Effect of Oil Pollution and Ice Formation on Microbial Community of Seawater from Ofotfjorden, Norway

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Abstract

Oil spill in ice-covered waters can become entrapped in sea ice and may be subjected to biodegradation by sea ice microorganisms. The extent of the hydrocarbon biodegradation, and microorganisms involved in such process in sea ice are not well understood. In this study, we performed ice formation in lab-tanks (125 l) with unpolluted seawater from Ofotfjorden, Norway. The tanks were insulated at the perimeter and bottom with Styrofoam and heated from the bottom so that ice only grew from the tank surface. Troll B crude oil was injected underneath the ice, forming an oil lens which later was encapsulated in the ice as the ice continued growing. The ice was harvested and stored at -14°C for 3 months following encapsulation. Metagenomic analysis of the microbial communities in the ice samples which formed in the lab showed changes in the microbial community structure with dominance of *Alpha-* and *Gammaproteobacteria*. At the same time, *Archaea*, *Bacteroidetes* and *Actinobacteria* reduced significantly in the ice compare to the original seawater. No significant change of the microbial community in the ice was observed in the presence of the oil. However, a slight increase in abundance of some bacterial genera such as *Cowellia*, *Glaciecola* and *Acrobacter* was detected among the phylotypes of the oil-contaminated ices. Member of genera *Cowellia* and *Glaciecola* are common sea-ice inhabitants and have been known for their n-alkanes and aromatic hydrocarbons metabolism capacity. Despite this development, no significant loss of the oil or change of n-C_{17}/Pristane or n-C_{18}/Phytane ratio was detected. But a slight reduction of water soluble PAHs was observed that may results from microbial activity in the ice.

1. Introduction

Further development of oil exploration activity into Arctic areas which have seasonal ice cover presents challenging conditions for oil spill response. Oil spills may happen in ice-covered waters or drift into the ice-covered areas where they can become encapsulated in the ice in winter. In spring, the oil may permeate to the surface through brine channels within the sea ice. Drifting ice can further transport the pollution and release it far from the source following melting of the ice. Understanding how sea-ice microorganism response in the presence of oil would help predicting the fate of spilled oil in the ice as well as helping the preparation of a relevant remediation strategy in the event of the oil release.

Sea ice is an ideal habitat for both psychrophilic and psychrotrophic microorganisms. There is evidence that microbial activity can occur in Arctic sea ice at -20 °C (Junge et al., 2004). But very few studies have focused on biodegradation of oil in sea ice and how the sea-ice microorganism response in the presence of oil (Gerdes et al., 2005; Brakstad et al., 2008; Greer et al., 2014; Garneau et al., 2016). Some of the studies revealed that *Gammaproteobacteria* became predominant phylotypes in the oil-contaminated sea ices from Svalbard (Gerdes et al., 2005; Brakstad et al., 2008) or from bottom sea ices from Canadian Arctic Archipelago (Greer et al., 2014). Bacterial genera such as: *Marinobacter, Shewanelle,* and *Pseudomonas*

were found predominated in the melted sea ices which were incubated with crude oil for one year (Gerdes, et al., 2005). While *Colwellia*, *Marinomonas* and *Glaciecola* were the predominant phylotypes in the oil-contaminated ice cores from Svalbard (Brakstad et al., 2008). Garneau et al., (2016) compared the ice with the sub-ice water from Nunavut, Canada, and found that *Bacteroidetes* became dominated in the oil-contaminated microcosm of melted ice while *Epsilonproteobacteria* increased in the oil-contaminated microcosm of the sub-ice water (Garneau et al., 2016).

Most of the mentioned studies were performed with natural ice by adding oil to melted ice, to the surface of the ice or in a hole in the ice. In this study, for the first-time oil biodegradation was performed with laboratory-formed sea ice. Short-gun metagenomic sequencing technique was applied for comparing the microbial community of the starting seawater with the formed ice, and the ice which had been exposed to crude oil to study the effect of ice formation as well as oil encapsulation on the microbial community of seawater.

2. Materials and Methods

2.1. Materials

Five hundred litres of surface seawater were collected from near shore areas: Taraldsvika (68.44743° N; 17.43525° E) and Kvitvika (68.44208° N 17.38917° E), in Ofotfjorden. Two individual experiments were conducted from each seawater.

The Troll B type crude oil was used for all experiments. The crude oil was pasteurized at 65 °C for 30 min, for three consecutive days and pre-cooled at 4 °C overnight prior to use, to eliminate any contamination in the oil.

2.2. Chemical Analyses

Nitrate and ammonium concentration in the seawater and sea ice were determined spectrometrically with nitrate test kit, and ammonium cell test kit (Merck, Darmstadt Germany) followed the manufacturer's instructions after diluting the seawaters. Total nitrogen and total organic carbon (TOC) content of the seawater were analysed by Akvaplan niva. The soluble phosphorous was determined using spectrometric method described by Murphy and Riley (1962).

The pH of the seawater and melted sea ice was measured using pH/Cond 340i meter (Wissenschaftlich-Technische Werkstätten GmbH). The salinity of the seawater was measured with salinity meter YSI 30-25FT (YSI incorporated, USA).

2.3. Sea Ice Mesocosm Setup

Ice formation in mesocosm has been demonstrated in Norut's cold climate laboratory as described in an earlier study (Petrich and Arntsen, 2013). About 120 L of seawater were filled into a pre-cleaned tank made from Plexiglass. The tank was insulated at perimeter and bottom with Styrofoam which was 5 cm thick (Fig 1A). Heating elements and fan were placed underneath the tank to maintain a constant heat flux into the water beneath the ice. Cleaned thermocouples were installed to monitor the temperature in the growing ice and in the water. The lab temperature was set at -1 °C for two days to pre-cool the water in the tank. To start the ice growing process, the lab temperature was decreased to -15 °C.

For each experiment, two tanks were prepared. Tank A was used for growing clean sea ice. Tank B was set up similar to tank A but was injected with Troll B crude oil. As the ice was about 7-8 cm, an amount of 250 ml of well mixed crude oil was injected aseptically underneath the ice (Fig 1B). The sea ice was then allowed to grow about 5 cm thicker under the oil lens. The final ice thickness was about 12-14 cm before being harvested. Ice samples of 9-

10 kg with encapsulated oil were then remove from the tanks and kept at -14 °C for 3 months prior to melting for DNA isolation and subsequently metagenomic sequencing.

For oil analysis, several smaller oil lenses (3 ml each) were introduced in the ice. The ice with oil lens was subsequently cored and kept frozen at similar condition as the big ice and analysed for total hydrocarbon content (THC) and n-C17/Pristane and n-C18/Phytane ratios.

2.4. Oil Analysis

The ice core containing oil was kept frozen in a closed-cap bottle before it was melted at room temperature and extracted with 3 volumes of dichloromethane (DCM). The DCM was then concentrated by evaporation. The concentrated extract was purified by solid phase extraction through a silica column and further evaporated before gas chromatography analysis. The total hydrocarbon content (THC) of the sample was determined by GC-FID analysis (Agilent 7890A model gas chromatograph with a flame ionisation detector) using a Varian Wcot Fused Silica column (25 m × 0.32 mm id, coating CP-sil 8CD.CP7452), with hydrogen as carrier gas (flow 2.5 ml/min) and the temperature program of 55°C (3 min) - 25°C/min → 300°C (10 min) splitless in 1 min.

Sixteen PAHs according to EPA standard were analysed with GC-MS by Akvaplan Niva using standard method. The concentration of 16-PAHs in the samples were nomalized against chrysene to evaluate their reduction over 3 months. Chrysene was chosen among the analysed PAHs because of its resistance to biodegradation (Alimi et al., 2003)

2.5. DNA Isolation

The ices of about 9-10 kg were melted at room temperature in sterile artificial seawater (Instant Ocean) at ratio 1:1 (w/w). The melted water was then filtered aseptically through 0.2 μm Sterivex filter SVGPL10RC (Milipore). The Sterivex filters contains DNA were then stored at -80°C until DNA extraction. The DNA extraction was conducted using PowerWater® Sterivex™ DNA isolation kit (MOBIO laboratories, Inc) followed the instruction of the manufacturer. Genomic DNA samples were quantified using the Qubit 2.0 (Invitrogen), along with the Qubit dsDNA HS assay kit (Thermo Fisher Scientific).

2.6. Generation of Sequencing Libraries and Sequencing

The Indexed pair-ended libraries were prepared using Nextera DNA sample preparation kit (Illumina) and Nextera DNA sample preparation index kit (Illumina) as described by the manufacturer with minor modifications: fifty nanogram genomic DNA was tagmented at 55°C for 10 min. The tagmented DNA was amplified with two primers from Nextera DNA sample preparation Index Kit. Each PCR reaction contained 5μl index 1 primer (N7xx), 5μl index 2 primer (N5xx), 15μl NPM (Nextera PCR master Mix), 5μl PPC (PCR primer cocktail) and 20μl tagmented DNA. PCR amplification was carried out as follows: 72°C x 3min ,98°C x 30s, 8 cycles of 98°C x 10s, 63°C x 30s, 72°C x 3min and held at 10°C. PCR products were cleaned using Agencourt AMPure XP beads (Beckman Coulter) and the purified PCR products were quantified using Qubit dsDNA HS assay kit. The sizes of the fragmented libraries were analyzed using Agilent 2100 Bioanalyser. The samples were pooled at concentration of 4nM and denatured with 0.2N NaOH, then diluted to 10pM with HT1 (hybridization buffer). Samples were sequenced on MiSeq (Illumina) sequencing platform, using 2 x 300 cycle V3 kit (Illumina), following the standard Illumina sequencing protocols.

2.7. Bioinformatics Analysis

Sequence reads were processed using a Galaxy version of META-pipe (doi: 10.12688/f1000research.10443.1). In short, sequencing reads were filtered using

PRINSEQ (doi: 10.1093/bioinformatics/btr026). The filtered reads were used as input for rRNASelector, a tool for selecting rRNA sequences from metagenomics shotgun reads (doi: 10.1007/s12275-011-1213-z) (Table 1). Selected rRNA sequences were annotated using LCAClassifier with default parameters (LCA relative range: 2%; minimum bit score: 155), using the manually curated Silva*Mod* database (doi: 10.1371/journal.pone.0049334). Taxonomic analysis was performed using MEGAN4 (doi: 10.1101/gr.120618.111).

3. Results

3.1. Seawater and Ice Characteristics

The chemical characteristics of the collected seawaters and ice samples are summarised in Table 2. The salinity of the collected seawaters was about 27.8 ppt, lower than usual for seawater, which properly due to the rain during the weeks prior to seawater sampling. The seawaters had also low TOC, nitrogen, ammonium, nitrate and orthophosphate. No significant differences were observed for the seawaters collected from the two locations, except that sample from location 1 contained slightly higher TOC concentration, 2.76 instead of 2.1 mg/l.

Analysis of the ice samples after harvesting showed a consistent salinity ranging from 9.4-9.6 ‰, which is a typical magnitude for fast-growing and relatively thin sea ice.

3.2. Microbial Community in Seawater and Sea Ice

The microbial community in seawater was dominated by *Alphaproteobacteria* (36.9-41.9%), *Gammaproteobacteria* (11.2%), *Bacteroidetes* (15.7-16.7%) and *Actinobacteria* (13.8-18.1%) (Fig 2). Dominance of *Alphaproteobacteria* in the Arctic surface seawater has been reported by Garneau et al., (2006) and Alonso-Sáez et al., (2008).

In comparison to the seawater, the microbial community of the ice shifted toward enrichment of *Alpha*- and *Gammaproteobacteria*, 59.2-62.4% and 16.4-19%, respectively. Other groups such as *Archaea, Bacteroidetes* and *Actinobacteria*, in the contrary reduced significantly in the ices. *Archaea* reduced from 2.8-3.4 % to 0-0.3 % in the ice; *Bacteroidetes* from 15.7-16.7 % to 6-6.9 %; *Actinobacteria* from 13.8-18.1 % to 0-2.4%. The seawater and the ice samples from two locations did not exhibit significant difference in their microbial community structure (Fig 2).

The most abundant OUTs in the seawater, sea ice and oil-encapsulated ice samples are depicted in Table 3. Within the *Alphaproteobacteria* and *Gammaproteobacteria*, bacterial groups which became dominant in the ice are members of the SAR11 clade (41-48 %), *Rhodosprillaceae* (4-5%), *Rhodobacteraceae* (6.6-7.7%) and the SAR86 clade (7.2-8.6 %) (Table 4).

3.3. Oil Degradation and Microbial Response to the Oil

Analysis of the oil which was encapsulated in the ice did not show any change in the oil quantity. GC-FID chromatogram of the starting oil and the oil in the ice for 3 months did not show any obvious change (Figure 3). Both n-C17/Pristane and n-C18/Phytane ratios of the encapsulated oil (0.3 and 0.5 respectively) were unchanged compared to the starting oil suggesting no significant biodegradation of n-alkanes > C_{17} occurred in the ice. However, whether n-alkanes < C_{17} was biodegraded or not we don't know.

The GC-MS analysis of 16 PAHs according to EPA exhibited a reduction of some 2 and 3-rings PAHs after 3 months (Figure 4). This result suggests a slow biotransformation of small aromatic hydrocarbons might have occurred in the ice.

Metagenomic comparison of sea ice samples which contain encapsulated crude oil with the clean sea ice did not showed any difference in the microbial community structure (Figure

2). Both clean ice and oil-encapsulated ice samples showed similar microbial community pattern, which were predominant by the *Alphaproteobacteria* (47.8-62.4 %), *Gammaproteobacteria* (13.8-27 %) and Bacteroidetes (4.4-6.9 %). However, a comparison of the samples at genus level revealed greater similarity between the two oil-encapsulated ices. Genera *Glaciecola*, and *Arcobacter* seemed to increase in the two oil contaminated samples, while *Colwellia* increased in one of the oil contaminated ice (Figure 4).

4. Discussion

4.1. Microbial Community in Seawater and Sea Ice

It is generally known that bacteria become enriched in sea ice in association with algae (Grossmann and Diekmann 1994; Helmke and Weyland 1995; Riedel et al., 2007). Higher bacteria abundance in newly formed sea ices compared to the initial seawater was reported for sea ice formed in mesocosm (Rasimus et al., 2014) as well as for ice cores from first-year sea ice in Franklin Bay, Canada (Collins et al., 2010). Collins and other authors observed a greater abundance of the *Alpha-* and *Gammaproteobacteria* in first year winter sea ice compared to the seawater through clone library sequencing of the microbial community (Collins et al., 2010), which coincides with our results. In our experiment, the bacteria increased from 97% of the microbial community in the original seawaters to 98-99% in the ices with the dominance of *Alpha-* and *Gammaproteobacteria*. The presence of algae, however, was very low in our sea ice possibly because the experiment was conducted mostly in the dark.

Bacterioplankton belonging to the SAR11 clade of *Alphaproteobacteria* which was found predominant in our lab formed sea ice (41-48 %), have been found distributed throughout the world's ocean and represent about one quarter of all rRNA genes identified in the clone libraries from marine environments (Morris et al., 2002). They were also found represent at 47 % of the prokaryotic phenotype in first year winter sea-ice in Franklin Bay (Collins et al., 2010). These organisms have the smallest genome known for free-living heterotrophic cells, results of genome streamlining and reduction driven by the selection for efficient growth in oligotrophic ocean habitats (Giovannoni et al., 2005).

4.2. Effect of Oil

Almost no change of the microbial community was observed in the ice after introduction of the oil lens in the ice. The ice samples were kept at -14 °C for 3 months, which led to low microbial activity in the ice. Having a nominal pour point of -15 °C, at -14 °C Troll B oil would have been very viscous. The oil geometry imposed another constraint on biodegradation, as activity is limited to the surface of the oil lens. In additional to that, encapsulated oil was not subjected to evaporation, and at such high concentration, volatile hydrocarbons can be toxic to the sea-ice microorganisms which could explain almost zero biodegradation of the oil and no significant alteration of the microbial communities. Brakstad et al., (2008) when studying biodegradation of an oil lens which was frozen into an ice core during winter months in Svalbard, also observed a slow biotransformation of soluble naphthalene in the top ice layer, while no change of n-C17/Pristane ratio was observed. But the n-C17/Pristane ratio showed reduction in the bottom part of the ice where the oil concentration was much lower. In another study, Gerdes (2004) did not find any significant biodegradation of crude oil which was spread on the ice surface for 2 months at -20 to -30 °C in Van Mijenfjorden.

Genera *Cowellia* and *Glaciecola* have been found being enriched in bottom ice formed in mesocosm from seawater of North Sea, near Heligoland (Rasimus et al., 2014). Moreover, members of those genera have been identified in both Arctic and Antarctic sea ice (Deming, 2009), suggesting they are sea-ice inhabitants. Many species of *Glaciecola* have been known for their hydrocarbon-degrading ability (Yakimov et al., 2004; Deppe et al., 2005; Brakstad et

al., 2008; Chronopoulou et al., 2015). Genus *Glaciecola* have been identified in oil contaminated Arctic and Antarctic seawater (Brakstad and Lødend, 2005; Yakimov et al., 2004) as well as in oil contaminated sea ices (Brakstad et al., 2008). Some *Glaciecola* isolates showed capability of degrading n-alkanes (Chronopoulou et al., 2015). *Colwellia* have been associated with oil-contaminated cold marine environments such as Antarctic and Arctic seawater and sea ice (Yakimov et al., 2004; Brakstad et al., 2008; Greer et al., 2014; Brakstad et al., 2015). They have been found dominant in Deepwater horizon after the oil spill (Redmond and Valentine, 2012; Mason et al., 2014). There were direct and indirect evidences about their capability of degrading gaseous alkanes (Redmond and Valentine 2012; Mason et al., 2014; Brakstad et al., 2015). In addition to gaseous hydrocarbons, there was also data supporting the uptake of aromatic hydrocarbons by *Cowellia* (Redmond and Valentine, 2012).

Genus *Arcobacter* were identified by sequencing analysis of the water phase of water-flooded oil reservoir (Wang et al., 2014), and seawater contaminated with hydrocarbons (Yakimov et al., 2004; Pradagaran et al., 2007). Their preferred growth substrates are mostly the water-soluble intermediates of organic matter degradation such as petroleum hydrocarbons (Wang et al., 2014).

The depletion of the small molecular weight PAHs in the ice after 3 months may be the result of microbial activity. However, we do not have continuous data to show the development of those bacteria in the ice overtime.

5. Conclusion

Artificial sea ice was grown in lab tanks from Atlantic seawater to investigate the development of sea-ice borne microbial communities. Ice properties resembled naturally-grown sea ice, and the ice temperature ranged from -15 to -1.8 °C (from surface to bottom) during the experiment. Metagenomic analysis of the microbial communities in the ice samples which formed in the lab showed changes in the microbial community structure with dominance of *Alpha-* and *Gammaproteobacteria.* At the same time, *Archaea*, *Bacteroidetes* and *Actinobacteria* reduced significantly in the ice compare to the original seawater.

An oil lens was introduced beneath the ice in one of the tanks and overgrew subsequently. Over the course of three months at -14 °C, the oil did not cause a significant change in the microbial community of the ice. However, a slight increase in abundance of some bacterial genera such as *Cowellia*, *Glaciecola* and *Acrobacter* was detected among the phylotypes of the oil-contaminated ices. Member of genera *Cowellia* and *Glaciecola* are common sea-ice inhabitants and have been known for their hydrocarbon-degrading capacity. Despite of this development, no significant loss of the total hydrocarbon content or change in n-C_{17}/Pristane and n-C_{18}/Phytane ratios was detected after three months. But the observed reduction of 2 and 3-ring PAHs in the ice may have been result of biotransformation. Biodegradation may have been inefficient due to a low surface area-to-volume ratio and limited accessibility of hydrocarbons due to high viscosity at low temperatures.

6. Acknowledgment

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7. References

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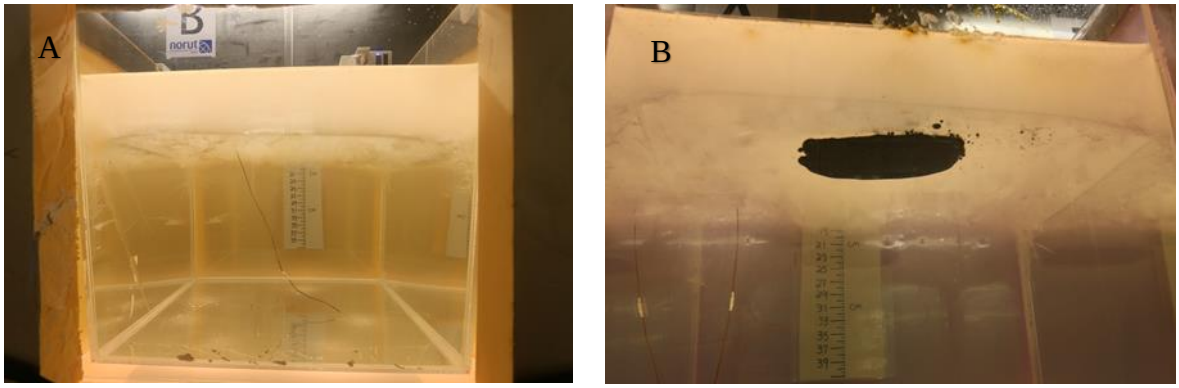

Figure 1. Sea ice mesocosm setup. A: Before oil injection; B: After oil injection

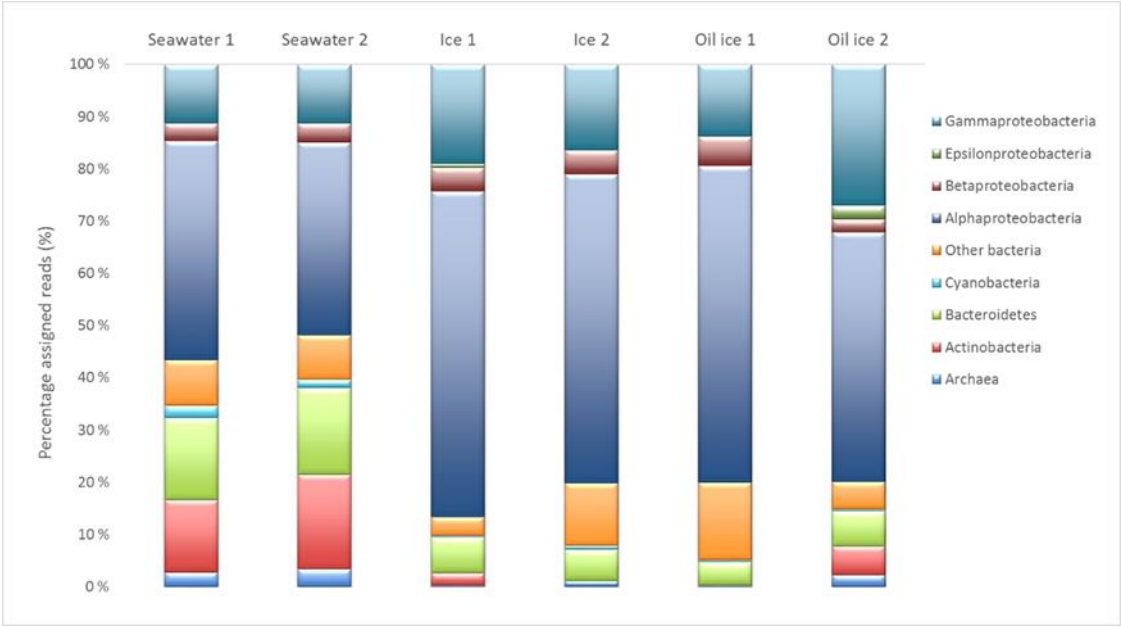

Figure 2. Relative abundance of microorganisms in original seawater compared to lab grown sea ice (not taken in account unidentified organisms)

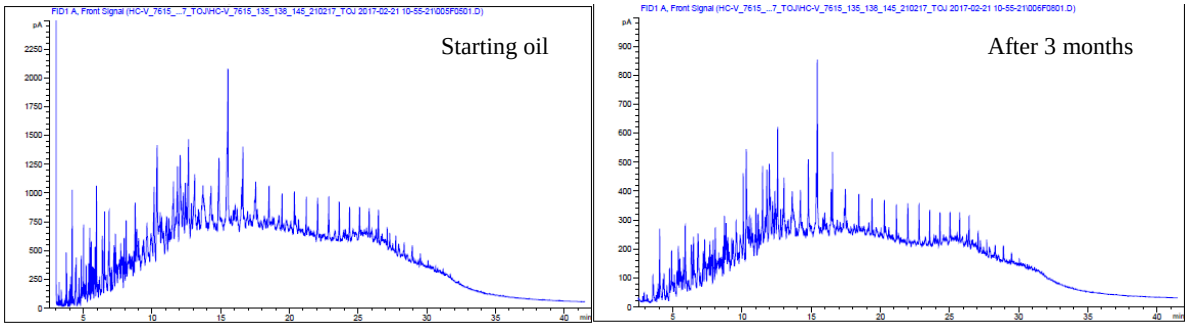

Figure 3. GC-FID chromatogram of starting Troll B oil and the encapsulated oil at -14 °C for 3 months.

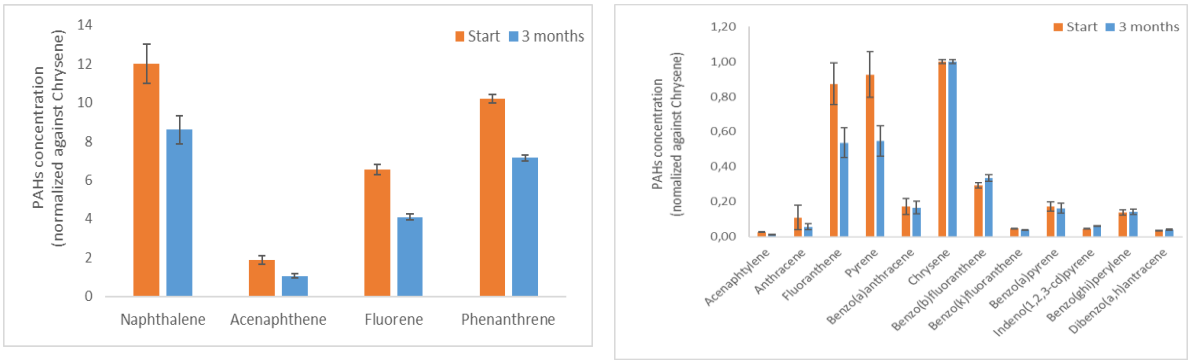

Figure 4. Depletion of 16 EPA aromatic hydrocarbons in the ice after 3 months. The depletion of the PAHs war determined after normalizing the targeted PAH against Chrysene.

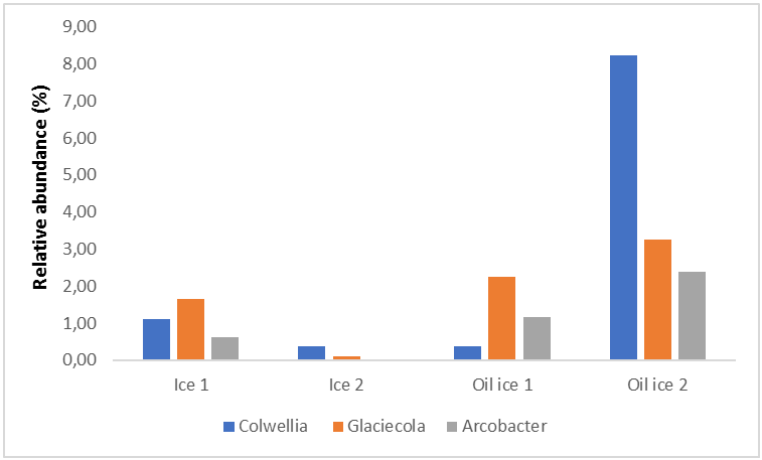

Figure 5. Increased abundance of some bacterial genera in the oil-contaminated ice compared to clean ice samples.

Table 1. Number of reads, contigs and predicted 16S rRNA sequences

Sample ID	Reads			Sequences
	Total paired reads	Filtered	Discarded	Predicted 16S rRNA
Seawater 1	9 577 768	2 991 347	8 645	13 378
Seawater 2	5 018 744	2 451 066	1 915	5 938
Ice 1	5 959 978	2 026 956	3 232	8 837
Ice 2	6 256 830	3 123 900	3 586	8 834
Ice + oil 1	4 349 480	1 113 093	3 252	6 995
Ice + oil 2	4 500 830	2 335 813	6 149	3 894

Table 2. Temperature and chemical characteristics of seawater and formed ice

Sample	Temperature (°C)	Salinity (ppt)	pH	TOC (mg/l)	Total nitrogen (mg/l)	Ammonium (mg/l)	Nitrate (mg/l)	Orthophosphate (mg/l)
Seawater 1	8	27.7 ± 0.2	7.94 ± 0.05	2.76 ± 0.02	0.5 ± 0.01	0.06	3.65 ± 0.21	0.003
Seawater 2	8	27.8 ± 0	7.97 ± 0.1	2.1 ± 0.01	0.5 ± 0.01	0.06	3.7 ± 0.28	0.003
Ice 1	-2 to -15	9.4 ± 0.7	7.8 ± 0.1			0.06	3.65 ± 0.07	0.003
Ice 2	-2 to -15	9.65 ± 0.9	8.0 ± 0.1			0.06	3.9 ± 0.14	0.003

Table 3. Summary of the bacteria and *Archaea* from the taxonomic assignment of starting seawater, formed ice and oil contaminated ice samples. Reads from the dataset predicted with rRNASelector was used for taxonomic classification with LCAClassifier using the Silva*mod* database.

Name	Reads assigned					
	Seawater 1	Seawater 2	Ice 1	Ice 2	Oil ice 1	Oil ice 2
All bacteria	11692	5166	8278	8269	6720	3538
Proteobacteria						
Alphaproteobacteria						
SAR11 clade	3145	1265	3973	3445	2964	1008
Rhodospirillaceae	405	159	338	419	362	129
Rhodobacteraceae	897	372	551	646	485	460
SAR116 clade	203	66	121	154	102	50

Other *Alphaproteobacteria*	395	112	193	281	177	82
Gammaproteobacteria						
SAR86 clade	618	276	712	605	388	103
Alteromonadaceae	249	116	254	160	253	173
Oceanospirillaceae	78	27	248	86	24	220
Other *Gammaproteobacteria*	404	182	363	517	267	482
Betaproteobacteria	403	198	380	384	374	90
Deltaproteobacteria	43	26	11	33	28	13
Epsilonproteobacteria	6	0	59	2		96
Other *Proteobacteria*	124	36	88	82	199	35
Bacteroidetes						
Flavobacteria	1796	858	558	474	285	228
Other	94	34	15	26	12	17
Other groups						
Cyanobacteria	292	82	23	62	26	12
Actinobacteria	135	66	62			
Verrucomicrobia	130	40	7	23	6	22
SAR406 clade	133	88	16	28	26	15
Candidate division OD1	147	61	28	22	5	18

All *Archaea*	336	180	24	98	22	83
Thaumarchaeota	40	21	10	76	22	66
Euryarchaeota	296	159	14	22		17

Table 4. Comparing relative abundance of several bacterial groups in *Proteobacteria* class between seawater and formed ice samples (in %)

Bacterial phylotype	Seawater 1	Seawater 2	Ice 1	Ice 2
SAR11 clade	26.1	23.7	47.9	41.2
Rhodospirillaceae	3.4	3.0	4.1	5.0
Rhodobacteraceae	7.5	7.0	6.6	7.7
SAR86 clade	5.1	5.2	8.6	7.2