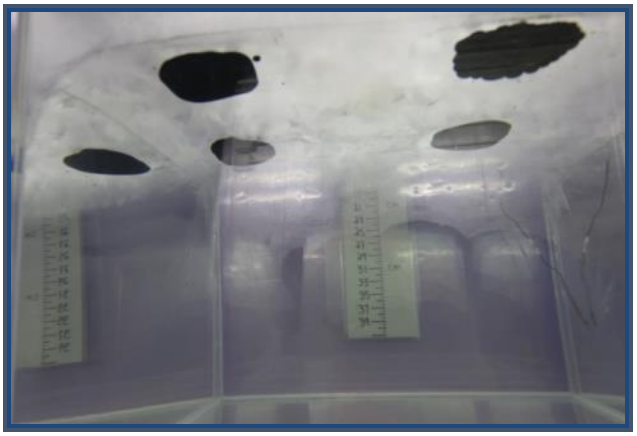
GRACE grant no 679266

Report on specific properties of oil biodegradation in sea water-ice interface

D2.2

WP2: Oil biodegradation and bioremediation



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Executive Summary

Biodegradation experiments with seawater and lab-grown ice were conducted using Norwegian Troll B (North Sea naphthtenic) crude oil and water accommodated fraction of dispersed oil (WAF+CE). The purpose of the study was to monitor the biodegradation rate of the oil and dispersed oil, and to identify the marine microorganisms involved in biodegradation process of different hydrocarbon compounds at sea ice and water interface and their biodegradation pathways. The experiment with seawater from Svalbard which was incubated with 1% (wt) Troll B crude oil at 4 °C after 8 months showed that about 30% of the total hydrocarbon compounds (THC) and 70% of 16 EPA-PAHs reduction in the seawater was due to physical loss. Biostimulation with inorganic fertilizer only slightly increased the reduction of THC and 16 EPA-PAHs concentrations by 10% and 15% respectively compared to sterile control microcosms. The ice formation experiment with seawater from Ofotjorden, Norway when incubated with WAF+CE at the concentration of 25 mg/kg, demonstrated significant depletion of volatile compounds such as BTEX and n-alkanes (nC_5-C_9) in both sterile control mesocosms and WAF+CE mesocosms. Semivolatile compounds such as C_0-C_4 decalines, 2 ring-PAHs and low-range MW n-alkanes (nC_{10}-nC_{15}) also were reduced in the ice of both types of mesocosm but with lesser extent. No reduction occurred for the medium- and heavy-range MW n-alkanes (nC_{16}-nC_{21} and nC_{22}-nC_{36}), and 3 ring-PAH/DBT and 4,5 ring-PAHs. Hence, the depletion of hydrocarbon compounds occurred also in the sterile control mesocosms, making it difficult to estimate the biodegradation of the hydrocarbons in the ices. The THC concentration in the ice of both WAF+CE and sterile control mesocosm showed reduction of about 34% and 43% after 90 days. But higher reduction was observed for the bottom water of the WAF+CE mesocosms (88%), compared to the sterile control mesocosms (35%). Marine microorganisms which involved in the biodegradation processes of hydrocarbon compounds and their degradation pathways are still under investigation. The results will be available by the end of the project.

1. Background

1.1. Previous studies on oil biodegradation in sea ice

Delille et al. (1997) were one of the first to study the biodegradation of oil in sea ice, focusing on the long term effects of diesel fuel and crude oil on microbial community in Antarctic land-fast ice located around Terre Ade'lie for over 9 months [2]. The bacterial counts increased three orders of magnitude for sea ices contaminated with diesel and crude oil, and the proportion of oil-degrading bacteria increased from <0.001% to 10% in the community after 30 weeks of contamination. Brakstad et al, (2008) conducted a field experiment at Van Mijen fjord, by introducing paraffinic oil into holes on ice during winter for 112 days [1]. The ice samples were taken and microbial community was analysed with FISH and DGGE methods. There was evidence of stimulation of microbial growth in oil contaminated ice and the microbial community became dominated by γ-Proteobacteria. The enriched microorganisms belonged to the genera *Colwellia*, *Marinomonas* and *Glaciecola*. A slow oil degradation took place at the deeper part of the ice where the oil concentration was low.

A similar result was reported by Gerdes et al, (2005) when studying the effect on crude oil on Arctic sea ice samples in microcosm experiment at 1 °C [4]. A shift in microbial community structure toward γ-Proteobacteria was observed. Gerdes (2004) also studied the effect of oil bioremediation in sea-ice [5]. In the lab experiment, the sea ice samples from different depth were incubated at -2 to -9 °C with Statfjord crude oil. The water from the gap layer which is typically formed in the Antarctic summer month was incubated with crude oil at 0 °C. The result of the gap water experiment showed that the addition of inorganic nutrient increased biodegradation of n-alkanes up to the range of C_{24}. Seeding with oil-degrading bacteria and fertilizer addition enhanced the biodegradation of n-alkanes to C_{28}, but longer chain n-alkanes and heavy PAHs were resistant to the biodegradation. Another experiment with [^{14}C]-hexadecane in sea ice at -3 °C, showed significant mineralization of [^{14}C]-hexadecane to CO_2. About 47-59% increase of ^{14}C evolved to CO_2 with addition of inorganic fertilizer and 47-62% while both inorganic fertilizer and inoculum were added to the ice. The GC-MS analysis of the crude oil incubated ice did not show a significant degradation of hydrocarbons. The author suggested that the crude oil might have inhibited the sea ice microorganisms. In addition, the exposure of the oil to the atmosphere caused weathering of the oil making it less bioavailable [5].

The field experiment was conducted with crude oil and supplemented with oleophilic fertilizer Inipol or fish meal, at Van Mijenfjorden with temperature from -30 to -2 °C [5]. Similar microbial communities were found in oil treated samples and oil plus Inipol samples. The highly viscous oil and the Inipol fertilizer which became solid when spread on the cold ice surface did not show significant effect on the bacterial sea ice community. But there was a significant change of microbial community in experiment treated with oil and fish meal. Strong reduction in the microbial diversity and increase of γ-Proteobacteria with member of *Pseudoalteromonas* sp., were observed. No significant oil degradation was observed after 2 months in any of the treated samples. The experiment treated with oil and fish meal showed high amount of microbial biomass and produced extracellular substances suggesting a significant microbial activity, hence the author suggested that the insignificant reduction of the oil components may be due to the unhomogenized sampling of crude oil at low temperature.

Faksness et al (2011) studied biodegradation of oil in artificial brine at 5, 0, -5 and -10 °C [3]. Two biodegradation experiments were conducted with: i) water soluble fractions (WSFs) of oil which was generated by stirring paraffinic crude oil in seawater and ii) n-alkane fractions of the same oil were immobilized to a hydrophobic adsorbent. In WSFs experiment at 5 and 0 °C, except for C_0-C_4 phenols, C_0-C_4 naphthalene and 2-3 rings PAHs showed rapid depletion. The biodegradation of WSFs also occurred at -5 and -10 °C. With the immobilized oil, no oil reduction was observed at those temperatures. Experiment with inorganic nutrients did not show any significant difference in biodegradation of both WSFs and the immobilized oil compared to the samples with no nutrients.

McFarline et al (2011a, 2011b) studied biodegradation of Alaska North Slope crude oil using indigenous microbes collected from Beaufort and Chukchi Seas [6,7]. Biodegradation of Alaska North Slope crude was significant in all treatment at -1 and 2 °C and dispersants enhanced it.
The most extensive and very recent study was under the Arctic Oil Spill Response Technology – Joint Industry Programme. It was the first in-ice semi open mesocosm experiment and was conducted in Svalbard, Norway. These experiments investigated the fate of the oil, oil plus dispersant and the role of microorganisms in the natural oil biodegradation process in the Arctic [8]. The results of the study showed that the majority of the oil seems to be trapped in the ice during the winter. There was a diffusion of dissolved hydrocarbon compounds from the top section of the ice until the ice-water interface and was subjected to biodegradation. For the oil trapped in the top section of the ice, biodegradation of light alkanes was observed. The Arctic microbial communities were shifted in response to the oil spill within the first month of exposure, but the changes were more visible on the activity of microorganism than on the total population.
Hence, there is evidence of oil biodegradation occurring at subzero temperatures but to what extent and which microorganisms are involved/contribute to the biodegradation process in the ice are still not known.

2.2. Objectives and activities of task 2.2

The objectives of this task are
1) assessment of the natural degradation rate of crude oil in seasonal sea-ice covered water (in seawater and sea-ice) of the Barents Sea.
2) identification of key microbial species and metabolic pathways responsible for the degradation of oil.

The study used uncontaminated seawater from Isfjord (Barents Sea) and Ofotfjord (Norwegian Sea) and Troll B (North Sea naphthenic) crude oil. Microcosm experiments with the collected seawater were performed to determine the degradation rate of crude oil as well as key microbial species and metabolic pathways responsible for degradation of the oil in the water phase. Ice mesocosms were performed in the lab with two scenarios i) oil encapsulated in ice and ii) dispersed oil incorporated in ice. With the use of dispersant, the oil was dispersed into the water and ice, hence enhancing the bioavailability of oil to sea ice microorganisms. In addition, the oil concentration was kept low to simulate real conditions, to avoid inhibition effects of oil on microorganisms, and limit of the nutrient level. In the first scenario, the response or change of sea ice-microbial communities in the presence of oil was studied. In the second scenario, both the oil degradation and change of sea ice-microbial communities in the presence of dispersed oil were studied to identify the key microbial species and metabolic pathways responsible for degradation of the oil in the water-ice interface.

2. Experiment with seawater from Svalbard

2.1. Materials & Methods

Seawater

About 100 L of surface seawater was collected at 3 locations on the Barentsburg cruise on 13 April 2016 (Fig 1). The seawater was maintained at 4 °C during transportation and storage until being used for preparing microcosms and mesocosms.

Figure 1. Location of seawater collection sites in Svalbard

Microcosm of seawater

The microcosms were conducted in 1L - Schott Duran bottles which were covered with cotton stopper. 900 ml of seawater was filled in each bottle and supplemented with 9g Troll B crude oil which was heated to 70 ºC for 30 min in 3 consecutive days for sterilizing. The biostimulation microcosm was supplemented with NPK fertilizer (22-3-10), at the concentration of 4.5 g/900 ml (130 mg/l P, 500 mg/l NO_3^-, 580 mg/l NH_4^+ as final concentrations). The microcosms were incubated at 4 ºC in the dark without agitation. The samples were collected for amplicon based and shot-gun metagenomic sequencing after 4 and 8 months.
In parallel with the microcosm of seawater, a biodegradation experiment using seawater was prepared in smaller volume, 200 ml for each setup, but using oil concentration and incubation conditions similar to those of the microcosm experiment. The biodegradation experiment was subjected to oil analysis to assess the oil biodegradation rate.

Mesocosm of sea-ice

16L seawater was filled in 18L - Plexiglass tanks. The tank was insulated at perimeter and bottom with Styrofoam, and heated from the bottom, so that the ice only grew from the surface (Fig 2).

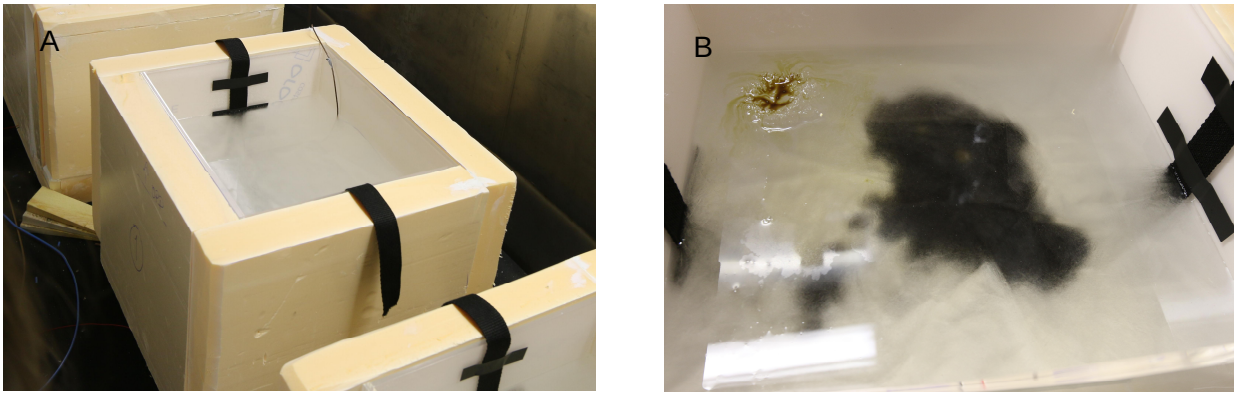


Figure 2. Ice tank mesocosms A. Clean ice tank; B. Oil encapsulated in the ice

The lab temperature was reduced to -10ºC for the ice to grow. As the ice reached 3-4 cm, 120 ml of the crude oil was injected underneath the ice. The ice continued to grow and encapsulate the oil. The tanks were than incubated at -10 ºC for 4 months in the dark.

2.2. Chemical analyses of seawater and microcosms

The seawaters from three sampling locations (SWL1, SWL2 and SWL3) had very similar characteristics, from the salinity, pH to the nutrient levels (Table 1).
There was no difference between the sterile control microcosm (MCC4 & MCC8) and oil microcosms (MCO4 & MCO8) with regard the seawater properties and nutrient concentrations. However, nitrate, ammonium and total phosphate decreased in both control and oil microcosms after 4 and 8 months compared to the starting seawater.
In the micocosms which were amended with fertilizer (MCF4 & MCF8), the nutrient levels were still rather high after 4 and 8 months of incubation, thus the availability of inorganic nutrients was not the rate-limiting factors for the oil biodegradation. The pH of those microcosms was lower, about 6.8 compared to 8-8.2 for other microcosms, suggesting stronger growth of microorganisms in the fertilizer supplemented microcosms.

2.3. Biodegradation kinetic

The total hydrocarbon concentration in the biodegradation experiment showed about 10 and 30% reduction after 4 and 8 months respectively, for the sterile control sample. Sample with only oil and fertilizer addition (NPK sample) showed almost identical results with 20 and 40% reduction of THC after 4 and 8 months respectively. Biostimulation did not show a significant effect on THC reduction compared to the sample with no treatment.
The result of 16 EPA-PAH analysis showed a high reduction in the sterile control sample. The total PAHs reduction was 65 and 70% for 4 and 8 months, respectively. The biostimulation sample showed slightly lower PAH concentration (70 and 85%) than both sterile control and oil samples.
Abiotic degradation contributed about 30 and 70% reduction of the THC and 16 EPA-PAHs, respectively. Biostimulation with NPK fertilizer did not exhibited a profound effect on oil degradation rate in our experiment. Other factors than nutrients could be the rate-limiting factors of the oil biodegradation, such as low temperature and low oil solubility/bioavailability.

Table 1. Characteristics of the collected seawater from Svalbard and the microcosms

Sample	Description	pH	Dissolved* Oxygen (mg/l)	Temperature (°C)	Salinity (ppt)	Nitrate (mg/l)	Ammonium (mg/l)	Total nitrogen (mg/l)	TOC (mg/l)	Orthophosphate (mg/l)	Total phosphate (mg/l)
SWL1	Seawater location 1	7.9	nd	2	35.5	0.4	0.078	<0.5	2.03	< 0.1	0.15
SWL2	Seawater location 2	7.95	nd	2	34.9	0.3	0.059	<0.5	2.33	< 0.1	0.12
SWL3	Seawater location 3	7.88	nd	2	35.3	0.4	0.055	<0.5	2.23	< 0.1	0.16
MCC4	Microcosm Control 4 months	7.96	10.53	4	34.6	0.67	0.036	nd	nd	nd	0.06
MCO4	Microcosm Oil 4 months	8	10.5	4	33.8	0.66	0.018	nd	nd	nd	nd
MCF4	Microcosm Oil+NPK 4 months	6.82	8.3	4	35.2	42.8	255	445	18.3	152	nd
MCC8	Microcosm control 8 months	8.23	11.8	4	35	<0.3	0.033	nd	nd	0.03	0.05
MCO8	Microcosm Oil 8 months	8.16	nd	4	34.6	<0.3	0.01	nd	nd	0.01	0.04
MCF8	Microcosm Oil+NPK 8 months	6.85	10.55	4	36	22	262	487	18	105	nd

2.4. Microbial community abundance and structure in seawater microcosms

Microbial DNA was extracted from water samples using DNeasy PowerWater Sterivex Kit (Qiagen). The qPCR assay of the 16S rDNA copy number were performed on RotorGene® Q applying RotorGene Series Software version 2.0.2. 16S rDNA amplicons were obtained from each water/ice sample and these amplicons were sequenced on Illumina MiSeq on 2x300 bp mode. DNA yield and 16S rRNA gene copy numbers are given in Table 2.

Table 2. DNA yield and 16S rRNA gene copy numbers in sea water samples from micro-and mesocosm experiments.

Sample description	DNA concentration ng/ul	16S rDNA concentration (copy/ml)
Seawater from location 3	<3	3.10×10^{4}
Seawater from location 2	<3	2.36×10^{4}
Sea-ice from Sveagruva no oil top 0-18 cm	2.0	2.44×10^{5}
Sea-ice from Sveagruva no oil bottom 18-35 cm	14.5	3.51×10^{5}
Sea-ice from Sveagruva no oil, top 0-18 cm	<3	5.14×10^{3}
Sea-ice from Sveagruva no oil, bottom 18-35 cm	<3	2.42×10^{4}
Seawater composit 1,2,3 location, replicate 1	3.1	2.32×10^{6}
Seawater composit 1,2,3 location, replicate 2	0.5	2.28×10^{6}
Seawater Svalbard beach (Marina collected 11 May, 2016), rep. 1	<3	8.91×10^{3}
Seawater Svalbard beach (Marina collected 11 May, 2016), rep.2	<3	1.47×10^{4}
Seawater Svalbard beach (Marina collected 11 May, 2016), rep. 3	<3	4.25×10^{3}
4 months microcosm seawater - control no oil, replicate 1	<3	8.17×10^{4}
4 months microcosm seawater - control no oil, replicate 2	<3	6.47×10^{4}
4 months microcosm seawater - control with oil, rep.1	<3	8.57×10^{4}
4 months microcosm seawater - control with oil, rep.2	<3	1.69×10^{5}
4 months microcosm seawater +NPK, replicate 1	46.2	2.34×10^{7}
4 months microcosm seawater +NPK, replicate 2	57.6	4.27×10^{7}
4 months mesocosm sea ice - control no oil	88.5	6.09×10^{7}
4 months mesocosm sea ice - with oil, replicate 1	26.0	9.65×10^{6}
4 months mesocosm sea ice - with oil, replicate 2	31.3	2.03×10^{7}
8 months microcosm seawater - control no oil, replicate 1	24.2	8.61×10^{6}
8 months microcosm seawater - control no oil replicate 2	10.7	3.24×10^{6}
8 months microcosm seawater - control with oil, rep. 1	8.1	3.68×10^{6}
8 months microcosm seawater - control with oil, rep. 2	8.5	5.87×10^{6}
8 months microcosm seawater +NPK_ replicate 1	44.2	1.52×10^{7}
8 months microcosm seawater +NPK_ replicate 2	74.7	2.23×10^{7}

3. Experiment with seawater from Narvik

3.1. Materials and methods

Sampling

About 200L of surface seawater was collected at Kvitvika, Narvik (68.44208° N 17.38917° E), on 8th May and 11th October 2017 (Fig 3). Preparation of water accommodated fractions of dispersed oil (WAF+CE) started about 1h after sample collection.

Figure 3. Map of the seawater sampling location in Narvik.

Preparation of water accommodated fraction with dispersant

In the first round, each mesocosm tank was filled with 13L of seawater. Crude oil and dispersant Finasol 51 was added to the seawater at concentrations of 1g oil and 0.1g Finasol 51 per 1 liter of seawater. The mixture was vortexed at 400 rpm at 5°C for 20 h. After vortexing, the mixture was allowed to separate at 4°C for 2 h in a bottom-tap glass bottle (Fig 4A). The aqueous phase was withdrawn through the tap.

In the second round, 5 g of the oil and dispersant mixture was added to 1L of seawater instead of 1g. The mixture was vortexed at 400 rpm at 4°C for 40 h. After vortexing, the mixture was allowed to separate at 4°C for 2 h in a bottom-tap glass bottle. For the mesocosm, 1.2kg WAF+CE was diluted with 14.8 kg seawater to make up 16 kg.

A

B

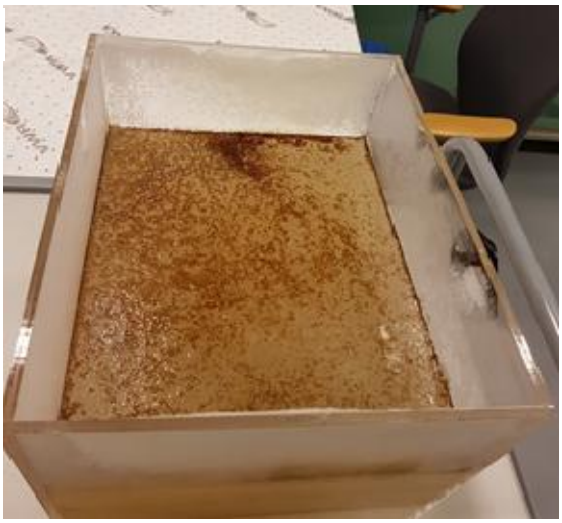


Figure 4. Ice mesocosm of water accommodated fraction and dispersant (WAF+CE). A. Bottom tap bottle with WAF+CE; B. Ice formation from WAF+CE.

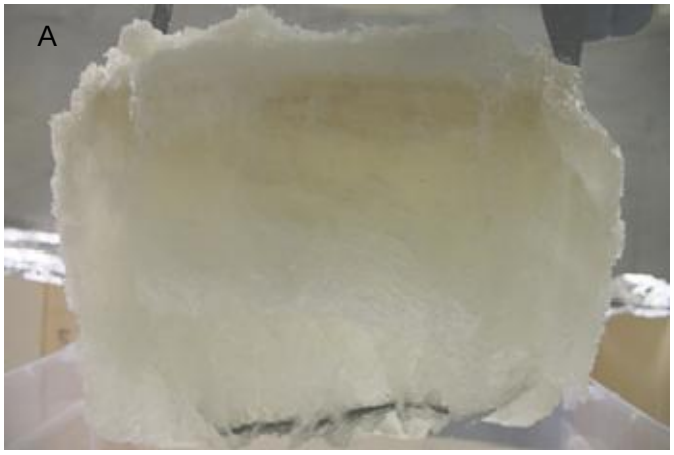
Mesocosms with dispersed oil for metagenomic sequencing

In each mesocosm, 16L of WAF+CE or sterile control WAF+CE were added. For the sterile control, the seawater was filtered prior use. The tanks were insulated as described previously but without heating from the bottom.

The lab temperature was reduced to -1°C for 24 h to precool the seawater. Before the temperature adjustment, about 5L of seawater from each tank was collected as the starting point. Afterward, the temperature was further reduced to -10°C for the ice to grow (Fig 5). The ice temperature ranged from -10 to -2 °C from the top to the bottom ice. The ice was sampled after 12 and 28 days (from the day the temperature was set at -10°C) by cutting half of the tank each time. Since large ice samples were required for metagenomic sequencing, the ice could not be sampled more frequently. After 12 days, the ice was 13cm in the center and 21 cm at the edges and corners. After 28 days, the ice was about 21 cm thick (Fig 5A).

From each setup about 5L of the seawater was sampled for DNA analysis, just right before lowering the temperature to -1°C. The ice samples were melted at room temperature for 24 h for biomass collection.

A



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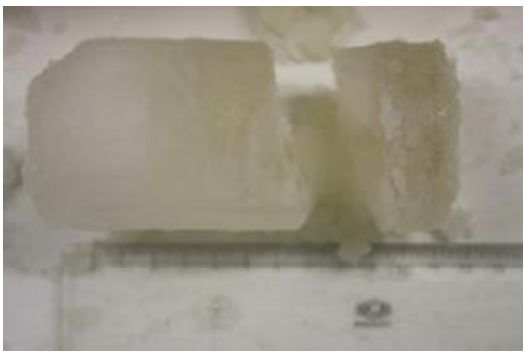

Figure 5. Ice samples from mesocosms. A. Large ice sample; B. Ice core sampled with corer.

Mesocosms with dispersed oil (oil analysis and 16S amplicon sequencing)

The mesocosms were setup as described above but with heating at the bottom so the ice did not grow to the bottom of the tank (free floating ice). The ice was grown at -15 °C instead of -10 °C (with bottom heating, the ice grew slower than without heating therefore, the room temperature was reduced). Both the ice and water temperature were monitored over time. The temperature profile of the ice ranged from -10 to -2 °C, from the top to the bottom ice. The water temperature was about -1 to -2 °C. Ice cores were sampled at 0, 10, 20, 34 and 90 days for both oil analysis and 16S rDNA amplicon sequencing. To avoid the draining of brine from the ices due to the lowering of the water level after sampling, sterile bags filled with autoclaved artificial seawater were put into the sampling holes to replace the sampled ice.
The ice was melted in the same volume of filtered-sterile artificial seawater at 4 °C overnight.

Figure 6. Ice tank mesocosms with water accommodated fractions of dispersed oil (WAF+CE). A. Ice tanks in the cold lab; B. Sterile control tank with water accommodated fractions of dispersed oil.

3.2. Biodegradation of dispersed oil

Analysis of the total hydrocarbon compounds (THC) in the ice after 90 days showed a reduction from 25 mg/kg to 14 and 16 mg/kg (57 and 66%) in the sterile control mesocosms and WAF+CE mesocosms, respectively. But much higher THC reduction was observed in the bottom water of the WAF+CE mesocosms compared to the sterile control mesocosms. The THC decreased from 25 mg/kg to 2.9 mg/kg (removal efficiency 87.4%) in the bottom water of the WAF+CE mesocosms but only reduced to 16.2 mg/kg (removal efficiency 35.2%) in the sterile control mesocosms, suggesting a stronger biodegradation occurred in the under-ice water.
Depletion of semivolatile (SVOC) nC_{10}–nC_{36} alkanes, non-alkylated and alkyl-substituted decalines, naphthalenes, fluorenes, phenanthrenes, dibenzothiophenes, fluoranthenes and chrysenes were recorded in the ice after normalization against the persistent biomarker 17a(H),21b(H)-Hopane (30ab Hopane), commonly used in biodegradation studies [9,10,11]. The depletions were determined as percentages of normalized data at the start of the experiment (day 0) for each target oil compound. The results of SVOCs analysis of the ice samples at 10, 20, 34 and 90 days did not show any changes of the medium and heavy-range molecular weight (MW) n-alkanes (nC_{16}-nC_{36}), and medium heavy-range MW PAH-compounds (3 ring PAH/DBT and 4,5 ring-PAHs).
Low range MW n-alkanes (nC_{10}-nC_{15}) in the ices reduced to 68-71% of the starting concentration for both control and WAF+CE mesocosms after 90 days. Low-range MW PAH-compounds (2 ring-PAH) reduced to 39% of the starting concentration in the control mesocosms but to 63% in the WAF-CE mesocosms. C_0-C_4 decalines reduced to 39% of the starting concentration in the control mesocosms and 66% in the WAF-CE mesocosms.
Analysis of volatile compounds (VOC) BTEX and n-alkane (nC_5-nC_9) in the ice samples showed significant depletion. BTEX reduced to 10% for control mesocosm and 40% for WAF+CE mesocosm, after 34 days. n-alkane (nC_5-nC_9) reduced to 29 and 10% for control samples and WAF+CE samples respectively.

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