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Biodegradation of petroleum hydrocarbons in seawater at low temperatures (0–5 °C) and bacterial communities associated with degradation

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Abstract

In this study biodegradation of hydrocarbons in thin oil films was investigated in seawater at low temperatures, 0 and 5 °C. Heterotrophic (HM) or oil-degrading (ODM) microorganisms enriched at the two temperatures showed 16S rRNA sequence similarities to several bacteria of Arctic or Antarctic origin. Biodegradation experiments were conducted with a crude mineral oil immobilized as thin films on hydrophobic Fluortex adsorbents in nutrient-enriched or sterile seawater. Chemical and respirometric analysis of hydrocarbon depletion showed that naphthalene and other small aromatic hydrocarbons (HCs) were primarily biodegraded after dissolution to the water phase, while biodegradation of larger polyaromatic hydrocarbons (PAH) and C_{10}–C_{36} *n*-alkanes, including *n*-hexadecane, was associated primarily with the oil films. Biodegradation of PAH and *n*-alkanes was significant at both 0 and 5 °C, but was decreased for several compounds at the lower temperature. *n*-Hexadecane biodegradation at the two temperatures was comparable at the end of the experiments, but was delayed at 0 °C. Investigations of bacterial communities in seawater and on adsorbents by PCR amplification of 16S rRNA gene fragments and DGGE analysis indicated that predominant bacteria in the seawater gradually adhered to the oil-coated adsorbents during biodegradation at both temperatures. Sequence analysis of most DGGE bands aligned to members of the phyla *Proteobacteria* (*Gammaproteobacteria*) or *Bacteroidetes*. Most sequences from experiments at 0 °C revealed affiliations to members of Arctic or Antarctic consortia, while no such homology was detected for sequences from degradation experiment run at 5 °C. In conclusion, marine microbial communities from cold seawater have potentials for oil film HC degradation at temperatures ≤5 °C, and psychrotrophic or psychrophilic bacteria may play an important role during oil HC biodegradation in seawater close to freezing point.

Introduction

Exploration, production, and transport of oil are increasing in the cold-water regions, including the Norwegian and Barents Seas. In these environments (e.g. from 60° N to the ice margins) the average surface seawater temperatures range from 10 °C to <0 °C, while temperatures below the thermoclines are permanently 5 °C or colder (http://www.cdc.noaa.gov). Vast petroleum reserves exist in these regions, and the needs for studies of environmental impacts of oil spills are therefore increasingly important.

In seawater several weathering processes contribute to the natural attenuation of discharged oil, including surface evaporation, hydrocarbon (HC) dissolution from oil films and droplets, photo-oxidation, emulsion, dispersion, and biodegradation, and several of these processes are affected by seawater temperatures. Weathering

processes in cold environments will be further affected by oil characteristics like wax content and viscosity (Walker & Colwell 1974). For instance, long-chain HC-compounds precipitate as waxes in cold environments, reducing the bioavailability of the oil.

Several studies have focused on hydrocarbon biodegradation in seawater or soil under Arctic or Antarctic conditions (e.g. Colwell et al. 1978; Siron et al. 1995; Cavanagh et al. 1998; Delille et al. 1998; Aislabie et al. 2000; Eriksson et al. 2001; Delille & Pelletier 2002; Garret et al. 2003). Thus, biodegradation as an oil attenuation process in cold environments is well documented.

The prevalence for HC biodegradation exists in pristine cold environments, and alkane monooxygenases and hydrocarbonoclastic bacteria have been detected and characterized in both Arctic and Antarctic environments (Whyte et al. 1996; Delille et al. 1997; Fiala & Delille 1999; Whyte et al. 2002; Yakimov et al. 2003). Studies of oil-polluted Polar environments have shown abundance of *Alphaproteobacteria*, *Gammaproteobacteria* and *Actinobacteria* (Grossman et al. 1999; Aislabie et al. 2000, Juck et al. 2000; Yakimov et al. 2003).

In this study we investigated seawater biodegradation of petroleum HCs associated with thin oil films at temperatures of 0 and 5 °C. These films were generated by immobilization to solid matrices, as recently described (Brakstad et al. 2002; Brakstad et al. 2004). HC depletion from the oil films were determined as dissolution or biotransformation of natural oil compounds, while mineralization was examined with radiolabelled compounds spiked in the oil. Microbial communities associated with the oil films were characterized during the biodegradation experiments.

Materials and methods

Seawater source

Seawater was collected from a depth of 90 m in a non-polluted Norwegian fjord (Trondheimsfjord; 63°26′ N, 10°26′ E), in January 2004. The water was collected from a continuous pipeline seawater supply system, and was filtered (50 μm) to remove coarse particles. The following seawater parameters were recorded at the time of collection: Temperature (5.8 °C), dissolved oxygen (9.07 mg/l), salinity (33.8%), *o*-PO_4^{3-} (25.8 mg/l), NH_4^+ (<5 mg/l), the sum of NO_3^- and NO_2^- (136.8 mg/l), dissolved organic carbon (1.1 mg/l) and colony-forming units (CFU) in Marin Agar 2216 (1.39×10^4 CFU/ml).

Enrichment cultures of heterotrophic and oil-degrading microorganisms

Fresh seawater (2 ml) was inoculated in autoclaved 250 ml Erlenmeyer flasks with 100 ml of Marine Broth 2216 (Difo Laboratories, Detroit, Mi, U.S.A.) for enrichment of heterotrophic microorganisms (HM), while oil-degrading microoganisms (ODM) were enriched in Bushnell-Haas broth (Difco), supplemented with 30 g/l NaCl, and 0.1% (vol/vol) of a crude Statfjord oil as carbon source (Statfjord batch no. 97–0264; specific density 0.859 g/cm^3). The vials were incubated at 0 or 5°C for 28 days with continuous shaking (150 r.p.m).

Epifluorescence and fluorescence in-situ *hybridization (FISH) microscopy*

Total numbers of microbial cell were enumerated by epifluorescence microscopy (DAPI), while distributions of bacteria and archaea were determined by FISH analysis with the Cy3-labelled domain-specific probes EUB338 and ARCH915, or a nonsense probe (NON33), as previously described (Glöckner et al. 1999).

Biotransformation experiments with immobilized oil

The crude Statfjord oil was heated to 30 °C for 90 min in a closed bottle to melt wax particles, equilibrated at 20 °C for 2 h, and 50 μg oil carefully applied to the surface of 100 ml autoclaved (121 °C; 15 min) seawater in a beaker (i.d. 8.5 cm). The oil immediately generated a thin film across the surface. The oil was immobilized on hydrophobic Fluortex adsorbents (Sefar Inc., Thal; Switzerland; production reference 09–150/36), consisting of ETFE monofilaments (polymer of tetrafluoroethylene and ethylene). The adsorbents were cut in squares of 1 cm^2 (1 × 1 cm), washed in dichloromethane (DCM), rinsed in sterile seawater, and applied on the oil film surface for 60 min. Excess oil was removed from the adsorbents by careful washes in two baths of sterile seawater. A

thin fishing line (0.030 mm) with knots in the end (prewashed in DCM and rinsed in sterile seawater) was carefully forced through the filament structure of each adsorbent. Fresh seawater was acclimated to test temperatures (0 or 5 °C) for 5–7 days and aerated with sterile air. Previous results from static seawater experiments in our lab showed that HC biodegradation was increased by 40% if a mineral solution was added (not published), and a solution of inorganic nutrients (8.5 mg/l KH_2PO_4, 21.8 mg/l K_2HPO_4, 33.3 mg/l Na_2HPO_4, 100 mg/l NH_4NO_3, and 0.25 mg/l $FeCl_3 \times 6H_2O$) was therefore supplied. Acclimated and nutrient-supplemented seawater (50 ml) was dispensed into 100 ml autoclaved infusion bottles with butyl rubber septa. Individual adsorbents with immobilized oil were submerged into the water of each bottle with the aid of the fishing lines, and the bottles capped. Bottles with immobilized oil in sterilized seawater (50 mg/l $HgCl_2$) were used as sterile controls. Biodegradation experiments were conducted at 5 and 0°C for 56 days. Adsorbents were removed in duplicate for chemical analysis at days 0, 1, 2, 4, 7, 10, 14, 21, 28, 35, 42, and 56. In addition, duplicate samples of adsorbents were withdrawn after 0, 7, 14, 28, and 56 days for extraction of nucleic acids.

Mineralization experiments with ^{14}C-labelled hydrocarbons

The fresh Statfjord oil was spiked with 4 μCi naphthalene UL–^{14} C (specific activity 31.3 mCi/mmol), 2 μCi phenanthrene-9–^{14}C (specific activity 8.2 mCi/mmol), or 2 μCi *n*-hexadecane-1–^{14}C (specific activity 12 mCi/mmol) (Sigma Chemical Co., St. Louis, Mo, U.S.A). Generation of thin oil films and immobilization on fabrics were essentially performed as described for the biotransformation experiments, except that the oil films were generated with 100 μg oil. The seawater used as inoculum was supplemented with inorganic nutrients as described above.

Bottles, supplemented seawater (50 ml), sterile controls, and submerged adsorbents were as described above (biotransformation experiments). For mineralization experiments a CO_2 trap was included in each bottle, consisting of a 5 ml glass tube with 1.0 ml 1 M KOH. The bottles were capped and incubated at 0 or 5 °C for 0–56 days, and $^{14}CO_2$ measured in triplicate at days 0, 1, 2, 4, 7, 10, 14, 21, 28, 35, 42, and 56. CO_2 in the water was released to the headspace by acidifying the solution (50 ml) by injecting 1.0 ml 1 M HCl into the capped bottles 4 h before measurement. Trapped $^{14}CO_2$ was counted by retrieving the KOH solution to a glass scintillation vial with 10 ml Hionic Fluor scintillation cocktail (Packard BioScience B.V., Groningen, The Netherlands).

Chemical analysis

Chemical analyses were performed mainly as described by Hokstad et al. (1999). Briefly, adsorbents were placed in 50 ml DCM. The solvent was dried (Na_2SO_4), filtered (glass wool), and evaporated to volumes of 0.5–1.0 ml in 2 ml GC vials on a TurboVap 500 closed cell concentrator (Zymark Co., Hopkinton, Ma, U.S.A.). A surrogate recovery standard of *o*-terphenyl was added to a final concentration of 20 μg/ml. Determination of C_{10}–C_{36} total extractable organic carbon (TEOC), C_{10}–C_{36} *n*-alkanes and *n*-hexadecane in DCM extracts was performed by GC–FID analysis (HP Model HP5890II gas chromatograph with a flame ionisation detector; Agilent Technologies), using a Durabond DB-5 (15 m × 0.25 μm i.d.) column, hydrogen carrier gas (flow 2.2 ml/min), and a temperature programme of 40 °C (5 min) −6 °C/min −310 °C (10 min) splitless in 40 s. Aromatic compounds were determined by GC–MS analysis (HP 6890 gas chromatograph with HP 5973 mass selective detector; Agilent) with a HP–5MS (60 m × 0.25 μ m) column, helium carrier gas (flow 1.0 ml/min), a temperature programme in the column of 40 °C (1 min) −°C/min −300 °C (20 min), and with an injector temperature of 300 °C. GC–MS analysis included naphthalene, phenanthrene, and the HC-compound groups Naph-1 (C_0-C_1 naphthalenes), Naph-2 (C_2-C_3 naphthalenes), PAH-1 (C_4-naphthalenes, biphenyl, acenaphthylene, acenaphthene, dibenzofurane, C_0- to C_1-fluorenes, C_0- to C_1-phenanthrenes/anthracenes, C_0- to C_1- dibenzothiophenes), and PAH-2 (C_2- to C_3-fluorenes, C_2- to C_4- phenanthrenes/ anthracenes, C_2- to C_4-dibenzothiophenes, fluoranthrene, pyrene, C_1- to C_3- fluoranthrenes/pyrenes, benz[a]anthracene, C_0- to C_4-crysenes, benzo[b,k]fluoranthene, benzo[e, a] pyrene, perylene, dibenzo[a, h]anthracene, benzo[g, h, i]perylene, indeno[1,2,3-c, d]pyrene).

PCR and denaturing gradient gel electrophoresis (DGGE)

Enrichment cultures (1 ml) were pelleted by centrifugation (14,000 × *g*; 5 min), while seawater samples were filtered through Durapore filters (Millipore, Bedford, Ma, U.S.A.) with 0.22 μm exclusion limit. Pellets and filters were extracted by GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) as described by the manufacturer. Nucleic acids on the Fluortex adsorbents were extracted by hot phenol–chlorophorm–isoamylalcohol (Sambrook & Russel 2001). Recovered nucleic acids were quantified by ethidium bromide (Sambrook & Russel 2001) and stored at −20 °C until analysis.

PCR amplification of bacterial 16S rRNA gene fragments was performed with the domain-specific primers Bac341f (5′-CCT ACG GGA GGC AGC AG-3′) and Bac907r (5′-CCC CGT CAA TTC CTT TGA GTT-3′) (Muyzer et al. 1993; Teske et al. 1996), yielding a PCR fragment of 550 bp (Muyzer et al. 1993). For DGGE a 40-mer GC-clamp (5′- CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′) was added to the 5′-end of the Bac341f primer (Muyzer et al. 1993). The amplification was conducted as a "touchdown" PCR to reduce formation of spurious by-products (Don et al. 1991), as previously described (Teske et al. 1996). Annealing temperature was initially set at 65 °C, then decreased by 1°C every cycle until 55 °C, at which point 25 additional cycles were carried out.

DGGE was performed with a continuous gradient of 20–70% of the denaturing agents urea and formamide (100% denaturants corresponded to 7 M urea and 40% deionised formamide), essentially as described by Teske et al. (1996). Each well contained 0.5–1.0 μg DNA. DGGE was run at 60 °C in a DCode Universal Mutation Detection System (Bio-Rad, Hercules, Ca, U.S.A.) at 150 V constant voltage for 4.5 h. Gels were stained for 20–30 min with SYBR Gold (Molecular Probes, Leiden, The Netherlands), and stained gels were scanned in a GelDoc system (Bio-Rad).

Cloning and sequencing

PCR fragments from enrichment cultures were purified (Perfectprep Gel cleanup kit; Eppendorf AG, Hamburg, Germany) from standard agarose gels after electrophoresis (150 V, 1.5–2 h). Selected DGGE bands were carefully cut out from the gels with sterile scalpels, eluted overnight at 4°C in 50 μl sterile water, and PCR re-amplified. The amplicons were cloned with the Qiagen PCR CloningPlus Kit (Qiagen GmbH, Hilden, Germany), as described by the manufacturer. Cells were spread on LB agar plates with ampicillin (100 μg/ml agar), IPTG (50 μM) and X-Gal (80 μg/ml), and 5–10 putative positive clones from each DGGE band were transferred to LB broth with ampicillin. Plasmids were prepared by GeneElute Plasmid Miniprep kit (Sigma), and plasmids stored at −20 °C.

Plasmid inserts of 16S rRNA gene fragments were amplified using primers for the M13 sites on the plasmid. These primers targeted sequences flanking the inserts on the plasmids (Sambrook & Russel 2001). M13 PCR products were subjected to restriction fragment length polymorphism (RFLP) analysis with *Hae*III and *Rsa*I (Massana et al. 1997), and selected amplicons were submitted for partial sequence analysis (Eurogentec, Ivoz-Ramet, Belgium). Sequence alignments were performed by the BLAST program of the National Centre for Biotechnology Information (Altschul et al. 1997).

The 16S rRNA gene sequences were submitted to GenBank and have been assigned to the following accession numbers: AY831470 to AY831476 (ODM cultures) and AY831477 to AY831482 (representative sequences from DGGE bands).

Statistical analysis

Parametric two-tailed *t*-test analysis was performed by GraphPad Prism 4.0 (GraphPad Software, Inc., San Diego, Ca, U.S.A.).

Results

Enrichments of HM and ODM from the seawater

Cultures of HM and ODM were enriched at 0 and 5 °C (Figure 1). HM cultures reached stationary phases after 7 (5 °C) or 21 (0°C) days, with concentrations of 1.09×10^9 (SD $\pm 0.28 \times 10^9$) cells ml^{-1} at 0 °C and 1.72×10^9 (SD $\pm 0.25 \times 10^9$) cell ml^{-1} at 5 °C, while the concentrations of ODM at

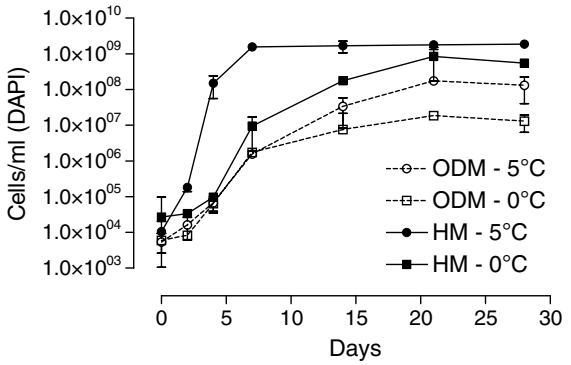

Figure 1. Growth curves at 0 and 5 °C of heterotrophic (HM) and oil-degrading microorganisms (ODM) from seawater collected at 90 m depth (Trondheimsfjord). Triplicate samples of inocula were incubated in Marine Broth 2216 and marine Bushnell-Haas medium with Statfjord oil as carbon source for enrichments of HM and ODM, respectively. Error bars represent standard deviations.

the stationary phase (21 days) was lower, 1.04×10^7 (SD $\pm 0.17 \times 10^7$) cells ml^{-1} at 0 °C and 1.77×10^8 (SD $\pm 0.31 \times 10^8$) cells ml^{-1} at 5 °C. Comparison of the HM and ODM growth curves at 0 and 5°C (Figure 1) by two-tailed *t*-test analysis revealed that all curves differed significantly ($p < 0.05$), except for the ODM cultures ($p = 0.15$).

Bacterial and archaeal fractions of the prokaryotic populations in the original seawater, and in enrichment cultures after 14 days of incubation, were determined by epifluorescence (DAPI) and FISH (domain-specific DNA probes) microscopy (Table 1). The distribution of bacteria and archaea corresponded well to the DAPI counts in the original seawater. Enrichment cultures showed lower fractions of archaea than in seawater, and these culture media therefore stimulated growth of bacterial rather then archaeal microbes. Table 1 also shows that large portions of the microbes were not detected by any of the domain-specific probes in the cultures at 0 °C.

A clone library ($n = 21$) of PCR amplified bacterial 16S rRNA gene fragments was generated from each of two ODM enrichment cultures incubated at 0 or 5 °C for 28 days. The clones from 0 °C ($n = 15$) and 5 °C ($n = 6$) ODM cultures were separated in 3 and 4 RFLP types, respectively. No RFLP types appeared in clones from both cultures. The results from sequence analysis of clones representing each RFLP type are shown in Table 2. Three sequences from the 0 °C (representing 16 clones), and one sequence from the 5 °C ODM culture (representing 1 clone), showed similarities to Arctic or Antarctic bacteria, including the *Gammaproteobacteria Marinomonas protea*, *Pseudomonas syringae*, Arctic sea ice bacterium ARK1003, and *Pseudoalteromonas* sp. CAM36 (Ray et al. 1998; Brinkmeyer et al. 2003; Gilbert et al. 2004; Nichols et al. 2004). Also bacteria of the genus *Acinetobacter* have been frequently reported from Antarctic or Arctic environments (Luz et al. 2004; Whyte et al. 2002).

Depletion of HC compound groups from adsorbents

The average amount of C_{10}–C_{36} TEOC immobilized on individual Fluortex adsorbents was 393 μg (95% confidence interval of 51 μg TEOC) when oil films of 50 μl in seawater were generated (see Materials and methods). Depletion rates of HC compound groups (described in Materials and methods) from oil films were examined at the end of the biodegradation tests (56 days) for experiments run at both 0 and 5 °C (Figure 2). For all compound groups the depletion was higher in

Table 1. Comparison of DAPI and FISH counts in original seawater, and in HM and ODM enrichment cultures after 14 days of incubation at 5 and 0 °C

Sample	Total cell count (10^6 ml $^{-1}$) (± SD)	Fraction (%) of total cells (mean ± SD)[a]		
		EUB338	ARCH 915	NON338
Seawater	0.72 ± 0.09	81.3 ± 4.5	20.5 ± 0.5	7.5 ± 3.1
ODM-5°C	27.3 ± 9.0	70.7 ± 19.2	2.5 ± 1.2	1.0 ± 0.4
ODM-0°C	12.1 ± 2.1	27.9 ± 7.3	3.7 ± 1.4	0.7 ± 0.4
HM-5°C	1640 ± 21.9	51.3 ± 12.3	3.6 ± 2.6	1.3 ± 0.9
HM-0°C	168 ± 17.9	29.2 ± 8.2	3.7 ± 1.3	1.0 ± 0.2

[a]percentage of corresponding DAPI concentrations

Table 2. Alignment of bacterial 16S rRNA gene fragments originating from enrichment cultures of ODM cultured at 5 or 0 °C. Sequenced fragments represented separate RFLP types

Culture (°C)	Clones (no.)[a]	Phylum or class	Closest match (% similarity)
0	5	*Gammaproteobacteria*	*Marinomonas protea*[b] (94)
0	6	*Gammaproteobacteria*	*Pseudomonas syringae* pv. atropurpurea[b] (98)
0	4	*Gammaproteobacteria*	Arctic sea ice bacterium ARK10032[b] (96)
5	2	*Alphaproteobacteria*	*Rhodocista* sp. AT2107 (98)
5	2	*Gammaproteobacteria*	*Acinetobacter* sp., DSM590 (95)
5	1	*Gammaproteobacteria*	*Pseudoalteromonas* sp. CAM36[b] (99)
5	1	*Alphaproteobacteria*	*Paracoccus* sp. DY19 (98)

[a]No. clones representing individual RFLP types
[b]Sequences reported from Arctic or Antarctic consortia

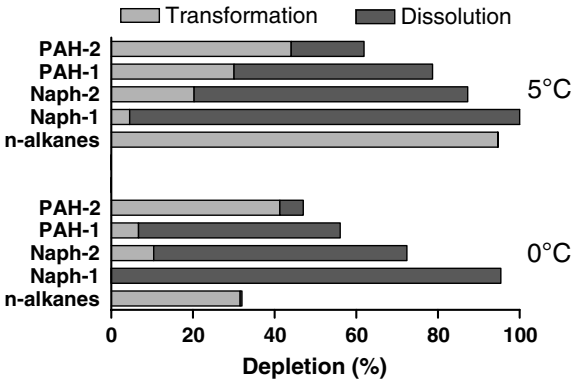

Figure 2. Depletion of C_{10}–C_{36} *n*-alkanes, Naph-1, Naph-2, PAH-1, and PAH-2 from oil-coated Fluortex adsorbents. The results are calculated as the distribution between dissolution and biotransformation after incubation in 56 days at 0 or 5 °C.

biotic seawater at 5 °C (ranging from 61.8 to 100%) than at 0 °C (ranging from 31.6 to 89.5%). We anticipated that biotransformation was the major process representing the differences between total HC depletion in nutrient-enriched seawater (biotic system) and dissolution from the oil films measured in the sterile waters. Using this assumption the biotransformation contributed to the depletion of HCs from adsorbents at 5 and 0 °C according to the trend *n*-alkanes > PAH-2 > PAH-1 > Naph-2 > NAPH-1. Transformation was the major process in the PAH-2 (alkylated 3-ring and 4- to 5-ring PAH) and *n*-alkane groups, contributing to 71.2–100% of the depletion at both temperatures. In sterile systems the mean differences between oil HC reductions at 0 and 5 °C were not significant ($p > 0.05$), indicating that the temperature differences had only moderate or negligible influences of dissolution.

Biodegradation and dissolution of single HC compounds

Biodegradation and dissolution of the HC compounds *n*-hexadecane, naphthalene and phenanthrene, immobilised on the oil-coated adsorbents, were determined at 0 and 5 °C. These compounds represented HCs with different water-solubilities, and depletion curves are shown in Figure 3. Naphthalene was rapidly depleted from the adsorbents in both nutrient-enriched and in sterile seawater at both water temperatures, and more than 80% depletion from the adsorbents was measured in all waters after 1 day of incubation in both biotic and abiotic systems. *T*-test analysis did not show significant differences ($p > 0.05$) between any of the curves. Thus, depletion in biotic and abiotic systems was comparable at both 0 and 5 °C incubation temperatures, and dissolution was the major depletion process for this compound. With phenanthrene considerable dissolution appeared at the end of the experiment in abiotic systems (29% at 0 °C and 47% at 5 °C). However, depletion was higher in biotic seawater (90% at 5 °C and 61% at 0 °C), and the differences to the corresponding abiotic systems were significant ($p < 0.05$ at both 5 and 0 °C). Thus, biodegradation accelerated the depletion of phenanthrene from the adsorbents. In addition, significant differences related to temperature were determined for phenanthrene depletion in both biotic and abiotic seawater ($p < 0.05$). *n*-Hexadecane depletion was exclusively caused by biotransformation, since dissolution in abiotic systems was negligible (Figure 3). An elevated lag-phase was measured at 0°C, but at the end of the depletion period (day 56) the depletion was 99% at 5 °C and 89%

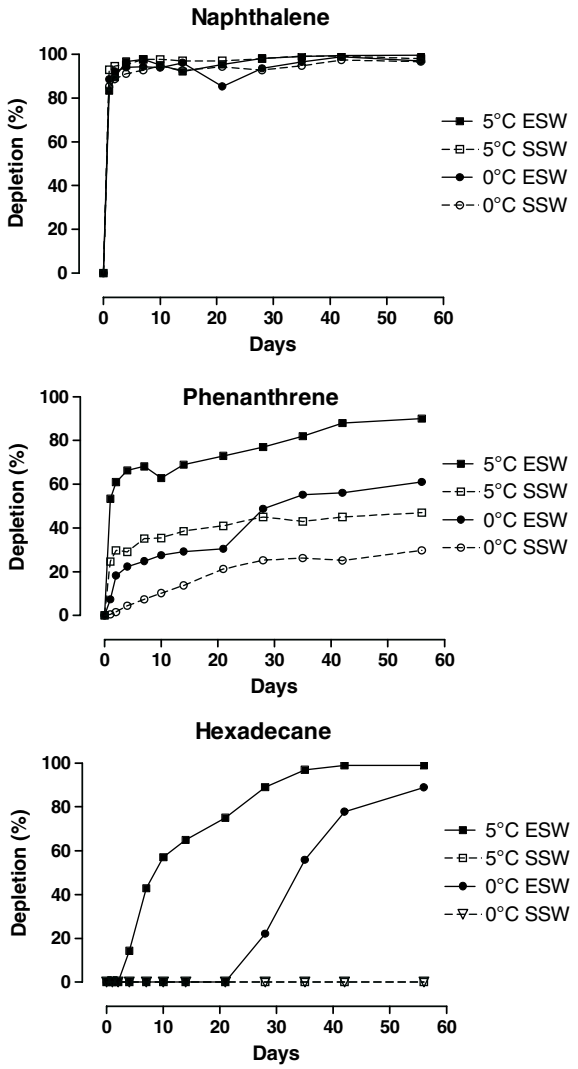

Figure 3. Depletion of naphthalene, phenanthrene, and *n*-hexadecane from oil-coated Fluortex adsorbents (mean values of two replicates) in nutrient-enriched (ESW) and sterile (SSW) seawater during static experiments run at 0 or 5 °C.

at 0 °C, showing that transformation was only delayed at the lower temperature.

The depletion results at the end of the tests for the single compounds were also compared with their respective HC groups (see Figure 2), i.e. *n*-hexadecane to C_{10}–C_{36} *n*-alkanes, naphthalene to Naph-1, and phenanthrene to PAH-1. Dissolution and biotransformation results did not differ significantly ($p > 0.05$) between single compounds and their HC groups.

Mineralization of *n*-hexadecane, naphthalene and phenanthrene was determined with ^{14}C-labelled compounds which were spiked separately in the crude oil before immobilisation. Determination of $^{14}CO_2$ in seawater at 0 or 5 °C (Figure 4) showed that naphthalene and phenanthrene mineralization were both temperature-related. Naphthalene mineralization reached 60 and 19% after day 56 at 5 and 0 °C, respectively, and the curves differed significantly ($p < 0.05$), while phenanthrene mineralization could only be recorded at 5 °C. Mineralization of phenanthrene at 5 °C was slower than for naphthalene at the same temperature. *n*-Hexadecane mineralization was not temperature-related. At the end of the test period mineralization of this HC was 53% at 5 °C and 67% at 0 °C, and

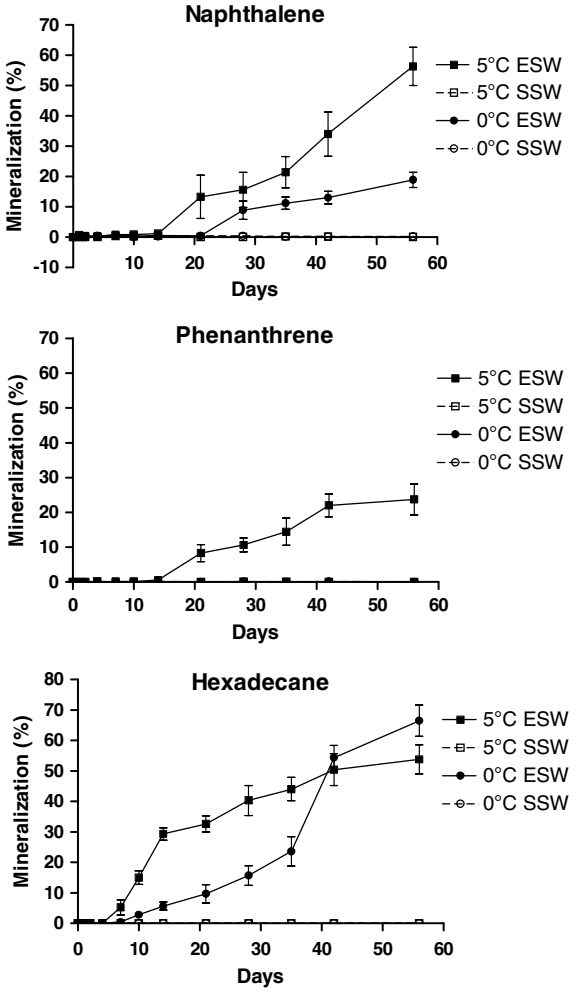

Figure 4. Mineralization of naphthalene, phenanthrene, and *n*-hexadecane from oil-coated Fluortex adsorbents seawater during biodegradation experiment at 0 and 5 °C. Error bars represent standard deviations of results with triplicate samples. For further explanations, see Figure 3.

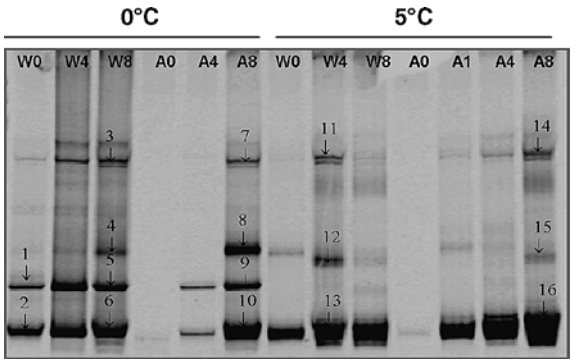

Figure 5. PCR–DGGE analysis of bacterial 16S rRNA genes extracted from oil-coated Fluortex adsorbents (A) or seawater (W) during biodegradation experiments at 0 and 5°C. Samples were analysed at weeks 0 (W0, A0), 4 (W4, A4) and 8 (W8, A8) of the experiments. Extract from adsorbents at 5°C was also analysed at week 1 (A1). DGGE-bands removed for cloning and sequencing are numbered 1–16.

the curves did not differ significantly ($p < 0.05$), although a slight delay of the process was measured at the lower temperature.

Community structures in seawater during biodegradation of immobilized oil

Bacterial communities associated with oil-coated adsorbents and seawater were examined by PCR amplification of 16S rRNA gene fragments and DGGE analysis (Figure 5). Aging of seawater at 0 or 5 °C apparently affected the bacterial DGGE banding patterns, and predominant bands in seawater at the two temperatures showed different mobilities at the start of the experiment (W0 lanes). The predominant DGGE bands in the W0 samples (bands 1 and 2 from lane at 0 °C, and bands 11, 12 and 13 at 5 °C) also showed similar mobilities to DGGE bands which emerged in amplified samples from the oil-coated adsorbents after 4–8 weeks of biodegradation (Figure 5). Thus, bacteria which became associated the oil films seemed to originate from the predominant members of the bacterial communities in the aged seawater.

A number of 16 bands were eluted from the DGGE gel (Figure 5), PCR re-amplified, and the products cloned and subjected to RFLP analysis. One clone representing the predominant RFLP type of each DGGE band was sequenced. Results from BLAST studies are shown in Table 3. Most DGGE bands from 0 or 5 °C aligned to GenBank sequences of *Gammaproteobacteria* or *Bacteroidetes*, respectively. Two exceptions (bands 7 from 0 °C and band 14 for 5 °C) both aligned to the *Epsilonproteobacterium Arcobacter* sp. There was good agreement between DGGE mobility and sequence alignment.

Table 3. Alignment of bacterial 16S rRNA gene fragments originating from DGGE bands during biodegradation. Sequenced fragments represented predominant RFLP types of each DGGE band

DGGE band[a]	Closest match[b]	Phylum or class	Similarity (%)
1	*Psychromonas arctica*[c]	*Gammaproteobacteria*	97
2	*Pseudoalteromonas* sp. CAM 36[c]	*Gammaproteobacteria*	99
3	*Oleospira antarctica* strain R[c]	*Gammaproteobacteria*	95
4	Marine psychrotrophic bacterium Mst37[c]	*Gammaproteobacteria*	98
5	*Psychromonas arctica*[c]	*Gammaproteobacteria*	99
6	Uncultured bacterium clone ARKDMS-13[c]	*Gammaproteobacteria*	97
7	*Arcobacter* sp. KT0913	*Epsilonproteobacteria*	98
8	Marine psychrotrophic bacterium Mst37[c]	*Gammaproteobacteria*	98
9	*Psychromonas arctica*[c]	*Gammaproteobacteria*	98
10	Uncultured bacterium clone ARKDMS-13[c]	*Gammaproteobacteria*	97
11	Not identified	–	–
12	Uncultured *Cytophaga* sp.	*Bacteroidetes*	93
13	Uncultured bacterium SB-9/21-CS	*Bacteroidetes*	90
14	*Arcobacter* sp. KT0913	*Epsilonproteobacteria*	95
15	Uncultured *Cytophaga* sp.	*Bacteroidetes*	93
16	Uncultured bacterium SB-9/21-CS	*Bacteroidetes*	93

[a] See Figure 5
[b] Sequence representing selected RFLP type
[c] Sequences reported from Arctic or Antarctic consortia

Several DGGE bands with equal mobilities in the gel showed sequence identities (Figure 5), e.g. the bands 1, 5, and 9 (97–99% similarities to *Psychromonas arctica*), the bands 4 and 8 (98% similarities to marine psychrotrophic bacterium Mst37), bands 12 and 15 (93% identities to uncultured *Cytophaga* sp.) and bands 6 and 10 (97% identities to uncultured bacterium clone ARKDMS-13). Sequences belonging to the predominant bacterial class (*Gammaproteobacteria*) showed different migrations in the gel, and this class could not be separated from the other phyla or classes with respect to mobility. Most of the DGGE fragments from 0 °C experiment (9 of 10) aligned to sequences from Arctic or Antarctic consortia, while none the DGGE fragments from the 5 °C experiment showed such homologies. It was also noted that none of the DGGE band sequences showed complete homology to ODM culture sequences, although the DGGE band 2 sequences showed 99% similarity to the ODM sequence matching *Pseudoaltermonas* sp. CAM36 (Table 2). Thus bacterial communities from biodegradation experiments and ODM cultures differed.

Discussion

We recently showed that the Fluortex adsorbents, originally recommended for cleanup of oil films (Greimann et al. 1995), could be used for dissolution and biodegradation studies in static or flow-through systems (Brakstad et al. 2002; Brakstad et al. 2004). The results of the study presented here confirmed that HC depletion processes at 0 and 5 °C followed similar trends as in experiments conducted at higher temperatures. Biodegradation studies performed at 13 °C also showed that the predominant oil film depletion mechanisms varied with HC compound partitioning between oil and water. Thus, biodegradation became the predominant oil film depletion process for oleophilic compounds with low or moderate water solubility (Brakstad et al. 2002) like *n*-alkanes and alkylated 3-ring/4- to 5-ring PAH (PAH-2). Dissolution from oil films was more important with smaller PAH compounds and naphthalenes. The seawater temperatures used in this study (5 and 0 °C) did not affect HC dissolution significantly, but biotransformation and mineralization differed significantly between these temperatures. For many HC compounds which partition between oil and water phases, dissolution from oil films may therefore become increasingly important depletion mechanisms when seawater temperatures are reduced.

Several studies have shown that bacteria isolated from cold environments exhibit decreased metabolisms at low water temperatures. Two psychrotrophic bacterial strains, isolated from Antarctic seawater, degraded HC with slower rates at 4 °C than at 20 °C (Michaud et al. 2004), and in a survey of cold-adapted microbes phenol degradation was decreased from temperatures of 20 to 1 °C (Margesin et al. 2003). Psychrotrophic/psychrophilic bacteria from Polar seas were reported to have substrate limitations at low temperatures, showing lower affinities for substrate uptake at 2 than at 16 °C (Nedwell & Rutter, 1994). Several studies have also indicated that oil seawater HC degradation in the winter is slower than in summer seasons (Minas 1986; Siron et al. 1995; Piehler & Paerl 1996).

However, microbes from several cold environments have significant potentials for HC biodegradation at seawater temperatures close to 0 °C, including both bacterial and fungal species (Cooney et al. 1985; Whyte et al. 1996; Margesin et al. 2003). This was substantiated for alkanes in our studies, including *n*-hexadecane. This compound was highly biotransformed and mineralized at both 5 and 0 °C. At the lower temperature degradation was delayed but at the end of the experiments degradation was similar at both temperatures. This was in agreement with the results from a sztudy of crude oil bioremediation at low temperature, suggesting that low temperatures only delayed the remediation process (Gibb et al. 2001).

The physical and chemical characteristics of the oils are important to consider during biodegradation at low temperatures. Both increased oil viscosity and wax content may contribute to reduced biodegradability. This may also delay the dissolution of volatiles and other water-soluble compounds to the seawater (Leahy & Colwell 1990). The crude Statfjord oil used in these experiments had a pour point of 8 °C (Moldestad, personal communication), indicating that the oil could be more solidified at 0 than at 5 °C.

The distribution of bacterial and archaeal assemblages in seawater collected from 90 m depth showed a large fraction of archaea, and in a recent

study we showed that the archaeal fraction of the prokaryotic assemblages was higher in the near-bottom used here than in surface seawater (Brakstad et al. 2004). This was in agreement with investigations of vertical distributions of microbial domains, showing that archaeal concentrations in seawater increased by depth (Massana et al. 1997). Previous studies have also shown that the distribution of bacteria and archaea in cold water (e.g. Antarctic coastal water) varied with season, with considerably higher archaeal contribution to the picoplankton assemblages in the winter than in the summer (Murray et al. 1998). We showed that predominantly bacterial growth was stimulated by the enrichment media at low temperatures, including the medium for propagation of ODM. This indicated that primarily bacteria rather than archaea were involved in the HC degradation of immobilised oil. Bacteria are considered to be the predominant agents of biodegradation, and microcosm studies have shown that beach sediment oil-pollution resulted in dramatic decreases of archaeal assemblages (Röling et al. 2004). Thus, only bacterial communities were further examined in this study.

In HM and ODM cultures at 0 °C the fractions of bacteria enumerated by the EUB338 probe was lower than in the cultures at 5 °C (Table 1). Whether this was the results of low ribosome content (below detection limit) or the presence of microbes lacking target sites for the FISH probes in cultures at 0 °C is not known. However, recent studies in our lab of ODM cultures enriched at 0 °C from surface seawater showed higher fractions (>50%) of cell hybridizing to the EUB338 probe (not published).

The results of the sequencing and DGGE analysis indicated temperature-related differences of the bacterial communities associated with oil, and showed that predominant phyla in the seawater adhered to the oil-coated adsorbents. Both characterization of community members from ODM enrichment cultures and DGGE bands from biodegradation experiments showed predominance of proteobacterial sequences. However, the sequence analysis did not show identity among bacterial populations in the ODM cultures and the DGGE bands. One reason for this may be that the conditions for the culture and biodegradation experiments differed significantly. For instance, the seawater aging prior to the biodegradation experiments affected the community structures. Further, separate batches of seawater were used for ODM inoculation and seawater biodegradation experiments. At 0 °C all clones except one belonged to *Gammaproteobacteria* and aligned to sequences reported from Arctic or Antarctic consortia (Brinkmeyer et al. 2003; Gilbert et al. 2004; Ray et al. 1998; Nichols et al. 2004). However, at 5 °C both members of *Alphaproteobacteria, Gammaproteobacteria* and *Bacteroidetes* were associated with oil biodegradation, and typical cold environment sequences were not abundant. Results from experiments with HC degradation in temperate marine environments have revealed prevalence of *Alphaproteobacteria*, the *Bacteroidetes* and the genera *Alcanivorax*/*Fundibacter* of *Gammaproteobacteria* (Chang et al. 2000; Kasai et al. 2002; MacNaughton et al. 1999; Röling et al. 2002). Studies of Antarctic communities from HC-polluted soil or seawater exposed to crude oil showed predominance of *Alphaproteobacteria* and *Gammaproteobacteria* (Aislabie et al. 2000; Yakimov et al. 2004).

In conclusion, microbial communities from cold seawater have the potential of oil films HC degradation at 0–5 °C. The community members associated with biodegradation at low temperature (0 °C) seemed to be related to psychrophilic of phychrotrophic bacteria.

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