ELSEVIER

FEMS Microbiology Ecology 53 (2005) 129–139

FEMS MICROBIOLOGY Ecology

www.fems-microbiology.org

Influence of crude oil on changes of bacterial communities in Arctic sea-ice

Birte Gerdes [a,*], Robin Brinkmeyer [b], Gerhard Dieckmann [a], Elisabeth Helmke [a]

[a] *Alfred Wegener Institute for Polar and Marine Research, Am Handelshafen 12, 27570 Bremerhaven, Germany*
[b] *Department of Marine Biology, Texas Institute of Oceanography, Texas A&M University at Galveston, 5007 Avenue, U / POB 1675 Galveston, TX 77551, USA*

Received 14 July 2004; received in revised form 15 October 2004; accepted 3 November 2004

First published online 8 December 2004

Abstract

The danger of a petroleum hydrocarbon spillage in the polar, ice-covered regions is increasing due to oil exploration in Arctic offshore areas and a growing interest in using the Northern Sea Route (NSR) as an alternative transportation route for Arctic oil and gas. However, little is known about the potential impact of accidental oil spills on this environment. We investigated the impact of crude oil on microbial community composition in six different Arctic sea-ice samples incubated with crude oil at 1 °C in microcosms for one year. Alterations in the composition of bacterial communities were analyzed with the culture-independent molecular methods DGGE (denaturing gradient gel electrophoresis) and FISH (fluorescence in situ hybridization). DGGE, FISH and cultivation methods revealed a strong shift in community composition toward the γ-proteobacteria in sea-ice and melt pool samples incubated with crude oil. *Marinobacter* spp., *Shewanella* spp. and *Pseudomonas* spp. were the predominant phylotypes in the oil-treated microcosms. The ability of indigenous sea-ice bacteria to degrade hydrocarbons at low temperature (1 °C) was tested using four representative strains cultivated from sea-ice enriched with crude oil. [^{14}C]Hexadecane was degraded by the sea-ice isolates at 20–50% capacity of the mesophilic type strain *Marinobacter hydrocarbonoclasticus*, a known hydrocarbon degrader, incubated at 22 °C.

Keywords: Sea-ice; Hydrocarbons; Oil degradation; Low temperature

1. Introduction

The risk of petroleum hydrocarbon pollution in the Arctic is increasing due to ongoing exploration for oil and gas in Arctic off-shore areas and a growing interest in developing the Northern Sea Route (NSR) as an alternative transportation route for oil and gas from Russian Arctic regions to Europe and other markets.

Natural oil reserves may serve either as substrates for specific microorganisms or alternatively can be extremely toxic for many microbes or other organisms. An extensive portion of the Arctic Ocean is covered by sea-ice for most of the year. Sea-ice constitutes an important and extreme ecosystem harbouring highly active sea-ice microbial communities (SIMCO) [1–3], which play a significant role in the marine food webs of Polar regions [4–6]. It is therefore important to assess the influence of crude oil contamination on bacterial communities inhabiting Arctic sea-ice and to elucidate their potential to degrade petroleum hydrocarbons under cold temperatures. Sea-ice microbial communities live mainly in brine channels and in pockets in the ice matrix, which arise during its formation [7]. This microhabitat is an extreme environment with salinities ranging

* Corresponding author. Tel.: +49 0471 4831 1842; fax: +49 0471 4831 1425.
E-mail address: bgerdes@awi-bremerhaven.de (B. Gerdes).

0168-6496/$22.00
doi:10.1016/j.femsec.2004.11.010

from 0 up to 150 psu and temperatures from −1 to −50 °C in winter. The volume of the brine channels and pockets is directly dependent upon the in situ temperature of the ice varying from 1% to 30%.

Most sea-ice bacteria are psychrophilic [2,8,9] and differ in size, activity and taxonomy from free-living bacteria in the underlying sea water [2,9,10].

The diversity of sea-ice microbial communities has been studied mainly in the Antarctic [5,9–11]. Only recently has the diversity of Artic sea-ice bacteria been investigated by Brown et al. [5] and Brinkmeyer et al. [10], combining both cultivation and cultivation-independent methods.

Biodegradation of petroleum hydrocarbons by autochthonous cold-adapted bacteria at low temperatures has been observed in soils of the Arctic [12–15], the Antarctic [13,16–21] and Alpine regions [22,23]. Studies in cold marine environments have focussed mainly on seawater, sediments or beaches in the Arctic Ocean [24–33] and in the Southern Ocean [34–37]. Only few studies have been performed on sea-ice [36,38–41]. These studies address mainly the effect of the ice cover on oil degradation whereas Delille et al. [42] investigated the impact of oil contamination on total and specific bacterial communities in Antarctic sea-ice using culture-dependent methods.

This is the first study to assess changes in bacterial communities in Arctic sea-ice and surface melt pools as influenced by crude oil and it combines culture-based and culture-independent molecular methods. Microcosm experiments with different Arctic sea-ice samples were incubated with crude oil at 1 °C for one year. Bacteria isolated from oil-enriched sea-ice samples were tested for their ability to degrade [^{14}C]hexadecane and [^{14}C]toluene.

2. Materials and methods

2.1. Sample site, sampling and microcosm experiments

Samples were collected by drilling ice cores (9 cm in diameter) in multi-year Arctic pack ice during the R.V. Polarstern cruise ARK-XVI/2 (July–August 2000) in an area northeast of Svalbard (Table 1). Samples were collected and processed as described by Helmke and Weyland [2]. Ice cores were immediately cut into 20 cm segments and subsequently allowed to melt in sterile plastic containers with an equal volume of sterile filtered seawater to avoid disruption of cells during the melting process.

To study the influence of crude oil on the bacterial communities, six microcosm experiments were set up using sterile 50 ml glass flasks each containing 25 ml of melted sea-ice from different ice cores and different depths (Table 1) and 500 μl of crude oil (Roth, Germany). Replicate samples of each experiment without oil served as controls. The microcosm experiments were incubated at 1 °C for 12 months in the dark to avoid the activity of phototrophic organisms.

Nutrients are an important factor in the degradation process and were expected to reach limiting levels in the microcosm experiments during the course of several months. After one year of incubation with crude oil, nutrients (0.5% [w/v] peptone and 0.1% [w/v] yeast extract) were supplied to sample A and sample E of the microcosm experiments. The nutrient amended microcosms were re-incubated at 1 and 10 °C for 6 months.

2.2. Isolation

Bacteria from the oil-contaminated microcosms were isolated by plating 100 μl on Marine agar (Difco 2216). The plates were incubated at 1, 10 and 22 °C for few days up to several weeks depending on the time that it took for colonies to become visible. Single colonies were picked with an inoculating needle and re-streaked twice to obtain pure cultures.

2.3. Fluorescence in situ hybridization (FISH)

FISH analysis was used to examine community structure of bacteria in natural, uncontaminated Arctic sea-ice samples and samples collected from the microcosm experiments after one-year incubation at 1 °C. Samples were fixed with buffered paraformaldehyde solution (final concentration, 2–4% [w/v]), immobilized on white polycarbonate filters (Nucleopore; diameter 47 mm; pore size, 0.2 μm), and then rinsed with 10 ml each of phosphate-buffered saline and distilled water. Air-dried filters were stored at −20 °C until further processing.

FISH analysis was conducted according to the method of Glöckner et al. [43] using Cy3-labeled oligonucleotide probes (final concentration 5 ng/μl; Interactiva, Ulm, Germany) specific for large phylogenetic groups and characteristic for sea-ice communities [10]. The specificity of the probes used ranged from domain to species level (Table 2). The probe Non338 [44] was used to test for non-specific probe binding.

For counterstaining, air-dried hybridized samples were mounted in a mixture of four parts Citifluor AF1 (Citifluor Ltd., London, UK) and one part Vecta Shield (Vector Laboratories, Burlingame, CA) amended with 4′,6′-diamidino-2-phenylindole (DAPI; final concentration 1 μM). Samples were then evaluated under an Axioplan2 epifluorescent microscope (Carl Zeiss, Jena, Germany) equipped with appropriate filter sets for Cy3 and DAPI fluorescence. Between 600 and 800 DAPI-stained objects were counted per probe and sample.

Table 1
Sample locations and sample depths

Station no.	Coordinates	Ice core/Pool no.	Ice thickness	Sample	Sample depths
57/196-1	78°58.4N	Core 6	220 cm	C	40 cm
	04°04.1W				
57/227-1	79°18.8N	Pool 2		D	Pool[a]
	13°36.1W	Core 5	160 cm	H	Bottom[b]
57/247-1	79°18.8′N	Core 11	215 cm	A	Bottom[c]
	O1°54.1E	Core 1	215 cm	B	80 cm
		Core 10	215 cm	E	Bottom[c]

[a] Melt water pond on top of ice flow.
[b] Algae accumulation.
[c] Sediment inclusions.

Table 2
Oligonucleotide probes used in this study

Probe	Specificity	Reference
NON338	Non-specific probe binding, complementary to EUB338	[44]
EUB338	Most *Bacteria*	[64]
EUB338-II	*Planctomycetales*	[65]
EUB338-III	*Verrucomicrobiales*	[65]
HGC69a[a]	Gram-positive (*Actinobacteria*)	[66]
HGC69c	Competitor for HGC69a	[66]
CF319a[a]	*Cytophaga flavobacterium* -group of the *Bacteroidetes*	[67]
CF319c	Competitor for CF319a	[67]
ALF968	α-Proteobacteria	[68]
BET42a[a]	β-Proteobacteria	[69]
BET42c	Competitor for BET42a	[69]
GAM42a[a]	γ-Proteobacteria	[69]
GAM42c	Competitor for GAM42a	[69]
MB-ICO22[a]	*Marinobacter* sp. strain ICO22 group	[10]
MB-ICO22c	Competitor for MB-ICO22	[10]
SF825	*Shewanella frigidimarina*	[10]
PS56a[a]	*Pseudomonas* spp.	[70]
PS56c	Competitor for PS56a	[70]

[a] Probes HGC69a, CF319a, BET42a, GAM42a, MB-ICO22, and PS56a were used with the competitors cited.

2.4. DNA extraction and PCR amplification for 16S rRNA gene analysis

Bacterioplankton of melted sea-ice samples and samples from microcosm experiments were collected on polycarbonate filters (pore size, 0.2 μm) and stored at −20 °C for later extraction of nucleic acids. Total community nucleic acids from filtered samples were extracted using the DNeasy tissue kit (Qiagen, Hilden, Germany) with additional lysozyme (final concentration 1 mg/ml in 20 mM Tris–HCl, pH 8,0; 2 mM EDTA and 1.5% Triton X-100) pre-incubations for 30 min at 37 °C. DNA from isolates was extracted by four freeze–thaw cycles. The extracted DNA was then purified with a PCR-Purification kit (Qiagen, Hilden, Germany).

Nearly full-length 16S rRNA gene sequences were amplified from nucleic acid extracts of the isolates from both microcosm experiments and environmental samples (approximately 100 ng) by PCR with a thermal cycler (Eppendorf, Hamburg, Germany). The *Bacteria*-specific primer 8F (5′-AGAGTTTGATCCTGG CTCAG-3′) and the universal primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) were used. PCR was performed in 50 μl reaction mixtures containing 1 μM of each primer, 12.5 mM each dATP, dCTP, dGTP. dTTP, 1 U red *Taq*-polymerase (Sigma), 10 mM Tris–HCl (pH 8.3), 50 mM KCl and 1.5 mM $MgCl_2$ under the following conditions: 95 °C for 5 min, followed by 29 cycles of 94 °C for 2 min, 50 °C for 3 min, and 72 °C for 4 min and a final elongation consisting of 72 °C for 10 min.

2.5. Nested PCR amplification and DGGE

To increase the sensitivity of denaturing gradient gel electrophoresis (DGGE) analysis, a nested PCR technique was applied. The first PCR was conducted as described above. The nested PCR was performed using

Bacteria -specific primers GM5-GC (corresponding to positions 341–358 of the *Escherichia coli* 16S rRNA) [45] and 907RM (corresponding to positions 907–927 of the *E. coli* 16S rRNA) [45] in 100 μl reaction mixtures as described above with the addition of 1 μl of PCR product from the first amplification reaction as template DNA. The template DNA was denatured in a thermal cycler for 5 min at 95 °C followed by a 'touchdown'-PCR as described by Muyzer et al. [45] to increase the specificity of amplification and to avoid the formation of spurious by-products. PCR-products were analyzed by DGGE, based on the protocol of Muyzer et al. [46] using a gradient-chamber.

Approximately, 30–40 μl of the PCR products were loaded onto 1-mm-thick 6% [w/v] polyacrylamide (37.5:1 acrylamide–bisacrylamide) gels containing a 20–70% linear denaturing gradient (where 100% denaturant is 7 M urea and 40% [v/v] formamide). Gels were run in 1× TAE buffer (40 mM Tris–acetate and 1 mM Na-EDTA, pH 8.0) at 60 °C and 100 V for 18 h. Gels were stained in 1× TAE containing SYBR Gold (diluted 1:10,000; Sigma) and immediately photographed under UV transillumination. Digitized DGGE profiles were straightened and aligned with the Bionumerics Gelcompare software (Applied Maths, Sint-Martens-Latern, Belgium).

Significant bands from the DGGE pattern were selected and, after excision from the gel, resuspended in 100 μl of MilliQ water for 1 h at room temperature, then reamplified by PCR under the following conditions: initial denaturation at 95 °C for 4 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and elongation at 72 °C for 3 min.

Before sequencing, the PCR products were purified using a PCR Purification kit (Qiagen, Hilden, Germany).

2.6. ARDRA and phylogenetic analysis

Nearly full-length 16S rRNA gene fragments of the isolates were amplified by PCR using the primers 8F and 1492R as described above. Amplified ribosomal DNA restriction analysis (ARDRA) [47] was used to characterize the 16S rRNA gene diversity within the collection of 25 isolates from the microcosm experiments. After simultaneous digestion (3 h, 37 °C) with the restriction enzymes *Rsa*I and *Hae*III (5 U of each) (Promega), according to the manufacturer's instructions, digestion products were separated on a 9% polyacrylamide gel, and the resulting restriction pattern was photographed under UV light. The ARDRA patterns were clustered with the Bionumerics Gelcompare software (Applied Maths, Sint-Martens-Latern, Belgium) using Ward and Pearson correlation method.

Two representative strains of the ARDRA pattern groups were selected for sequencing. Sequences were compared to those deposited in the GenBank using the BLAST algorithm [48].

Sequence data were analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de). A phylogenetic tree was reconstructed using maximum-likelihood analyses. Only 16S rRNA gene sequences from the isolates containing at least 1400 bases were used for the tree construction. Partial sequences were added to the existing tree by a special algorithm included in the ARB software, without allowing changes of the tree topology based on almost complete sequences.

2.7. [^{14}C]Hexadecane and [^{14}C]toluene incubation experiments

Bacterial utilization of hexadecane and toluene of four isolates and one reference strain (*Marinobacter hydrocarbonoclasticus* (DSM no. 8798; ATCC49840)) was determined by measurement of the formation of [^{14}C]CO_2 converted from [^{14}C]hexadecane or [^{14}C]toluene. Two replicates of each isolate, the reference strain, and one control (killed with 4% formalin) of 20 ml autoclaved melted sea-ice were inoculated with cells of enrichment cultures from sea-ice bacteria isolated from oil contaminated samples, final concentration approximately 4×10^7 cell ml^{-1}. The aliquots were then treated with 20 μl [^{14}C]toluene ([$^{14}C_6H_5CH_3$]; specific activity: 2.8 mCi mmol^{-1}; 25 μCi ml^{-1}; Sigma) or 20μl 1:50 diluted [^{14}C]hexadecane ([$CH_3[CH_2]_{14}{}^{14}CH_3$]; specific activity: 12 mCi mmol^{-1}; 1 mCi ml^{-1}; Sigma) with non-radiolabeled hexadecane. The aliquots were incubated on a rotary shaker for one month at 1 °C in the dark. Each aliquot was equipped with a glass vial containing 2 ml of 1 M KOH to trap CO_2 released to the headspace. The aliquots (100 ml glass vials) were sealed with silicon septa.

At weekly samplings, 500 μl KOH of each aliquot was removed with a syringe and transferred into scintillation cocktail (Lumasafe, Mumac-LSC, B.V.). The radioactivity was determined using a TriCarb spectrophotometer 2550 TR/LL (Perkin Elmer). After sampling the CO_2-trap was refilled with freshly prepared 500 μl of 1 M KOH. The dilution effect was taken into account when calculating the formation of [^{14}C]CO_2. All counts were corrected for background values, which were measured with 500 μl of 1 M KOH in Lumasafe scintillation cocktail. Before the last sampling, after 29 days of incubation, the bacterial suspension of the aliquots were acidified with 100 μl of $4n H_2SO_4$ to sparge the dissolved CO_2 into the headspace.

The incorporation of ^{14}C into biomass was not determined due to the high residue of non-incorporated hexadecane and toluene on the polycarbonate filters after filtration and several washing steps.

2.8. Nucleotide sequence accession numbers

Nearly full-length 16S rRNA gene sequences from eight isolates were deposited in GenBank under the Accession Nos. AY770006–AY770013.

3. Results

3.1. FISH analysis of natural sea-ice samples and oil-contaminated samples

FISH analysis was applied to samples of the microcosm experiments before addition of crude oil and after one-year incubation with crude oil to determine the natural bacterial community structures and subsequent changes after oil addition.

Percent values of DAPI-stained cells detected with the negative control probe NON338 varied from 0% to 2% and were subtracted from the percentages detected with specific probes. High background fluorescence was obtained in oil-contaminated samples due to strong auto-fluorescence of the crude oil.

The percentage of DAPI-stained cells detected with the *Bacteria*-specific probes EUB338, EUB338-II and EUB338-III and the percentage of group-specific probes of DAPI-stained cells are presented in Fig. 1. The percentages of detectable cells with the *Bacteria*-specific probe were high, ranging from 56% to ~85%, in the natural sea-ice samples before incubation. After one-year incubation these decreased in both the samples without oil (~47% to ~69%) and in those contaminated with oil (~35% to ~79%).

The results of FISH analysis showed a clear shift in the bacterial community structures induced by oil contamination. The group γ-proteobacteria was abundant in all natural samples from sea-ice with the exception of the melt pool sample. However, after one-year incubation with crude oil, γ-proteobacteria was the predominant group in all oil-contaminated samples, with percentages ranging from ~58% to 87% of detectable cells using *Bacteria*-specific probes (data not shown). To specify this group more closely, species-specific probes were applied to the oil-incubated samples. Percentage distributions that hybridized with these probes are presented in Table 4. The *Marinobacter* sp. strain ICO22 group was most abundant among the γ-proteobacteria group with relative abundances from ~16 to ~39% of cells detected by DAPI staining.

Gram-positive bacteria were below the detection limit in most samples except for the samples A, E and C of the natural sea-ice where bacteria were detected by the group-specific probe HGC69a with abundances of 1%, 0.3% and 0.7% of DAPI-stained cells, respectively. After one year of oil-free incubation the percentages of 0.3%,

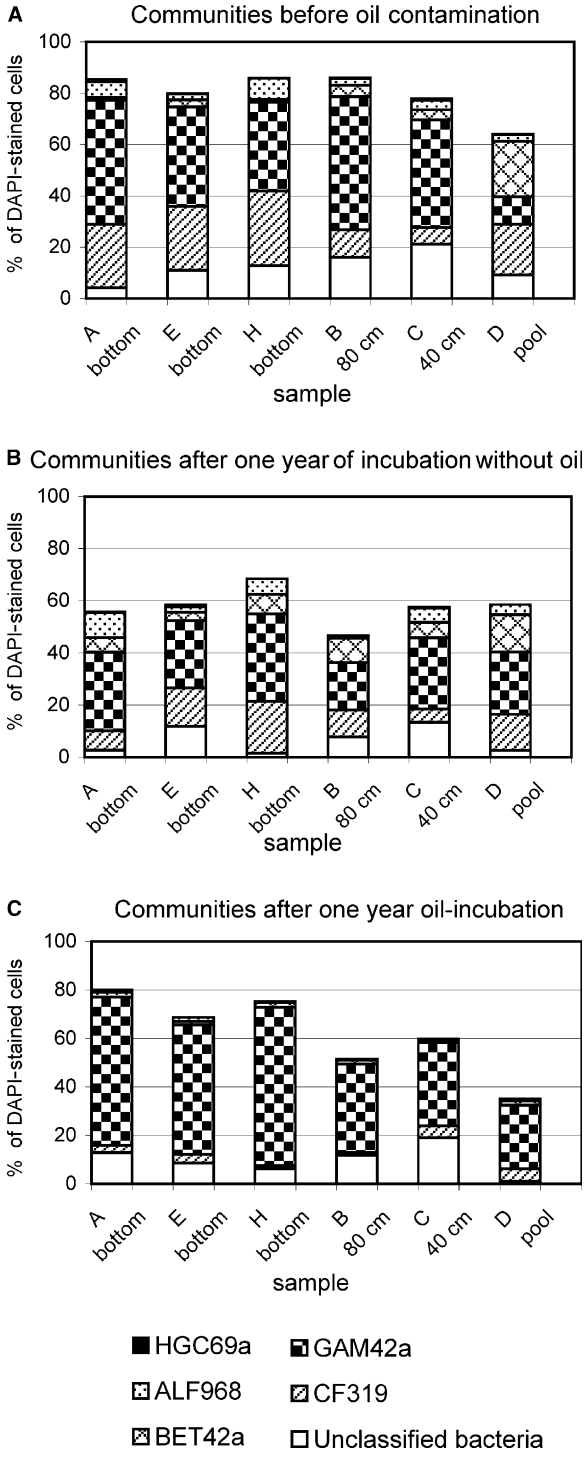

Fig. 1. Percentages of DAPI-stained bacteria detected by FISH using the probes HGC69a for gram-positive (*Actinobacteria*), ALF968 for α-proteobacteria, BET42a for β-proteobacteria, GAM42a for γ-proteobacteria, and CF319a for the *Cytophaga Flavobacteria* of the *Bacteroides* group. The percentage of DAPI-stained bacteria detected with the *Bacteria*-specific probes EUB338, EUB338-II, and EUB338-III corresponds to the maximum height of the bars. The white sections of the bars indicate the percentage of cells, which were not detected with genus-specific groups.

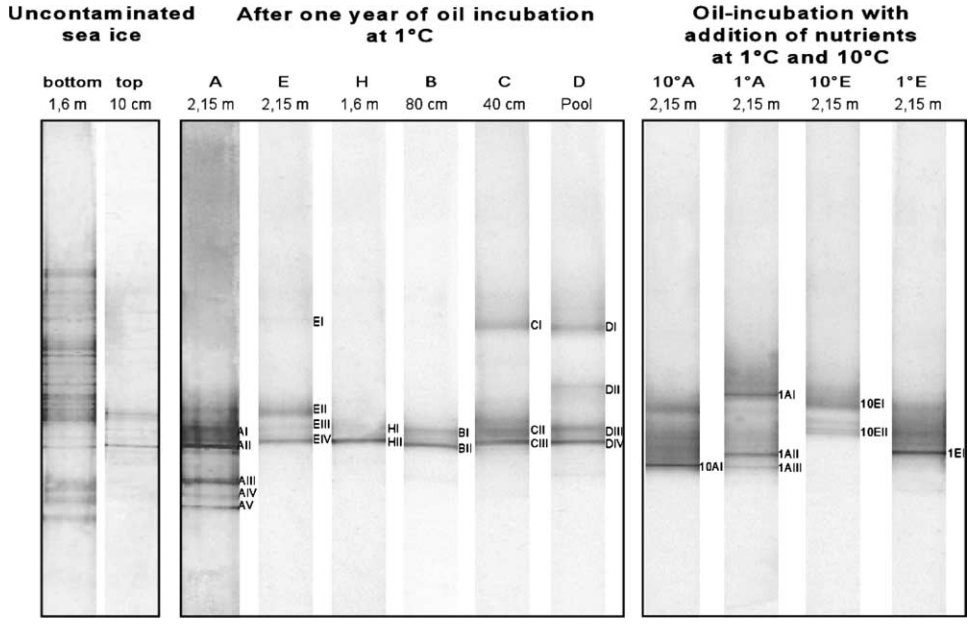

Fig. 2. DGGE profile of 16S rRNA gene fragments of non-contaminated sea-ice samples, samples from oil-treated microcosm experiments after one year of incubation at 1 °C, and samples from oil-treated microcosm experiments after 6 months of incubation at 1 and 10 °C. Numbering on the right hand side of the lanes indicates the names of excised DGGE bands listed in Table 3 with their closest relative in the GenBank database.

0.2% and 0.5% of DAPI-stained bacteria were detected for the same samples, respectively.

3.2. DGGE analysis of 16S rRNA genes

DGGE profiles of PCR-amplified 16S rRNA gene fragments were obtained from two different non-contaminated sea-ice samples as well as the six microcosm experiments (A, E, H, B, C and D) after one-year incubation with crude oil in order to compare bacterial diversity and community structure (Fig. 2). Some of the major bands of the contaminated samples were excised and sequenced. Table 3 lists the dominant bands and their closest relatives in the GenBank database.

Table 3
Sequence similarity of excised DGGE bands that appear in Fig. 2

DGGE band	Closest relative in GenBank database (Accession No.)	% Identity	Alignment length	Phylogenetic group	Genus
A-I	Bacterium str. S36-W1 (U14584)	98.9	543	*γ-Proteobacteria*	*Marinobacter*
A-II	Bacterium str. S36-W1 (U14584)	98.0	551	*γ-Proteobacteria*	*Marinobacter*
A-III	*Halomonas* sp. YIM-kkny11 (AY121436)	94,0	535	*γ-Proteobacteria*	*Halomonas*
A-IV	Arctic seawater bacterium R7102 (AJ293827)	99.5	551	*γ-Proteobacteria*	*Halomonas*
A-V	Arctic seawater bacterium R7102 (AJ293827)	98.7	544	*γ-Proteobacteria*	*Halomonas*
B-I	*Variovorax* sp. D63 (AF250422)	92.3	415	*β-Proteobacteria*	*Variovorax*
B-II	Bacterium str. S36-W1 (U14584)	98.6	502	*γ-Proteobacteria*	*Marinobacter*
C-I	*Flavobacterium* sp. ST-82 (AB075230)	98.2	502	*Flavobacteria*	*Flavobacterium*
C-II	Bacterium str. S36-W1 (U14584)	95.8	477	*γ-Proteobacteria*	*Marinobacter*
C-III	Bacterium str. S36-W1 (U14584)	96.0	444	*γ-Proteobacteria*	*Marinobacter*
D-I	Uncultured Polaribacter Arctic96B-11 (AF354621)	98,9	462	*Flavobacteria*	*Polaribacter*
D-II	Uncultured α proteobacterium (AF100168)	94.5	494	*α-Proteobacteria*	*Sulfitobacter*
D-III	Uncultured β proteobacterium (AF100168)	93.7	427	*β-Proteobacteria*	*Comamonadaceae*
D-IV	Bacterium str. S36-W1 (U14584)	96.5	489	*γ-Proteobacteria*	*Marinobacter*
E-I	Uncultured β proteobacterium (AY133088)	93.9	358	*β-Proteobacteria*	*Delftia*
E-II	Bacterium str. S36-W1 (U14584)	97.2	466	*γ-Proteobacteria*	*Marinobacter*
H-I	Bacterium str. S36-W1 (U14584)	92.3	338	*γ-Proteobacteria*	*Marinobacter*
H-II	Bacterium str. S36-W1 (U14584)	98.7	457	*γ-Proteobacteria*	*Marinobacter*
10A-I	Bacterium CS117 (AY124341)	99.4	503	*Actinobacteria*	*Actinomycetales*
1A-I	*Clostridium bowmanii* (AJ506120)	99.1	463	*Firmicutes*	*Clostridium*
1A-II	*Agreia* sp. 37-4 (AF513393)	97.0	473	*Actinobacteria*	*Agreia*
1A-IIII	Bacterium CS117 (AY124341)	83.3	430	*Actinobacteria*	*Actinomycetales*
10E-I	*Pseudomonas anguilliseptica* strain (AF439803)	98.0	488	*γ-Proteobacteria*	*Pseudomonas*
10E-II	*Shewanella* sp. Ko704 (AF550589)	90.2	418	*γ-Proteobacteria*	*Shewanella*
1E-I	*Agreia* sp. 37-4 (AF513393)	99.6	495	*Actinobacteria*	*Agreia*

Table 4
Percentages of DAPI-stained cells by species-specific probes, which group into the γ-proteobacteria

Probe target group	Probe	After one year oil incubation at 1 °C					
		A	E	H	B	C	D
γ-Proteobacteria	GAM42a	69.5 ± 7	53.6 ± 10	65.4 ± 5	36.6 ± 7	31.6 ± 13	26.3 ± 11
Marinobacter sp. strain ICO22 group	MB-ICO22	38.5 ± 8	18.2 ± 9	32.8 ± 5	31.3 ± 7	26.6 ± 5	16.3 ± 8
Shewanella frigidimarina	SF825	3.4 ± 13	8.1 ± 6	3.6 ± 4	0	<1	<1
Pseudomonas spp.	PS56a	7.8 ± 5	7.2 ± 9	3.2 ± 12	2.6 ± 18	2.2 ± 8	<1

Values represent the mean percentage ± standard deviation.

Similar to the FISH analysis, DGGE profiles indicated a highly reduced diversity in sea-ice bacterial communities after one year of oil incubation at 1 °C as compared to natural bottom sea-ice assemblages. Bacteria of the dominant bands belonged most frequently to the γ-proteobacteria (Table 3).

The addition of nutrients after one year of oil incubation resulted in a broad shift towards the group of Gram-positive bacteria after 6 months of incubation at 1 °C (Fig. 2). Sequences retrieved from DGGE bands were closely related to members of *Actinomycetales* (10A-I and 1A-III), *Agreia* (1A-II and 1E-I) and *Clostridium* (1A-I). However, after 6 months of incubation at 10 °C, bacteria in sample E (10°E) were still dominated by γ-proteobacteria (Table 3, DGGE bands 10E-I and 10E-II).

3.3. Phylogenetic analysis of isolates from oil-contaminated samples using ARDRA

From the oil-contaminated microcosm experiments, 25 bacterial strains were isolated at incubation temperatures of 1, 10 and 22 °C. These isolates were screened for taxonomic affiliation by ARDRA. The restriction patterns clustered into three groups. From each group at least two representatives were selected for sequencing of almost full-length 16S rRNA gene fragments. Sequences were compared to those deposited in the GenBank using the BLAST algorithm. Isolates from the predominant ARDRA-pattern group belonged to the genus *Marinobacter* (14 isolates), a group of 6 isolates belonged to the genus *Shewanella* and the smallest group of 5 isolates to the genus *Pseudomonas*. The phylogenetic distance to their closest relatives of the GenBank database and to *M. hydrocarbonoclasticus* as well as *M. aquaeolei* is shown in Fig. 3.

3.4. Degradation of [^{14}C]hexadecane and [^{14}C]toluene

Four representative isolates (at least one of each ARDRA-pattern group) were selected to study their potential to degrade hydrocarbon at low temperature (1 °C) and tested for their ability to utilize [^{14}C]hexadecane and [^{14}C]toluene. To evaluate the degradation capability of the sea-ice isolates, the type strain *M. hydrocarbonoclasticus*, a well-known degrader of hydrocarbons [49], was included for comparison in the experiments. *M. hydrocarbonoclasticus* is a mesophilic, marine bacterium and tests with this strain were performed at room temperature of 22 °C.

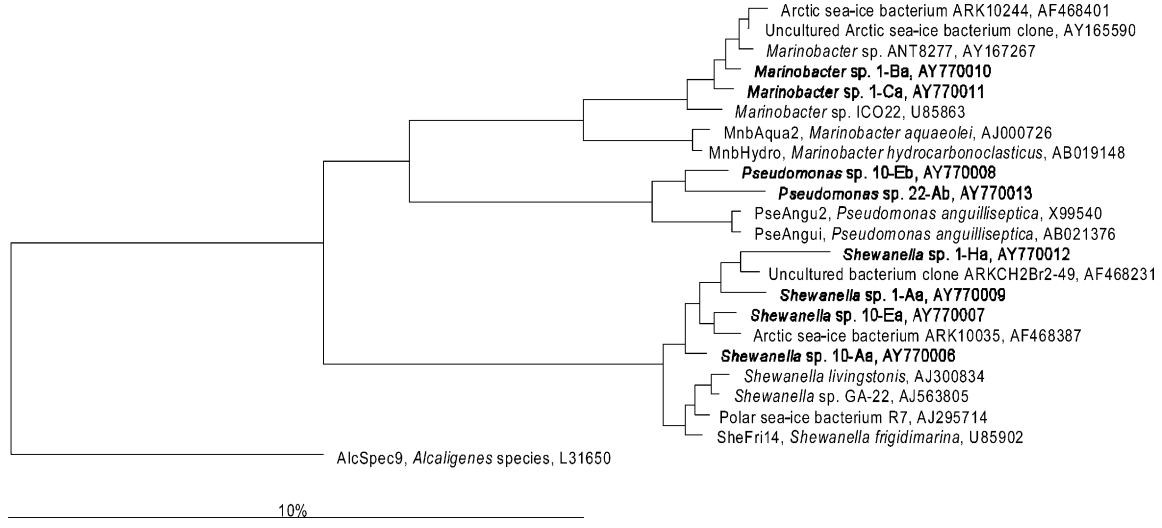

Fig. 3. 16S rRNA gene-based phylogenetic tree showing the relation of eight isolates cultivated from oil-contaminated Arctic sea-ice samples to their closest relatives in the GenBank as well as their phylogenetic distance to *M. hydrocarbonoclasticus* and *M. aquaeolei*. The tree was reconstructed using maximum-likelihood (FastDNAml) analysis. The scale bar indicates 10% estimated sequence divergence. An *Alcaligenes* sp. of the β-proteobacteria was selected to root the tree.

Table 5
[^{14}C]CO_2 production from [^{14}C]hexadecane without additional nutrient supply by sea-ice isolate at 1 °C and by *M. hydrocarbonoclasticus* at 22 °C

Strain/isolate		Temperature (°C)	% [^{14}C]CO_2 of the initial [^{14}C]hexadecane after				
			3 days	10 days	17 days	22 days	29 days
M. hydrocarbonoclasticus	Reference strain	22	0.37	1.47	3.16	5.44	10.72
10-Eb (*Pseudomonas* sp.)	Isolate	1	0.01	0.63	0.85	1.22	5.40
1-Ba (*Marinobacter* sp.)	Isolate	1	0.05	0.43	0.92	1.49	3.40
10-Da (*Marinobacter* sp.)	Isolate	1	0.05	0.34	0.55	1.10	2.27
10-Aa (*Shewanella* sp.)	Isolate	1	0.01	0.29	0.40	0.69	1.70

Toluene was not degraded significantly by any of the isolates (data not shown). The degradation of hexadecane is presented in Table 5. The reference strain *M. hydrocarbonoclasticus* indicated a higher degradation rate of hexadecane at 22 °C (10.7% [^{14}C]CO_2 of the initial [^{14}C]hexadecane) than the isolates at 1 °C. The isolates showed a longer lag period prior to the onset of hexadecane utilization compared to the reference strain. However, after 29 days of incubation at 1 °C the degradation of hexadecane by the isolates 10-Eb, 1-Ba, 10-Da and 10 Aa clearly differed from the control with 5.4%, 3.4%, 2.5% and 2.2% [^{14}C]CO_2 production from the initial [^{14}C]hexadecane, respectively. Degradation rates of the isolates were 20–50% of the rates observed with *M. hydrocarbonoclasticus* incubated at 22 °C.

4. Discussion

This study assessed the impact of crude oil contamination on bacterial community composition in Arctic sea-ice. Bacterial isolates from the ice matrix as well as from a surface melt pool were tested for their ability to tolerate crude oil and to utilize hexadecane and toluene, which are major components of crude oil.

4.1. Impact of crude oil on Arctic sea-ice community composition

FISH analysis of the ice matrix samples at the start and the end of the incubations (Fig. 1) revealed a markedly reduced diversity within microbial communities of the ice matrix and melt pool samples after one-year incubation with crude oil. A shift in community composition occurred mostly from the *Cytophaga-Flavobacterium* group, α-proteobacteria, and γ-proteobacteria to predominantly γ-proteobacteria. Further analysis with group-specific probes indicated the predominance of *Marinobacter* spp., *Shewanella* spp., and *Pseuodomonas* spp. (Table 5), all within the γ-proteobacteria. DGGE analysis of non-contaminated sea-ice and samples incubated with crude oil confirmed FISH data. A comparison of DGGE bands (Fig. 2), each band representing a 16S rRNA phylotype, showed highly reduced diversity in community composition as well as a shift towards the γ-proteobacteria with the predominant phylotype *Marinobacter*. Interestingly, despite the depth of the sample within the ice floe or even in the melt pool, the same bands or bacterial phylotypes were prominent in the DGGE gel after the incubations indicating an increase in abundance. β-Proteobacteria, shown to be abundant in surface melt pools on Arctic pack ice [10], were also detectable with DGGE and FISH in the treated and non-treated microcosm incubations. Some slight shifts in the distribution of bacterial groups were observed with FISH analysis in the non-treated microcosms (Fig. 1), however, when compared to the treated microcosms it was obvious that the addition of crude oil had a marked impact on community composition.

Marinobacter spp., *Shewanella* spp. and *Pseudomonas* spp. have been repeatedly detected in Arctic as well as Antarctic sea-ice [5,9,10,50]. Temperature tolerance tests showed that *Marinobacter* spp. and *Shewanella* spp. were psychrophilic rather than psychrotolerant [9; Helmke, E., unpublished data] whereas psychrotolerant isolates belonged to the genera of *Pseudomonas*. Therefore, all three genera are well adapted to the sea-ice environment.

In other marine environments, *Shewanella* spp. and *Pseudomonas* spp. are often involved in the degradation of hydrocarbons [26]. A psychrophilic hydrocarbon degrading *Shewanella* strain was, for example, identified in Antarctic sea water by Gentile et al. [51].

Marinobacter is one of the recently described genera of hydrocarbonoclastic bacteria (HCB) [49,52–56]. HCB are oil-degrading bacteria, which affiliate phylogenetically to the γ-proteobacteria and are ubiquitously distributed in the marine environment. Similar to the oil-treated sea-ice microcosms, observations in various marine environments have shown increases in γ-proteobacteria after oil contamination [31,57]. The close affiliation of the oil-treatment sea-ice phylotypes, *Marinobacter* spp., *Shewanella* spp. and *Pseudomonas* spp., to known hydrocarbon-degrading bacteria from other marine environments suggests a similar role of these groups in sea-ice.

The addition of nutrients to some of the oil-treated microcosms resulted in very different final community compositions. Rather than γ-proteobacteria, Gram-positive bacteria represented the prominent bands in sample A and E incubated with oil and added nutrients at 1 °C. Gram-positive bacteria were not detectable with

DGGE in oil-contaminated microcosms without nutrients, but were detected by FISH in low abundances (1.0% and 0.3%) in samples A and E, respectively. Within the group of Gram-positive bacteria predominantly the actinomycete genus *Rhodococcus* harbours hydrocarbonoclastic species. Whyte at al. [58] isolated a psychrotolerant *Rhodococcus* strain from a freshwater environment, which was examined for its ability to degrade variable-chain-length alkanes at low temperature. Oddly, in the 10 °C incubation of sample E, only a shift within the genus of the γ-proteobacteria group was observed from *Marinobacter* to members of *Pseudomonas* and *Shewanella*. Obviously *Marinobacter* spp. are replaced in microcosms with nutrient addition by more eurybiotic types.

4.2. Tolerance of Arctic sea-ice bacteria to crude oil

The high percentages of DAPI-stained cells detected with FISH and *Bacteria*-specific probes (up to 82%) in natural sea-ice samples indicated the presence of active sea-ice communities at the time of sampling in summer 2000. These results agree with FISH analyses of adjacent ice cores sampled on the same ice floe [10]. High concentrations of dissolved organic matter (DOM) produced by ice algae in Arctic sea-ice have been reported [59]. The sea-ice DOM is highly bioavailable [60] leading to increased microbial growth and activity in sea-ice relative to the surface seawater. Decreased detection yields with *Bacteria*-specific probes (~62%) and some weak hybridization signals were not surprising. The inhibited growth of algae due to the long incubation in the dark and reduced exchange of nutrients may have caused starvation conditions for bacteria, resulting in low rRNA content and thus weak hybridization signals. However, even after incubation with and without crude oil (~58%) the FISH-detection yields of the microcosms compared well with detection yields of bacterioplankton in various marine environments [61]. Although we did not directly measure bacterial activity, the FISH data indicate that a significant fraction of bacteria in the microcosm experiments was active or growing in the presence of crude oil.

4.3. Degradation of hexadecane and toluene at low temperature

Four *Marinobacter* spp., *Shewanella* spp. and *Pseudomonas* spp. isolates cultivated from oil-contaminated Arctic sea-ice samples were tested for their ability to degrade hexadecane and toluene; an aliphatic and an aromatic compound of crude oil, respectively, without addition of nutrients. Only hexadecane was degraded by the isolates. No significant degradation of toluene was observed. Similarly, Fought et al. [62] demonstrated that most of 200 bacterial strains degraded either the aliphatic [^{14}C]hexadecane or the aromatic [^{14}C]phenanthrene but not both. The degradation of only the aliphatic hexadecane by all three groups of sea-ice isolates suggests a similarity of functional enzymes. Aliphatics are generally considered to be the most readily degraded components in a petroleum mixture [63] and are therefore mineralized faster than aromatic hydrocarbons on contaminated sites. Despite incubation at 1 °C, the Arctic sea-ice isolates degraded the hexadecane at a remarkable 20–50% capacity compared to that of the mesophilic reference strain *M. hydrocarbonoclasticus*, incubated at room temperature. Hexadecane becomes solid at temperatures below 18 °C and is therefore less bioavailable. The reduced bioavailability of hexadecane at low temperature may have caused the initial lag phase of the isolates before onset of utilization and may have also contributed to lower rates of hexadecane mineralization. After 29 days of incubation in melted sea-ice the degradation of hexadecane appeared to be entering the exponential phase; however, the extent of the utilization was not determined in this study. The addition of nutrients may have resulted in higher rates of degradation for both the reference strain *M. hydrocarbonoclasticus* and the isolates.

5. Conclusions

This study provided a first look at the ability of microbial communities in Arctic sea-ice to tolerate and even degrade components of crude oil. In particular, three γ-proteobacterial groups, *Marinobacter* spp., *Shewanella* spp. and *Pseudomonas* spp., were shown with cultivation-independent and cultivation-based methods to be potential hydrocarbon degraders in the Arctic sea-ice environment. The question whether these bacteria have developed the ability to degrade hydrocarbons due to increased exposure to oil contamination or they possess the ability to degrade a variety of naturally occurring aliphatic compounds is intriguing and should be investigated further. However, the presence of hydrocarbon-degrading bacteria in Arctic sea-ice is promising for the potential bioremediation of oil spills, the frequency of which is highly likely to increase due to oil exploration in Arctic offshore areas and transport of Arctic oil and gas along the Northern Sea Route.

Acknowledgments

This study was funded by the European Union (ARCOP-Project) and the Alfred Wegener Institute for Polar and Marine Research Foundation.

We thank our fellow scientists who volunteered with ice coring and sample collection. In particular, K. Reuter for assisting with sample collection and processing during the R.V. Polarstern cruise ARKXVI/2. We are

grateful to J. Jürgens for technical assistance in the home laboratory.

Special thanks go to B. Meon for critical reading of the manuscript and J. Schwarz for proof reading.

References

[1] Grossmann, S. and Dieckmann, G.S. (1994) Bacterial standing stock, activity, and carbon production during formation and growth of sea ice in the Weddell Sea, Antarctica. Appl. Environ. Microbiol. 60, 2746–2753.
[2] Helmke, E. and Weyland, H. (1995) Bacteria in sea ice and underlying water of the eastern Weddell Sea in midwinter. Mar. Ecol. Prog. Ser. 117, 269–287.
[3] Junge, K., Eicken, H. and Deming, J.W. (2004) Bacterial Activity at −2 to −20 °C in Arctic wintertime sea ice. Appl. Environ. Microbiol. 70, 550–557.
[4] Staley, J.T., Junge, K. and Deming, J.W. (2002) In: Biodiversity of Microbial Life: Foundations of Earth's Biosphere (Staley, J.T. and Reysenbach, A.-L., Eds.), pp. 423–438. Wiley, New York.
[5] Brown, M.V. and Bowman, J.P. (2001) A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). FEMS Microbiol. Ecol. 35, 267–275.
[6] Smith, R.E. and Clement, P. (1990) Heterotrophic activity and bacterial productivity in assemblages of microbes from sea ice in the high arctic. Pol. Biol. 10, 351–357.
[7] Thomas, D.N. and Dieckmann, G.S. (2003) In: Sea Ice: An Introduction to its Physics, Chemistry, Biology and Geology (Thomas, D.N. and Dieckmann, G.S., Eds.), p. 402. Blackwell Science, Oxford.
[8] Gosink, J. and Staley, J. (1995) Biodiversity of gas vacuolate bacteria from Antarctic sea ice and water. Appl. Environ. Microbiol. 61, 3486–3489.
[9] Bowman, J., McCammon, S., Brown, M., Nichols, D. and McMeekin, T. (1997) Diversity and association of psychrophilic bacteria in Antarctic sea ice. Appl. Environ. Microbiol. 63, 3068–3078.
[10] Brinkmeyer, R., Knittel, K., Jurgens, J., Weyland, H., Amann, R. and Helmke, E. (2003) Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. Appl. Environ. Microbiol. 69, 6610–6619.
[11] Sullivan, C.W. and Palmisano, A.C. (1984) Sea ice microbial communities: Distribution, abundance, and diversity of sea ice bacteria in McMurdo Sound, Antarctica, in 1980. Adv. Appl. Microbiol. 47, 788–795.
[12] Braddock, J.F., Ruth, M.L., Walworth, J.L. and McCarthy, K.A. (1997) Enhancement and inhibition of microbial activity in hydrocarbon-contaminated arctic soils: implications for nutrient amended bioremediation. Environ. Sci. Technol. 31, 2078–2084.
[13] Whyte, L.G., Bourbonniere, L., Bellerose, C. and Greer, C.W. (1999) Bioremediation assessment of hydrocarbon-contaminated soils from the High Arctic. Bioremed. J. 3, 69–79.
[14] Mohn, W.W. and Stewart, G.R. (2000) Limiting factors for hydrocarbon biodegradation at low temperature in Arctic soils. Soil Biol. Biochem. 32, 1161–1172.
[15] Mohn, W.W., Radziminski, C.Z., Fortin, M.C. and Reimer, K.J. (2001) On site bioremediation of hydrocarbon-contaminated Arctic tundra soils in inoculated biopiles. Appl. Microbiol. Biotechnol. 57, 242–247.
[16] Aislabie, J., Foght, J. and Saul, D. (2000) Aromatic hydrocarbon-degrading bacteria from soil near Scott Base, Antarctica. Pol. Biol. 23, 183–188.
[17] Aislabie, J., Fraser, R., Duncan, S. and Farrell, L. (2001) Effects of oil spills on microbial heterotrophs in Antarctic soils. Pol. Biol. 24, 308–313.
[18] Aislabie, J., McLeod, M. and Fraser, R. (1998) Potential for biodegradation of hydrocarbons in soil from the Ross Dependency, Antarctica. Appl. Microbiol. and Biotechnol. 49, 210–214.
[19] Braddock, J.F., Walworth, J.L. and McCarthy, K.A. (1999) Biodegradation of aliphatic vs. aromatic hydrocarbons in fertilized Arctic soils. Bioremed. J. 3, 105–116.
[20] Mac Cormack, W. and Fraile, E.R. (1997) Characterization of a hydrocarbon degrading psychrotrophic Antarctic bacterium. Antarctic Sci. 9, 150–155.
[21] Delille, D. and Pelletier, E. (2002) Natural attenuation of diesel-oil contamination in a subantarctic soil (Crozet Island). Pol. Biol. 25, 682–687.
[22] Margesin, R., Labbe, D., Schinner, F., Greer, C.W. and Whyte, L.G. (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine Alpine soils. Appl. Environ. Microbiol. 69, 3085–3092.
[23] Margesin, R. and Schinner, F. (1997) Efficiency of indigenous and inoculated cold-adapted soil microorganisms for biodegradation of diesel oil in Alpine soils. Appl. Environ. Microbiol. 63, 2660–2664.
[24] Atlas, R.M. and Budosh, M. (1976) In: 3rd International Biodegradation Symposium (Sharpley, J.M. and Kaplan, A.M., Eds.), pp. 79–85. Applied Science Publishers, London.
[25] Atlas, R.M. (1977) In: Symposium (Wolfe, D.A., Anderson, J.W., Button, D.K., Malins, D.C., Roubal, T. and Varanasi, U., Eds.), pp. 261–269. Pergamon Press, Seattle, Washington.
[26] Floodgate, G. (1984) In: Petroleum Microbiology (Atlas, R.M., Ed.), pp. 355–397. Macmillan Publishing Company, New York USA.
[27] Swannell, R.J.P., Lee, K., Basseres, A. and Merlin, F.X. (1994) Seventeenth Arctic Marine Oil Spill Program Technical Seminar, pp. 1273–1286. Environment Canada, Ottawa, Canada.
[28] Sendstad, E., Hoddo, T., Sveum, P., Eimhjellen, K., Josefsen, K., Nilsen, O. and Sommer, T. (1982) Proceedings of the Fifth Artcic Marine Oilspill Program Technical Seminar, pp. 331–340. Edmonton, Alberta.
[29] Sergy, G.A., Guenette, C.C., Owens, E.H., Prince, R.C. and Lee, K. (1998) 21st Arctic and Marine Oil Spill Program Seminar, pp. 873–889. Environment Canada, Canada.
[30] Sveum, P. and Ladousse, A. (1989) In: International Oil Spill Conference, pp. 439–446. American Petroleum Institute, Washington DC.
[31] Grossman, S. et al. (1999) In: 8th International Symposium on Microbial Ecology (Bell, C., Brylinsky, M. and Johnson-Green, Eds.). Atlantic Canada Society for Microbial Ecology, Halifax Canada.
[32] Bragg, J.R., Prince, R.C., Harner, E.J. and Atlas, R.M. (1994) Effectiveness of bioremediation for the Exxon Valdez oil spill. Nature 368, 413–418.
[33] Prince, R.C. and Bragg, J.R. (1997) Shoreline bioremediation following the Exxon Valdez oil spill in Alaska. Bioremed. J. 1, 97–104.
[34] Cavanagh, J.E., Nichols, P.D., Franzmann, P.D. and McMeekin, T.A. (1998) Hydrocarbon degradation by Antarctic coastal bacteria. Antarctic Sci. 10, 386–397.
[35] Delille, D. and Vaillant, N. (1990) The influence of crude oil on the growth of subantarctic marine bacteria. Antarctic Sci. 2, 655–662.
[36] Delille, D., Basseres, A. and Dessommes, A. (1998) Effectiveness of bioremediation for oil-polluted Antarctic seawater. Pol. Biol. 19, 237–241.
[37] Yakimov, M.M., Gentile, G., Bruni, V., Cappello, S., D'Auria, G., Golyshin, P.N. and Giuliano, L. (2004) 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. 49, 419–432.

[38] Atlas, R.M., Horowitz, A. and Busdosh, M. (1978) Prudhoe crude oil in Arctic marine ice, water, and sedment ecosystems: degradation and interactions with microbial and benthic communities. Recovery potential of oiled marine northern environments. J. Fish. Res. Board Canada 35, 585–590.
[39] Atlas, R.M. (1983) Biodegradation of petroleum hydrocarbons in continental shelf regions of the Bering Sea. Oil Petrochem. Pol. 1, 85–96.
[40] Siron, R., Pelletier, E., Delille, D. and Roy, S. (1993) Fate and effects of dispersed crude oil under icy conditions simulated in mesocosms. Marine Environ. Res. 35, 273–302.
[41] Siron, R., Pelletier, E. and Brochu, C. (1995) Environmental factors influencing the biodegradation of petroleum hydrocarbons in cold sea water. Arch. Environ. Contamin. Toxicol. 28, 406–416.
[42] Delille, D., Basseres, A. and Dessommes, A. (1997) Seasonal variation of bacteria in sea-ice contaminated by diesel fuel and crude oil. Microb. Ecol. 33, 97–105.
[43] Glöckner, F.O., Amann, R., Alfreider, A., Pernthaler, J., Psenner, R., Trebesius, K. and Schleifer, K.-H. (1996) An in situ hybridization protocol for detection and identification of planktonic bacteria. Syst. Appl. Microbiol. 19, 403–406.
[44] Wallner, G., Amann, R. and Beisker, W. (1993) Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 14, 136–143.
[45] Muyzer, G., de Waal, E. and Uitterlinden, A. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.
[46] Muyzer, G., Brinkhoff, T., Nübel, U., Santegoeds, C., Schäfer, H. and Wawer, C. (1997) In: Molecular Microbial Ecology Manual (Akkerman, S.B., Van Elsas, J.D. and De Bruijn, F.J., Eds.), Vol. 3.4.4. Kluwer Academic Publisher, Dordrecht, Netherlands.
[47] Massol-Deya, A.A., Odelson, D.A., Hickey, R.F. and Tiedje, J.M. (1999) In: Molecular Microbial Ecology Manual (Akkermans, A.D.L., VanElsas, J.D. and DeBruijn, F.J., Eds.), Vol. 3.3.2, pp. 1–8. Kluwer Academic Publishers, Dordrecht, Netherlands.
[48] Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J. (1990) Basic local alignment search tool. J. Mol. Biol. 215, 403–410.
[49] Gauthier, M., Lafay, B., Christen, R., Fernandez, L., Acquaviva, M., Bonin, P. and Bertrand, J. (1992) *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. Int. J. Syst. Bacteriol. 42, 568–576.
[50] Junge, K., Imhoff, F., Staley, J. and Deming, J.W. (2002) Phylogenetic diversity of numerically important arctic sea-ice bacteria cultures at sub zero temperature. Mircob. Ecol. 43, 315–328.
[51] Gentile, G., Bonasera, V., Amico, C., Giuliano, L. and Yakimov, M.M. (2003) *Shewanella* sp. GA-22, a psychrophilic hydrocarbonoclastic antarctic bacterium producing polyunsaturated fatty acids. J. Appl. Microbiol. 95, 1124–1133.
[52] Yakimov, M., Golyshin, P., Lang, S., Moore, E., Abraham, W., Lunsdorf, H. and Timmis, K. (1998) *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. Int. J. Syst. Bacteriol. 48, 339–348.
[53] Dyksterhouse, S., Gray, J., Herwig, R., Lara, J. and Staley, J. (1995) *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon- degrading bacterium from marine sediments. Int. J. Syst. Bacteriol. 45, 116–123.
[54] Hedlund, B. and Staley, J. (2001) *Vibrio cyclotrophicus* sp. nov., a polycyclic aromatic hydrocarbon (PAH)-degrading marine bacterium. Int. J. Syst. Evol. Microbiol. 51, 61–66.
[55] Golyshin, P.N., Chernikova, T.N., Abraham, W.R., Lunsdorf, H., Timmis, K.N. and Yakimov, M.M. (2002) *Oleiphilaceae* fam. nov., to include *Oleiphilus messinensis* gen. nov., sp. nov., a novel marine bacterium that obligately utilizes hydrocarbons. Int. J. Syst. Evol. Microbiol. 52, 901–911.
[56] Yakimov, M.M. et al. (2003) *Oleispira antarctica* gen. nov., sp. nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. Int. J. Syst. Evol. Microbiol. 53, 779–785.
[57] Roling, W.F.M., Milner, M.G., Jones, D.M., Lee, K., Daniel, F., Swannell, R.J.P. and Head, I.M. (2002) Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Appl. Environ. Microbiol. 68, 5537–5548.
[58] Whyte, L.G., Hawari, J., Zhou, E., Bourbonniere, L., Inniss, W.E. and Greer, C.W. (1998) Biodegradation of variable-chain-length alkanes at low temperatures by a psychrotrophic *Rhodococcus* sp. Appl. Environ. Microbiol. 64, 2578–2584.
[59] Thomas, D.N., Lara, R.J., Eicken, H., Kattner, G. and Skoog, A. (1995) Dissolved organic matter in Arctic multi-year sea ice during winter: major components and relationship to ice characteristics. Pol. Biol. 15, 477–483.
[60] Amon, R.M.W., Fitznar, H.-P. and Benner, R. (2001) Linkages among the bioreactivity, chemical composition, and diagenetic state of marine dissolved organic matter. Limnol. Oceanogr. 46, 287–297.
[61] Glöckner, F.O., Fuchs, B.M. and Amann, R. (1999) Bacterioplankton Compositions of Lakes and Oceans: a First Comparison Based on Fluorescence In Situ Hybridization. Appl. Environ. Microbiol. 65, 3721–3726.
[62] Foght, J.M., Fedorak, P.M. and Westlake, D.W.S. (1990) Mineralization of [^{14}C]hexadecane and [^{14}C]phenanthrene in crude oil: specificity among bacterial isolates. Can. J. Microbiol. 36, 169–175.
[63] Atlas, R.M. (1981) Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol. Rev. 45, 180–209.
[64] Amann, R., Binder, B.J., Olson, R.J., Chrisholm, S.W., Devereux, R. and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 56, 1919–1925.
[65] Daims, H., Bruhl, A., Amann, R., Schleifer, K.-H. and Wagner, M. (1999) The domain-specific probe EUB338 is insufficient for the detection of all Bacteria: development and evaluation of a more comprehensive probe set. Syst. Appl. Microbiol. 22, 434–444.
[66] Roller, C., Wagner, M., Amann, R., Ludwig, W. and Schleifer, K. (1994) In situ probing of gram-positive bacteria with high DNA G + C content using 23S rRNA-targeted oligonucleotides. Microbiology 140, 2849–2858.
[67] Manz, W., Amann, R., Ludwig, W., Vancanneyt, M. and Schleifer, K.-H. (1996) Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga-Flavobacteria-Bacteroides* in natural environment. Microbiology 142, 1097–1106.
[68] Neef, A., 1997. Anwendung der in situ-Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozönosen Ph.D. thesis. Technische Universität München, München, Germany.
[69] Manz, W., Amann, R., Ludwig, W., Wagner, M. and Schleifer, K.-H. (1992) Phylogenetic oligonucleotide probes for the major subclasses of *Proteobacteria*: problems and solutions. Syst. Appl. Microbiol. 15, 593–600.
[70] Schleifer, K.-H., Amann, R., Ludwig, W., Rothemund, C., Springer, N. and Dorn, S. (1992) In: *Pseudomonas*: Molecular Biology and Biotechnology (Galli, E., Silver, S. and Witholt, B., Eds.). American Society for Microbiology, Washington DC.