UNIVERSITY OF TARTU

Faculty of Science and Technology

Institute of Technology

Farheen Fathima

The Response of Microbial Community to Dispersed Oil Contamination in Arctic Sea-ice

Master's Thesis (30 ECTS)

Curriculum Bioengineering

Supervisor:

Hiie Nõlvak, PhD

Tartu 2022

The Response of Microbial Community to Dispersed Oil Contamination in Arctic Sea-ice

Abstract

Oil spills can be very harmful to marine biota. The risk of oil spillages is increasing especially in the Arctics where the presence of ice in seawater further complicates spill-response actions. Bioremediation relying on microbial biodegradation is the only effective remediation option when spilled oil gets encapsulated into sea-ice. A three-month mesocosm experiment was conducted to determine the response of the microbial community to dispersed oil contamination in sea-ice. The results indicated that the dispersed oil contamination in ice caused the microbial community to shift towards dominance of *Gammaproteobacteria*. Bloom and succession tendency was noted on genus level: early community dominants like *Oleispira* were later replaced by other genera, like *Pseudoalteromonas*. The quantitative assays were developed for *Oleispira*- and *Pseudoalteromonas*-specific 16S rRNA gene abundance determination, which confirmed the tendencies of their dynamics in time. However, despite the structural shift of the microbial community towards known oil-degraders, the general abundance of the prokaryotic community in oil contaminated sea-ice decreased substantially throughout the experiment. This leaves the efficiency of oil bioremediation in Arctic sea-ice in doubt.

Keywords: Crude Oil, Dispersants, Biodegradation, Arctic seawater, Sea-ice

CERCS: T270 - Environmental technology, pollution control.

Mikroobikoosluse reaktsioon dispergeeritud naftale arktilises merejääs

Kokkuvõte

Naftareostus on mere ja ookeanide elustikule ohtlik. Naftareostuse oht on viimastel aastatel eriti kasvanud Arktikas, kus jää olemasolu vees muudab enamiku reostuse likvideerimise meetoditest raskesti kasutatavateks. Kui merevette sattunud nafta jääb jää tekkel sellesse lõksu, peetakse ainsaks puhastusvõimaluseks nafta lagundamist jääs sealsete mikroobide poolt. Käesolev töö põhineb kolmekuisel mesokosmi-katsel ja selle töö eesmärgiks oli uurida mikroobikoosluse muutusi jääs vastusena jäässe talletunud dispergeeritud naftale. Töö tulemused näitasid, et naftareostus põhjustas mikroobikoosluse struktuuri muutust: jää koosluses muutusid domineerivaks klassi *Gammaproteobacteria* esindajad. Perekonna tasemel täheldati ka koosluse suktsessiooni ajas: mõned perekonnad nagu näiteks *Oleispira* reageerisid naftareostusele kiire arvukuse tõusuga, katse hilisemas faasis nende arvukus langes ja koosluses muutusid domineerivaks muud bakteriperekonnad, eriti *Pseudoalteromonas*. *Oleispira* ja *Pseudoalteromonase* perekondade spetsiifiliste 16S rRNA geenide arvukuse määramiseks töötati välja kvantifitseerimismeetodid, mille rakendamine kinnitas nende perekondade ajalisi dünaamikaid. Vaatamata mikroobikoosluse struktuuri nihkele naftalagundajate domineerimise suunas, langes kogu koosluse arvukus reostunud jääs kolme kuu jooksul märgatavalt. See tendents seab nafta biodegradatsiooni ulatuse reostunud jääs küsitavaks.

Märksõnad: Toornafta, dispersant, biodegradatsioon, arktiline merevesi, merejää

CERCS: T270 - Keskkonnatehnoloogia, reostuskontroll

TABLE OF CONTENTS

TERMS, ABBREVIATIONS AND NOTATIONS

16Stot	total prokaryotic 16S rRNA gene abundance
API	American Petroleum Institute degrees
A16S	archaeal 16S rRNA genes
B16S	bacterial 16S rRNA genes
BTEX	benzene, toluene, ethylbenzene, xylene
BW	water from beneath the ice
C/N/P	carbon, nitrogen, phosphorus
CE	chemical dispersant
Ct	number of amplification cycles needed to reach fluorescence threshold
E	amplification efficiency
$N_{0,A}$	the concentration of target gene in the sample in arbitrary fluorescence units
$N_{0,B}$	the concentration of target gene in the standard dilution in arbitrary fluorescence units
Nt	the fluorescence threshold
PAHs	polyaromatic hydrocarbons
qPCR	quantitative polymerase chain reaction
ROV	remotely operated vehicle
rrnDB	the ribosomal RNA operon copy number database
SW	Arctic seawater used for mesocosm setup
US EPA	United States Environmental Protection Agency
US NOAA	United States National Oceanic and Atmospheric Administration
WAF	water accommodated fraction of crude oil
WAF+CE	experiment variant where chemically dispersed crude oil is encapsulated into forming ice

INTRODUCTION

Contamination of the environment with crude oil or its derivative products is a long-standing problem (Muthukamalam et al., 2017). An oil spill is the accidental or intentional discharge of petroleum hydrocarbons into the environment, especially frequently to the marine ecosystem (Doshi et al., 2018). Oil hydrocarbons release comes from activities like drilling, manufacturing, storing and transporting of crude oil and its products (Hassanshahian et al., 2020). Major oil spills can be caused either by machinery malfunctions and accidents, or by natural catastrophes like hurricanes and storms. Most often the spilled crude oil stays afloat on the surface of seawater and spreads across to form a layer called oil slick. But depending on the spilled oil characteristics, it can also form water-oil emulsions or get submerged inside the water column (Ossai et al., 2020; NOAA, 2022). Depending on the circumstances, oil spills can be very harmful to marine biota, including birds, sea turtles and mammals, as well as major trading category fish and shellfish. Oil destroys the insulating ability of fur-bearing mammals, such as sea otters, and the water-repelling abilities of a bird's feathers, exposing them to the harsh elements. Many birds and animals also swallow oil and are poisoned when they try to clean themselves or when eating oiled prey (NOAA, 2022).

In recent years, the risk of oil spillages is increasing especially in the Arctic marine environment. The warming climate conditions resulting in reduced ice coverage have enabled intensified oil exploration, production and transport activities and consequently also heightened probability of accidental oil spillages in this region (Boylan, 2021; Lofthus et al., 2021). However, the Arctic conditions still remain harsh which makes oil weathering processes slow and spill cleanup and oil recovery difficult (Wilkinson et al., 2017). The presence of ice in seawater further complicates spill-response actions as most of the physicochemical methods are seriously hindered in icy conditions (Bullock et al., 2019). Consequently, biological cleanup methods relying on the ability of microbes to degrade petroleum hydrocarbons have become increasingly relevant in such conditions (Mapelli et al., 2017). However, the knowledge of microbial community response to Arctic oil spills, its abundance dynamics, key players and petroleum hydrocarbons degradation potential is still rather limited.

1 LITERATURE REVIEW

1.1 Oil pollution in cold marine environments

1.1.1 Crude oil composition and classification

Petroleum hydrocarbons exist in nature in the form of gases (natural gas), semisolids (wax or asphaltite) and liquids (crude oil) (Ossai et al., 2020). Crude oil mainly consists of carbon (83-87%), hydrogen (10-14%), sulphur (0.04-8%), oxygen (0.1-5%), nitrogen (0.1-2%) and several metals (Sama et al., 2018). It is a complex mixture of different hydrocarbons, such as straight and branched chain alkanes (n-alkanes), cycloalkanes, monoaromatic hydrocarbons like benzene, toluene, ethylbenzene, xylene (BTEX), polycyclic aromatic hydrocarbons (PAHs), as well as compounds such as waxes, tars and nitrogen-sulphur-containing heterocompounds (Ossai et al., 2020).
Crude oils of different origins vary to a great extent in their composition and viscosity. According to American Petroleum Institute degrees (API), crude oils are broadly classified as:

1) Light crude oils - low viscosity and density, API >31.1 degrees
2) Medium crude oils - medium viscosity and density, API >22.3 and <31.1 degrees
3) Heavy crude oils - high viscosity and density, API of <22.3 degrees
4) Extra heavy crude oils - API <10.0 degrees.

In general light crude oils are more viscous than water but still easily extracted and poured and can flow through porous rocks. The heavy crude oils are more viscous in nature and flow less easily through pores in rocks which results in difficulties in extraction.
United States Environmental Protection Agency (US EPA) offers a slightly different classification of crude oils:

Class A Light volatile oils (light crudes): These oils are highly fluid and clear, flammable, with a strong odour and high evaporation rate, they spread vigorously on solid and water surfaces and do not tend to adhere to them. These oils are highly toxic to humans, animals and other organisms.
Class B Non-sticky oils (medium and heavy paraffin oils): These are waxy oils which attach to the surfaces more firmly than the *class A* oils. The evaporation of volatiles from these oils leads to *class C* and *D* oils.

Class C Heavy, sticky oils (residual fuel oils and medium to heavy crudes): These oils are brown or black in colour, sticky and viscous. Their density is similar to water in which they often sink. The weathering or evaporation of volatiles from these oils leads to *class D* oils. The toxicity of these oils is low but endangers wildlife through smothering.
Class D Nonfluid oils (residual oils, heavy crude oils, weathered oils): These brown and black oils are relatively nontoxic but at higher temperatures can melt and attach to the surfaces as coats which makes cleanup difficult (US EPA, 2022).
For spilled oils these classifications are dynamic as the oil behavioural changes depend on the environmental conditions, especially temperature.

1.1.2 Oil contamination in cold marine environment

Crude oil has been part of the marine environment for millions of years due to natural seeps which contribute nearly 600,000 tonnes of oil to marine environment annually (Hazen et al., 2016). However, the next largest amount (in some estimations around 10 %, (Mapelli et al., 2017)) of oil hydrocarbons release comes from human activities like drilling, manufacturing, storing and transporting of crude oil and its products (Hassanshahian et al., 2020). The risk of oil spillages is increasing especially in the Arctic marine environment. The undiscovered oil deposits are estimated to be up to 90 billion barrels in the Arctic region (Bird et al., 2008; Lofthus et al., 2021) which becomes more accessible due to warming climate conditions and has elevated the interest of exploration and production of oil in this region. The reduced ice coverage also has caused prolonged and more frequent transport activities along the Arctic sea-routes (Boylan, 2021). The increased oil and gas exploitation and ship traffic activities in the Arctic region have majorly increased the probability of accidental release of oil which cleanup and recovery are difficult in the harsh Arctic conditions.
Arctic oil spills can happen in partly ice-covered areas, and the spilled oil from open waters can also drift with winds and predominant streams into the ice covered areas (Wilkinson et al., 2017). There the oil can get trapped either beneath the ice or upon ice formation encapsulated within it (Vergeynst et al., 2019). Physical weathering of oil spills in ice-covered environments is very low due to reduced evaporation. Also, oil which gets encapsulated in ice can travel with it and get released far from the original spill site when the ice melts (Lofthus et al., 2021). In the ice, oil is exposed to a hypersaline environment in brine channels which lowers the solubility of many oil hydrocarbons like alkanes and aromatic compounds (Vergeynst et al., 2018). Most oil spill response actions become impossible after the oil has frozen into sea ice, leaving bioremediation relying on microbial ability within ice to degrade oil compounds as a main remediation option.

1.2 Methods for combating oil pollution in marine environment

Current emergency response techniques to oil spills in marine environments include mechanical containment and recovery of spilled oil, usage of chemical dispersants and physical cleanup of shorelines (Mapelli et al., 2017). The remaining petroleum hydrocarbons in the marine environment after usage of these physicochemical cleanup methods are expected to be mineralized by microorganisms. The choice of oil spill response technique derives from the amount and type of spilled oil, spill location along with its environmental conditions, as well as availability and cost of response technique equipment (Bullock et al., 2019).

1.2.1 Physicochemical methods

The first emergency response actions to marine oil spills mostly deploy various physical and chemical cleanup methods (Fig. 1).

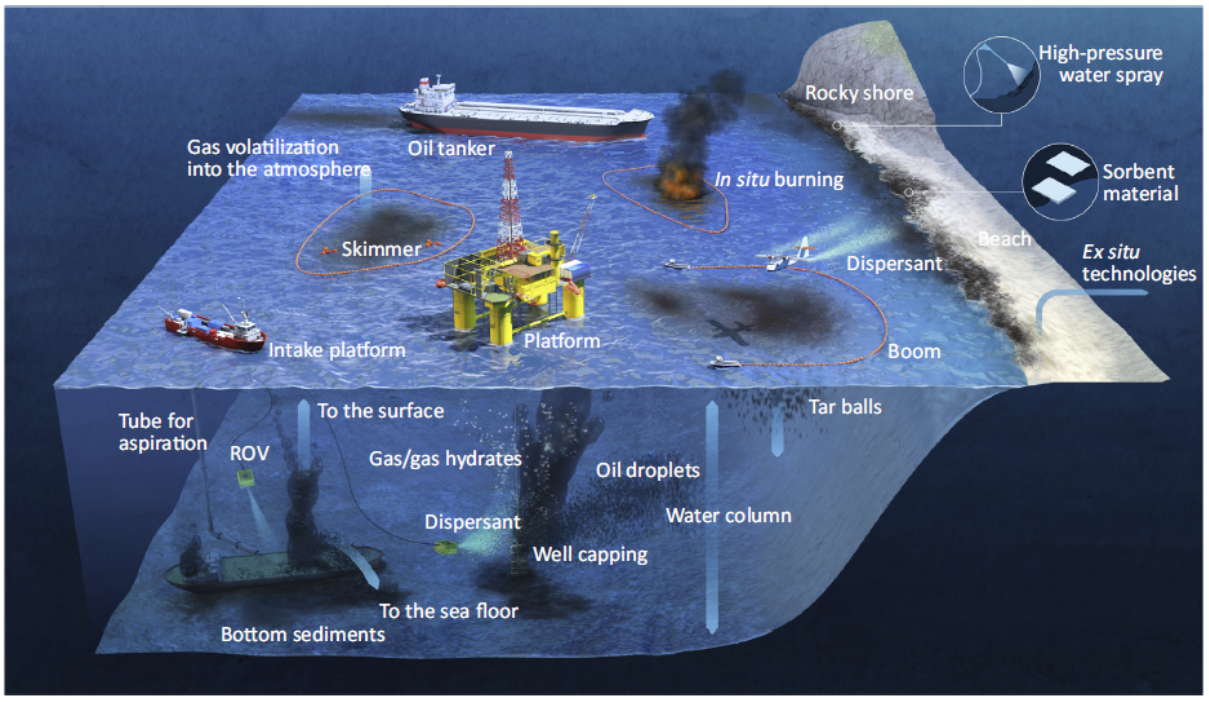


Fig. 1. Physicochemical methods for emergency responses to oil spills in marine environments. ROV - remotely operated vehicle. Figure reproduced from Mapelli et al., 2017.

Spilled oil is mechanically contained and stopped from spreading using different types of booms (fences, curtains, inflatable booms). Thicker oil layers can be recovered by harnessing various types of skimmers (weir, oleophilic, suction skimmers) while smaller spills or residual oil after skimmer usage can be combated by using natural or synthetic sorbents that adsorb oil

internally or to their surface (Mapelli et al., 2017; Ossai et al., 2020). These techniques are generally rather energy- and labour-intensive and therefore quite costly. In some cases *in situ* burning - a technique in which the oil spills are burnt at the location of the spill, are also used. This technique reduces up to 95% of the oil amount on water and lowers the environmental effects of oil spill if done in controlled matter in the environment (US NOAA, 2022). However, the downside of this technique is a release of secondary pollution from burning in the form of carbon dioxide and solid particles to the environment (Doshi et al., 2018).

Chemical oil spill response actions mainly consist of the usage of dispersants, which are amphiphilic compounds or chemical mixtures which break the oil into smaller droplets with the help of the waves and winds (Mapelli et al., 2017). Smaller oil droplets have a higher surface-to-volume ratio compared to oil slicks which allows more of the oil surface to be exposed to microorganisms, which have the potential to naturally degrade hydrocarbons (Prince et al., 2013). Dispersant usage also enables transferring the oil from the sea surface to the water column which helps to avoid drifting and contaminating the shoreline (Wilkinson et al., 2017). The main constituents of chemical dispersants are surface active agents called surfactants which have the ability to break down the oil as they have oil soluble hydrocarbons and water soluble groups; they reduce surface and interfacial tension within an oil slick (Ossai et al., 2020). The use of chemical dispersants in oil spill response actions relies on the trade-off between making finer droplets more accessible and increasing the toxicity of the contaminants to marine life (Mapelli et al., 2017). Hence, the goal of technological development is replacing toxic chemical surfactants with nontoxic biosurfactants.

Oil spills in Arctic regions in general are persistent and poorly self-dispersing due to low temperatures as well as low light intensity (Carpenter et al., 2018). Additional challenges for spill response actions in Arctics are set by poorly accessible locations and extreme weather, including ice conditions, which can complicate or often negate the use of mechanical techniques such as booms, skimmers, and pumping systems (Wilkinson et al., 2017) or reduce the effectiveness of dispersants (Lewis and Daling, 2007).

1.2.2 Biological methods

Biological oil spill response techniques or in other words bioremediation techniques are based on the removal of contaminants with the help of microorganisms like bacteria, fungi, archaea and algae that use pollutants as a source for carbon and energy by breaking them into smaller and simpler molecules (mostly fatty acids and carbon dioxide) through the metabolism (Mapelli et al., 2017; Agarwal, 2021). Bioremediation can be performed by indigenous

microbial communities whose progress can be either simply monitored (monitored natural attenuation) or enhanced (biostimulation), or by allochthonous microorganisms added to the polluted system (bioaugmentation) (Fig. 2; Lawniczak et al., 2020). In general, bioremediation is a long procedure which takes from months to even years, and is often used as a secondary cleanup strategy for residual oil pollution after quicker solutions like booms and skimmers or sorbents have been used (Agarwal, 2021).

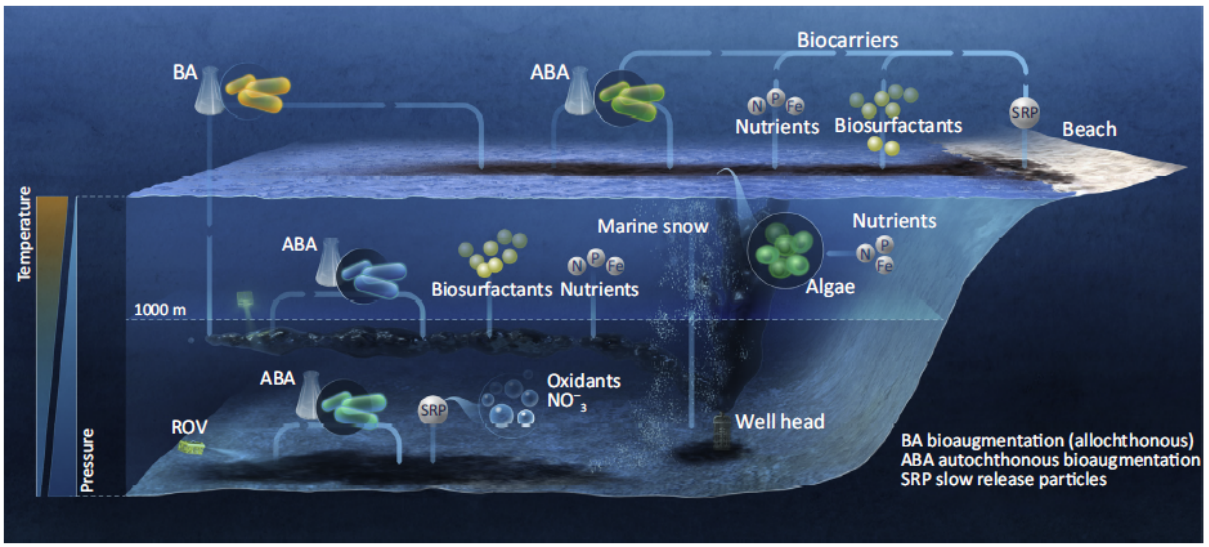


Fig. 2. Biotechnological oil-hydrocarbons remediation approaches in polluted marine environments. ROV - remotely operated vehicle. Figure reproduced from Mapelli et al., 2017.

(Monitored) Natural attenuation of spilled oil, the simplest of bioremediation techniques, relies on the indigenous microbial community ability at the spill site to degrade oil hydrocarbons (Mapelli et al., 2017). It reduces the toxicity and/or mobility of the contaminant without human interference. Three evidences are sought via monitoring to indicate the efficiency of this process: decreased contamination at the spill location, proven ability of the microorganisms from the spill location to degrade the contaminants, and proof that the biodegradation potential has been realised (Smets and Pritchard, 2003; Rügner et al., 2006). Monitored natural attenuation is used for limited spills at low risk locations where usage of higher-cost methods are deemed unfeasible.

The natural microbial oil degradation processes in marine environments are often inhibited by low availability of nutrients and electron acceptors or donors as well as low solubility of pollutants at the contaminated location. These limitations are addressed using biostimulation technique which modifies the environmental conditions to favour the metabolism of microorganisms capable of bioremediation (Mapelli et al., 2017). The addition of nutrients, electron donors/acceptors or (bio)surfactants to the contaminated area helps to increase the

microbial abundance and activity and increase oil hydrocarbons degradation (Fig. 2). Biostimulation has been shown to be among the most effective biological oil spill response methods in marine environments, since it improves the carbon/nitrogen/phosphorous (C/N/P) ratio that is unbalanced after oil spills (Mapelli et al., 2017, Lawniczak et al., 2020). Biostimulation via nutrient additions in marine oil spill areas have been complicated as the additives can be washed away by the wave action or settle from the water column before the microbes are able to utilise them (Tyagi et al., 2011). Nutrient microencapsulation within slow-release particles like alginate beads have been proposed as a solution for this particular biostimulation limitation (Shan et al., 2016). Low bioavailability of oil hydrocarbons is combated with additions of biosurfactants. These surface-active macromolecules act analogously to chemical dispersants, but are more convenient than the chemical surfactants as they are more environment friendly, less toxic, have better biodegradability, and prolonged activity under extreme temperature, salinity and pH values (Kapadia Sanket and Yagnik, 2013; Silva et al., 2014). However, their routine production for large scale usage is still an issue in this field.

Another option for oil spill biological treatment is bioaugmentation, a practice of adding cultured active microorganisms with specialised metabolic capacities to enhance oil hydrocarbons degradation to the spill site (Mapelli et al., 2017). The more traditional approach of bioaugmentation with site-allochthonous (foreign) microbes has been diminishing as the bioremediation through this process is temporary as the inserted microorganisms population often fails to survive and proliferate at the contamination site (Lawniczak et al., 2020). Instead, site-autochthonous microbes (indigenous to the spill site), isolated from the spill site, grown in laboratory in high quantity and then reintroduced into the spill site, have shown good survival and good effect on oil hydrocarbons removal, especially in combination with biostimulation (Nikolopoulou et al., 2013).

1.3 Potential of microbes to biodegrade oil constituents

Crude oil has been part of the marine environment for millions of years due to natural seepages enabling microbes to adapt to use its rich source of energy and carbon (Hazen et al., 2016). Microbes are able to degrade oil hydrocarbons in both aerobic and anaerobic conditions in marine habitats (Head et al., 2014, Prince et al., 2007), but most are degraded in aerobic marine waters with a half-life of days to months (Hazen et al., 2016). Oil hydrocarbons degrading ability is a widespread trait among (micro)organisms as it has been recorded from all three domains of life (Fig. 3). The vast majority of oil degraders belong to various bacterial taxa. More than 350 prokaryotic genera (mostly bacterial but also several

archaeal) are known to be capable of degrading oil hydrocarbons (Nõlvak et al., 2021), including a variety of marine psychrophilic and psychrotolerant bacterial genera performing these actions in Arctic environments.

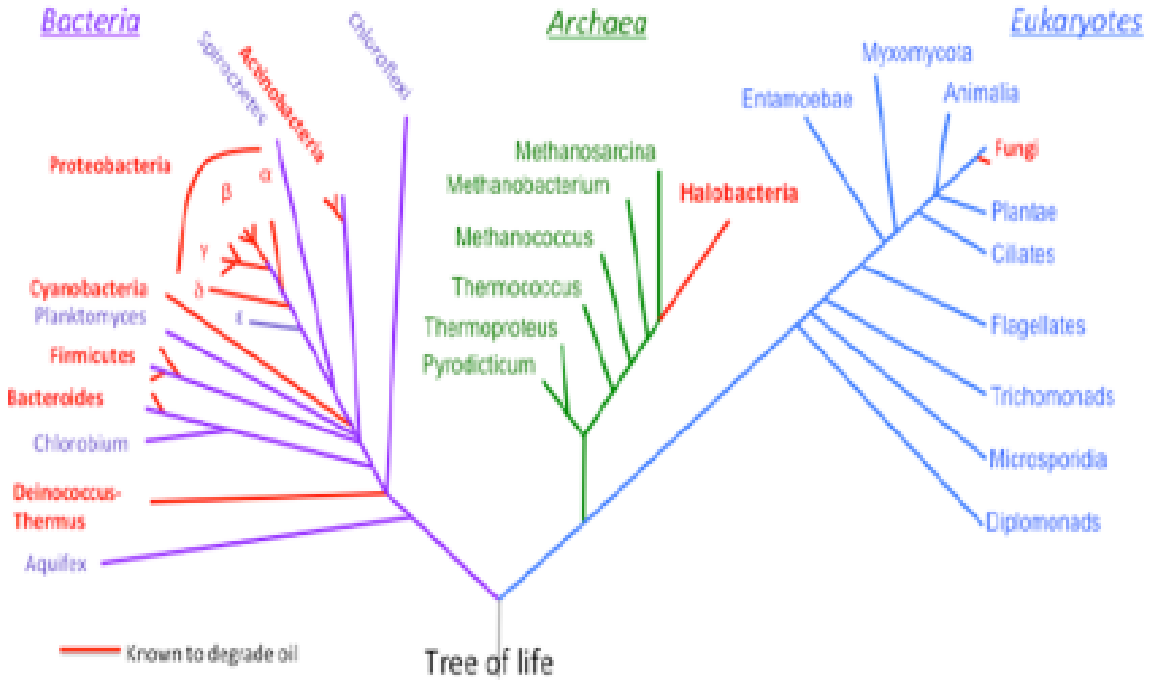


Fig. 3. Spread of taxa involving organisms capable of oil hydrocarbons degradation (marked in red) within the tree of life . Figure reproduced from Hazen et al., 2016.

The majority of oil hydrocarbons degrading bacteria in both the temperate and arctic seawater, as well as in marine ice belong to phylum *Proteobacteria* (or phylum *Pseudomonadota* according to recently proposed classification (Oren and Garrity, 2021)), more specifically to classes *Alphaproteobacteria* and *Gammaproteobacteria* (Bowman and McCuaig, 2003; Yakimov et al., 2004; Deppe et al., 2005; Gerdes et al., 2005; Brakstad et al., 2008; Bagi et al., 2014; McFarlin et al., 2014; Garneau et al., 2016). In arctic seawaters influenced by oil spills, genera belonging to class *Gammaproteobacteria* tend to become dominant (Brakstad et al., 2018; Nõlvak et al., 2021). *Gammaproteobacterial* genera *Colwellia*, *Cycloclasticus*, *Marinobacterium*, *Marinomonas*, *Glaciecola*, *Oleispira*, and *Pseudoalteromonas* are the genera involving oil degraders which have been associated with cold marine habitats the most (Yakimov et al., 2004; Deppe et al., 2005; Brakstad et al., 2008, 2015, Yergeau et al., 2017, Lofthus et al., 2021, Nõlvak et al., 2021).

The complex composition of crude oil is matched by a complex community that degrades it, and this community changes as biodegradation proceeds. In a process of bloom and

succession the preferred substrates are consumed, the microbes specialised to consume those compounds die off and are replaced by microbes able to utilise the remaining compounds (Hazen et al., 2016). Aliphatic hydrocarbons (like alkanes), an attractive source of energy and easiest to break down (Lawniczak et al., 2020), are consumed first, in Arctic seawater often by *Oleispira* and *Colwellia* (Yakimov et al., 2003; Brakstad et al., 2015; Krolicka et al., 2019). Upon depletion of aliphatic hydrocarbons in cold seawater the community gets first overtaken by organisms degrading simpler aromatic compounds like (among others) some *Neopseudomonas* species (Nõlvak et al., 2021) along with some *Colwellia* species (Vergeynst et al., 2019). These are succeeded by organisms capable of PAHs degradation, with genera like *Cycloclasticus*, *Marinobacter* and *Marinomonas* becoming dominant in oil-contaminated arctic seawater at this stage (Brakstad et al., 2015, Nõlvak et al., 2021).

Sea ice is an even more challenging environment for microbes than Arctic seawater: they are exposed to sub-zero temperatures and high salinity in the liquid brine channels formed when the ice grows (Junge et al., 2017). When spilled oil gets entrapped into sea-ice, only a small fraction of the surface area of the oil is in direct contact with brine (oil-brine interface) and its biodegradation is thought to mostly depend on the biofilm mediated process (Brakstad and Bonaunet, 2006). So far it has been shown that the changes in the ice microbial community are related to oil spills: the proportions of *Alphaproteobacteria* (*Pelagibacter, Octadecabacter*), *Gammaproteobacteria* (*Glaciecola*), *Bacteroidetes* and *Actinobacteria* that were seen in clean ice showed a shift towards almost exclusively *Gammaproteobacteria* (genera *Colwellia*, *Marinobacterium*, *Marinomonas*, *Glaciecola*, and *Pseudoalteromonas*) in oil contaminated samples (Brakstad et al., 2008). In addition, increased proportions of genera like *Oleispira*, *Colwellia*, *Peredibacter*, *Bacteriovorax* and *Arcobacter* have been shown after exposure to oil in sea ice (Vergeynst et al., 2019). It seems that bacteria in sea-ice possess the ability to degrade oil hydrocarbons likewise to bacteria present in seawater, even if they have big variations in the microbial community composition and structure between them (Boccadoro et al., 2018).

2 THE AIMS OF THE THESIS

The general aim of this research was to get insights about the dynamics of microbial community abundance and structure in response to dispersed crude oil contamination in sea-ice. The thesis is based on a three-month-long experiment that was carried out at controlled laboratory conditions on mesocosm scale.

The specific aims of the thesis were:

- to characterise the dynamics of microbial community structure in dispersed oil containing sea-ice during a 89-day study period;
- to determine the abundance dynamics of prokaryotic community within dispersed oil containing sea-ice based on bacterial and archaeal 16S rRNA gene abundances;
- to develop and optimise qPCR assays for determining the abundance of community dominants *Oleispira* and *Pseudoalteromonas* based on their 16S rRNA genes;
- to determine the abundance dynamics of microbial community dominants *Colwellia*, *Oleispira*, and *Pseudoalteromonas* in dispersed oil containing sea-ice during a 89-day study period.

The data collected in this thesis serves as a first step of estimating the microbial oil degradation potential in Arctic sea ice within a larger project.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

All procedures described in sections 3.1-3.3 were performed in SINTEF's Cold Climate Laboratory in Narvik, Norway and analysis described in sections 2.4-2.6 in Laboratory of Environmental Microbiology and Biotechnology in University of Tartu. The author of the thesis is responsible for data analysis of the experiment described in section 2.5 and conducting all laboratory work and analysis of experiments described in section 2.6 and its subsections.

3.1.1 Experiment setup

A 89-days long ice formation mesocosm experiment was conducted in SINTEF's Cold Climate Laboratory (Narvik, Norway) to estimate the microbial community structure and potential for oil hydrocarbons degradation in sea ice and the microbial community response to dispersed crude oil encapsulation into sea ice. The experiment incorporated the following treatment variants (Fig. 4):

- Chemically dispersed crude oil encapsulated in forming sea-ice (WAF+CE; 2 replicates);
- Dispersant control (dispersant encapsulated in forming sea-ice);
- Clean ice control (2 replicates);
- Sterilised control with chemically dispersed crude oil (2 replicates).

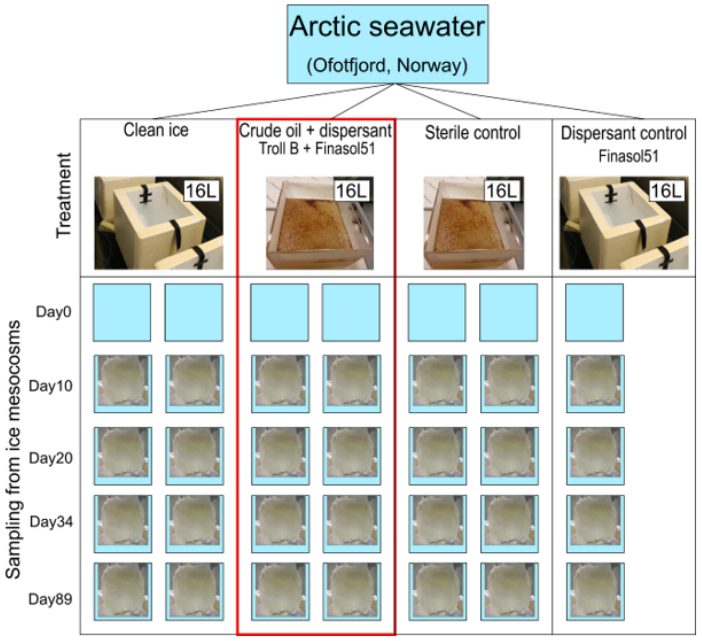


Fig. 4. The scheme of the conducted ice-mesocosm experiment. The experiment variant analysed in this thesis is marked in red.

3.1.1.1 Materials

The experiment was carried out using Arctic seawater (SW), water accommodated fraction of crude oil (WAF) and chemical dispersant (CE).

The seawater (~200 L) used to set up the mesocosms was collected as a grab sample with sterile plastic containers from Ofotfjord, from the beach of Kvitvika, Narvik (68.44208° N 17.38917° E) on 11th October 2017. The salinity of SW was ~32.6‰ and temperature ~7 °C. SW was maintained at 5 °C while it was transported to the laboratory (~1 h) where it was immediately used for the preparation of water accommodated fraction with crude oil (WAF) and dispersant.

The Troll B type crude oil (North Sea naphthenic type) provided by Statoil was used. The Troll B oil had the following characteristics: viscosity of 6 cSt at 20 °C, density of 0.845 g/cm^3, and pour point of -15 °C.

The dispersant Finasol® OSR 51 (TotalEnergies, Courbevoie, France) was used for preparation of the dispersed oil.

The water accommodated fraction with crude oil and dispersant was prepared as follows. The dispersant Finasol® OSR 51 and crude oil were well mixed in a mass ratio of 1:10 (dispersant:oil). This mixture at a concentration of 5 g/L was then added to SW. The mixture of SW with crude oil and dispersant was then vortexed at 400 rpm at 4 °C for 40 hours. Post vortex, the mixture was allowed to separate at the same temperature for 2 hours in a bottom-tap glass bottle. Subsequently, 1.2 kg of the mixture aqueous phase was collected from the tap and used with 14.8 kg of clean SW to set up the ice formation mesocosm (WAF+CE). In case of preparation of water accommodated fraction with crude oil and dispersant for sterilised control mesocosms, SW was filtered using 0.2 µm Sterivex filter SVGPL10RC (Merck Millipore, Darmstadt, Germany) so that the microorganisms were removed before applying the described protocol for the preparation of the mixture.

3.1.1.2 Ice-mesocosms setup

Sterilised 18 L plexiglass tanks, insulated from perimeter and bottom with 5.5 cm of styrofoam were used for mesocosm setup. The heating elements were placed at the tank bottom and walls to ensure that the ice would grow only from the tank surface (free-floating). Each tank was filled either with

1) 16 kg of clean seawater,
2) 14.8 kg of SW and 1.2 kg of dispersed oil mixture (WAF+CE),
3) 16 kg of SW and dispersant (CE) as a control,
4) 14.8 kg of filtered SW and 1.2 kg of sterile dispersed oil mixture.

After the mesocosms setup, the lab temperature was set at -1 °C overnight and then changed to -15 °C for the ice to grow. The thickness of the ice ranged from 12-15 cm and ice temperature ranged from -15 °C at the top to -2 °C at the bottom. The mesocosm tanks were covered with aluminium foil to reduce evaporation of water.

3.1.2 Mesocosm sampling

At the start of the experiment (day 0), 2000 mL of SW and dispersed oil mixture (WAF+CE) were taken as grab samples from the mixed mesocosms. The ice from each mesocosm (413-786 g) was sampled using a sterile core drill at days 10, 20, 34, and 89 of the experiment. To avoid the draining of brine from the ice due to lowering the water level after sampling, sterile bags filled with autoclaved artificial seawater were put into the sampling holes to replace the removed ice. At the end of the experiment (day 89), 536-611 mL of water from beneath the ice (BW) was sampled as a grab sample after removal of ice blocks from the mesocosms.

The ice samples were melted overnight in an equal mass of sterilised artificial seawater at 4 °C. The melted ice and water samples (from day 0 and BW from day 89) were filtered through 0.2 μm Sterivex SVGPL10RC filters (Merck Millipore) which were stored at -80 °C until DNA extraction.

3.1.3 Measurement of temperature, pH and salinity

The temperature of ice in mesocosms throughout the experiment was monitored by a temperature probe. The pH of the seawater and melted ice samples were determined by pH/Cond 340i meter (WTW Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) and the salinity with salinity meter YSI 30-25FT (YSI Inc., Yellow Springs, Ohio, USA).

3.1.4 DNA extraction

The DNA was extracted from the biomass of SW, day 0 mesocosms water samples, melted ice (days 10, 20, 34, and 89) and BW (day 89) which was collected on Sterivex SVGPL10RC filters using DNeasy PowerWater Sterivex Kit (Qiagen, Foster City, CA, USA). The quality and quantity of DNA extracts were determined using Infinite M200 (Tecan AG, Grödig, Austria). The extracted DNA was stored at -20 °C until further analyses.

3.1.5 Shotgun Metagenomic Sequencing

The prokaryotic community composition and structure in SW, SW and dispersed oil mixture in mesocosms on day 0, dispersed oil containing ice on days 10, 20, 34, and 89, and BW on day 89 was assessed using whole genome shotgun sequencing analysis. For the preparation of DNA libraries, the DNA from two parallel mesocosms of the same treatment was pooled. The pooled DNA samples were purified and concentrated with NucleoSpin® Gel and PCR Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany) according to the manufacturer's protocol. Paired-end sequencing libraries (2 × 150 bp) were constructed using the Nextera XT DNA Library Preparation kit (Illumina) according to the manufacturer's instructions and sequenced using the NovaSeq 6000 system (Illumina).

The quality of obtained raw metagenomic sequences was controlled using FastQC v 0.11.7 (Andrews, 2010). Reads shorter than 35 bp and poly-G tails were removed and bases with quality scores lower than 20 were trimmed with Cutadapt v 1.16 (Martin, 2011). Coverage and diversity metrics of the quality-controlled metagenomic sequences were estimated using Nonpareil v 3.3.3 (Rodriguez et al., 2018). Bacterial and archaeal communities were classified to species level using Kaiju v 1.7.3 (Menzel et al., 2016) with the NCBI-nr database. The technical analysis of whole genome shotgun sequencing data was conducted by doctoral student Angela Peeb from Environmental Microbiology and Biotechnology laboratory; the author of the thesis is responsible for analysis of taxonomic data deriving from the Kaiju analysis.

3.1.6 Quantitative PCR

The quantitative polymerase chain reaction (qPCR) was used to determine the abundances of 16S rRNA genes which are specific to bacteria (B16S), archaea (A16S), and genera *Oleispira*, *Colwellia*, and *Pseudoalteromonas* from SW, melted ice, and BW samples.

3.1.6.1 General conditions of qPCR

The qPCR assays were run on a RotorGene® Q machine using RotorGene Series Software v 2.0.2 (Qiagen). The qPCR reactions were performed in 10 µL volume containing 5 µL of Maxima SYBR Green Master Mix reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA), 0.2 µL of target specific forward and reverse primers, 3.6 µL of sterile water, and 1 µL of template DNA. The used primers and the optimised conditions of each qPCR assay are described in Table 1.

Table 1. The characteristics of primer pairs and amplification programs used in qPCR.

Target gene	Primer	Primer sequence 5´-3´	Amplicon size (bp)	Primer conc (µM)	Amplification program	Primer reference
Bacterial 16S rRNA	Bact 517F	GCCAGCAGCCGCGGTAA	530	0.6	95°C 10 min; 35 cycles: 95°C 30 s; 60°C 45 s; 72°C 45 s	Liu et al., 2007
	Bact 1028R	CGACARCCATGCASCACCT*				Dethlefsen et al., 2008
Archaeal 16S rRNA	Arc 519F	CAGYCGCCRCGGTAA*	393	0.6	95°C 10 min; 35 cycles: 95°C 15 s; 56°C 30 s; 72°C 30 s	Espenberg et al., 2016
	Arch 910R	GCYCCCCCGCCWATTC*				
Colwellia 16S rRNA	Col 134F	CCTTATGGTGGGGGACAACA	96	0.6	95°C 10 min; 35 cycles: 95°C 15 s; 56°C 30 s; 72°C 30 s	Modified from Krolicka et al., 2014
	Col 209Rm	AATCAAATGGCGARAGGTCC*				
Oleispira 16S rRNA	Olea 339Fm	TGGRCGAAAGYCTGATGCAGCCATG*	186	0.6	95°C 10 min; 35 cycles: 95°C 15 s; 60°C 30 s, 72°C 30 s	Modified from Krolicka et al., 2014
	Olea 520R	TCCGATTAACGCTTGCACCTTTAGT				
Pseudoalteromonas 16S rRNA	PsALT F2	GCATTTCGAACTGGCAAACT	205	0.8	95°C 10 min; 40 cycles: 95°C 15 s; 57°C 30 s; 72°C 30 s	This study
	PsALT 815 Rm	CCSAGCTYCTAGTAGACATCGTT*				Modified from Holmes et al., 2001

* R - (A/G); S - (G/C); Y - (C/T); W - (A/T)

Immediately after the qPCR assay the melting curve analysis was performed by increasing the temperature from 70 °C to 90 °C (0.35 °C/3 s) with continuous fluorescence recording. All

target genes from all samples were amplified in triplicate, and multiple negative controls (including all reaction mixture components aside from template DNA) were included in every qPCR run.

3.1.6.2 The optimization of qPCR assay conditions for *Oleispira* and *Pseudoalteromonas* genera specific 16S rRNA genes quantification

Three *Oleispira* and four *Pseudoalteromonas* 16S rRNA gene specific primers (Table 2) were tested in order to choose the best primer combinations and optimise quantification conditions for these qPCR targets. *Oleispira antarctica* (DSM14852) and *Pseudoalteromonas arctica* (DSM18437) strain DNA were used as positive control in this experiment and sample "N5" from a previous study (microbial community DNA from a four-month biostimulated arctic seawater; Nõlvak et al., 2021) was used as environmental positive control.

Table 2. The tested *Oleispira* and *Pseudoalteromonas* genera specific 16S rRNA gene targeting primers.

	Primer sequence 5´-3´	**Primer reference**
Oleispira genus specific 16S rRNA gene		
OLEA 339 F	TGGACGAAAGTCTGATGCAGCCATG	Krolicka et al., 2014
OLEA 339 Fmod	TGGRCGAAAGYCTGATGCAGCCATG*	Modified from Krolicka et al., 2014
OLEA 520 R	TCCGATTAACGCTTGCACCTTTAGT	Krolicka et al., 2014
Pseudoalteromonas genus specific 16S rRNA gene		
Ps Alt Eub 341 F	CCTACGGGAGGCAGCAG	Holmes et al., 2001
Ps Alt 815 Rmod	CCSAGCTYCTAGTAGACATCGTT*	Modified from Holmes et al., 2001
Ps ALT_F1	GTGTAGCGGTGAAATGCGTA	This study
Ps ALT_F2	GCATTTCGAACTGGCAAACT	This study

* R - (A/G); S - (G/C) ; Y - (C/T)

A reaction mixture as described in 3.1.6.1 was used in all conducted experiments. At first, single primers in qPCR reaction mixture were tested to determine any nonspecific amplification by single primers. For primers passing that test, concentration optimization (at

0.4, 0.6, and 0.8 μM of primers) test was applied with number of amplification cycles needed to reach fluorescence threshold (Ct), amplification efficiency (E) and melting curve position recording. Thirdly, amplifications using the optimised primer concentration were applied to find the optimal annealing temperature; once again Ct, E, and melt curve position estimations were used in this analysis.

3.1.6.3 The creation of standard curves

To create standard curves for qPCR assays, the stocks of 10^9 gene copies per μL of target sequence containing plasmid vectors pEX-A2 (for B16S and A16S) and pEX-A128 (for *Colwellia*-, *Oleispira*-, and *Pseudoalteromonas*-specific 16S rRNA genes) were used (Eurofins MWG Operon, Ebersberg, Germany). Ten-fold serial dilutions in the range of 10^8 to 100 copies/μL of target sequence containing plasmids were created for each target gene and amplified using the optimised program conditions (Table 1).
The author of this thesis prepared standards for *Oleispira*- and *Pseudoalteromonas*-specific 16S rRNA gene quantification assays.

3.1.6.4 Determination of the abundance of target genes from seawater, ice, and under-ice water

Quantification data were first analyzed with the RotorGene Series Software v 2.0.2 (Qiagen) and amplifications showing either irregular or deviating amplification and melting curves were omitted from further analysis. Subsequently, the amplification efficiency of each sample was estimated using LinRegPCR program v 2020.0 (Ruijter et al., 2009). The target gene abundance was calculated through the estimation of the fold difference between a sample and multiple data points from the standard curve. First, the fold difference between target gene concentration (A) and the concentration of single points of the respective standard curve (B) was calculated according to the following formula (Ruijter et al., 2009):

$$\text{Fold difference} = N_{0,A}/N_{0,B} = (N_{t,A}/E_{ACt,A}) / (N_{t,B}/E_{BCt,B}),$$

where:
$N_{0,A}$ - the concentration of target gene in the sample in arbitrary fluorescence units
$N_{0,B}$ - the concentration of target gene in the standard dilution in arbitrary fluorescence units
E - amplification efficiency
N_t - the fluorescence threshold
Ct - number of amplification cycles needed to reach fluorescence threshold.

To calculate the estimate of target gene abundance in the sample per standard point, this fold difference was multiplied with the concentration of the respective standard curve point. Next, the average of several estimates (n=3-5) of target gene abundances respective to different standard curve points was calculated. Finally, the abundance of the target gene in an environmental sample was found by averaging the abundances of three amplification parallels.

The target gene abundances are presented as gene copy numbers per mL of analysed water. The total prokaryotic 16S rRNA gene abundance (16Stot) was calculated by summing the bacterial and archaeal 16S rRNA gene abundances (B16S+A16S). The relative abundances (%) of archaeal 16S rRNA genes in the prokaryotic community and targeted genera (*Oleispira, Colwellia, Pseudoalteromonas*) specific 16S rRNA genes in the prokaryotic community were also calculated.

3.2 RESULTS

The 89-day dispersed oil containing ice mesocosm experiment was carried out mainly to evaluate the potential of natural degradation of dispersed crude oil by the microorganisms in arctic sea ice through identification and quantification of the dominant microorganisms potentially involved in this degradation process.

3.2.1 Physicochemical characteristics

The arctic seawater used to set up the ice mesocosms was characterised by temperature of 4 °C, salinity of 32.6 ± 1.1 ‰ and pH of 8.28 ± 0.3 (Table 3).

Table 3. The average and standard deviation of temperature, pH and salinity in initial seawater (SW), in dispersed crude oil containing ice (WAF+CE) mesocosms and sterile ice mesocosms throughout the experiment as well as in under-ice water (BW) at the end of the experiment. N=2; nd - not detected.

Sample	Treatment	Temperature (°C)*	Salinity (‰)	pH
SW, day 0	-	4 ± 0	32.6 ± 1.1	8.28 ± 0.3
Ice, day 10	WAF+CE	-2 to -15	12.7 ± 1.0	7.89 ± 0.05
	Sterile control		11.4 ± 1.0	7.90 ± 0.02
Ice, day 20	WAF+CE	-2 to -15	nd	8.15 ± 0.05
	Sterile control		11.7 ± 0.0	7.90 ± 0.10
Ice, day 34	WAF+CE	-2 to -15	10.0 ± 1.2	7.99 ± 0.17
	Sterile control		8.2 ± 0.7	7.88 ± 0.08
Ice, day 89	WAF+CE	-2 to -15	6.5 ± 1.1	7.84 ± 0.21
	Sterile control		5.4 ± 1.2	7.95 ± 0.05
BW, day 89	WAF+CE	4 to -2	66.5 ± 2.5	7.97 ± 0.05
	Sterile control		63.0 ± 3.7	7.98 ± 0.02

* Ice temperature range is given from the bottom to the top of the ice in the mesocosms.

The temperature in ice in both WAF+CE and sterile control ice-mesocosms ranged from -2 to -15 °C (from bottom to top) throughout the experiment. The salinity in ice decreased throughout the experiment, slightly more in sterile control compared to WAF+CE; by the end of the experiment the salinity in ice had dropped to 6.5 ± 1.1 ‰ in WAF+CE treatment and to 5.4 ± 1.2 ‰ in sterile treatment (Table 3). The pH in ice was slightly lower than in initial SW and fluctuated in time in the range of 7.63-8.20 in WAF+CE treatment and 7.80-8.00 in the sterile treatment.

The water from beneath the ice (BW) at the end of the experiment was characterised by high salinity in both sterile treatment (63.0 ± 3.7 ‰) and in WAF+CE treatment (66.5 ± 2.5 ‰). The pH of BW was close to 8 in both analysed treatments (Table 3).

3.2.2 Prokaryotic community structure in seawater and dispersed oil containing ice mesocosms

The prokaryotic community structure in SW, in WAF+CE ice mesocosms, and in BW was estimated using whole-genome shotgun sequencing analysis. The technical characteristics of gained metagenomes along with coverage and diversity metrics are presented in Annex 1.

The prokaryotic community of SW, used to set up the ice mesocosms, was dominated by bacteria (85.6 %) while archaea formed (14.4 %) of the community. The structure of the prokaryotic community of SW at the phylum level (phylum *Proteobacteria/Pseudomonadota* was included into analysis on class level) was quite diverse with *Bacteroidetes* (11.9 %), *Alphaproteobacteria* (26.74 %), *Gammaproteobacteria* (20.1%) and *Euryarchaeota* (12.85 %) being the dominant taxa (Fig. 5). At genus level the SW community was also very diverse with only three genera comprising >3 % of the prokaryotic community noted: *Alphaproteobacterial Ca. Pelagibacter*, *Gammaproteobacterial Ca. Thioglobus*, and *Cyanobacterial Synechococcus* (Fig. 6). Of those, genus *Ca. Pelagibacter* was predominantly composed of species *Ca. Pelagibacter ubique* (Table 4) and *Ca. Thioglobus* majorly of species *Ca. Thioglobus singularis* (Table 4) while genus *Synechococcus* was more diverse in species composition.

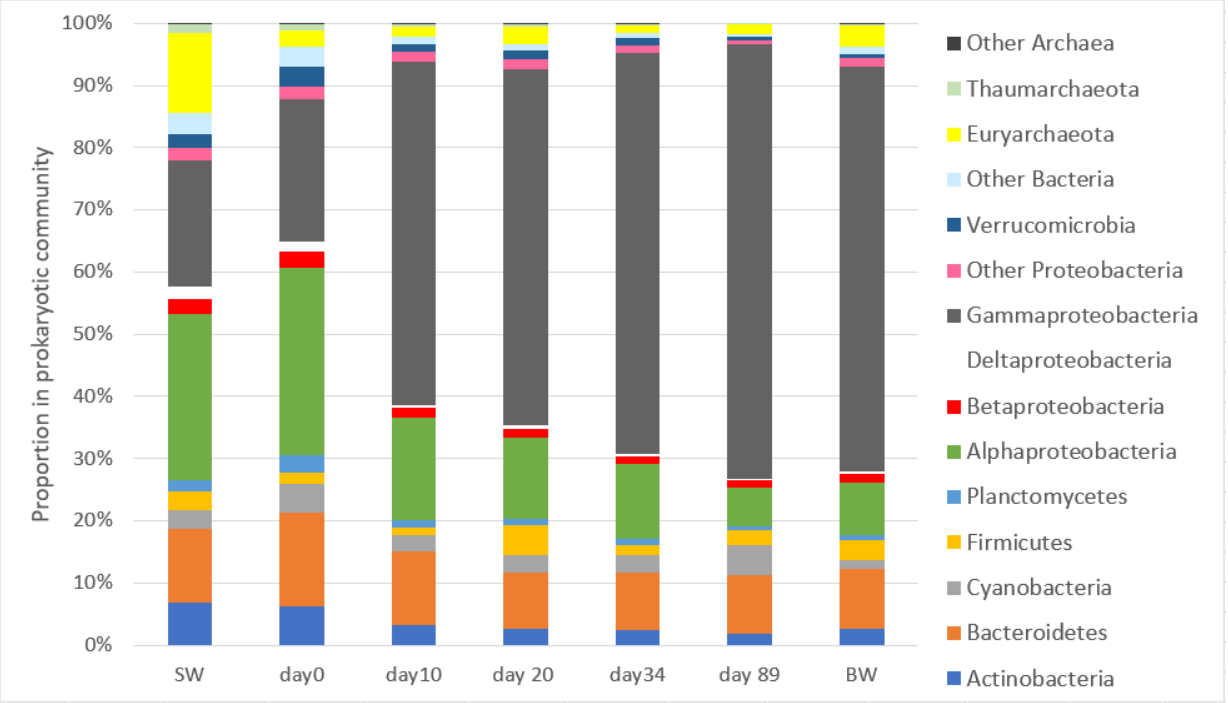


Fig. 5. The prokaryotic community structure at phylum level in initial seawater (SW), in dispersed crude oil containing ice (WAF+CE) mesocosms throughout the experiment as well as in under-ice (bottom) water at the end of the experiment. Phylum *Proteobacteria/Pseudomonadota* is presented on class level.

The archaea proved sensitive to dispersed oil, as their proportion dropped from 14.4 % in SW to 3.8 % following mixing and setup of ice mesocosms on day 0 and decreased further throughout the experiment (Annex 1). Consequently, the prokaryotic community of WAF+CE ice mesocosms throughout the experiment were dominated by bacteria (>96 %) and the community diversity decreased in time (Annex 1). At phylum level the structure of the prokaryotic community of WAF+CE mesocosms on day 0 was quite similar to SW with the exception of substantially lower *Euryarchaeota* proportion. However, by day 10 *Gammaproteobacteria* had become clear community dominants (>50 %), especially at the expense of *Alphaproteobacteria*, and its proportion increased in time up to 70 % by the end of the experiment (Fig. 5). The prokaryotic community of WAF+CE ice mesocosms at genus level were dominated by less than 10 genera which showed different behavioural patterns in time. The proportions of *Ca. Pelagibacter* and *Ca. Thioglobus*, found abundantly in SW, decreased in ice throughout the experiment down to <1 % by day 89 (Fig. 6). Genera *Bermanella* and especially *Oleispira* bloomed in ice already by day 10 (up to almost 14 % of the community in case of *Oleispira*) which was followed by a decrease in their proportion throughout the rest of the experiment. While the proportion of *Colwellia* had also increased

substantially by day 10, no substantial decrease in proportions was noted throughout the rest of the experiment for this genus. On the other hand, the proportions of genera *Pseudoalteromonas*, *Paraglaciecola,* and *Marinomonas* increased in WAF+CE ice throughout the experiment (Fig. 6). Of those, *Pseudoalteromonas* became a clear community dominant with a proportion of 44.8 % of the ice prokaryotic community by the end of the experiment. The prokaryotic community analysis on species level revealed that early-blooming genus *Oleispira* was represented solely by single species (*O. antarctica*) and genus *Bermanella* was majorly represented by species *B. marisrubri* (Table 4). On the other hand, the genera showing increase in proportion throughout the experiment like *Pseudoalteromonas* were diverse in species composition without any clearly dominating species.

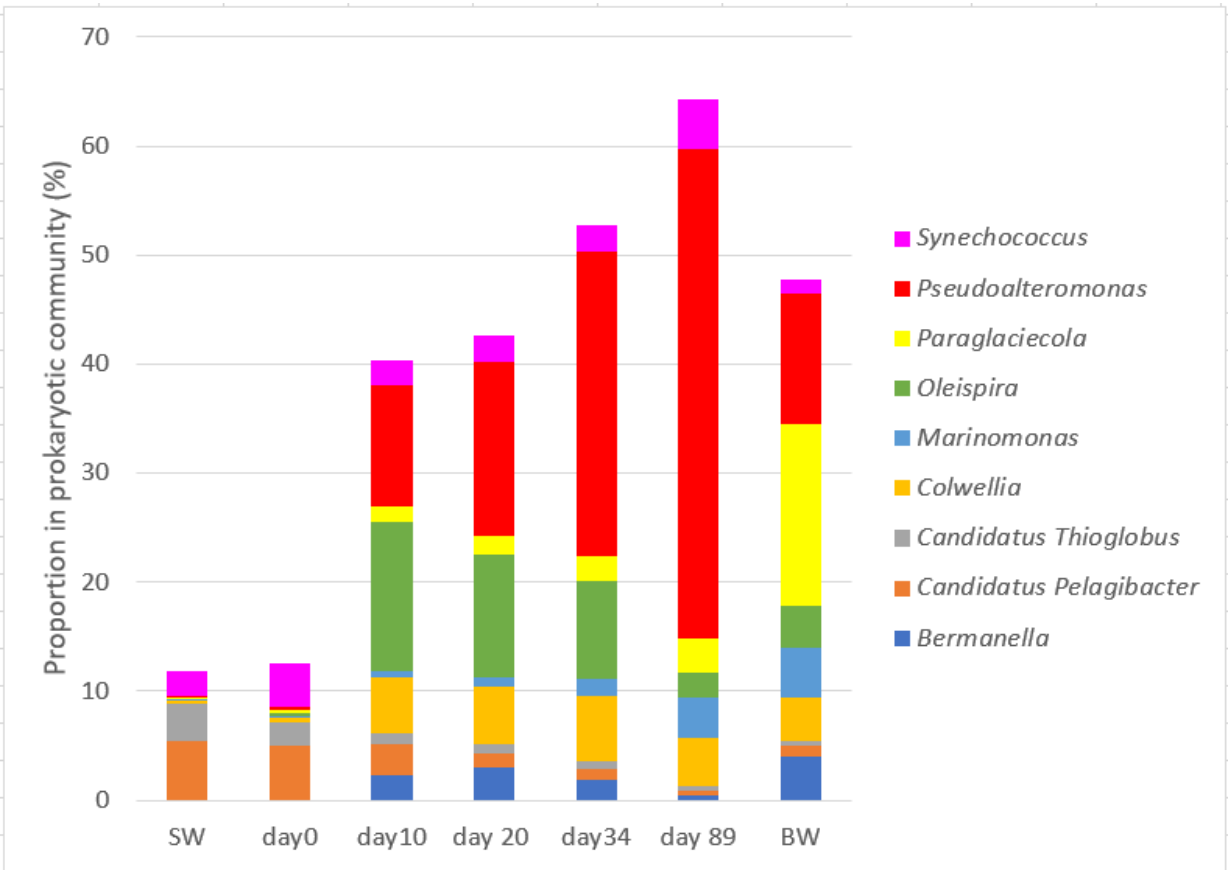


Fig. 6. The proportion of dominant genera (>2.5% in at least one sample) in the prokaryotic community of initial seawater (SW), in dispersed crude oil containing ice (WAF+CE) mesocosms throughout the experiment as well as in under-ice (bottom) water at the end of the experiment.

Table 4. The proportion of dominant species (>1% in at least one sample) in the prokaryotic community of initial seawater (SW), in dispersed crude oil containing ice (WAF+CE) mesocosms throughout the experiment as well as in under-ice water (BW) at the end of the experiment.

Species	Species proportion in prokaryotic community (%)						
	SW	**day0**	**day10**	**day20**	**day34**	**day89**	**BW**
Bermanella marisrubri	0.01	0.05	1.21	1.51	0.95	0.24	2.01
Ca. Pelagibacter ubique	3.52	3.23	1.69	0.68	0.56	0.17	0.33
Ca. Poseidoniales archaeon	7.62	0.92	0.25	0.14	0.09	0.02	0.04
Ca. Thioglobus singularis	2.52	1.43	0.58	0.36	0.24	0.10	0.31
Clostridium difficile	1.39	0.45	0.49	2.01	0.64	0.96	1.49
Colwellia sp. Arc7-635	0.00	0.01	1.19	1.67	0.87	1.81	0.36
Euryarchaeotal archaeon TMED129	1.78	0.80	0.91	2.00	0.87	0.87	2.56
Gammaproteobacteria bacterium	2.16	1.92	0.70	0.57	0.43	0.16	0.46
Oleispira antarctica	0.02	0.28	13.69	11.23	9.02	2.25	3.81
Pseudoalteromonas arctica	0.04	0.06	0.27	0.39	0.68	1.20	5.21
Pseudoalteromonas psychrophila	0.02	0.03	0.14	0.19	0.29	0.51	2.30
Paraglaciecola sp. D3211	0.07	0.12	0.53	0.58	0.49	0.29	3.71
Paraglaciecola sp. MB-3u-78	0.02	0.04	0.16	0.22	0.35	0.60	2.69
Pseudoalteromonas sp. EB27	0.00	0.00	0.63	0.89	1.68	2.69	0.69

In the BW of the WAF+CE ice mesocosms at the end of the experiment the prokaryotic community comprised 96.1 % of bacteria and 3.9 % of archaea. The structure of the BW prokaryotic community at phylum level was quite similar to ice on day 89 with clear dominance of *Gammaproteobacteria* (65.0 %) (Fig. 5). At genus level the BW community was dominated by a handful of *Gammaproteobacterial* genera, like *Paraglaciecola* and

Pseudoalteromonas, followed by smaller proportions of *Marinomonas, Bermanella* and *Oleispira* (Fig. 6). Of those genera, especially *Paraglaciecola* (16.64 %) was characteristic to the BW environment surpassing the proportion in day 89 ice prokaryotic community 5-fold. Two major (>2.5 %) *Paraglaciecola* species were identified in BW, namely *Paraglaciecola sp. D3211* and *Paraglaciecola sp. MB-3u-78* (Table 4). Also notably different from day 89 ice community, in BW the genus *Pseudoalteromonas* was on species level represented mainly by two dominant species (*P. arctica* and *P. psychrophila*) (Table 4).

3.2.3 Prokaryotic 16S rRNA gene abundance in dispersed oil containing ice mesocosms

The quantitative analysis of bacterial and archaeal 16S rRNA gene abundances confirmed that the prokaryotic community of SW, used to set up the ice mesocosms, was dominated by bacteria ($2.34*10^6$ copies/mL) while archaea ($2.71*10^5$ copies/mL) formed 12.7 % of the community according to this analysis.

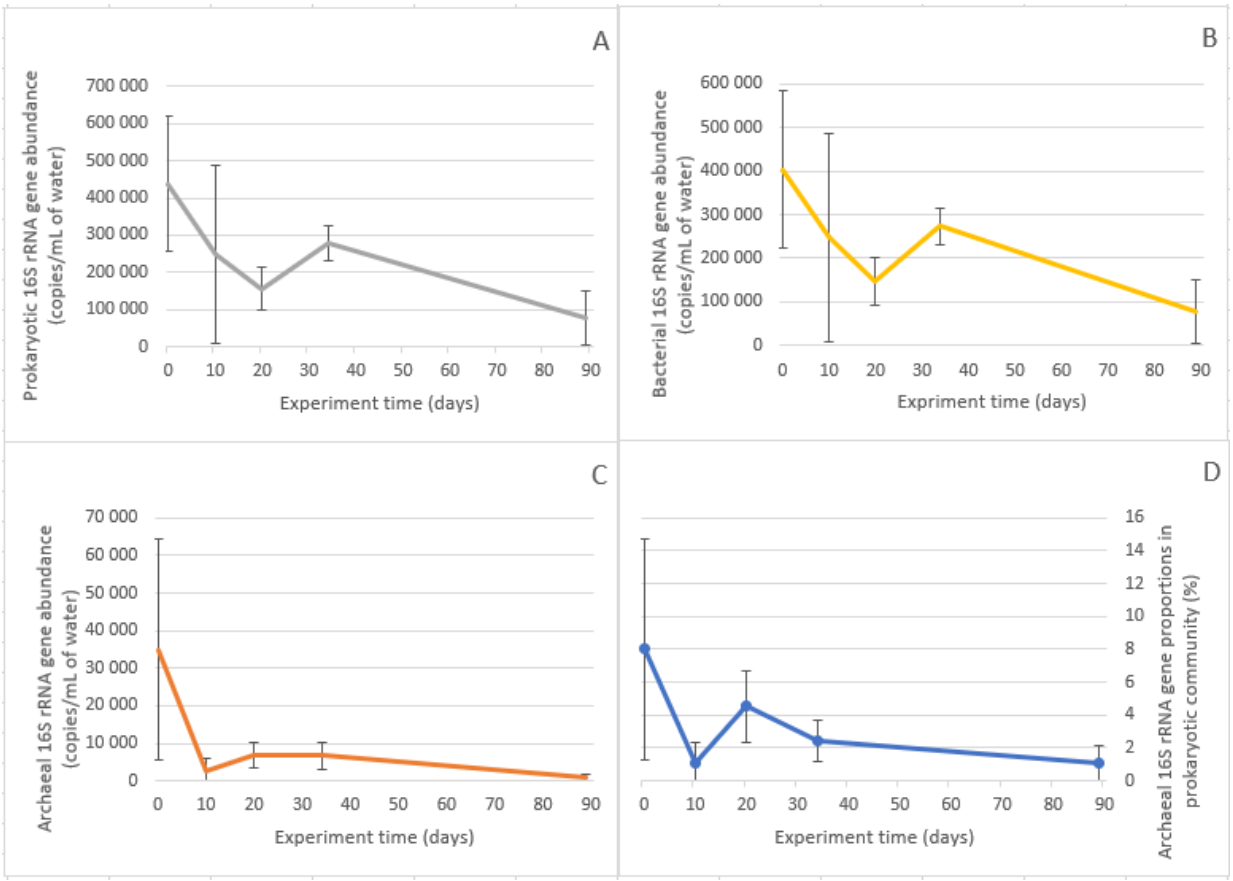


Fig. 7. The dynamics of (A) prokaryotic 16S rRNA gene abundance, (B) bacterial 16S rRNA gene abundance, (C) archaeal 16S rRNA gene abundance, and (D) the proportion of archaea in the prokaryotic community in dispersed crude oil containing ice (WAF+CE) mesocosms throughout the experiment. N=2.

The quantification of prokaryotic 16S rRNA gene abundance in WAF+CE mesocosms indicated that upon setup the parallel mesocosms of the same treatment had received quite deviant microbial community amount ($4.38*10^5 \pm 1.82*10^5$ copies/mL on day 0), of which archaea formed 8.0 ± 6.7 % (Fig. 7A, D). Despite some abundance fluctuations at day 34 for bacteria (Fig. 7B) and days 20 and 34 for archaea (Fig. 7C), a generally decreasing trend in WAF+CE ice was noted for all these abundances culminating in the prokaryotic community abundance of $7.73*10^4 \pm 7.34*10^4$ copies/mL by the end of the experiment (Fig. 7A). The archaeal 16S rRNA gene abundances had dropped <1000 copies/mL and relative abundances in the prokaryotic community to 1 % by day 89 (Fig. 7C-D).

The prokaryotic 16S rRNA gene abundance in BW at day 89 was $1.71*10^5$ copies/mL, of which archaeal 16S rRNA genes formed 0.31 % ($5.29*10^2$ copies/mL).

3.2.4 The determination of abundance of dominant genera *Colwellia*, *Oleispira*, and *Pseudoalteromonas* specific 16S rRNA genes

3.2.4.1 Optimization of *Oleispira* and *Pseudoalteromonas* 16S rRNA gene specific quantification assays

At first, all *Oleispira*- and *Pseudoalteromonas*-specific 16S rRNA genes targeting primers (Table 2) were run singularly in reaction mixture to verify, whether any of them gave nonspecific amplification on their own. Primers OLEA339F, PsALT_Eub341F and PsALT_F1 resulted in nonspecific amplification from either the respective reference strain DNA or environmental DNA known to contain targeted genera (Annex 2). These primers were omitted from further analysis.

As a second step, primers were tested for optimal concentration in qPCR reaction mixture yielding lowest Ct value, highest amplification efficiency (E), and stable melting curve position and shape (Table 5). The optimal primer concentration, showing the best combination of tested parameters, for *Oleispira* 16S rRNA gene specific qPCR assay is 0.6 µM, and for *Pseudoalteromonas* 16S rRNA gene specific qPCR assay 0.8 µM. These primer concentrations were used for further assay optimizations.

Finally, optimal annealing temperatures were found for *Oleispira* and *Pseudoalteromonas* 16S rRNA gene specific qPCR assays also by aiming at the combination of lowest Ct value, highest amplification efficiency, and stable melting curve position and shape when using the optimised primer concentration (Table 6). The optimal annealing temperatures, showing the

best combination of tested parameters, were 60 °C for *Oleispira* 16S rRNA gene specific primers and 57 °C for *Pseudoalteromonas* 16S rRNA gene specific primers.

Table 5. The result of the concentration optimization of (A) *Oleispira* 16S rRNA gene specific primers (OLEA339Fmod/OLEA520R), and (B) *Pseudoalteromonas* 16S rRNA gene specific primers (PsALT_F2/PsAlt815Rmod). Ct - cycle threshold, E - amplification efficiency, Melt - melting curve position. N=3.

A: *Oleispira* 16S rRNA gene						
Primers concentration (μM)	*Oleispira antarctica*			"N5"		
	Ct	E	Melt (°C)	Ct	E	Melt (°C)
0.4	10.33 ± 0.31	1.931 ± 0.071	82.3	20.45 ± 0.17	1.921 ± 0.053	83.0
0.6	10.09 ± 0.15	2.018 ± 0.039	82.0	19.59 ± 0.29	2.040 ± 0.409	82.3
0.8	9.68 ± 0.31	1.995 ± 0.029	82.3	19.00 ± 0.07	1.855 ± 0.104	83.0
B: *Pseudoalteromonas* 16S rRNA gene						
Primers concentration (μM)	*Pseudoalteromonas arctica*			"N5"		
	Ct	E	Melt (°C)	Ct	E	Melt (°C)
0.4	10.93 ± 0.07	1.888 ± 0.075	82.7	20.75 ± 0.23	1.770 ± 0.127	83.0
0.6	11.55 ± 0.50	1.871 ± 0.045	82.7	20.36 ± 0.19	1.810 ± 0.116	83.0
0.8	10.41 ± 0.41	1.915 ± 0.065	82.7	19.08 ± 0.12	1.847 ± 0.149	82.7

Table 6. The result of the annealing temperature optimization of (A) *Oleispira* 16S rRNA gene specific primers (OLEA339Fmod/OLEA520R), and (B) *Pseudoalteromonas* 16S rRNA gene specific primers (PsALT_F2/PsAlt815Rmod). Ct - cycle threshold, E - amplification efficiency, Melt - melting curve position. N=3.

A: *Oleispira* 16S rRNA gene						
Annealing temperature (°C)	*Oleispira antarctica*			"N5"		
	Ct	E	Melt (°C)	Ct	E	Melt (°C)
59	10.78 ± 0.09	1.889 ± 0.030	82.7	19.47 ± 0.21	1.787 ± 0.007	83.5
60	9.98 ± 0.08	2.010 ± 0.055	82.5	19.38 ± 0.0	1.853 ± 0.071	83.0
61	10.08 ± 0.28	1.911 ± 0.017	83.0	19.56 ± 0.06	1.812 ± 0.127	83.5
B: *Pseudoalteromonas* 16S rRNA gene						
Annealing temperature (°C)	*Pseudoalteromonas arctica*			"N5"		
	Ct	E	Melt (°C)	Ct	E	Melt (°C)
56	10.41 ± 0.14	1.915 ± 0.065	82.7	19.08 ± 0.12	1.847 ± 0.149	82.7
57	10.51 ± 0.33	1.962 ± 0.031	83.0	18.49 ± 0.23	1.893 ± 0.043	83.0
58	9.92 ± 0.14	1.972 ± 0.044	83.2	19.49 ± 0.12	1.995 ± 0.112	83.2

The optimised qPCR reaction conditions were used to quantify *Oleispira* and *Pseudoalteromonas* genera specific 16S rRNA gene abundances from the samples of seawater, WAF+CE ice mesocosms, and bottom water of ice mesocosms.

3.2.4.2 The abundance of *Colwellia*, *Oleispira*, and *Pseudoalteromonas* genus-specific 16S rRNA genes

The quantitative analysis of *Colwellia, Oleispira,* and *Pseudoalteromonas* genus-specific 16S rRNA gene abundances indicated their relatively low abundance in the community of SW used to set up the ice mesocosms. The recorded abundances were $9.41*10^3$ copies/mL, $6.93*10^3$ copies/mL and $2.81*10^3$ copies/mL for *Colwellia*-, *Oleispira*-, and *Pseudoalteromonas*-specific 16S rRNA genes, respectively. Based on quantitative analysis, *Colwellia-, Oleispira*- and *Pseudoalteromonas*-specific 16S rRNA genes formed 0.53 %, 0.27 %, and 0.14 %, respectively, of the total prokaryotic community 16S rRNA gene counts.

Colwellia, *Oleispira*, and *Pseudoalteromonas* specific 16S rRNA genes were quantified from dispersed crude oil containing ice mesocosms at days 0, 10, and 89; day 20 and 34 samples could not be targeted, as the DNA of these samples had been depleted in order to ensure the success of sequencing analysis. At day 0, the abundances of all genera-specific 16S rRNA genes were lower compared to SW in WAF+CE mesocosms and remained in the range of 1000 to 3000 copies/mL (Fig. 8A-C). This corresponded to 0.67 % of the prokaryotic 16S rRNA gene counts in case of *Colwellia* and <0.30 % in case of the two other targeted genera (Fig. 8D).

Throughout the experiment, two different types of temporal dynamics were recorded for genera-specific 16S rRNA gene abundances in dispersed oil containing ice. By day 10 the abundances of *Colwellia*- and *Oleispira*-specific 16S rRNA genes had increased substantially to $1.56*10^4 \pm 1.36*10^4$ copies/mL and $5.53*10^4 \pm 3.66*10^4$ copies/mL, respectively. This was followed by a decrease culminating in *Colwellia* and *Oleispira* average abundances of $8.95*10^2$ and $2.21*10^3$ copies/mL, respectively, by the end of the experiment (Fig. 8A-B). The temporal dynamics of relative abundances of *Colwellia* and *Oleispira* 16S rRNA genes followed the same pattern: an increase by day 10 was followed by substantial decrease by day 89 (Fig. 8D). The abundance of *Pseudoalteromonas* specific 16S rRNA genes showed different kind of temporal dynamics: while the increase of its abundance by day 10 was lesser than *Colwellia* and *Oleispira*, its abundance in dispersed oil containing ice continued to grow throughout the experiment culminating on average at $6.36*10^4$ copies/mL on day 89 (Fig. 8C). This abundance on day 89 translates to 78.85 ± 7.25 % of the total prokaryotic 16S rRNA gene abundance at the end of the experiment (Fig. 8D).

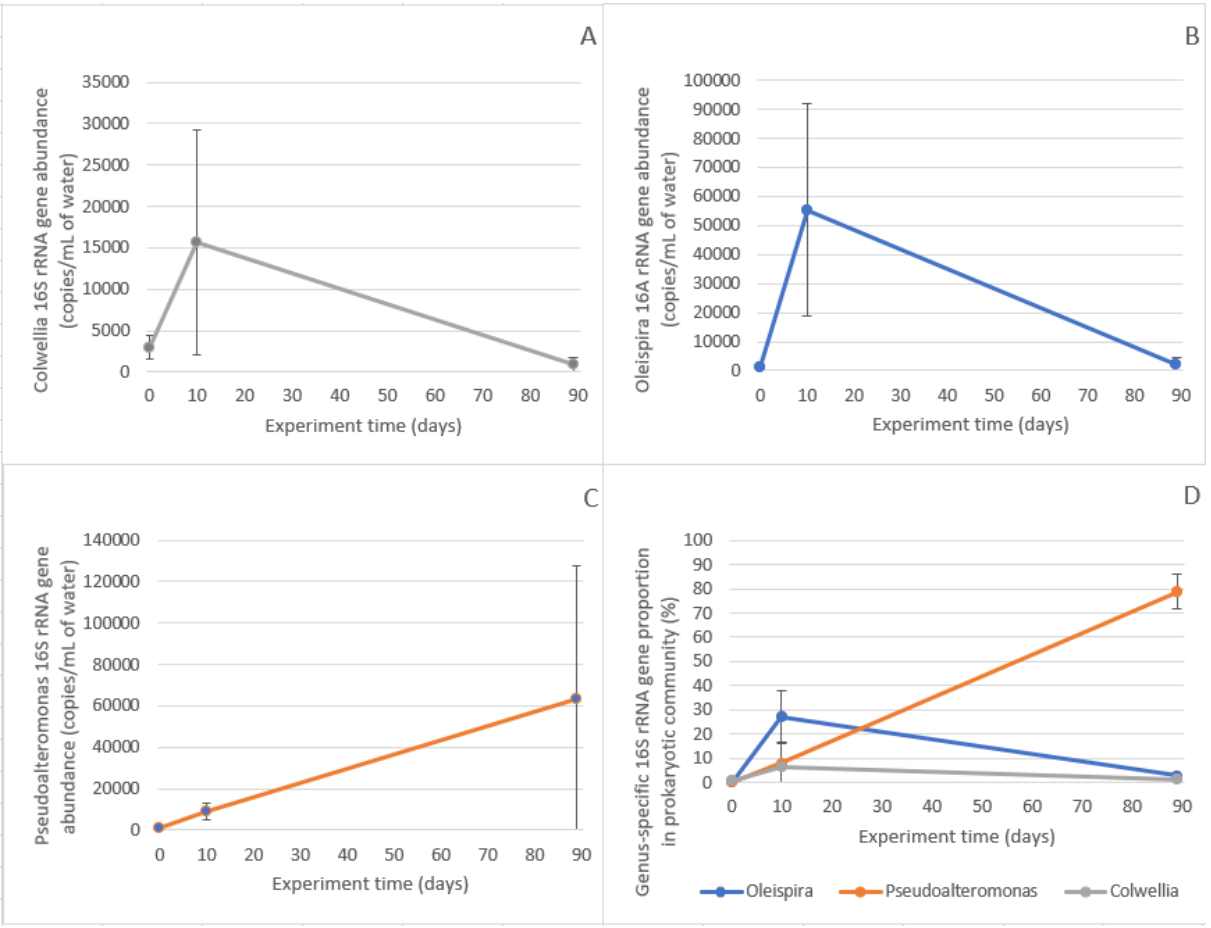


Fig. 8. The dynamics of (A) genus Colwellia specific 16S rRNA gene abundance, (B) genus Oleispira specific 16S rRNA gene abundance, and (C) genus Pseudoalteromonas specific 16S rRNA gene abundance in dispersed crude oil containing ice (WAF+CE) mesocosms throughout the experiment. N=2.

The *Colwellia, Oleispira,* and *Pseudoalteromonas* genus-specific 16S rRNA gene abundances in BW at day 89 were $5.28*10^3$, $1.62*10^3$ and $1.38*10^4$ copies/mL, respectively. This corresponded to 3.09 %, 0.95 %, and 8.06 % of the total prokaryotic community 16S rRNA genes in case of *Colwellia*, *Oleispira*, and *Pseudoalteromonas*, respectively.

3.3 DISCUSSION

Accidental spills of crude oil in the Arctic sea are challenging to manage due to harsh climate conditions and remote locations. The presence of ice in seawater negates the use of most physicochemical spill-response actions and when oil gets encapsulated in the ice, only bioremediation methods based on the ability of microbes to degrade petroleum hydrocarbons can be relied on (Wilkinson et al., 2017; Bullock et al., 2019). As the knowledge in this field is still rather limited, this thesis addressed the microbial community structure and abundance dynamics in dispersed crude oil containing sea-ice as a first step of estimating the microbial oil degradation potential in Arctic sea-ice.

The abundance of the prokaryotic community of Arctic seawater from Ofotfjorden, used to set up the ice mesocosms, was in similar range as reported from Svalbard seawater; also similarly the community was dominated by bacteria while archaea formed 10-15 % of the seawater community (Nõlvak et al., 2021). The seawater microbial community was dominated by class *Alphaproteobacteria* organisms, followed by class *Gammaproteobacteria.* This coincides with previous reports from Ofotfjorden seawater (Peeb et al., 2022) but differs from reports on Canadian Arctic seawater (Yergeau et al., 2017) and Svalbard seawater (Nõlvak et al., 2021) where *Gammaproteobacteria* dominated. However, this difference might not only be caused by location differences but also seasonal dynamics of seawater microbial communities - all Ofotfjorden seawater samples were collected in autumn (October-November), opposed to spring (April-May) in the other studies. If the seawater prokaryotic community structural differences are indeed seasonal, the precondition for oil degradation in forming sea-ice is not the best: most proficient oil degrading organisms known from Arctic seawater and sea-ice belong to class *Gammaproteobacteria* (Boccadoro et al., 2018; Gutierrez et al., 2013; Brakstad et al., 2018; Krolicka et al., 2019).

Dispersant usage for breaking oil slicks into small droplets is one of the most common spill-responses in cold marine environments. Upon ice formation these oil droplets get encapsulated within the growing ice along with microbes from the contaminated seawater. Any oil biodegradation happening in sea-ice takes place in a hypersaline environment of liquid brine channels formed in ice and only a small fraction of encapsulated oil is exposed to this environment (Junge et al., 2017; Brakstad and Bonaunet, 2006). Such conditions proved unsuitable for most of the archaea in this experiment. Also, both the bacterial community abundance and diversity decreased in dispersed oil containing sea-ice. The abundance of microbial community is tied with the potential oil compounds degradation rate in a

contaminated environment which in some cases has proven insufficient in cold environments like Arctic seawater (Nõlvak et al., 2021). The recorded prokaryotic community abundance in sea-ice in this study was even an order of magnitude lower than in the latter case.

Despite low abundances, the microbial community responded to dispersed oil contamination in ice: *Gammaproteobacteria,* which are identified as the main players of oil degradation in cold marine environments (Brakstad et al., 2008; Garneau et al., 2016; Lofthus et al., 2021), became community dominants already by day 10 of the experiment. The bloom and succession tendency in the ice microbial community was well visible on genus level. The known alkane degraders at low temperatures like *Oleispira* and *Bermanella* (Yakimov et al., 2003; Brakstad et al., 2008; Lofthus et al., 2018) bloomed on day 10, followed by their proportion as well as abundance withering till the end of the experiment. Genus *Colwellia* abundance followed similar dynamics while its proportion in the microbial community remained similar from day 10 onward till the end of experiment. This might be explained by the ability of some *Colwellia* strains to also use aromatic hydrocarbons (Gutierrez et al., 2013). The increased appearance of *Colwellia* and members of order *Oceanospirillales* (both *Bermanella* and *Oleispira* belong to this order) have been noted also during the Deepwater Horizon oil spill (Valentine et al., 2010; Mason et al., 2014). On the other hand, genera like *Pseudoalteromonas*, *Paraglaciecola* and *Marinomonas* showed continuous increase in proportions throughout the experiment. *Marinomonas* and *Paraglaciecola* strains are known to be able to degrade harder to access petroleum hydrocarbons like PAHs and long-chain n-alkanes, respectively (Melcher et al., 2002; Vergeynst et al., 2019). However, these genera were in oil contaminated sea-ice clearly overshadowed by dominance of *Pseudoalteromonas.*

According to metagenomic data *Pseudoalteromonas* proportions in sea-ice microbial community increased to ~45 % by the end of the experiment; according to quantitative analysis the dominance of *Pseudoalteromonas* genus was even more profound, as its 16S rRNA genes formed ~80 % of the total prokaryotic 16S rRNA gene counts in sea-ice at the end of the experiment. The discrepancy between these estimates arises from two sources: the metagenomic taxonomic classifier performance and ecological truthfulness of the results vary according to the sample type, taxa present, and composition of the reference database used (Ye et al., 2019) and different *Pseudoalteromonas* strains possess 5-12 copies of 16S rRNA genes (rrnDB, 2022). However, it is clear that genus *Pseudoalteromonas* became an undisputed community dominant in dispersed oil containing sea-ice by the end of the experiment. Members of *Pseudoalteromonas* genus are nutritionally diverse bacteria - different strains have been shown to degrade either linear alkanes, branched alkanes, or

various PAHs (Deppe et al., 2005; Chronopoulou et al., 2015). Nutritional diversity also probably explains the lack of emergence of few dominant *Pseudoalteromonas* species in this experiment - the dominant section of the sea-ice community consisted of numerous different *Pseudoalteromonas* species and strains, all with slightly different functional profiles towards petroleum hydrocarbons degradation. Increased *Pseudoalteromonas* abundances have also been noted in the later successional stages of microbial community following Deepwater Horizon Spill (Dubinsky et al., 2013).

The bottom water samples of the mesocosms unfortunately do not adequately represent the marine under-ice conditions, as the enclosed mesocosm keeps under-ice water hypersaline, which is not such an extreme case in real-life. The mesocosm bottom water microbial communities were dominated by genus *Paraglaciecola*, which supports the notion that these bacteria can be prominent and transcriptionally active in hypersaline brine channels (Rapp et al., 2021).

It has been noted that dispersants themselves might have several different effects on the microbial community. Dispersants can negatively affect and reduce the impact of some oil degrading bacteria, while other bacteria have the ability to use dispersant-derived compounds as growth substrates (Kleindienst et al., 2015). The magnitude of dispersant effect in this experiment remains to be estimated in further analysis based on dispersant-control treatment variant data.

SUMMARY

In this study it was found that the microbial communities of Arctic seawater and ensuing dispersed crude oil encapsulating sea-ice were substantially different. The microbial community responded to oil contamination as *Gammaproteobacteria* became dominant in oil-contaminated sea-ice with genera *Bermanella*, *Colwellia*, and especially *Oleispira* blooming early in the experiment. These genera were replaced by *Marinomonas*, *Paraglaciecola* and especially *Pseudoalteromonas* as microbial community dominants in sea-ice in latter stages of the experiment. Quantitative PCR assays for determining the abundance of *Oleispira*- and *Pseudoalteromonas*-specific 16S rRNA genes were successfully developed, optimised and harnessed in this study. The quantitative analysis confirmed the early bloom and later dieoff dynamics for *Oleispira* and continuously increasing dynamics for *Pseudoalteromonas* genera in dispersed oil containing sea-ice.

However, even though the microbial community structure responded to oil contamination in ice, its overall abundance decreased substantially during the experiment sparking a concern that the microbial abundance in oil contaminated sea-ice might be just too low for notable biodegradation of the pollutant. This needs to be verified in further in depth analysis of the experiment data.

The future work based on the conducted mesocosm experiment encompasses determination of normalised abundance dynamics of oil degradation related genes and the construction of metagenome-assembled genomes from metagenomic data and comparison of the data in context of all mesocosm experiment treatment variants. Further research on the microbial oil degradation potential in arctic sea ice could use biosurfactants and added nutrients in form of slow release particles that get encapsulated into ice along with the contaminant to enhance the response to complex oil hydrocarbons.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Hiie Nõlvak for advice and support. I am grateful to members of SINTEF's Cold Climate Laboratory in Narvik who conducted the mesocosm experiment setup. I would also like to thank doctoral student Angela Peeb from the Laboratory of Environmental Microbiology and Biotechnology in the Institute of Molecular and Cell Biology for metagenomic data analysis.

This work has received funding from the European Union's Horizon2020 Programme under Grant N.679266, and the Estonian Research Council grant PRG548.

REFERENCES

1. Agarwal, M. 10 Methods for Oil spill Cleanup at Sea. **2021**, https://www.marineinsight.com/environment/10-methods-for-oil-spill-cleanup-at-sea/ (accessed on 20.05.2022).
2. Andrews, S. FastQC: A Quality Control Tool for High Throughput Sequence Data. **2010**.Available online: http://www.bioinformatics.babraham.ac.uk/projects/fastqc (accessed on 10. October 2021).
3. Bagi, A.; Pampanin, D.M.; Lanzén, A.; Bilstad, T.; Kommedal, R. Naphthalene biodegradation in temperate and arctic marine microcosms. *Biodegradation*, **2014**, 25, 111–125.
4. Bird, K.J.; Charpentier, R.R.; Gautier, D.L.; Houseknecht, D.W.; Klett, T.R.; Pitman, J.K.; Moore, T.E.; Schenk, C.J.; Tennyson, M.E.; Wandrey, C.R. Circum-Arctic resource appraisal: estimates of undiscovered oil and gas north of the Arctic Circle. *In: US Geological Survey Fact Sheet.* **2008**, 3049, 4p.
5. Boccadoro, C.; Krolicka, A.; Receveur, J.; Aeppli, C.; le Floch, S. Microbial community response and migration of petroleum compounds during a sea-ice oil spill experiment in Svalbard. *Mar. Environ. Res.* **2018**, 142, 214–233.
6. Bowman, JP.; McCuaig, RD. Biodiversity, community structural shifts, and biogeography of prokaryotes within Antarctic continental shelf sediment. *Appl. Environ.* **2003,** 69, 2463–2483.
7. Boylan, MB. Increased maritime traffic in the Arctic: Implications for governance of Arctic sea routes. *Marine Policy*, **2021**, 131, 104566.
8. Brakstad, O.G.; Bonaunet, K. Biodegradation of petroleum hydrocarbons in seawater at low temperatures (0–5°C) and bacterial communities associated with degradation. *Biodegradation,* **2006,** 17: 71–82.
9. Brakstad, O.G.; Nonstad, I.; Faksness, L.G.; Brandvik, P.J. Responses of microbial communities in Arctic sea ice after contamination by crude petroleum oil. *Microb. Ecol.* **2008**, 55(3), 540−552.
10. Brakstad, O.G.; Throne-Hoist, M.; Netzer, R.; Stoeckel, D.D.; Atlas, R.M. Microbial communities related to biodegradation of dispersed Macondo oil at low seawater temperature with Norwegian coastal seawater. *Microb. Biotechnol*. **2015**, 8(6), 989-998.
11. Brakstad, O.G.; Farooq, U., Ribicic, D., Netzer, R. Dispersibility and biotransformation of oils with different properties. *Chemosphere,* **2018,** 191, 44–53.

12. Bullock, R.J.; Perkins, R.A.; Aggarwal, S. In-situ burning with chemical herders for Arctic oil spill response: Meta-analysis and review. *Sci. Total Environ.* **2019**, 675, 705-716.
13. Carpenter, A. Oil pollution in the North Sea: The impact of governance measures on oil pollution over several decades. *Hydrobiologia*, **2018**, 845, 109–127.
14. Chronopoulou, P-M.; O'Sanni, G.; Silas-Olu, D.I.; van der Meer, J.R.; Timmis, K.N.; Brussaard, C.P.D.; McGenity, T.J. Generalist hydrocarbon-degrading bacterial communities in the oil-polluted water column of the North Sea. *Microb. Biotechnol.* **2015**, 8(3), 434-447.
15. Deppe, U.; Richnow, H-H.; Michaelis, W.; Antranikian, G. Degradation of crude oil by an arctic microbial consortium. *Extremophiles.* **2005**, 9, 461–470.
16. Dethlefsen, L.; Huse, S.; Sogin, M.L.; Relman, D.A. The pervasive effects of an antibiotic on the human gut microbiota. as revealed by deep 16S rRNA sequencing. *PloS biol*. **2008**, 6(11), 2383–2400.
17. Doshi, B.; Sillanpää, M.; Kalliola, S. A review of bio-based materials for oil spill treatment. *Water Res*. **2018**, 135, 262-277.
18. Dubinsky, E.A.; Conrad, M.E.; Chakraborty, R.; Bill, M.; Borglin, S.E.; Hollibaugh, J.T., Mason, O.U.; Piceno, Y.M.; Reid, F.C.; Stringfellow, W.T.; Tom, L.M.; Hazen, T.C.; Andersen, G.L. Succession of hydrocarbon-degrading bacteria in the after-math of the Deepwater Horizon oil spill in the Gulf of Mexico. *Environ. Sci. Technol.* **2013**, 47, 10860–10867.
19. Espenberg, M.; Truu, M.; Truu, J.; Maddison, M.; Nõlvak, H.; Järveoja, J.; Mander, Ü. Impact of reed canary grass cultivation and mineral fertilisation on the microbial abundance and genetic potential for methane production in residual peat of an abondoned peat extraction area. *Plos ONE.* **2016**, 11(9), e0163864.
20. McFarlin K.; Leigh MB.; Perkins R. Biodegradation of oil and dispersed oil by Arctic marine microorganisms. *In: International Oil Spill Conference Proceedings. American Petroleum Institute*.**2014,** vol 1, p 300317.
21. Garneau, M-E.; Michel, C.; Meisterhans, G.; Fortin, N.; King, T.L.; Greer, C.W.; Lee, K. Hydrocarbon biodegradation by Arctic sea-ice and sub-ice microbial communities during microcosm experiments, Northwest Passage (Nunavut, Canada). *FEMS Microbiol Ecol.* **2016,** 92, fw130.

22. Gerdes B.; Brinkmeyer R.; Dieckmann G.; Helmke E. Influence of crude oil on changes of bacterial communities in Arctic sea-ice. *FEMS Microbiol Ecol*, **2005**, 53, 129–139.
23. Hassanshahian, M.; Amirinejad, N.; Behzadi, M.A. Crude oil pollution and biodegradation at the Persian Gulf: A comprehensive and review study. *J. Environ. Health Sci.* **2020,** 18, 1015–1027.
24. Hazen, T.C.; Prince, R.C.; Mahmoudi, N. Marine Oil Biodegradation. *Environ. Sci. Technol.* **2016**, 50, 2121–2129.
25. Head, I.M.; Gray, N.D.; Larter, S.R. Life in the slow lane; biogeochemistry of biodegraded petroleum containing reservoirs and implications for energy recovery and carbon management. *Front. Microbiol*. **2014**, 5, 23.
26. Holmes, A.J.; Tujula, N.A.; Holley, M.; Contos, A.; James, J.M.; Rogers, P.; Gillings, M.R. Phylogenetic structure of unusual aquatic microbial formations in Nullarbor caves, Australia. *Environ. Microbiol.* **2001**, 3(4), 256-264.
27. Junge, K.; Krembs, C.; Deming, J.; Stierle, A.; Eicken, H. A microscopic approach to investigate bacteria under in situ conditions in sea-ice samples. *Ann. Glaciol*. **2017**, 33, 304–310.
28. Kapadia Sanket, G.; Yagnik, B.N. Current trend and potential for microbial biosurfactants. *Asian J. Exp. Biol. Sci*. **2013**, 4(1).
29. Kleindienst, S.; Seidel, M.; Ziervogel, K.; Grim, S.; Loftis, K.; Harrison, S.; Malkin, S.Y.; Perkins, M.J.; Field, J.; Sogin, M.L.; Dittmar, T.; Passow, U.; Medeiros, P.M.; Joye, S.B. Chemical dispersants can suppress the activity of natural oil-degrading microorganisms. *PNAS*, **2015**, 112(48), 14900-14905.
30. Krolicka, A.; Boccadoro, C.; Maeland, M.; Preston, C.M.; Birch, J.; Scholin, C.; Baussant, T. Detection of oil leaks by quantifying hydrocarbonoclastic bacteria in cold marine environments using the environmental sample processor. In *Proceedings of the 37th AMOP Technical Seminar on Environmental Contamination and Response*, Environment Canada, **2014**, pp. 791–807.
31. Krolicka, A.; Boccadoro, C.; Nilsen, M.M.; Demir-Hilton, E.; Birch, J.; Preston, C.; Scholin, C.; Baussant, T. Identification of microbial key-indicators of oil contamination at sea through tracking of oil biotransformation: An Arctic field and laboratory study. *Sci. Total Environ*. **2019**, 696, 133715.

32. Lawniczak, L.; Karczewska, MW.; Loibner, AP.; Heipieper, H.J.; Chrzanowski, L. Microbial degradation of Hydrocarbons-Basic principles for bioremediation: Review. *Molecules*, **2020**, 25, 856.
33. Lewis, A.; Daling, P.S. A review of studies of oil spill dispersant effectiveness in Arctic conditions. *SINTEF Materials and Chemistry, Oil in ice JIP report*, **2007**, 11, 26p.
34. Liu, Z.; Lozupone, C.; Hamady, M.; Bushman, F.D.; Knight, R. Short pyrosequencing reads suffice for accurate microbial community analysis. *Nucleic Acids Res*. **2007**, 35(18), e120.
35. Lofthus, S.; Netzer, R.; Lewin, A.S.; Heggeset; T.M.B.; Haugen, T.; Brakstad, O.G. Biodegradation of n-alkanes on oil–seawater interfaces at different temperatures and microbial communities associated with the degradation. *Biodegradation,* **2018,** 29, 141–157.
36. Lofthus, S.; Bakke, I.; Greer, C.W.; Brakstad, O.G. Biodegradation of weathered crude oil by microbial communities in solid and melted sea ice. *Mar. Pollut. Bull*. **2021**, 172, 112823.
37. Mapelli, F.; Scoma, A.; Michoud, G.; Aulenta, F.; Boon, N.; Borin, S.; Kalogerakis, N.; Daffonchio, D. Biotechnologies for marine oil spill cleanup: Indissoluble ties with microorganisms. *Trends Biotechnol*. **2017**, 35, 860–870.
38. Martin, M. Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet J.* **2011**, *17*, 10.
39. Mason, O.U.; Han, J.; Woyke, T.; Jansson, J.K. Single-cell genomics reveals features of a Colwellia species that was dominant during the Deepwater Horizon oil spill. *Front. Microbiol*. **2014**, 5, 332.
40. Melcher, R.J.; Apitz, S.E.; Hemmingsen, B.B. Impact of irradiation and polycyclic aromatic hydrocarbon spiking on microbial populations in marine sediment for future aging and biodegradability studies. *Appl. Environ. Microbiol.* **2002**, 68, 2858–2868.
41. Menzel, P.; Ng, K.L.; Krogh, A. Fast and sensitive taxonomic classification for metagenomics with Kaiju. *Nat. Commun.* **2016**, *7*, 11257.
42. Muthukamalam S.; Sivagangavathi S.; Dhrishya D.; Sadras S.R. Characterization of dioxygenases and biosurfactants produced by crude oil degrading soil bacteria. *Brazilian Journal of Microbiology*. **2017,** 48(4).

43. Nikolopoulou, M.; Pasadakis, N.; Kalogerakis, N. Evaluation of autochthonous bioaugmentation and biostimulation during microcosm-simulated oil spills. *Mar. Pollut. Bull.* **2013**, 72, 165–173.
44. NOAA.GOV, oil spills major marine ecosystem threat https://www.noaa.gov/explainers/oil-spills-major-marine-ecosystem-threat (accessed on 10.5.22)
45. Nõlvak, H.; Dang, N.P.; Truu, M.; Peeb, A.; Tiirik, K.; O'Sadnick, M.; Truu, J. Microbial community dynamics during biodegradation of crude oil and its response to biostimulation in Svalbard seawater at low temperature. *Microorganisms*, **2021**, 9, 2425.
46. Oren, A.; Garrity, G.M. Valid publication of the names of forty-two phyla of prokaryotes. *Int. J. Sys. Evol. Microbiol*., **2021**, 71(10), 005056.
47. Ossai, I.C.; Ahmed, A.; Hassan, A. Remediation of soil and water contaminated with petroleum hydrocarbons: A review. *Environmental Technology & Innovation*, **2020**, 17, 100526.
48. Peeb, A.; Dang, N.P.; Truu, M.; Nõlvak, H.; Petrich, C.; Truu, J. Assessment of hydrocarbon degradation potential in microbial communities in Arctic sea ice. *Microorganisms*, **2022**, 10, 328.
49. Prince, R.C.; Walters, C.C. Biodegradation of oil and its implications for source identification. In Oil Spill Environmental Forensics; Wang, Z.; Stout, S. A., Eds. ; *Academic Press: Burlington, MA*, **2007**; pp 349−379.
50. Prince, R.C.; McFarlin, K.M.; Butler, J.D.; Febbo, E.J.; Wang, F.C.; Nedwed, T.J. The primary biodegradation of dispersed crude oil in the sea. *Chemosphere*, **2013**, 90, 521–526.
51. Rapp, J.Z.; Sullivan, M.B.; Deming, J.W. Divergent genomic adaptions in the microbiomes of Arctic subzero sea-ice and cryopeg brines. *Front. Microbiol.* **2021**, 12, 701186.
52. Rodriguez, L.M.; Gunturu, S.; Tiedje, J.M.; Cole, J.R.; Konstantinidis, K.T. Nonpareil 3: Fast estimation of metagenomic coverage and sequence diversity. *mSystem*s, **2018**, *3*, e00039.
53. rrnDB, 2022, https://rrndb.umms.med.umich.edu/ (accessed on 25.05.2022).
54. Rügner, H.; Finkel, M.; Kaschl, A.; Bittens, M. Application of monitored natural attenuation in contaminated land management - A review and recommended approach for Europe. *Environ. Sci. Pol.* **2006**, 9(6), 568-576.

55. Ruijter, J.M.; Ramakers, C.; Hoogaars, W.M.H.; Karlen, Y.; Bakker, O.; van den Hoff, M.J.B.; Moorman, A.F.M. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. *Nucleic Acids Res*. **2009**, 37, e45.
56. Sama, S.G.; Barrere-Mangote, C.; Bouyssiere, B.; Giusti, P.; Lobinski, R. Recent trends in element speciation analysis of crude oils and heavy petroleum fractions. *Trend Anal Chem*. **2018**, 104, 69-76.
57. Shan, L.; Gao, Y.; Zhang, Y.; Yu, W.; Yang, Y.; Shen, S.; Zhang, S.; Zhu, L.; Xu, L.; Tian, B.; Yun, J. Fabrication and use of alginate-based cryogel delivery beads loaded with urea and phosphates as potential carriers for bioremediation. *Ind. Eng. Chem. Res*. **2016**, 55, 7655-7660.
58. Silva, E.J.; Rocha e Silva, N.M.P.; Rufino, R.D.; Luna, J.M.; Silva, R.O.; Sarubbo, L.A. Characterization of a biosurfactant produced by Pseudomonas cepacia CCT6659 in the presence of industrial wastes and its application in the biodegradation of hydrophobic compounds in soil. *Colloids and Surfaces B: Biointerfaces*, **2014**, 117, 36-41.
59. Smets, B.F.; Pritchard, P.H. Elucidating the microbial component of natural attenuation. *Curr. Opin. Biotechnol.* **2003**, 14(3), 283-288.
60. Tyagi, M.; da Fonseca, M.M.R.; de Carvalho, C. Bioaugmentation and biostimulation strategies to improve the effectiveness of bioremediation processes. *Biodegradation*, **2011**, 22, 231–241.
61. US EPA, **2022**, https://www.epa.gov/emergency-response/types-crude-oil (assesed on 16.5.2022).
62. Valentine, D.L.; Kessler, J.D.; Redmond, M.C.; Mendes, S.D.; Heintz, M.B., Farwell, C.; Hu, L.; Kinnaman, F.S.; Yvon-Lewis, S.; Du, M.; Chan, E.W.; Tigreros, F.G.; Villanueva, C.J. Propane respiration jump-starts microbial response to a deep oil spill. *Science*, **2010**, 330, 208–211.
63. Vergeynst, L.; Kjeldsen, K.U.; Lassen, P.; Rysgaard, S. Bacterial community succession and degradation patterns of hydrocarbons in seawater at low temperature. *J. Hazard. Mater.* **2018**, 353, 127–134.
64. Vergeynst, L.; Christensen, J.H.; Kjeldsen, K.U.; Meire, L.; Boone, W.; Malmquist, L.M.V.; Rysgaard, S. In situ biodegradation, photooxidation and dissolution of petroleum compounds in Arctic seawater and sea ice. *Water Res*. **2019,** 148, 459–468.
65. Wilkinson, J.; Beegle-Krause, C.; Evers, K.-U.; Hughes, N.; Lewis, A.; Reed, M.; Wadhams, P. Oil spill response capabilities and technologies for ice-covered Arctic

marine waters: A review of recent developments and established practices. *Ambio.* **2017**, 46, 423–441.

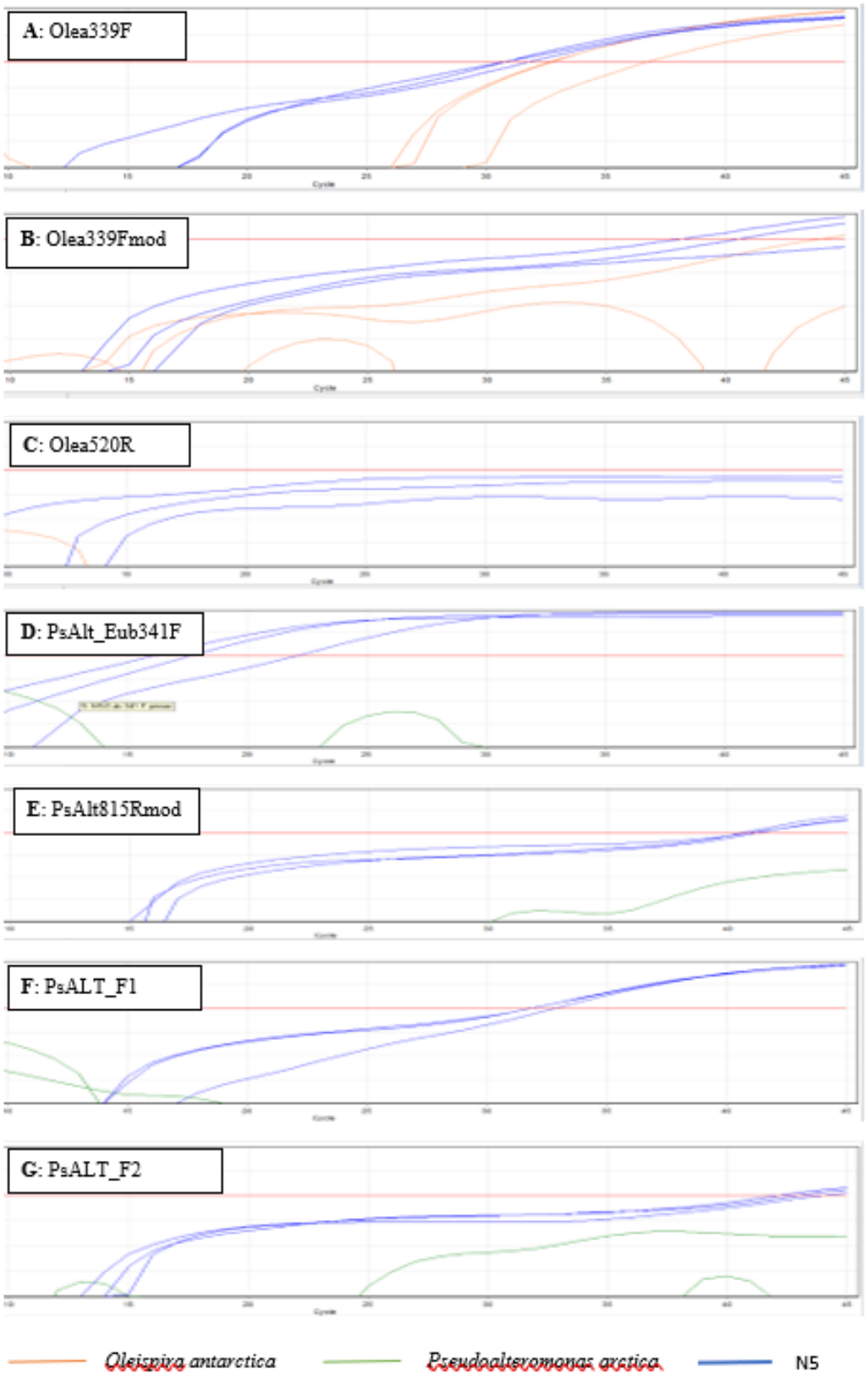
66. Yakimov, M.M.; Giuliano, L.; Gentile, G.; Crisaf, E.; Chernikova, T.N.; Abraham, W.R.; Lunsdorf, H.; Timmis, K.N.; Golyshin, P.N. Oleispira antarctica gen. nov., sp nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. *Int. J. Syst. Evol. Microbiol.* **2003,** 53, 779–785.
67. Yakimov, M.M.; Gentile, G.; Bruni, V.; Cappello, S.; D'Auria, G.; Golyshin, P.N.; Giuliano, L. Crude oil-induced structural shift of coastal bacterial communities of rod bay (Terra Nova Bay, Ross Sea, Antarctica) and characterization of cultured cold-adapted hydrocarbonoclastic bacteria. *FEMS Microbiol Ecol.* **2004,** 49; 419–443.
68. Ye, S.H.; Siddle, K.J.; Park, D.J.; Sabeti, P.C. Benchmarking Metagenomics Tools for Taxonomic Classification. *Cell,* **2019**, 178, 779–794.
69. Yergeau, E.; Michel, C.; Tremblay, J.; Niemi, A.; King, T.L.; Wyglinski, J.; Lee, K.; Greer, C.W. Metagenomic survey of the taxonomic and functional microbial communities of seawater and sea ice from the Canadian Arctic. *Sci. Rep*. **2017**, 7, srep42242.

ANNEXES

Annex 1. The numbers of total reads and reads after quality trimming, coverage and diversity metrics, the proportions of classified reads (%), the numbers of prokaryotic reads as well as proportions of archaeal and bacterial reads in prokaryotic reads in metagenomes of initial seawater (SW, n=2), in dispersed crude oil containing ice (WAF+CE) mesocosms on days 0, 10, 20, 34, and 89, as well as in under-ice water (BW) at the end of the experiment.

Sample	**Number of total reads**	**Number of reads after quality trimming**	**Coverage**	**Diversity**	**Classified reads (%)**	**Number of prokaryotic reads**	**Archaeal reads (%)**	**Bacterial reads (%)**
SW	63,933,179 ± 3,236,935	63,763,947 ± 3,400,112	0.75 ± 0.02	20.44 ± 0.37	61.23 ± 0.10	32,340,042 ± 2,580,980	14.4 ± 2.5	85.6 ± 2.5
WAF+CE day0	53,406,735	52,930,050	0.67	21.13	69.73	33,405,072	3.8	96.2
WAF+CE day 10	54,013,074	53,964,263	0.83	19.64	79.59	37,532,276	2.1	97.9
WAF+CE day 20	57,104,823	56,372,562	0.86	19.32	71.67	32,885,251	0.01	99.99
WAF+CE day 34	62,196,786	62,150,752	0.86	19.12	83.89	46,850,402	1.5	98.5
WAF+CE day 89	58,078,630	57,363,673	0.92	18.05	88.23	46,316,064	1.8	98.2
BW	54,552,677	54,171,141	0.92	18.27	75.39	29,697,729	3.9	96.1

Annex 2. The nonspecific amplification of single primers tested for *Oleispira*-specific 16S rRNA gene quantification (A-C) and *Pseudoalteromonas*-specific 16S rRNA gene quantification (D-G). The red line marks the fluorescence threshold at 0.1 units.



NON-EXCLUSIVE LICENCE TO REPRODUCE THESIS AND MAKE THESIS PUBLIC

I, Farheen Fathima,

1. grant the University of Tartu a free permit (non-exclusive licence) to reproduce, for the purpose of preservation, including for adding to the DSpace digital archives until the expiry of the term of copyright, my thesis The Response of Microbial Community to Dispersed Oil Contamination in Arctic Sea-ice, supervised by Hiie Nõlvak.

2. I grant the University of Tartu the permit to make the thesis specified in point 1 available to the public via the web environment of the University of Tartu, including via the DSpace digital archives, under the Creative Commons licence CC BY NC ND 4.0, which allows, by giving appropriate credit to the author, to reproduce, distribute the work and communicate it to the public, and prohibits the creation of derivative works and any commercial use of the work from 26/05/2022 until the expiry of the term of copyright,

3. I am aware that the author retains the rights specified in points 1 and 2.

4. I confirm that granting the non-exclusive licence does not infringe other persons' intellectual property rights or rights arising from the personal data protection legislation.

Farheen Fathima

26/05/2022