al.*, 2016) than if it were
spilled at shallower depths. The results of this study confirm the hypothesis that in the event
of an oil spill in the FSC, the microbial response would likely be lagged and the
biodegradation rates would be relatively slow due to the cold temperatures of the region.

3.4.2 Isolated strains of hydrocarbon-degrading bacteria
Based on 16S rRNA gene sequencing, the majority of the isolates obtained from crude oil
enrichment were identified to be closely related (>98% identity) to commonly known
hydrocarbonoclastic bacteria such as the genera *Alcanivorax*, *Pseudoalteromonas*,
Marinobacter, *Cycloclasticus*, *Halomonas*, *Pseudomonas*, *Glaciecola* and *Thalassolituus*
(Refer to Chapter 1, Table 1.2). Since the majority of microorganisms in environmental
samples cannot be cultivated in the laboratory, the composition of the isolate libraries (Figure
3.1) is not an accurate representation of their abundances in the FSC and nor in the crude oil
exposed cultures from which they were isolated. However, library composition analysis may

be used to assess the cultivation and activity preferences (or tolerance for such conditions) of the strains associated with different genera, which may be valuable information for assessing their biotechnological potential.

For example, one of the most well known hydrocarbonoclastic bacteria, the genus *Alcanivorax*, are known for their relatively easy laboratory cultivation, but only in the presence of nutrients and under moderate temperatures (Yakimov *et al.*, 2007). Concurrently, isolates belonging to this genus were consistently cultured in this study from CRO-EE/20°C microcosms (e.g. isolates CRO10, CRO1, HEX5, HEX2), but their presence in the CRO-EE/4°C isolate libraries was noticeably limited (Figure 3.1). Although *Alcanivorax* strains are found in various marine environments, including polar regions, their cultivation under low temperature conditions have thus far been a challenge (Yakimov *et al.*, 2007). Investigations focused on the temperature and activity adaptations of *Alcanivorax* isolates obtained from the CRO-EE/4°C samples (representatives not shown), may help confirm and understand psychrotolerant adaptations within the isolated strains (and subsequently the genus) and their reluctance to cultivation under such conditions. *Pseudoalteromonas* strains (e.g. HEX49, HEX50, CRO30) were isolated primarily from 4°C microcosms with hexadecane or crude oil as carbon source. Strains from this genus however, have been reported to exhibit PAH degradation activity as well (Chronopoulou *et al.*, 2015). In this study, *Pseudoalteromonas* FSC strains were not obtained from 4°C microcosms and with PAH as a carbon source, suggesting CRO-isolated strains (from water column samples) may not have been utilizing PAHs from within the crude oil. *Pseudoalteromonas* with suspected PAH-degrading activity was however, isolated from FSC surface-layer sediment exposed to crude oil at 20°C (Figure 3.1 and Table 3.5, e.g. isolate PHE3). Based on this, an intriguing interrogation into the PAH-degrading activity of the PHE3 isolate with change in temperature, may not only confirm the substrate utilization of this isolate, but also reveal if such activity is present at lower temperatures (more realistic for the FSC). *Marinobacter* species (e.g. CRO52, HEX12) were isolated on different alkane sources (CRO or HEX) from microcosms incubated under both temperature conditions, affirming their broad range of temperature adaptations characteristic for the genus (Yakimov *et al.*, 2007; Duran, 2010; Kostka *et al.*, 2011, Table 1.2). The genus *Cycloclasticus* is the paradigm for PAH-utilizing bacteria, which is most commonly found under moderate temperature conditions (Yakimov *et al.*, 2007). Strains related to the genus *Cycloclasticus* (e.g. PHE111, PHE91), however,

were obtained from the CRO-EE/4°C microcosms, thus suggestive of potential cold adaptation for these FSC isolated strains. Isolates PHE111 and PHE91 were identified as strains of the species *C. pugetii*, which has also been reported in other Nordic waters, such as the North Sea (Yakimov *et al.*, 2007), again suggestive of their cold adaptation. Further studies into the capacities and tolerance of such *Cycloclasticus* isolates, might be beneficial in understanding and even developing efficient psychrotolerant PAH-degrading strains. *Thalassolituus oleivorans* species are known obligate aliphatic hydrocarbon degraders capable of growth at low temperature conditions, such as the cultivation conditions under which isolate HEX65 (affiliated with this species) was isolated in this study (Yakimov *et al.*, 2004). Its presence in the isolate libraries derived from enrichments with hexadecane or crude oil at 4°C suggests also an adaptation to the cold environment of the FSC.

Isolates HEX16 and PHE6 affiliated with the species *Halomonas alkaliphila* were obtained from both AWC and NWC seawater treated with crude oil at moderate temperatures (CRO-EE/20°C) and with alkane as well as aromatic carbon sources. The species *H. alkaliphila* are known alkaliphilic halotolerant bacteria with moderate temperature growth preferences (Romano *et al.*, 2006), which is consistent with the temperature conditions of their cultivation in this study. *Halomonas* species have commonly been found in crude oil utilizing communities and are described as a generalist hydrocarbon degraders with some species also exhibiting psychrotolerant behaviour (Chronopoulou *et al.*, 2015; Giudice *et al.*, 2010, Table 1.2). Further work to determine the capacities of these FSC isolates affiliated to *H. alkaliphila* is recommended in order to gauge their hydrocarbon utilization potential and also for use in biotechnological applications since many halomonads have been reported to produce biomolecules (e.g. biosurfactants) of biotechnological potential (Romano *et al.*, 2006). Similarly to *Halomonas*, members of the genus *Pseudomonas* are known for their preference for alkaline and saline environments (Zhang *et al.*, 2015). Concurrently, *Pseudomonas*-affiliated isolates (e.g. HEX57 affiliated with *P. zhaodingensis*) were obtained from CRO-EE/4°C microcosms provided hexadecane as carbon source during isolations. Members of this genus are also known for their nutritional versatility, especially ability to degrade a variety of hydrocarbons and xenobiotic compounds in cold temperature regions (Chronopoulou *et al.*, 2015; Palleroni *et al.*, 2010). Although the species *P. zhaodongensis* has not thus far been reported as an alkane degrader (Zhang *et al.*, 2015), it is a fairly recently described species within the *Pseudomonas* genus (only 2015). Based on the

genomic features of the genus, as well as reported observations of such behaviour from multiple un-described *Pseudomonas* species, which could potentially be *P. zhaodongensis* (Palleroni *et al.*, 2010), it appears likely that such metabolic capacity was exhibited in this FSC isolated strain.

The genus *Glaciecola* has been commonly reported in pristine and hydrocarbon polluted cold marine environments (Brinkmeyer *et al.*, 2003; Rosenburg *et al.*, 2014; Bowman *et al.*, 1997; Chronopoulou *et al.*, 2015) and various species and strains have been successfully isolated from similar environments with crude oil as a sole carbon source (Yakimov et al. 2004; Prabagaran et al. 2007; Giudice et al. 2010; Chronopoulou et al. 2015). The isolates PHE13 and PHE9, affiliated with the species *Glaciecola mesophila*, however, were isolated in this study with phenanthrene as carbon source and specifically from sediment samples after exposure to crude oil. Phenanthrene activity has not been previously reported for this species (especially at 20°C), but agar-digesting abilities have been reported (Romanenko *et al.*, 2003). Therefore further work would be needed to prove whether these isolate strains, are indeed capable of utilising phenanthrene as a sole carbon source or their presence in the isolate libraries was due to agar-digesting abilities.

The genus *Sulfitobacter* has also been previously reported in various marine environments, including polar seawater (Prabagaran *et al.*, 2007; Pujalte *et al.*, 2014) and in association with sediment and even oil contaminated environments (Brakstad and Lødeng, 2005; Prabagaran *et al.*, 2007; Jung *et al.*, 2010). Therefore it is not surprising that isolates like PHE2 affiliated with the genus *Sulfitobacter* were obtained from near-zero FSC sediment samples when provided phenanthrene as sole carbons source. Although finding a *Sulfitobacter* strain in sediment samples is not surprising, they have thus far not been shown to exhibit hydrocarbon biodegradation activity (Prabagaran *et al.*, 2007; Brakstad and Lødeng, 2005), but rather sulfite oxidation and organic acid assimilation (which would explain their presence in oil contaminated communities) (Pujalte *et al.*, 2014). This could explain their common presence in crude oil contaminated environments(Brakstad and Lødeng, 2005; Prabagaran *et al.*, 2007; Jung *et al.*, 2010). Before disregarding their role as a hydrocarbon utilizer, however, it needs to be noted that analysis of *Sulfitobacter* genomes have revealed the presence of oxygenolytic ring-cleaving pathways which are broadly distributed among PAH-degrading microbes especially in soils (Buchan and González, 2010). This indicates a potential of PAH

degradation within the FSC isolated strains like isolate PHE2. Similarly, *Phaeobacter arcticus*, to which isolates like CRO81 were affiliated, are a known psychrophilic bacteria related to *Sulfitobacter* and commonly isolated from the Nordic marine ecosystems (Zhang *et al.*, 2008; Hahnke *et al.*, 2013). Their genomic makeup also reveals PAH-degradation pathways, yet such functional activity has not yet been confirmed (Buchan and González, 2010). Therefore, further studies of the isolates PHE2 and CRO81 would need to be implemented in order to confirm or disprove their hydrocarbonoclastic abilities.

The libraries of isolates obtained in this study represent strains that exist in the FSC water column and importantly add to knowledge about cultivable strains and their preferred conditions of cultivation and possible activity. Additionally, these strains were the first isolates obtained from the FSC with likely hydrocarbonoclastic activity. Hence, these bacterial libraries comprise a unique collection of known and putative FSC hydrocarbon degraders, amendable to cultivation in laboratory conditions under specific temperature and substrate conditions. These isolate libraries provide opportunities to directly study physiology and metabolic functionality of FSC cultivatable bacteria with apparent or suspected hydrocarbonoclastic activity, and to explore opportunities for their biotechnological applications.

3.4.3 Dynamics of the FSC microbial communities in response to crude oil exposure

As noted earlier, the baseline microbial communities extracted from FIM6a water column (T0W-Baseline original control samples) did not produce sufficient biomass (and hence DNA and PCR product) for microbial profiling, which was a limitation when assessing microbial response and community changes. The lack of sufficient biomass accumulation in the T0W-baseline treatments could have been due to many factors, the most likely of which was the relatively small volumes of the samples (10 mL) originating from an oligotrophic environment (esp. subsurface seawater), incubated for a prolonged period of time under laboratory conditions (6-8 weeks including the pre-setup storage) without the provision of any carbon or nutrient source. Consequently, the microbial profiles from each water mass were matched based on water characteristics to each missing T0W-Baseline treatment (Table 3.2). Only the MNAW mass was slightly discrepant from the characteristics of the FIM6a water column at depth 400 m, although, the general results from the study indicated that the

microbial profile of any indigenous non-perturbed microbial community from within the FSC would have contrasted significantly enough from profiles of the crude oil exposed FSC communities to be applicable as representatives of the FSC T0W-Baseline controls. This sharp contrast in profiles was consistent with the many studies exploring the microbial communities of a region prior, during and after exposure to crude oil (Yang *et al.*, 2014; Joye *et al.*, 2014; Lu *et al.*, 2012). The shortcomings of this experiment highlighted the necessity for appropriate microbial community baseline prior to any crude oil exposure as this would allow for more accurate measurements of the community development and dynamics occurring after crude oil exposure (whether *in vitro* or *in situ*).

Based on the microbial profiles of the CRO-EE/4°C microcosms, it could be inferred that the first microbial responders in case of an oil spill in the FSC, would likely be *Oleispira* (OHCB) and *Pseudoalteromonas* (GHCB). *Oleispira* are known obligate alkane degraders adapted to cold temperatures (Refer to Chapter 1, Table 2.1); therefore their 100-fold enrichment in all oil-exposed microcosms (from <0.5% in non-exposed cultures to ~51%) within only 2 weeks of exposure was an affirmative sign of hydrocarbon biodegradation within the microcosms. As discussed earlier, *Pseudoalteromonas* are psychrophilic, generalist broad-range hydrocarbon degraders requiring access to dissolved hydrocarbons (Chronopoulou *et al.*, 2015; Hedlund and Staley, 2006). The enrichment of these organisms was on average ~30 fold increase, primarily in the water samples from 700 m depth where the increase was at a maximum 60 fold. Considering the known hydrocarbon preference of these groups of bacteria, it could be postulated that alkane degradation was the primary activity during the 6 weeks of exposure to crude oil. Although the microbial community dynamics in the 20°C microcosms (CRO-EE/20°C) were not followed, the observations of the changes in the cultures leading to considerably reduced crude oil layer (Table 3.3), indicated that the activity within these microcosms included active degradation of not only aliphatic but also at least basic aromatic hydrocarbons. The predominance of primarily alkane-degraders within the 4°C microcosms, affirmed the observations of delayed onset of hydrocarbon degradation in 4°C cultures compared to 20°C.

Amongst the minority bacteria found in the 4°C cultures exposed to crude oil, *Colwellia*, *Thalassolituus* and *Alcanivorax* are known and well studied HCB (Refer to earlier discussion as well as Chapter 1, Section 1.4.3, 1.4.4 and Table 1.2). Based on their ubiquity in other

petroleum-polluted environments, these genera also are expected to play a role in the degradation of crude oil in case of an oil spill in the FSC. The family *Moraxellaceae* (of the order *Pseudomonadales*) and genus *Vibrio*, are known to contain members with hydrocarbonoclastic abilities (Beazley *et al.*, 2012). However, the role of these organisms groups in biodegradation of petroleum compounds has not been recognized as significant.

3.4.4 Response of Oleispira and Pseudoalteromonas oligotypes to crude oil exposure

Oligotyping was applied as a computational method to increase the resolution on the diversity of the two most enriched OTUs after exposure to crude oil, namely *Oleispira* and *Pseudoalteromonas*. Oligotyping was applied to address the question whether microdiversity changes occurred within each of the OTUs for these two taxa. Results suggest that there was no apparent shift in microbial diversity within the *Oleispira* oligotypes, however, the opposite appeared to be the case for the *Pseudoalteromonas* oligotypes. Before speculating on the significance of these oligotype shifts, however, it needed to be taken into consideration that the oligotypes identified were likely not representative of ecologically meaningful ecotypes (e.g. species or strains) as this hypothesis (postulated by the creators of the method) was disputed as being largely untested (Eren *et al.*, 2013; Berry *et al.*, 2017). It was argued by Berry *et al.* (2017) that due to the limitations of the 16S rRNA gene used for taxonomic assignment, phylogenically and ecologically informative results could not be produced through oligotyping of the 16S rRNA gene (Berry *et al.*, 2017). In short, these limitations primarily stem from the fact that the 16S rRNA gene is a slowly evolving gene, and therefore even at full length, cannot represent the more recent evolutionary diversification of an organism (such as speciation) and cannot resolve functional variations between species or strains within a genus. The author also emphasized that in order to produce finer taxonomic identification (beyond genus level), studies need to go beyond 16S marker gene analysis (Berry *et al.*, 2017). Additionally as discussed in Section1.5.3, gene transfer is a factor in degradation of crude oil, especially among psychrophilic species such as *Pseudoalteromonas* (Hedlund and Staley, 2006; Bej *et al.*, 2010; Cappello *et al.*, 2007; Luz *et al.*, 2010; Yakimov *et al.*, 2007; Head *et al.*, 2006). This trait complicates any associations that could be extrapolated between deep-level 16S rRNA gene -based taxonomic identification and species metabolic capacity. Therefore, despite the successful application of the oligotyping methodology here, it was decided as inaccurate to attempt to deduce

meaningful ecological information such as species or strain response, based on minute variations in 16S rRNA gene sequences (ecotypes). Oligotyping analysis appears a more appropriate tool for comparing the nucleic acid and even amino acid sequences of functional genes and gene clusters, such as those encoding for alkane hydroxylase systems (e.g. *alk*SB1GHJ gene cluster) and exploring the variable and conserved regions between species or paralogous gene copies (Berry *et al.*, 2017; Yakimov *et al.*, 2007).

CHAPTER 4: DNA-BASED STABLE ISOTOPE PROBING OF FSC MICROBIAL COMMUNITIES

4.1 Introduction

DNA-based stable isotope probing (DNA-SIP) is a method used in microbial ecology for identifying specific functional groups of microorganisms based on their ability to perform a particular metabolic function without the prerequisite of organism isolation or procurement of pure cultures (cultivation) (Whiteley *et al.*, 2007; Dumont and Murrell, 2005). Recently, the use of DNA-SIP with hydrocarbon substrates emerged as an important tool in understanding the *Deepwater Horizon* oil spill effect on microbial processes (Gutierrez, 2011; Mishamandani *et al.*, 2014; Redmond and Valentine, 2012; Gutierrez *et al.*, 2013). The technique involves providing a uniformly labelled ^{13}C source (in this case, a hydrocarbon) as a sole source of carbon to a microbial community (in this case, microbiota from the FSC). Any microorganism capable of primary utilization of the labelled hydrocarbon substrate incorporates the 'heavy' carbon into its biomass (e.g. RNA, proteins, fatty acids, DNA) during cultivation. In DNA-based SIP distinguishing which organisms incorporated the substrate, is done through exploration of the DNA from the community. Provided that incubation time with the labelled substrate has been sufficient enough for ^{13}C incorporation into the DNA of the primary substrate utilizers, yet not prolonged enough for secondary degradation to occur (hence transferring ^{13}C downstream to the entire community), DNA-based SIP is an unequivocal technique to link taxonomy to metabolic function. Tracking of the substrate's decreasing concentration in the culture solution during incubations, tends to be sufficient for estimating the end of this primary degradation phase and sufficient DNA incorporation (Sheppard *et al.*, 2013; Gutierrez, 2010). Subsequently, the DNA of the community is extracted and a CsCl-based ultracentrifugation separates the 'heavy' DNA of the primary utilizers from the DNA of the lingering non-utilizers ('light' DNA). Community fingerprinting techniques such as Denaturing Gradient Gel Electrophoresis (DGGE) have been commonly used to confirm the separation of the DNA, visualize and distinguish the two bacterial consortia as well as extract and identify specific microbial species (Sheppard *et al.*, 2013). Based on the protocol of Gutierrez *et al.* (2013), in this study the utilizers were identified via 16S rRNA gene assessments involving cloning and Sanger sequencing (Gutierrez, 2011; Gutierrez *et al.*, 2013). The schematic methodology of

the method is presented in Figure 4.1. In this SIP experiment, the chosen model ^{13}C-sources were hexadecane (HEX) as representative of alkane components of crude oil (CRO) and phenanthrene (PHE) as representatives for aromatic CRO components.
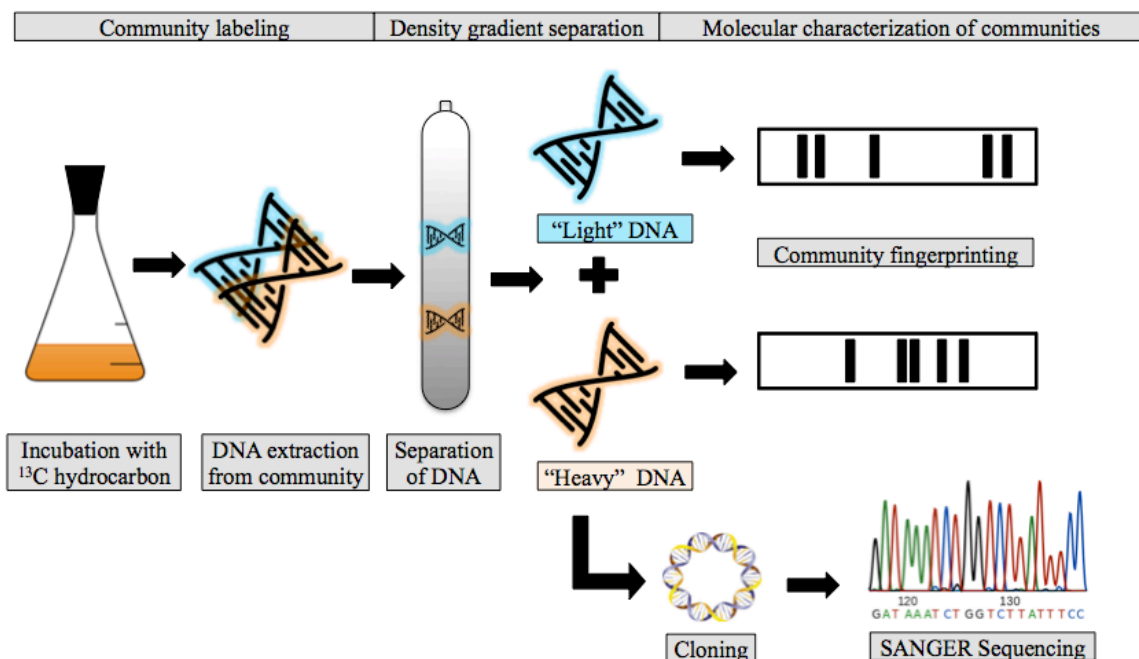
Figure 4.1. Schematic representation of the working principle of DNA-based SIP

Although DNA-based SIP methods have been successfully applied to understand hydrocarbon degraders in oil plumes and oil-affected waters (Gutierrez *et al.*, 2013; Sheppard *et al.*, 2013), exploring the microbial potential for hydrocarbon degradation using this method has not yet been conducted on waters of the NE Atlantic or West of Shetland region which includes the FSC. The method holds the potential to pinpoint naturally low-abundance uncultivable hydrocarbonoclastic bacterial groups in the microbial communities of the FSC. This method was utilized in this study in an attempt to reveal novel strains of hydrocarbonoclastic bacteria characteristic for the FSC, particularly those not easily amendable to cultivation. This further elucidated the biodegradative potential of the microbial community and provided DNA from FSC hydrocarbon utilizing consortium for further investigation into the functional and metabolic capacities of the FSC hydrocarbonoclastic communities (Dombrowski *et al.*, 2016; Lu *et al.*, 2012)

4.2 Materials & Methods

4.2.1 Sample collection

Water samples of volume 1 L were collected from location FIM6a (60° 38'N, 4° 54'W) and depths 5m (AWC) and 700m (NWC) during spring (April 24th- May 9th) and fall (September 23rd –October 8th) seasons of 2014 during monitoring research cruises to the FSC of MRV *Scotia*. Samples were stored at 4°C until ready for processing in laboratory conditions.

4.2.2 SIP incubations

Due to the oligotrophic pristine nature of the samples, and to remove any potential intrinsic carbon source, microbial communities within seawater were concentrated through filtration. This was performed as follows: From the collected 1 L samples, a volume of 800 mL was filtered through 47 mm diameter 0.22 μm pore gridded Mixed Cellulose Esters (MCE) filters (Millipore Sigma). Filters were submerged in sterile 40 mL synthetic seawater media ONR7a (Dyksterhouse *et al.*, 1995) and vigorously shaken for 30 min to dislodge collected bacteria. Using sterilized tweezers, the filters were then removed and the media was used as inoculum. For the setup of the SIP incubations uniformly labelled ^{13}C *n*-hexadecane (^{13}C-HEX) was used as model for alkane hydrocarbon. ^{12}C and ^{13}C *n*-hexadecane was obtained with purity >99% from Sigma-Aldrich. For SIP incubations with model aromatic hydrocarbon phenanthrene (PHE), uniformly labelled ^{13}C PHE and unlabelled ^{12}C PHE were obtained with purity >98% from Sigma-Aldrich. To setup the SIP incubations 16 mL sterile ONR7a was inoculated with 4 mL inoculum and amended with 1 μg of the labelled or unlabelled hydrocarbon substrate (C-source) as follows: 2 flasks (replicates) were amended with ^{13}C hydrocarbon (HEX or PHE) and 2 flasks (replicates) containing corresponding ^{12}C hydrocarbon were used as experimental cultures; 3 flasks (replicates) containing ^{12}C hydrocarbon were used as Live Controls, and 3 flasks (replicates) with ^{12}C hydrocarbon + 750 μL 85% H$_3$PO$_4$ (Fisher Scientific) were used as Dead Controls. Flasks were incubated at room temperature (20°C) in darkness with gentle horizontal shaking (~150 rpm) until time of termination. Time of termination was determined based on hydrocarbon levels within the Live and Dead Controls tracked as described in Section 4.2.3 below.

4.2.3 Hydrocarbon tracking and DNA extractions

On a daily basis 1 mL from each Live and each Dead Control was extracted, mixed with 1 mL ethyl acetate and analysed for HEX or PHE concentrations by GC or HPLC (courtesy of

HWU Chromatography facility). Another 1 mL was also collected from these controls on a daily basis and used to extract cell biomass by centrifugation (17,000 $\times g$ for 10 min) for subsequent qPCR analysis to gauge 16S rRNA gene copy number accumulation during incubations. The remaining ^{12}C and ^{13}C hydrocarbon cultures (the experimental cultures) were harvested 2 days after concentration measurements from the Live Controls consistently showed undetectable levels of HEX or PHE in the samples. Harvesting was performed via pelleting of bacterial mass in each culture by centrifugation for 15 min at 17,000 $\times g$. DNA from samples was extracted from SIP pellets based on the protocol of Tillet *et al.* (2000) also described in Chapter 2, Section 2.2.2.

4.2.4 CsCl gradient ultracentrifugations

A solution of CsCl (Fisher Scientific) of density 1.68 g/mL was prepared in 1× TE buffer to match refractive index 1.3937. Refractive index was accurately assessed with digital refractometer (Reichert AR200). Prior to ultracentrifugation of experimental SIP samples, multiple mock samples (in triplicates) containing ^{12}C, ^{13}C or ^{12}C+^{13}C DNA were ultracentrifuged in order to assess the distance of separation between the ^{12}C and ^{13}C DNA bands, their distance from the bottom of the tube (elevation within the tube), as well as their density-specific formation within the CsCl gradient. For the actual CsCl gradient separation, DNA extracted only from the experimental ^{12}C and ^{13}C incubations was used (not from the Live or Dead Controls which were only used for C-source tracking). Invitrogen SybrSafe (10,000×) of volume 20 μL was mixed with each sample of DNA, immediately before adding the mixture to 8 mL polyallomer tubes (Beckman Coulter) containing ~5 mL of the prepared CsCl solution. The tubes were equated in weight down to < 0.05 g difference with more of the CsCl solution. About 3 mL of mineral oil (Beckman Coulter) was added on top of the CsCl solution until tubes are almost full. Tubes were again equated in weight, but with mineral oil, then heat-sealed and placed into fixed-angle rotor 70.1Ti (Beckman Coulter). Ultracentrifugation was conducted at 187,000 $\times g$ (~43,800 rpm) at 20°C using a BC Optima L-100 XP ultracentrifuge (Beckman Coulter). Ultracentrifugation proceeded for 40 hours, employing maximum acceleration but no breaks during deceleration.

4.2.5 Fractionation

Produced CsCl gradient from experimental samples was collected in ~35 fractions of volume 150 μL accurately measured with the aid of a AL-4000 programmable syringe pump and 50 mL luer lock syringes (Williams Medical Supplies, World Precision Instruments). To

perform the fractionation distilled water was slowly pumped in ~150 μL volumes inside the polyallomer tube above the mineral oil through 22-gauge disposable needle (Farmline). Fractions were collected from the bottom of the tube inside 1.5 mL microcentrifuge tubes (Sarstedt). The refractive index of each fraction was measured and recorded for each fraction in order to assess the formation of the CsCl gradient.

4.2.6 DNA purifications & quantifications

DNA in each fraction was cleaned of SybrSafe and CsCl by precipitations with one volume of 100% molecular grade 2-propanol (Fisher Scientific). The mixture was incubated at -20°C for 30 min and centrifuged at 17,000 ×g for 30 min at 4°C. Supernatant was removed and 750 μL of 70% ethanol was added to wash the DNA pellet. Another centrifugation was performed under the same conditions. Ethanol was removed and DNA pellet was left to air dry. The nucleic acid pellet was resuspended in 50 μL 1 mM TE buffer. DNA in each fraction was quantified using a PicoGreen dsDNA dye (Thermo Scientific) and fluorospectrometer NanoDrop 3300 (Thermo Scientific) according to manufacturer's protocol. DNA quantities were recorded for each fraction.

4.2.7 Denaturing Gradient Gel Electrophoresis (DGGE)

4.2.7.1 16S rRNA gene fragment amplifications

16S rRNA gene fragment amplification was performed via touchdown PCR with general bacterial primers (341F: 5'-CCT ACG GGA GGC AGC AG- 3' and 534R: 5'-ATT ACC GCG GCT GC TGG-3'), the forward of which was amended with a 40 nucleotide GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') according to the protocol a modified version of Muyzer *et al* (1993). These primers amplified ~193 bp V3 region of the 16S rRNA gene. PCR of final volume 50 μL were prepared using BioLine MyTaq polymerase protocol, individually for each fraction. The reaction mixture contained 1× reaction buffer (BioLine), 4 μM of each primer, 1.5 U MyTaq polymerase (BioLine), 3% DMSO and ~1 μL DNA template. Touchdown PCR conditions are described in Table 4.1.

Table 4.1. Touchdown PCR conditions (adapted from Muyzer *et al.*, 1993)

Stage number	Number of Cycles	PCR steps		
		Denaturation	Annealing	Extension
1	1×	95°C (30 sec)		
2	4×	95°C (30 sec)	65°C (30 sec)	72°C (30 sec)
3	4×	95°C (30 sec)	63°C (30 sec)	72°C (30 sec)
4	4×	95°C (30 sec)	61°C (30 sec)	72°C (30 sec)
5	4×	95°C (30 sec)	59°C (30 sec)	72°C (30 sec)
6	4×	95°C (30 sec)	57°C (30 sec)	72°C (30 sec)
7	4×	95°C (30 sec)	55°C (30 sec)	72°C (30 sec)
8	1×	72°C (5 min)		
9	1×	4°C (hold)		

4.2.7.2 DGGE setup

DGGE gels were prepared based on the protocol of Strathdee & Free (2013) with 30-70% denaturing agent urea into 16×16 cm frames of DCode Universal Mutation Detection System (BioRad). From each SIP experiment, the PCR amplicons of 25 fractions were run through individual DGGE gels at 60V for 16 hours at 60°C, based on the protocol of Gutierrez *et al.* (2013). DGGE gels were stained with EtBr, imaged with InGenius3 gel imaging system (Syngene) and accompanying software was used to determine the community fingerprint of each fraction obtained during the ^{13}C hydrocarbon incubations. Several fractions containing only ^{13}C DNA were chosen to proceed to next steps based on intensity of bands and fingerprint pattern.

4.2.8 Full-length 16S rRNA gene amplifications and cloning

Selected fractions within each replicate were pooled together and amplified with 16S rRNA gene universal primers 27F and 1492R as described in Chapter 3, Section 3.2.4. Cloning of PCR products was performed using TOPO-TA cloning kit for sequencing (ThermoFisher Scientific) according to manufacturer's instruction manual. Ligation was performed using pUC19 plasmid vector (ThermoFisher Scientific) and plasmids were inserted into Transformation One Shot Mach1-T1 competent cells (Invitrogen) via heat shock at 42°C. Transformations were performed according to TOPO-TA cloning kit user manual. Efficiency of transformations was followed by white/blue screening of colonies grown on LB media amended with 50 μg/mL Kanamycin and 40 μg/mL X-gal. From each experimental replicate 48 white colonies were selected, converted to LB+ kanamycin 30% glycerol stocks and stored at -80°C. Simultaneously, plasmids were extracted from each clone with Plasmid

miniprep kit (QIAGEN) following the manufacturer's protocol. Insert size determination was performed via *Eco*RI enzymatic digest performed for 1 hour at 37°C and visualised by electrophoresis that was run through 1% agarose 1× TAE gel at 90V for 30min. Individual libraries passed quality control if they contained less than 5% blue colonies (95% transformation efficiency) and Eco*RI* digest produced banding of expected sizes (3.4 kbp vector size and 1.4 kbp insert size).

4.2.9 Analysis of Sanger 16S rRNA gene sequence data

Plasmids containing the inserted 16S rRNA genes obtained from the 'heavy' DNA fractions, were shipped to and sequenced by GeneWiz (UK) as described in Chapter 3, Section 3.2.4. Sequencing reactions were initially performed only in one direction with vector primer T7. Reads (per SIP experiment) were trimmed of low quality ends and manually curated using Consed/Phred/Phrap (Gordon and Green, 2013). High quality reads were clustered using USEARCH v9.0 (http://www.drive5.com) and taxonomic identification of resulting OTUs was performed manually using NCBI's BLAST against NCBI's 16S ribosomal RNA sequences (Bacterial and Archaea) database. OTUs with abundance < 5% were not analysed further. A representative clone from each OTUs of interest was selected for sequencing with M13 primer (reverse orientation). The reverse reads were manually curated similarly and merged with their T7-complementary read using Consed/Phred/Phrap (Gordon and Green, 2013). Taxonomic identification of full length 16S rRNA gene sequence was confirmed using BLAST and 16S ribosomal RNA gene sequences (Bacterial and Archaea) database. Phylogenic analysis was performed as described in Section 2.2.5. High quality sequence reads of length >1300 bp were submitted to GenBank.

4.2.10 qPCR

qPCR primers were designed specific for each OTU using AliView software (Larsson, 2014) as well as NCBI Primer Blast online tool (Ye J. *et al.*, 2012). Primer pairs were designed to amplify a region no bigger than 160 bp and with melting temperatures (T_M) varying no more than 2°C within the pair. Primers were checked for specificity to only the selected organism using NCBI's Primer BLAST. qPCR primers were optimized using gradient PCR performed as described above but with annealing temperature gradient range from 55-65°C. qPCR was performed on DNA extracted from cell pellets collected as described in Section 4.2.3 using SensiFAST™ SYBR kit (BioLine). qPCR reaction included 5 μL 2× SensiFAST™ SYBR reaction buffer (containing polymerase), 0.4 μL Primer Mix (4 μM each primer), 0.5 μL

DNA template (~20 ng/µL) and 4.1 µL molecular grade water. qPCR incubation conditions included a 1 min denaturation step at 95°C, 40 cycles of 95°C denaturation step of 10 sec followed by a 58°C annealing step of 10 sec. The reaction was completed with a 30 sec final extension step at 72°C and a melting curve step to assess the quality of the qPCR product. Standard curves were prepared using plasmid DNA from the corresponding organism with known concentration and C_T values determined in the qPCR reaction. DNA concentrations of all samples used in the qPCR were determined using Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).   Gene copy number accumulation for each selected OTU was calculated by correlating the acquired template-specific qPCR C_T values from the standards (plasmid with known concentration and therefore known amplicon copy number) to the C_T values of reciprocal template within the SIP total-DNA samples.

4.3 Results

4.3.1 SIP incubations and tracking

The SIP experiment comprised of 10 cultures: $2\times$ ^{13}C SIP cultures, $2\times$ ^{12}C SIP cultures (experimental cultures), $3\times$ Live Control incubations and $3\times$ Dead Control incubations per hydrocarbon source (HEX or PHE) and per depth (5m or 700m). Results from tracking the concentrations of provided carbon source in the Live and Dead Control samples (Figure 4.2) showed noticeably higher rates of disappearance of HEX in the Live Controls (within 3 to 4 days) compared to those in Dead Controls (concentrations did not reach zero in a 5 day incubation). Tracking of PHE concentrations in the SIP+PHE Control cultures was indicative of slow dissolution into aqueous phase by day 5, and then rapid disappearance of PHE by day7. This was observed in both Live and Dead Control cultures, indicative of potential biodegradation of PHE in the Dead Controls as well (Figure 4.2, bottom panel). Since complete degradation of HEX was observed within 3 days in Live Controls, experimental incubations were terminated on day 5. As PHE degradation occurred by day 7 in Live and Dead Controls, these experimental incubations were harvested on day 10. All Live Controls and experimental SIP cultures incubated with HEX produced visible turbidity during the incubation periods, while Dead Control samples remained clear. No SIP incubations with PHE produced turbidity. A slight appearance of yellowish hue was observed in all PHE SIP samples by day 10 of incubation (including Live and Dead Controls and experimental cultures).

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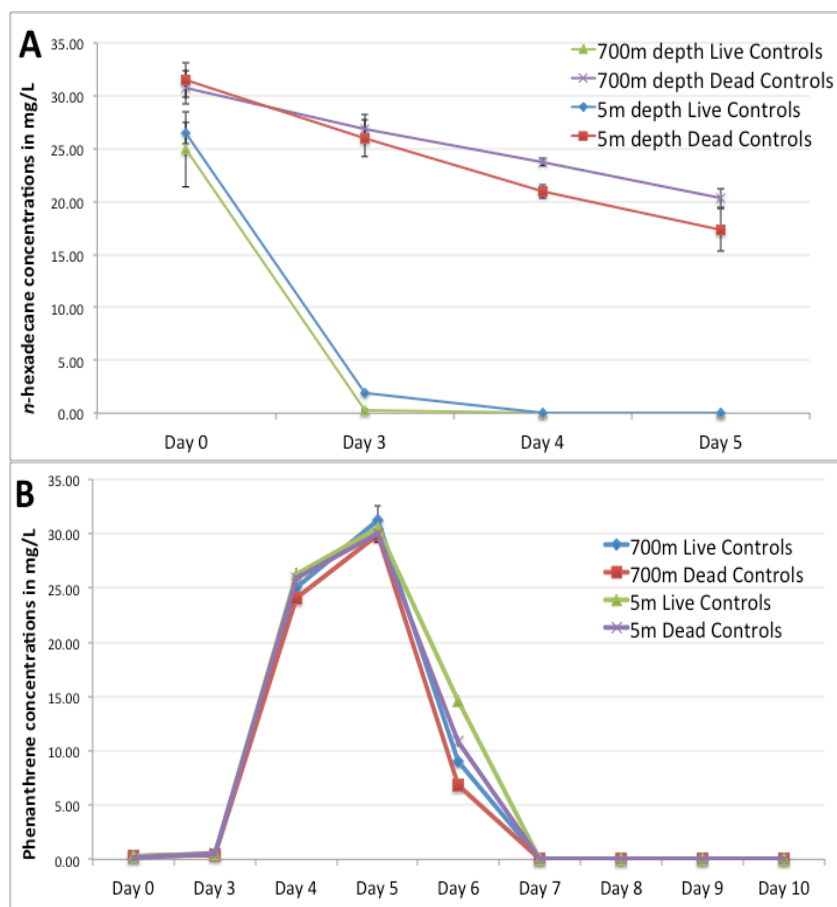
Figure 4.2. Hydrocarbon source concentrations detected with progression of incubation period in DNA-SIP. A: Incubations with uniformly labelled ^{13}C *n*-hexadecane (HEX). B: Incubations with uniformly labelled ^{13}C phenanthrene (PHE).

4.3.2 CsCl gradient ultracentrifugations

Mock samples from preliminary studies resulted in the consistent formation of CsCl gradient of 1.60 – ~1.73 g/mL, with unlabelled 'light' ^{12}C DNA consistently collecting into a single band at ~2.0 cm distance from the bottom of the polyallomer tube, and the ^{13}C-labelled 'heavy' DNA consistently collecting into a band of ~0.9-1.1 cm from the bottom of the tube. When mock samples containing both ^{12}C and ^{13}C DNA were ultracentrifuged, the gradient yielded a clear separation between the two bands of ~1 cm, with one DNA band matching the height of ^{13}C DNA (~1 cm from the bottom of the tube) and another band matching the height of the ^{12}C DNA band (~2.0 cm from the bottom of the tube) (data not shown). These metrics were used to distinguish ^{12}C DNA from ^{13}C DNA in the experimental SIP samples after ultracentrifugation.

CsCl gradient of ^{13}C incubations with HEX (both 5m and 700m SIPs) resulted in the formation of one fluorescent band in the polyallomer tubes which consistently appeared at ~1.0 cm distance from the bottom of the tube, coinciding with the expected position for ^{13}C-enriched DNA. No ^{12}C DNA bands were clearly visible in the ^{13}C-HEX incubations (2 replicates per SIP), however in ^{12}C-HEX samples (2 replicates per SIP) a band of ^{12}C DNA was observed around 2.0 - 2.1 cm from the bottom of the tubes. CsCl gradient separations of the ^{13}C-PHE incubations with surface water (5 m depth) resulted in the formation of two fluorescent bands in the polyallomer tubes with ~1 cm expected separation. Lower band appeared at 0.9 - 1.1 cm from the bottom of the each replicate tube while higher band – at ~2.0 - 2.1 cm from the bottom. The ^{12}C-PHE SIP incubations with surface water (all replicates) produced bands only at ~2.0 cm distance from bottom. The experimental ^{13}C-PHE cultures from the subsurface water (700 m depth) (which during incubations were observed to not produce turbidity, but exhibit slight yellowish hue; refer to Section 4.3.1) produced no visible bands in any of the incubations. Summary of band observation results can be viewed in Table 4.2.

Table 4.2. Observed bands in the CsCl gradient of each DNA-SIP sample, including sample name, expected content and band distance from the bottom of the tube.

Tube No.	Experimental sample name	DNA content	Observed bands	"Heavy" band	"Light" band
1	^{12}C-HEX 5mSIP Replicate1	^{12}C DNA only	1	NA	~2.1 cm
2	^{13}C-HEX 5mSIP Replicate1	^{12}C DNA + ^{13}C DNA	1	~1.1 cm	NA
3	^{12}C-HEX 5mSIP Replicate2	^{12}C DNA only	1	NA	~2.1 cm
4	^{13}C-HEX 5mSIP Replicate2	^{12}C DNA + ^{13}C DNA	1	~1.1 cm	NA
1	^{12}C-HEX 700mSIP Replicate1	^{12}C DNA only	1	NA	~2.1 cm
2	^{13}C-HEX 700mSIP Replicate1	^{12}C DNA + ^{13}C DNA	1	~1.1 cm	NA
4	^{12}C-HEX 700mSIP Replicate2	^{12}C DNA only	1	NA	~2.1 cm
3	^{13}C-HEX 700mSIP Replicate2	^{12}C DNA + ^{13}C DNA	1	~1.1 cm	NA
1	^{12}C-PHE 5mSIP Replicate1	^{12}C DNA only	1	NA	~2.1 cm
2	^{13}C-PHE 5mSIP Replicate1	^{12}C DNA + ^{13}C DNA	2	~1.1 cm	~2.1 cm
3	^{12}C-PHE 5mSIP Replicate2	^{12}C DNA only	1	NA	~2.1 cm
4	^{13}C-PHE 5mSIP Replicate2	^{12}C DNA + ^{13}C DNA	2	~1.1 cm	~2.1 cm
1	^{12}C-PHE 700mSIP Replicate1	^{12}C DNA only	0	NA	NA
2	^{13}C-PHE 700mSIP Replicate1	^{12}C DNA + ^{13}C DNA	0	NA	NA
3	^{12}C-PHE 700mSIP Replicate2	^{12}C DNA only	0	NA	NA
4	^{13}C-PHE 700mSIP Replicate2	^{12}C DNA + ^{13}C DNA	0	NA	NA

4.3.3 Fractionation

'Heavy' ^{13}C-DNA tested with mock samples was typically extracted within fractions 4-12 with corresponding CsCl density of ρ=1.72-1.70 g/mL, while "light" ^{12}C-DNA from mock samples tended to elute into fractions 14-24 (CsCl ρ=1.69-1.64 g/mL). These metrics were

observed in the mock samples and re-confirmed by the experimental SIP samples. Fraction densities in all SIP experimental samples showed consistent gradient formation and collection per fraction (Figure 4.3). Results from fractions obtained from the SIP samples incubated with ^{13}C-HEX confirmed the low levels of "light" DNA and high levels of 'heavy' DNA within the samples. Contrarily, all the ^{12}C-HEX incubated experimental samples (from surface, 5m and subsurface, 700 m seawater) exhibited high abundance in 'light' DNA, eluting in later fractions (>14) (Figure 4.3, panel A and B). Surface water (5m) SIP incubations with PHE confirmed the presence of ^{12}C and ^{13}C DNA (Figure 4.3, panel C) suggested by the observed DNA bands in the CsCl gradient (Table 4.2). Experimental incubations with subsurface water (700m) and PHE did not produce sufficient amounts of cell mass or DNA (Figure 4.3, panel D, Table 4.2 and Section 4.1).
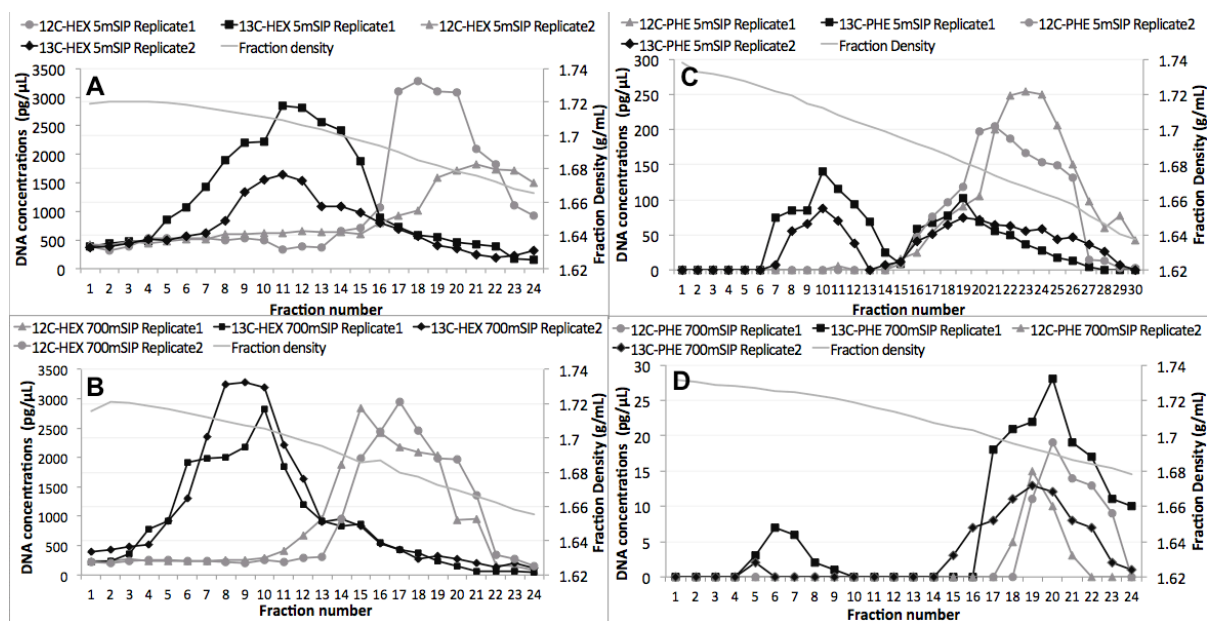
Figure 4.3. DNA concentration and CsCl density for fractions 1-24 in each experimental replicate (^{12}C and ^{13}C incubations) of each SIP experiment. A: SIP experiment with *n*-hexadecane (HEX) and surface water (5 m); B: SIP experiment with HEX and subsurface water (700 m); C: SIP experiment with phenanthrene (PHE) and surface water (5 m); D: SIP experiment with phenanthrene (PHE) and subsurface water (700 m).

4.3.4 DGGE results

Microbial community fingerprinting was successful for all SIP experiments except for the PHE-SIP with subsurface water (700 m), which did not produce sufficient DNA for PCR

amplifications (see Figure 4.3D). Therefore experiment 700mSIP+PHE was discontinued.
Microbial community fingerprints for 5mSIP+HEX and 700mSIP+HEX and 5mSIP+PHE
were produced and are presented in Figure 4.4. Results from 5mSIP+HEX and
700mSIP+HEX (Figure 4.4, panels A-D) affirmed high DNA concentrations in lower
numbered fractions (fraction numbers 2 - ~14 have highest band intensity) and coincided
with expected 'heavy' DNA fractions observed after fractionation (Figure 4.3). DGGE
imaging of SIP+HEX communities (Figure 4.4, panels A-D) also confirmed the low levels of
^{12}C-DNA in all HEX experiments (based on low band intensities for higher numbered
fractions, >16). Contrarily, DGGE results from the 5mSIP+PHE incubations showed
distinguishable switch in community fingerprints between the lower numbered fractions (7-
14, expected 'heavy' DNA) and higher number fractions (>15, expected 'light' DNA; Figure
4.4, panels E and F). Again, the more intense DGGE bands from the 5mSIP+PHE
incubations coincide with observed higher levels of eluted DNA during fractionation (Figure
4.3D).
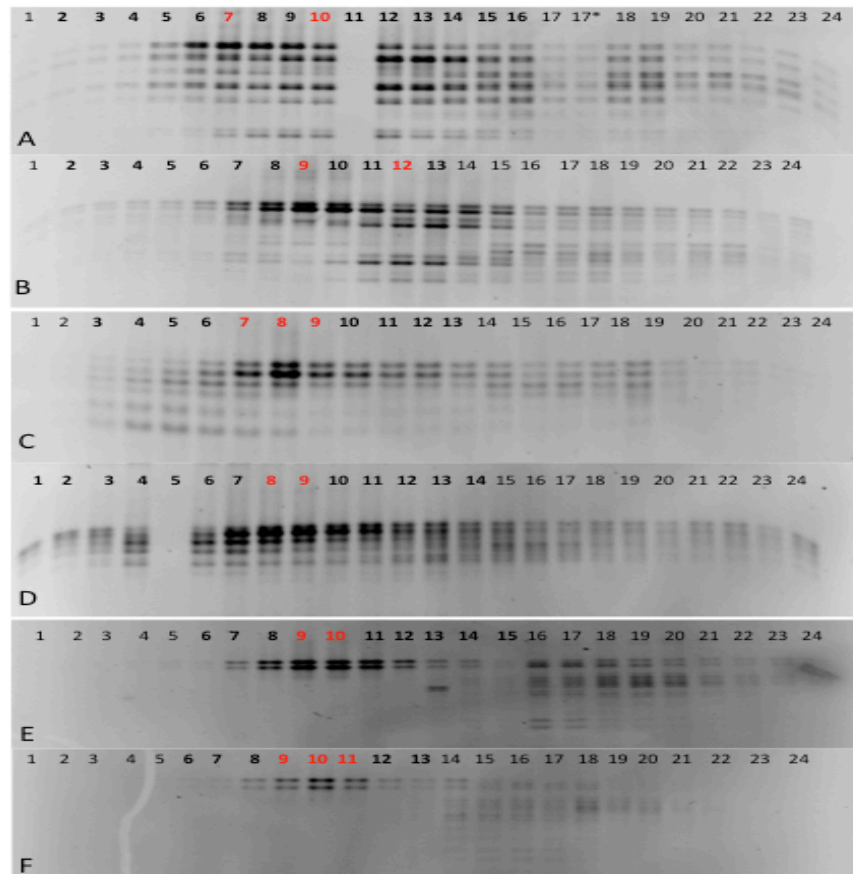
Figure 4.4. DGGE fingerprint of FSC microbial communities from various depths, formed after incubation with ^{13}C-HEX or ^{13}C-PHE. Images A & B: Replicates 1 and 2 (respectively) of 5 m FSC microbial communities formed after 5 day exposure to ^{13}C-HEX. Images C & D: Replicates 1 and 2 of 700 m FSC microbial communities formed after 5 days exposure to ^{13}C-HEX. Images E & F: Replicate 1 and 2 of 5 m microbial communities formed after 10 days exposure to ^{13}C-PHE. Fingerprints were based on 16S rRNA gene fragment sequences. Numbering indicates well/fraction numbers, with 'heavy' DNA fractions being labelled in bold font. Fraction numbers marked in red were selected for subsequent analysis. Blank lanes indicate undetectably low amounts of DNA.

4.3.5 Fraction selection and cloning

Based on the DGGE microbial fingerprints for 'light' and 'heavy' DNA communities, several 'heavy' DNA fractions from the low fraction numbers (7-11) were selected for pooling and proceeding to cloning. Samples from SIP experiment with ^{13}C-PHE and subsurface water (700 m) did not proceed to cloning step. Selected fractions can be viewed in Table 4.3 along with number of clones selected and names of each produced clone library.

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Table 4.3. SIP experiments and fraction numbers selected for cloning

Clone Library Name	Number of clones selected	SIP experiment sample name	Selected fractions
5mSIP+HEX	48	^{13}C-HEX 5mSIP Replicate1	7 + 10
	48	^{13}C-HEX 5mSIP Replicate2	9 + 12
700mSIP+HEX	48	^{13}C-HEX 700mSIP Replicate1	7 + 8 + 9
	48	^{13}C-HEX 700mSIP Replicate2	8 + 9
5mSIP+PHE	48	^{13}C-PHE 5mSIP Replicate1	9 +10
	48	^{13}C-PHE 5mSIP Replicate2	9 +10 + 11

Results from cloning showed all libraries had 98% transformation efficiency (<2% blue colonies). Gel images of Eco*RI* digests showed vector bands of size ~3.4kb and an insert bands of size of ~1.4kb across all randomly selected test clones from all libraries. Figure 4.5 presents a gel image of *Eco*RI digest from 16 clones from library 5mSIP PHE as an example of typical *Eco*RI digestion result.
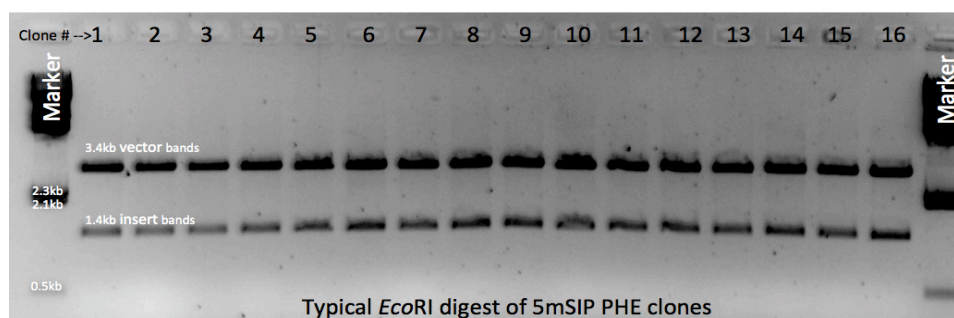
Figure 4.5. Gel image of 16 random clones from library 5mSIP PHE digested with *Eco*RI**.**

4.3.6 Sequence processing

Sequencing of clone libraries produced 288 sequences, of which 276 where one directional high quality Sanger sequences of ~900 bp length. Sequences were organized into corresponding libraries (96 clones each) and processed as such. The taxonomic composition of each library is presented in Table 4.4. The taxa with highest abundance in the 5mSIP+HEX library (SIP experiment proceeded with ^{13}C HEX and 5m seawater) included *Lentibacter algarum* (OTU15), *Thalassolituus oleivorans* (OTU17) and *Alcanivorax borkumensis* (OTU23). In library 700mSIP HEX, highest abundance taxa were *Marinobacter algicola* (OTU15), *Alcanivorax borkumensis* (OTU31) and *Oleibacter marinus* (OTU18). Library 5mSIP+PHE was dominated by *Cycloclasticus spirillensus* (OTU7). The

phylogenic tree relations between selected clones (representative of each OTU) and closest taxonomic relative is presented in Figure 4.6

Table 4.4. Description of each SIP cloned library including OTU number, abundance of OTU in the library, representative clone, BLAST ID with percentage, taxonomic lineage shorthand for each OTU, GenBank Accession number and clone length. Library names 5mSIP+HEX (top table): cloned library produced from SIP experiment performed with surface water (5 m) and ^{13}C-*n*-hexadecane (HEX). 700mSIP+HEX (middle table): cloned library produced from SIP experiment performed with subsurface water (700m) and ^{13}C-*n*-hexadecane (HEX). 5mSIP+PHE (bottom table): cloned library produced from SIP experiment performed with surface water (5 m) and ^{13}C-phenantherene (PHE).

5mSIP+HEX cloned library							
OTU	Relative abund.	Rep. Clone	BLAST ID	% ID	Lineage	GenBank Acc No	Clone length (bp)
OTU22	8.5%	2.4	*Oleibacter sp.*	96%	*c:γ; o:Oceanospirillales*	KY515284	1493
OTU15	14.9%	1.1	*Lentibacter algarum*	99%	*c:α; o:Rhodobacterales*	KY515280	1419
OTU17	22.3%	2.6	*Thalassolituus oleivorans*	99%	*c:γ; o:Oceanospirillales*	KY515286	1465
OTU23	31.9%	2.14	*Alcanivorax borkumensis*	99%	*c:γ; o:Oceanospirillales*	KY515282	1489

700mSIP+HEX cloned library							
OTU	Relative abund.	Rep. Clone	BLAST ID to	% ID	Lineage	GenBank Acc No	Clone length (bp)
OTU11	5.3%	4.12	*Dokdonia sp.*	95%	*c:Flv; o:Flavobacteriales*	KY515291	1476
OTU13	8.5%	3.32	*Glaciecola sp.*	95%	*c:γ; o:Alteromonadales*	KY515288	1497
OTU14	8.5%	4.30	*Phaeobacter arcticus*	98%	*c:α; o:Rhodobacterales*	KY515293	1465
OTU15	9.6%	3.15	*Marinobacter algicola*	99%	*c:γ; o:Alteromonadales*	KY515294	1498
OTU31	12.8%	4.22	*Alcanivorax borkumensis*	99%	*c:γ; o:Oceanospirillales*	KY515292	1505
OTU18	17.0%	3.27	*Oleibacter sp.*	96%	*c:γ; o:Oceanospirillales*	KY515287	1490

5mSIP+PHE cloned library							
OTU	Relative abund.	Rep. Clone	BLAST ID to	% ID	Lineage	GenBank Acc No	Clone length (bp)
OTU7	100%	6.11	*Cycloclasticus pugetii*	99%	*c:γ; o:Thiotrichales*	KY515295	1485
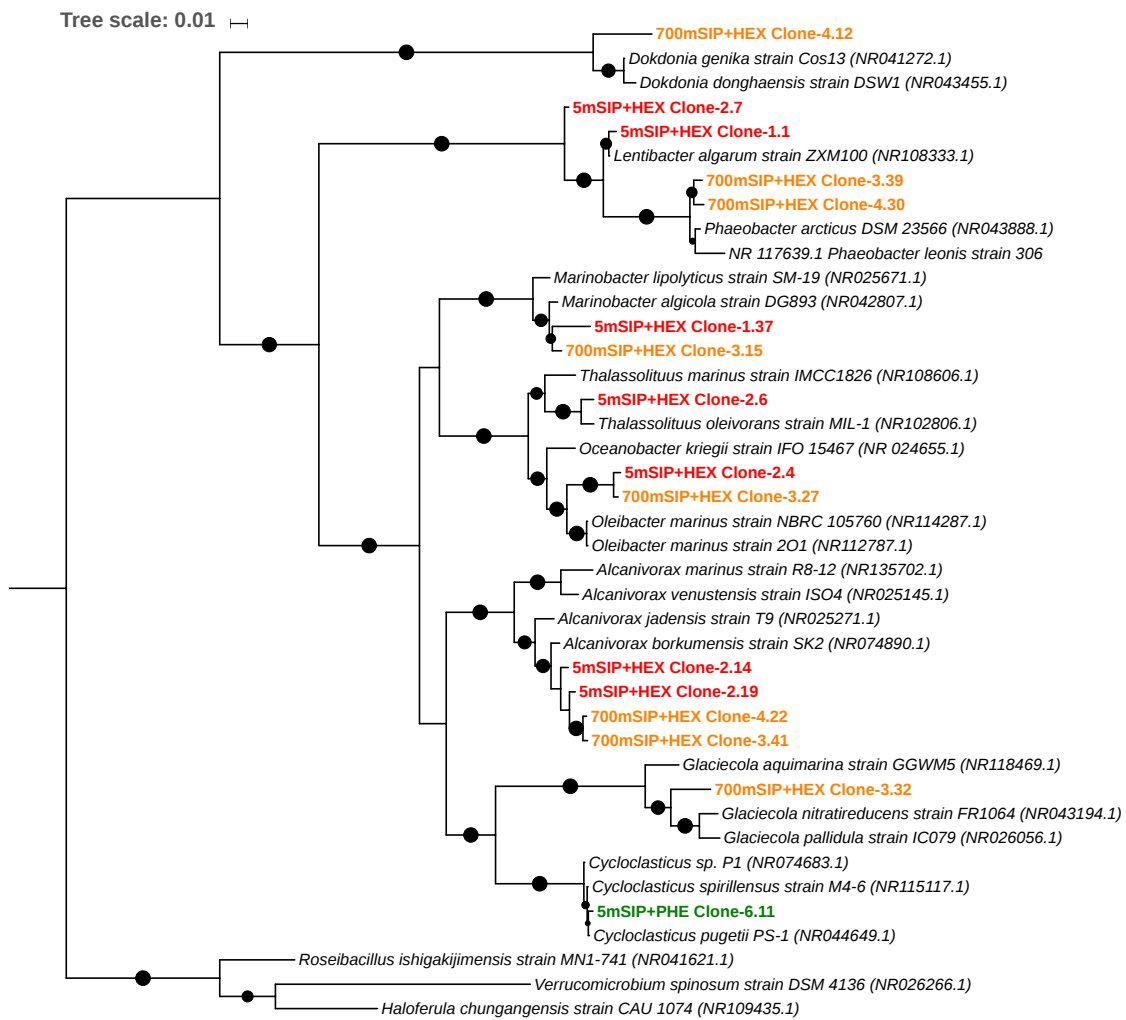
Figure 4.6. Phylogenic affiliations of representative clone sequences from each OTU in each SIP library. SIP libraries are: 5mSIP HEX (red), 700mSIP HEX (orange), 5mSIP PHE (green). Several type strain unrelated bacterial organism species from family *Verrucomicrobiaceae* were used to root tree. Tree was constructed by neighbour-joining method. Size of black dots corresponds to bootstrap confidence value of 70% - 100% based on 1,000 bootstrap replications. Scale bar represents substitution rate (nucleotide substitutions per site).

4.3.7 qPCR results

Accumulation of each OTU in the duration of the incubation was determined with qPCR. Results shown in Figure 4.7 present orders of magnitude increase in the 16S rRNA gene abundance of HEX-active organisms for the 5 m HEX library, such as *Oleibacter* sp. (6 orders of magnitude), *Alcanivorax borkumensis* (6 orders of magnitude)*, Thalassolituus*

oleivorans (4 orders of magnitude) and *Lentibacter algarum* (6 orders of magnitude). For the 700 m HEX library orders of magnitude increase in gene copy number were observed for *Oleibacter* sp. (6 orders of magnitude), *Alcanivorax borkumensis* (6 ord. mag) and *Marinobacter algicola* (3 ord. mag), *Glaciecola* sp (3 ord. mag) and *Phaeobacter arcticus* (3 ord. mag). The ^{13}C-PHE incubation for the 5mSIP resulted in a 6 orders of magnitude increase in gene copy number for *Cycloclasticus spirillensus* over the 10 day period. Other organisms of interest from the libraries also examined with qPCR, showed similar orders of magnitude increase, except for *Dokdonia* sp. from 700mSIP+HEX library, which only showed 2 orders of magnitude increase in copy number, primarily occurring in the last few days of incubation.
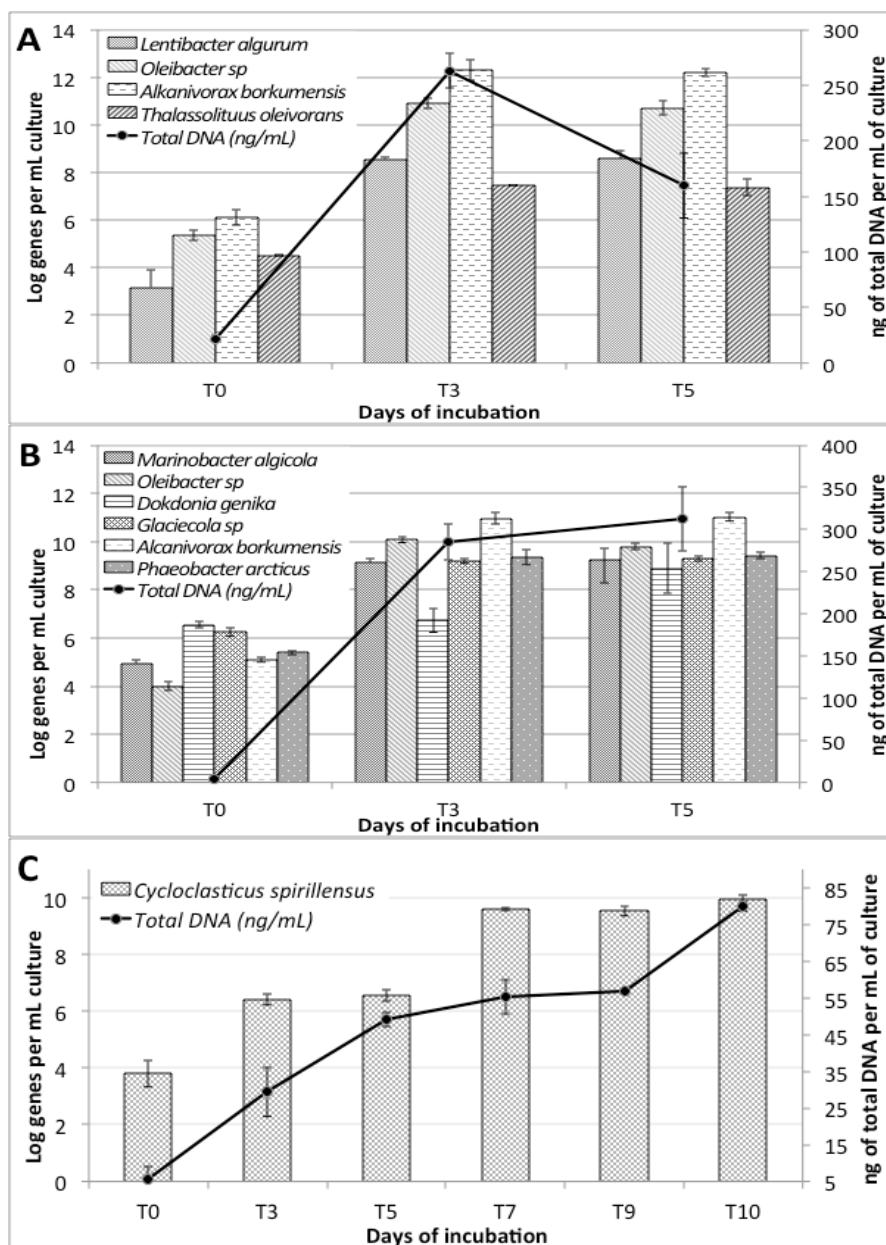
Figure 4.7. 16S rRNA gene accumulation over the time of incubation in 5m seawater SIP experiments with *n*-hexadecane (HEX) (A) or phenanthrene (PHE) (B) and 700m seawater SIP with *n*-hexadecane (C).

4.4 Discussion

4.4.1 Considerations and limitations of experiment

Before extrapolating meaning to the presence of bacterial strains within the SIP cultures, some factors, which may have influenced the results or their interpretation, needed to be taken into account. The first factor was the fast rate of disappearance of HEX observed in

the Live Controls during the HEX incubations of the SIP experiment. Due to technical limitations, tracking concentrations during incubations could not be performed on a daily basis. This resulted in absent data for the levels of the provided substrate between days 0 and day 3, where the majority of the substrate appear to have been removed from solution. This rapid reduction in HEX in the Live Control cultures and its relatively low reduction in the Dead Control cultures signified not only of the importance of the microbial factor in the removal of the HEX, but also the rate of this removal. As ^{13}C-HEX mineralization and its DNA incorporation could not be directly tracked, the duration of the incubation was based on the disappearance of the hydrocarbon. Additionally 2 extra days of incubation were allowed in order for bacterial members to properly incorporate the heavy carbons into their DNA during replication. Considering the observed fast rate of removal it could be argued that ^{13}C-HEX bacterial utilization and possibly the ^{13}C biomass incorporation has occurred within the first 3 days of incubation and the excess days of incubation may have increased the risk of secondary utilizers (bacteria, which does not directly utilize the provided hydrocarbon, but rather utilizes by-products of the degradation process such as shorter-carbon-chain alcohols). This could have produced ^{13}C incorporation from the entire SIP-culture community, rather than just the primary degraders, which would display as appearance of single heavy ^{13}C DNA band (and lack of ^{12}C DNA band) in CsCl gradient separation of the communities as well as single community fingerprint across all fractions examined with DGGE. In this experiment however, the relative dominance of only limited number of species within the cloned libraries (7 dominant species with 8-32% abundance within the libraries) as well as their phylogenic associations to known hydrocarbon degraders (e.g. *Alcanivorax*, *Thalassolituus*, *Marinobacter*, *Oleibacter*) suggest that secondary degradation of the provided hydrocarbon was not yet the primary activity within the cultures. For a more reliable interpretation of the results, bacteria with delayed enrichment into the community (e.g. after day 3 in the SIP+HEX incubations) and/or low abundance within the cloned libraries (e.g. below 8%) were not presumed as HEX degraders.

The second factor that needed to be taken into account was the temperature conditions of the incubations, which may have applied a selective force on the microbial community, promoting the advancement of more mesophilic bacterial species, while inhibiting potentially important psychrophilic members. Consequently, in order to produce more accurate assessment of the FSC degraders capable of degrading hydrocarbons under ambient FSC

conditions, the SIP incubation should have been amended to proceed at lower temperature (e.g. 4°C). This however would have had implications on the hydrocarbon's bioavailability, physicochemical properties and biodegradation rates, as those have shown to be significantly influenced by temperature (Chapter 1, Section 1.3 as well as (Hollebone, 2015; Tarr *et al.*, 2016; Brakstad *et al.*, 2010; Fingas, 2011; Atlas, 1975)). Such amendment would therefore produce complications requiring other protocol amendments such as increased culture volumes (due to daily volume extractions) and significantly prolonged incubation times (due to substrate precipitation, delayed response to substrate and/or delayed ^{13}C incorporation, *etc.*). Therefore low-temperature incubation amendments could not be implemented until further investigations into the feasibility of the amendment could be performed. Furthermore, the moderate temperature condition of the incubations did not appear to be detrimental to the presence of strains affiliated with known psychrophilic and psychrotolerant genera within the cultures (e.g. OTU22 and OTU17 of the 5mSIP+HEX library or OTU18 and OTU15 of the 700mSIP+HEX library), such as *Oleispira*, *Thalassolituus* and *Marinobacter* (Refer to Table 1.2). Frankly, it is still unclear whether the *Alcanivorax* strains isolated in this SIP experiment (OTU23 within the 5mSIP+HEX and OTU31 within the 700mSIP+HEX library) exhibited psychrophilic or psychrotolerant behaviour or they were merely enriched mesophilic strains. Therefore further investigation into the cold adaptations of the enriched strains of the FSC community (possibly via genome reconstruction and metabolic pathway examination) would be beneficial to affirm cold adaptations. Essentially, however, the chosen elevated incubation conditions of this SIP experiment (20°C) did not appear to affect the capacity of the technique to pinpoint microbial members within an indigenous community capable of specific the metabolic functionality of interest.

The third factor influencing the interpretation of this experiment's results, stems from the risk of assuming organism enrichment based only on the accumulation of 16S rRNA gene copy numbers determined through qPCR. As each bacterial organisms may have different 16S rRNA gene copy number (Vetrovsky and Baldrian, 2013), an artificial inflation of the gene's accumulation during incubations, may be produced during PCR amplifications. Therefore, the 16S rRNA gene accumulation results should not be considering without information about the abundance of the organism within the cloned library, replicate variability of qPCR replicates, the timeframe of turbidity formation during incubation, total DNA increase within the samples from different days and the timeframe of disappearance of the substrate.

Considering these three factors, the presence and enrichment of bacteria within the SIP cultures of this study can be explored.

4.4.2 Degradation of n-hexadecane by surface and subsurface microbiota

Results from this stable isotope probing experiment suggested that key players in the hydrocarbon degradation in the FSC water column would be members within the genera *Alcanivorax*, *Thalassolituus* and *Cycloclasticus* for the surface water (selected as representative of the Atlantic water core (AWC)), while hydrocarbons in the subsurface water (Nordic water core (NWC)) would be utilized primarily by *Marinobacter*, cold-adapted *Alcanivorax* strains and *Oleibacter* species. The phylogenic analysis indicated that the *Oleibacter* strain found dominant in the subsurface cultures (OTU18/Clone 4.27) were also present in the surface water (OTU22/Clone 2.4), although under lower levels of abundance (Figure 4.6). This could be due to the organism's water core association (NWC), preference for colder environments and/or higher pressure. The phylogenic analysis and alignment to known GenBank type strains, also showed that the strain of *Oleibacter* sp. identified from this SIP experiment could be a novel *Oleibacter* species as it only shows 96% sequence similarity to *Oleibacter marinus* type strains. This unclassified *Oleibacter* species (OTU18), however, has also shown 99% sequence similarity to a bacterium reported as an anaerobic aromatic biodegrader from the *Prestige* oil spill in a study published from 2013 (GenBank Acc JF344207) (Acosta-Gonzalez *et al.*, 2013), yet no species identity has yet been assigned. It is therefore considered possible that the discovered *Oleibacter* sp. were a novel psychrotolerant alkane and possibly aromatic degrading bacteria capable of both aerobic and anaerobic degradation (Teramoto *et al.*, 2011). The genus *Oleibacter* is known for its psychrophilic behaviour and its hydrocarbonoclastic activity (Chronopoulou *et al.*, 2015; Teramoto *et al.*, 2011). However, due to the mobility of hydrocarbon-degradation functional gene cassettes between strains, specialization of each strain is possible to specific class of hydrocarbons as substrate and even specific environmental conditions (Hedlund and Staley, 2006; Bej *et al.*, 2010; Cappello *et al.*, 2007; Luz *et al.*, 2010; Yakimov *et al.*, 2007; Head *et al.*, 2006). Therefore, the metabolic potential this and all other dominant FSC taxa identified (*Oleispira*, *Thalassolituus*, *Alcanivorax* and *Marinobacter*) should be further explored, potentially through genomic reconstruction and analysis of their genomic features.

Other bacteria revealed in this SIP experiment were found affiliated with the genera *Glaciecola* and *Doknonia* (Figure 4.6), which have previously also been reported at oil-contaminated polar environments (Prabagaran *et al.*, 2007; Zhang *et al.*, 2011; Qin *et al.*, 2014; Timmis, 2010). Despite this association, likely hydrocarbonoclastic activity and presence in this SIP experiment, their degradation activity of alkanes, such as hexadecane could not be unequivocally confirmed through this SIP experiment. The high rate of hydrocarbon disappearance in the Live Controls (within 3 to 4 days), coupled with the relatively low abundance of the *Dokdonia* strain (OTU11, only 5.4% of the 700mSIP+HEX library) and the delayed 16S rRNA gene accumulation of OTU11 during incubation (gene copy numbers increase on day 5, rather than 3 like to the other members of the assemblage) (Figure 4.7B) allows for a possibility for its appearance to be due to either slow doubling time or secondary activity within the cultures (e.g. degradation of hydrocarbon-degradation by-products such as alcohols). Therefore further investigation would be required in order to elucidate the role of this bacterium within the SIP cultures and its potential as a hydrocarbon degrader in the FSC. As mentioned in Chapter 1, Table 1.1 the genus *Glaciecola* has also been recognized as a (strictly aerobic, psychrotolerant) hydrocarbon degrader especially in polar marine environments (incl. sediment) (Zhang *et al.*, 2011). As gene transfer has shown to be a significant factor in the genome size as well as metabolic and environmental adaptations of a lot of species within this genus of bacteria (Qin *et al.*, 2014), substrate preference for the species and strains within this genus may be highly variable. *Glaciecola* species have exhibited pyrene degradation properties (Wang *et al.*, 2008) and appear capable of degrading water soluble hydrocarbons (Prabagaran *et al.*, 2007). In this study *Glaciecola*-affiliated strain (OTU13, ~8.5% relative abundance in 700mSIP+HEX library) had exhibited enrichment and abundance within the cloned libraries comparable to that of strains belonging to groups of known hydrocarbonoclastic bacteria such as *Marinobacter* (e.g. OTU15, 9.6% abundance). Additionally the bacterial strain was found within the colder subsurface FSC water, which is concurrent with its group's psychrophilic behaviour (Zhang *et al.*, 2011). It is therefore likely that the *Glaciecola*-affiliated strain observed in this study (originating from the FSC) possesses the capacity to degrade hexadecane and possibly other alkanes. In addition, due to the willingness of the members within this genus to accept functional genomic cassettes (Qin *et al.*, 2014), members of this bacterial group may be capable of utilizing a variety of other hydrocarbon compounds. The detected strain (OTU13) shows 95% affiliation with the genus *Glaciecola* suggesting it may be a novel species within the

genus, however isolation and characterization of the bacteria may be required in order to characterize it. Further investigation into the genomic determinants for hydrocarbon degradation of this FSC strain bacterium may also be beneficial for potential biotechnological applications.

Two *Alphaproteobacteria* belonging in the order *Rhodobacterales* were also observed in relatively high abundance in HEX cloned libraries and active enrichment during the first 3 days of incubation – a strain (OTU15 within the 5mSIP+HEX library) affiliated with the species *Lentibacter algarum* (99%) observed in the surface FSC water (belonging to the warmer AWC) and a strain (OTU14 within the 700mSIP+HEX library) affiliated with the species *Phaeobacter arcticus* (98%), observed in subsurface FSC water (colder, NWC). Members of the order *Rhodobacterales* have been known for exhibiting high levels of adaptability to various environments, use large variety of organic substrates, and some can even possess hydrocarbonoclastic activity (Hahnke *et al.*, 2013; Timmis, 2010). Due to an active gene transfer system, individual species and even strains within the *Rhodobacterales* order may exhibit unique physiological abilities (Hahnke *et al.*, 2013; Newton *et al.*, 2010). *Phaeobacter arcticus* was first described in 2008 as an psychrophilic bacteria isolated from the Arctic (Zhang *et al.*, 2008), then later on the genus was linked to hydrocarbon catabolism based on its genetic contents (Timmis, 2010). This study is another report for the alkane degradation abilities of a strain within the species *P. arcticus*. Further interrogations into the behaviour, genomic features, metabolic capacities and specific hydrocarbon preferences of this FSC *Phaeobacter arcticus* strains could provide more detail on its potential role in hydrocarbon degradation. The species *Lentibacter algarum* was first characterized as a species in 2012 by Li *et al.* as mesophilic, capable of oxidizing various sugars and living in association with green algae. Species within the *Lentibacter* genus (also closely associated with *L. algarum*), also have been found in the North Sea, affiliated with phytoplankton and interestingly exhibiting no growth on a variety of sugars (Li *et al.*, 2012; Hahnke *et al.*, 2013). It is also a commonly known fact that some algae produce *n*-alkanes in the form of waxes and paraffins, which may be utilized by associated bacteria such as *Lentibacter* sp. (Smits *et al.*, 2002; Blumer *et al.*, 1971; Radakovits *et al.*, 2010). This could explain the SIP-identified *Lentibacter algarum* strain presence in the alkane-incubated SIP cultures. Due to the accumulation of the 16S rRNA gene copy numbers from a strain associated with this species (e.g. OTU15/Clone1.1) within the first 3 days of incubation (Figure 7.4A), it can be

postulated that this strain is a primary alkane degrader, enriched during the SIP incubation on *n*-hexadecane (HEX) substrate. Additionally, this strain's presence only in the surface waters of the FSC (not found in 700m HEX libraries) is consistent with the group's algal associations defined by Li *et al.* (2012).

4.4.3 Degradation of phenanthrene by surface and subsurface microbiota

Contrary to HEX incubations, which showed considerable accumulation of bacterial biomass (indicated by turbidity formation), PHE cultures exhibited no turbidity during incubation interpreted as very limited cell mass accumulation. Additionally, the rate of disappearance of PHE was observed to follow the same pattern in the Live and Dead tracking controls, which indicates that potentially, the pattern of delayed increase of PHE concentrations in solution (occurring after day 3) and its rapid subsequent disappearance (occurring after day 6; see Figure 4.2B) could be due merely to physico-chemical factors such as photooxidation, limited dissolution in polar solutions such as salt water (Grimaud, 2010) or a combination of both. This result suggested complete lack of microbial activity in the Live controls and hence the experimental cultures or a potential procedural error causing the insufficient bacterial extermination in the Dead Control cultures. However, during incubations the appearance of slight yellowish hue was observed within the initially completely clear cultures. This was indicative of 2-hydroxymuconate semialdehyde formation produced by meta-cleavage of aromatic compounds (Prabhu and Phale, 2003; Sala-Trepat and Evans, 1971)) and was considered a sign of potential biodegradation. Additionally, during harvesting of the cultures through centrifugation, small specks of granulated yellow material were pelleted from the experimental cultures, from which DNA was successfully extracted. This result was concurrent with bacteria's necessity for attachment to the crystalline PHE substrate in order to utilize it (Section 1.4.2 of Chapter 1). Although PHE dissolution problems and photooxidation may have been factors during this experiment, the ^{13}C-PHE SIP experiment managed to successfully recognize a bacterial strain capable of obligate PHE utilization at 20°C; *Cycloclasticus*. To affirm potential psychrophilic adaptations of the SIP-identified FSC *Cycloclasticus* strain, as well as the role adaptations and activity of each identified OTU throughout this SIP experiment, a metagenomic analysis of the 'heavy' DNA obtained in this study, might be highly beneficial.

CHAPTER 5: DISCUSSION

5.1 Project summary

This research project investigated the spatiotemporal dynamics of FSC bacterioplankton and its potential to respond to exposure to crude oil, especially under *in situ* FSC temperature conditions. The project revealed water core-specific microbial communities, despite the presence of 2-3 distinguishable water masses within each water core. This community distinguishability appeared primarily produced by the general water origin (North Atlantic *vs* Nordic seas origin) and its physicochemical characteristics (primarily salinity and temperature). Additionally, a seasonal variation in community diversity and composition were revealed in both water cores of the region. Bacterial communities were primarily dominated by the *Alpha* and *Gamma –proteobacteria*, especially the orders *Rhodobacterales*, SAR11, *Alteromonadales* and *Methylococcales*. Depending on season and potentially environmental conditions, blooms of different genera within these orders were found to dominate the communities, namely *Lentibacter* of the *Rhodobacterales* and *Alteromonas* or *Pseudoalteromonas* of the order *Alteromonadales*. The findings of this study were concurrent with the findings of the surrounding water regions, with the AWC communities being similar to those of the North Atlantic while the NWC communities being similar to those of the Norwegian Seas (Agogué *et al.*, 2011; Jensen *et al.*, 2012). The results also indicated a dominant role of the water origin and thermal regime in the region over its ecology. The microbial profiles of the region also revealed a noticeably high abundance of obligate methanotrophs (orders SAR324 and *Methylococcales*), despite the lack of known hydrothermal vents in the FSC. The methanotrophs were hypothesized as a result from the transference effect of the strong currents going through the FSC and the presence of cold seeps and whale fall site in nearby waterways.

In this project the biological potential of the water column FSC microbiota to respond to crude oil was also examined in laboratory-based microcosms under moderate and average FSC temperature conditions. Observations on the changes occurring in microcosm cultures (both aqueous and oil layers) suggested that lower temperatures hindered community response to crude oil. Based on these results it was concluded that an oil spill in the AWC of the FSC would likely be degraded more rapidly than if the same oil spill were to occur in the colder NWC. The microbial profiles of communities exposed to CRO were found to sharply

and significantly differ in community diversity and composition from the baseline profiles from any water mass or season. Microbial members with recognized enrichment during crude oil exposure at 4°C were affiliated primarily with the genera *Oleispira* (order *Oceanospirillales*) and *Pseudoalteromonas* (order *Rhodobacterales*), although after 6 weeks of exposure some microcosms also exhibited enrichment in the genus *Thalassolituus* (also order *Oceanospirillales*). The presence of these genera in the crude oil exposed communities was concurrent not only with their known hydrocarbonoclastic activity but also with their psychrophilic behaviour (Yakimov *et al.*, 2007; Chronopoulou *et al.*, 2015). Unsurprisingly, a significant enrichment of both *Oceanospirillales* (*Oleispira* and *Thalassolituus*), was also detected in the deep-sea *Deepwater Horizon* oil plume of 2010, where the temperature conditions (4.7°C) were similar to those applied in this study (4°C) (Hazen *et al.*, 2010). FSC strains successfully isolated on CRO, PHE or HEX comprised the first ever collection of cultivation-amendable FSC strains with biotechnological potential for degrading hydrocarbons at moderate and/or *in situ* FSC temperatures. Isolated strains primarily belonged to genera commonly known for their hydrocarbonoclastic behaviour such as *Alcanivorax*, *Marinobacter*, *Pseudoalteromonas*, *Cycloclasticus*, *Halomonas*, *Thalassolituus* and *Pseudomonas* (Yakimov *et al.*, 2007; Chronopoulou *et al.*, 2015)*.* Other isolated FSC strains, although suspected in hydrocarbonoclastic behaviour (*Glaciecola, Sulfitobacter* and *Phaeobacter*) would require further testing to confirm such capacities.

Next, the hydrocarbon biodegradation potential of the FSC surface (5 m; AWC) and subsurface (700 m; NWC) waters was examined in a DNA-based SIP experiments. Results suggested that the majority of FSC bacterial community members were capable of utilizing aliphatic compounds, however aromatic compounds such as phenanthrene, may be efficiently utilized only by *Cycloclasticus* members. Recognized HCB from the ^{13}C-HEX SIP experiments were, unsurprisingly *Alcanivorax borkumensis*, *Marinobacter algicola* and *Thalassolituus oleivorans*. Members of the genera *Cycloclasticus*, *Alcanivorax* and *Marinobacter* have also been detected in DNA-based SIP experiments performed on *Deepwater Horizon* oil plume communities (Gutierrez *et al.*, 2013). The most prominent finding in the surface water communities of the FSC (AWC) was the activity of an Alphaproteobacterial strain from the species *Lentibacter algarum*, which was proven enriched and capable of utilizing aliphatic hydrocarbons such as HEX. Its activity and detection only in the surface FSC waters was speculated as due to the genera's association

with microalgae and ability to degrade algal aliphatic compounds (see Section 4.4.2). Amongst the prominent alkane utilizers of the subsurface waters (NWC) was a potentially new species of the genus *Oleibacter* (only 96% identity to *Oleispira* sp.). Although also detected in the surface FSC waters, the *Oleibacter* species appeared most active in the deeper colder layers of the FSC, converging with its expected psychrophilic behaviour (Yakimov *et al.*, 2007). In the NWC, an *Alphaproteobacteria* strain from the species *Phaeobacter arcticus* was also found enriched along with a potentially new species of the genus *Glaciecola* (a *Gammaproteobacteria*). Interestingly, strains from the same genera (and a few others such as *Thalassolituus*, *Alcanivorax*, *Marinobacter* and *Cycloclasticus*) were also isolated during the crude oil exposure experiments (CRO-EE), which raised the question about how related those strains could be.

5.2 Phylogenic relationships between CRO-EE isolates and SIP-identified strains

In an attempt to reveal the phylogenic relationships of isolates obtained in the CRO-EE (Chapter 3) to hydrocarbon degraders identified in the SIP experiment (Chapter 4) and their association to known hydrocarbonoclastic bacterial type strains, a 16S rRNA V3-V4 gene fragment alignment-based comparison was performed and presented as phylogenic tree (Figure 5.1). The tree indicated that some the bacteria isolated during the CRO-EE were very closely related to the hydrocarbon degraders found during SIP experimentation. For example, Clone-4.30 identified as alkane degrader from the 700 m seawater (NWC) SIP incubated with ^{13}C-HEX at 20°C, showed to be closely related to the isolate CRO81 obtained after crude oil exposure of 400 m FSC seawater at 4°C (BLAST v2.7.0+ (Zhang *et al.*, 2000), DNA alignment % = 99%). Both strains were affiliated with the psychrophilic species *Phaebacter arcticus* (Zhang *et al.*, 2008), which has only been postulated as hydrocarbon degrader based on its genomic makeup (Buchan and González, 2010). The phylogenic similarity between the SIP-confirmed degrader Clone-4.30 and the postulated crude oil degrader CRO81 (discussed in Chapter 3, Section 3.4.2), can be used to affirm the hydrocarbonoclastic activity of isolate CRO81. Additionally, since both strains are closely affiliated with *P. arcticus*, it can be asserted that the taxonomic group *P. acticus* was observed to exhibit hydrocarbonoclastic behavior. Similarly, due to close phylogenic associations to known hydrocarbonoclastic type strains and SIP-confirmed HCB strains, the hydrocarbon degradation activity can be extrapolated for CRO-EE isolates HEX65 (affiliated

with *Thalassolituus oleivorans* with 99% identity to it and to SIP Clone-2.6), HEX19, HEX12 and CRO52 (affiliated with *Marinobacter algicola* and SIP Clone 3.15 at >99% identity), PHE111 and PHE91 (>99% to *Cycloclasticus* sp. and each other) (Figure 5.1). For other CRO-EE isolates such as PHE9 and PHE13 (affiliated >99% with *Glaciecola mesophila*) and HEX61 (affiliated >99% with *Marinobacter arcticus*) it was noted that their phylogenic affiliations were sufficiently different from reciprocal SIP-identified clones 3.32 (alignment = 94%) and 3.15 (alignment = 94%), to not be suited for similar functionality extrapolation. As discussed in Chapter 3 (Section 3.4.2), the presence of PHE9 and PHE13 isolates in the CRO-EE/20°C isolate libraries may have been due to agar-digestive abilities (Romanenko *et al.*, 2003) and further analysis into their metabolic capacity would be required. Due to the attainment of all isolated strains in pure cultures, further studies into their metabolic functionality, nutritional requirements and genomic capacities would now be possible.

Based on the phylogenic associations, it was also noted that the SIP-identified clones 4.30, 2.6 and 6.11 were closely related to their reciprocal CRO-EE/4°C isolates, suggesting these SIP clones are psychrophilic strains, despite SIP-experiment incubations at 20°C. Contrarily, clone 3.15 (affiliated with genus *Marinobacter algicola*) was more closely affiliated (>99%) to the isolates obtained under moderate temperature conditions during the CRO-EE/20°C (isolates HEX19 and HEX12) (Figure 5.1). This variation suggested that not all isolates enriched during the 20°C SIP-experiment incubation were psychrophilic and that temperature of incubation affected the microbial consortia formed in SIP-experiment incubations. As discussed in Chapter 4 (Section 4.4.1), the DNA-SIP protocol may need adjustments to obtain more accurate assessment of the active hydrocarbonoclastic strains in colder marine environments.
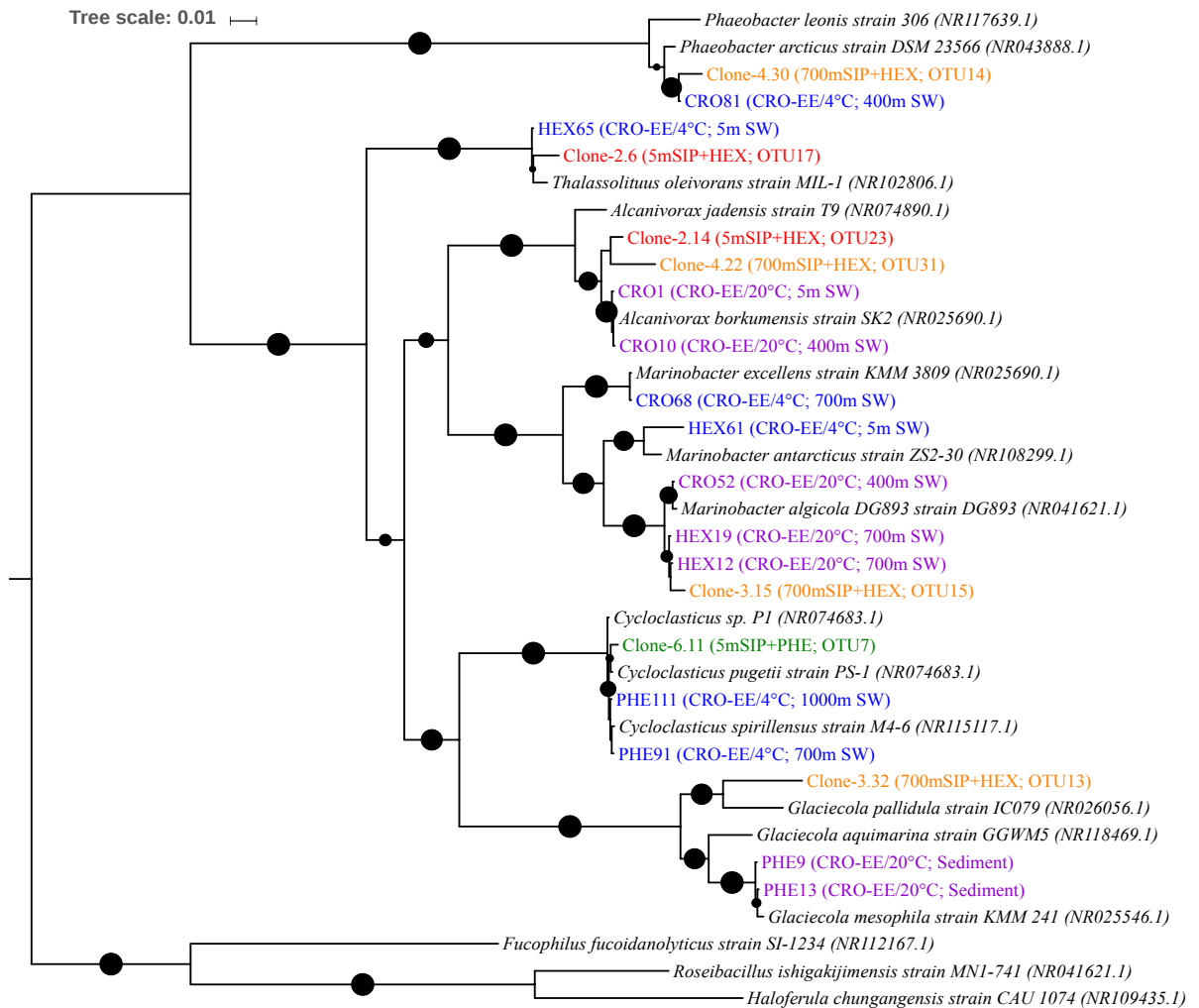
Figure 5.1. Phylogenic relationships between known type strains, FSC isolated strains and SIP-identified organisms in this project. The isolates and SIP clones are colour-coded based on experiment: Purple colour: isolates obtained from CRO-EE/20°C experiment (purple); blue: isolates obtained from CRO-EE/4°C; red: strains from surface seawater cultures (5 m; AWC) exposed to 13C-HEX; orange: strains from subsurface seawater cultures (700 m; NWC) exposed to 13C-HEX during SIP experiments; and green: strains from surface seawater cultures (5 m; AWC) exposed to 13C-PHE during the SIP experiments. Strains marked in black are known type strains obtained from NCBI GenBank. Tree was constructed by maximum-likelihood neighbour-joining method. Black circles indicate bootstrap confidence value of 70%-100% obtained over 1,000 repetitions. The tree was out-grouped and rooted with 16S rRNA gene sequences of the organisms from the order Verrucombicrobiales. Scale bar represents substitution rate (nucleotide substitutions per site).

5.2 Baseline abundance of hydrocarbonoclastic bacteria in FSC microbial communities

Based on the findings in this research project, the production of a FSC bacterioplanktonic community baseline, and the identification of various FSC strains that may or do have the capacity to degrade crude oil, a more comprehensive look into the hydrocarbon utilization potential of the FSC communities, can now be possible. Correlating the identified taxonomies during this study with their baseline abundance in the FSC, indicated that as much as 41% of the indigenous FSC microbiota holds the ability to degrade at least one hydrocarbon species (Table 5.1). The majority of this fraction however, (~17%) was primarily due to methanotrophs from the orders SAR324 and *Methylococcales* (discussed in Chapters 1 and 2). Aside from the methanotrophic bacteria of the FSC water column, the hydrocarbon active microbial fraction of the FSC water column was revealed to be ~24%. It is worth noting that the studies in this project explored only bacterial members involved in hydrocarbon utilization (based on the bacterial 16S rRNA gene), and that it is likely that the FSC microbiota contains hydrocarbonoclastic microorganisms from other phyla (e.g. algae, yeasts) which may also play a role during an oil spill (Leahy and Colwell, 1990; NRC, 2003; Head *et al.*, 2006). In accordance with previous research (Yakimov *et al.*, 2007; Head *et al.*, 2006), the results from this investigation showed that the natural abundance of most of the OHCB (individually) was below 1%, whereas the GHCB (capable of utilizing more than just hydrocarbons) maintained higher abundances within the total microbial abundance in the FSC water column. This bioprospecting analysis also showed that hydrocarbon-active microbial fraction of the unperturbed NWC microbiota was higher (~ 55%) than that of the AWC (~ 27%). However, as discussed in Chapter 1 (Section 1.4) as well as observed throughout this project (Chapters 3 and 4), the response of these microbial members to hydrocarbons would depend on physico-chemical factors such as temperature, oxygen, nutrients, hydrocarbon type, solubility (bioavailability) and molecular composition.

The hydrocarbon biodegradation potential of the FSC microbiota suggested an ample capacity to degrade hydrocarbons. However, based on the results of the study, it was determined that the response to hydrocarbons may be critically delayed and possibly proceed slower in the FSC than what could be expected in warmer regions. In terms of a hypothetical oil spill, these results signified that crude oil might persist in the sub-arctic environment such as the FSC, especially in the deep-sea layers. Other studies have signified that released oil within the region may also spread considerably to other regions and exhibit prolonged

toxicity (Main *et al.*, 2016; Hansen and Østerhus, 2000; Berx, 2012). Extremely high caution is therefore advised to all petroleum industries developing in the FSC. Baseline microbial community datasets, understanding of the microbial response in the FSC, as well as collections of FSC-specific hydrocarbonoclastic isolates, would be a valuable asset in case of a disaster in any Nordic marine region.

Table 5.1. Baseline abundance of hydrocarbon degraders within the AWC, NWC or the entire FSC water column, expected to be most active hydrocarbon utilizers in case of an oil spill in the region. Taxonomic groups were selected based on findings from the literature review, crude oil exposure experiments (CRO-EE) and DNA-based stable isotope probing experiments (DNA-SIP).

Order	Genus	Abundance within FSC (st.dev, n=40)	Abundance within AWC (st.dev, n=20)	Abundance within NWC (st.dev, n=20)
Class: *Alphaproteobacteria*				
Rhodobacterales	*Lentibacter*	13.8% (11.8)	12.7% (11.3)	15.0% (12.8)
	Phaeobacter	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
Class: *Gammaproteobacteria*				
Oceanospirillales	*Oleibacter*	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
	Thalassolituus	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
	Alcanivorax	0.2% (0.3)	0.1% (0.2)	0.2% (0.4)
	Oleiphilus	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
	Oleispira	0.2% (0.3)	0.1% (0.2)	0.2% (0.4)
	Halomonas	0.4% (1.3)	0.1% (0.1)	0.7% (1.8)
Methylococcales	All	8.1% (4.6)	7.6% (5.9)	8.7% (3.1)
Alteromonadales	*Marinobacter*	0.0% (0.0)	0.0% (0.0)	0.1% (0.1)
	Glaciecola	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
	Alteromonas	1.9% (7.0)	0.1% (0.2)	3.6% (9.8)
	Colwellia	0.4% (0.9)	0.1% (0.1)	0.7% (1.2)
	Pseudoalteromonas	6.0% (9.8)	1.4% (1.3)	10.8% (12.3)
	Psychromonas	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
	Shewanella	0.0% (0.1)	0.1% (0.2)	0.0% (0.0)
Pseudomonadales	*Pseudomonas*	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
	Moraxellaceae sp.	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
Thiotrichales	*Cycloclasticus*	0.5% (1.4)	0.6% (1.8)	0.3% (0.9)
	Methylophaga	0.0% (0.0)	0.0% (0.0)	0.0% (0.0)
Vibrionales	*Vibrio*	0.2% (0.3)	0.2% (0.4)	0.1% (0.2)
Class: *Deltaproteobacteria*				
SAR324	All	9.3% (6.5)	4.2% (2.0)	14.5% (5.3)
Total fraction of HCB with methanotrophs:		~41%	~27%	~55%
Total fraction of HCB without methanotrophs:		~24%	~15%	~32%

5.3 Future research

Despite the plethora of data produced in this study, some of its limitations could be addressed in future work in order to improve estimations in its hydrocarbon degradation potential. The study included a 2-year monitoring program of the FSC, which was the first study to explore the indigenous microbial communities and their vertical distribution throughout the water column. A prolonged systematic microbial monitoring program, however, would likely improve understanding into the seasonal and annual community variations of both water cores, improve their distinguishability and elucidate the observed seemingly irregular blooms of *Methylococcales*, *Alteromonadales* or *Flavobacteriales* species. Producing a better understanding of the spatiotemporal dynamics of the FSC microbiota, their controlling factors and habitat changes with industrial development in the region, may be crucial in case of an oil spill in this intense hydrodynamic region. Next, the microbial community response to crude oil can be further explored, not only in terms of temperature, but also in terms of effects by nutrient, oxygen and surfactants. The collection of FSC-specific isolates may hold a tremendous biotechnological potential, not only important for the FSC, but the entire sub-arctic marine region. The dietary and environmental preferences of each isolate could be assessed through behavioural or genomic explorations. Finally any obtained ^{13}C DNA from this study can provide pivotal information about the metabolic tools of different hydrocarbon degrading bacteria.

The development of novel molecular approaches in recent years has greatly improved the understanding of the complexity of hydrocarbon biodegradation, involved communities and their dynamics. Despite these significant contributions, the 16S rRNA gene-based surveys are known to have one major limitation: they miss the diversification of metabolic abilities within deeper-level taxonomic groups (Chadhain and Zylstra, 2010; Berry *et al.*, 2017; Hedlund and Staley, 2006). Therefore a primary enhancement of this 16S rRNA gene-based research project would be the addition of genome and metagenome-based functional assessments (e.g. genomic reconstruction and bioprospecting, metagenomic metabolic diversity investigation, *etc.*) in order to increase resolution into the ecological role of these FSC microbial communities and isolated strains.

5.4 Conclusion

This research project resulted in a few invaluable resources of information. Firstly the project elucidated the diversity and composition of FSC baseline microbial communities. Revealed was a water core-based specificity within the communities, most likely produced by the contrast in temperatures and origins of the inflow waters. An internal seasonal variation was also observed in both water cores. Next, this project revealed some of the potential of the baseline FSC microbiota to respond to crude oil by identifying multiple indigenous genera capable of responding to crude oil, even under the prevailing temperature conditions. Lastly, this study produced in a collection of FSC-specific hydrocarbon degraders with invaluable biotechnological potential. This research project was the first microbiological assessment of the FSC and its capacity to respond to and degrade hydrocarbons.

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