IMPACT OF OIL CONTAMINATION AND BIOREMEDIATION TREATMENTS ON THE COMPOSITION AND DEGRADATION EFFICIENCY OF POLAR BACTERIAL SEA-ICE COMMUNITIES

Dissertation

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Zusammenfassung

Die Gefahr einer Ölkontamination in den polaren meereisbedeckten Gebieten des Arktischen und Antarktischen Ozeans hat sich in den letzten Jahren durch zunehmenden Schiffsverkehr und Tourismus, durch ansteigende wissenschaftliche Aktivitäten sowie durch wachsendes Interesse an der Erschließung der im Arktischen Ozean lagernden Öl- und Gasvorkommen, die ca. ein Viertel der Weltrohölvorräte ausmachen, deutlich verstärkt.

In der vorliegenden Dissertation wurde der Einfluss von Rohöl auf die im Meereis des Arktischen und Antarktischen Ozeans lebenden bakteriellen Gemeinschaften untersucht. Daneben wurden Bioremediationsexperimente im Labor und Freiland durchgeführt, um die Wirkung von künstlich zugeführten Nährstoffen und Ölabbauern auf Abbauprozesse in kontaminiertem Meereis zu ermitteln. Getestet wurden die Nährstoffsubstrate: Inipol, Fischmehl und anorganische Stickstoff- und Phosphorverbindungen. Bioaugmentationsversuche wurden mit ölabbauenden Meereisbakterien aus eigenen Pilotexperimenten durchgeführt um zu klären, ob Beimpfungen die Anlaufphase bis zur Entwicklung der autochthonen ölabbauenden Flora verkürzen und Abbauprozesse beschleunigen können. Die Reaktionen der bakteriellen Meereis-Lebensgemeinschaften auf die unterschiedlichen Einwirkungen wurden mit den molekularbiologischen Methoden: DGGE (Denaturierende Gradienten Gelelektrophorese) und FISH (Fluoreszenz in situ Hybridisierung) sowie auf der Basis von Isolationen ermittelt. Der Abbau der Rohölsubstanzen wurde mit Hilfe der Gaschromatographie (GC-FID bzw. GC-MS) verfolgt.

Zugesetztes Rohöl reduzierte die bakterielle Diversität aller untersuchten Meereisgemeinschaften deutlich. Die ursprünglich diversen Gemeinschaften mit hohen Anteilen von Vertretern aus dem *Bacteroidetes* Phylum und den *Gamma-* und *Alphaproteobakterien Gruppen,* mit nur verschwindend geringen Anteilen an *Actinomyceten* bzw. *Betaproteobakterien*, entwickelten sich zu Gesellschaften mit einer Vorherrschaft von *Gammaproteobakterien,* wobei Bakterien der Gattungen *Shewanella*, *Marinobacter*, *Pseudomonas* und *Pseudoalteromonas* am häufigsten auftraten. Die bei den Fluoreszenz *in situ* Hybridisierungen (FISH) beobachteten schwachen Fluoreszenzsignale und das Vorkommen extrem kleiner Zellen lassen auf einen starken toxischen Effekt der Ölbestandteile auf die Organismen schließen.

In den Bioremediationsanwendungen mit Nährstoffzusatz reduzierte sich die Diversität ebenfalls deutlich. Obgleich sich, abhängig von der Ausgangsgemeinschaft, unterschiedlich zusammengesetzte Populationen entwickelten, war immer der gleiche Trend zu einer von *Gammmaproteobakterien* dominierten Gesellschaft zu erkennen. Jene Gattungen, die in den mit Öl kontaminierten Experimenten ohne Nährstoffzugaben verstärkt auftraten, dominierten auch die Ansätze mit Nährstoffen. Nur in wenigen Bioremediationsansätzen traten verstärkt auch Bakterien aus anderen als der *Gammaproteobakterien*-Gruppe auf. So entwickelten sich in den Bioremediationsansätzen mit ‚Gapwater' aus antarktischem Meereis auch *Alphaproteobakterien* und in jenen von arktischen

Schmelzwassertümpeln Bakterien der Gattung *Ralstonia* (*Betaproteobakterien*). Diese Gattungen waren jedoch bereits im unkontaminierten Meereis detektiert worden.

Die Experimente zum Ölabbau ergaben, dass unter Meereisbedingungen ohne Verabreichung von Nährstoffen kein signifikanter Abbau von Kohlenwasserstoffen stattfindet. Erst durch zusätzliche Nährstoffgaben wurde der Abbau von Kohlenwasserstoffen auch bei Temperaturen von 0°C und –3°C stimuliert. Der bakterielle Abbau von Rohölkomponenten bei Temperaturen unter 0°C blieb aber vergleichsweise langsam. Durch Bioaugmentation mit angereicherten ölabbauenden Meereisbakterien konnte der Dekontaminationsprozess weiter beschleunigt werden. Augenscheinlich überbrückten die zugesetzten allochthonen Bakterien die Entwicklungszeit der autochthonen ölabbauenden Flora. Die Experimente zeigten weiter, dass zumindest ein Teil der inokulierten Bakterien auch nach einem halben Jahr noch vorhanden war. Durchgesetzt hatten sich Vertreter aus den Gattungen *Oleispira*, *Pseudomonas, Shewanella* sowie *Rhodococcus*.

Für eingehendere Untersuchungen zum Abbaupotential von Kohlenwasserstoffen der Meereisbakterien wurde eine breites Spektrum an Bakterien aus unterschiedlichen Anreicherungsansätzen und Pilotexperimenten isoliert und auf ihre Fähigkeit zum Abbau von Kohlenwasserstoffen sowie auf ihre Temperatur- und Salztoleranz hin untersucht. *Gammaproteobakterien* aus den Gattungen *Oleispira*, *Pseudomonas und Psychrobacter* sowie Actinobakterien aus den Gattungen *Dietzia* und *Rhodococcus* erwiesen sich als potente Abbauer eines breiten Spektrums von kurz- und langkettigen Alkanen bei Temperaturen von 0°C und -3°C. *Pseudomonas* sp. 327 und *Marinobacter* sp. 81 konnten darüber hinaus verschiedene aromatische Verbindungen verwerten. Neben weiteren Isolaten aus der Gruppe der *Gammaproteobakterien*, die den Gattungen *Shewanella*, *Psychromonas* und *Marinomonas* angehören, verwerteten auch Isolate aus der Gruppe der *Alphaproteobakterien,* die den Gattungen *Loktanella* bzw. *der Gattung Jannaschia* nahe stehen, kurzkettige Alkane sowie die 2-Ring Aromaten Naphthalin und Acenaphthen .

Die Untersuchungen haben gezeigt, dass es autochthone Öl-abbauende Meereisbakterien gibt und dass durch Bioremediation in Form von gezieltem Nährstoff- und Mikroorganismenzusatz der Ölabbau in kontaminiertem Meereis deutlich beschleunigt werden kann.

Summary

The threat of a hydrocarbon contamination in the sea-ice covered areas is growing rapidly due to increasing human activities in the polar regions , which require petroleum as energy source, and due to a fast growing interest in exploiting the estimated one fourth of the world's oil reservoirs of the Arctic Ocean.

This dissertation assessed the influence of crude oil contamination on sea-ice microbial communities (SIMCO) in the Arctic and the Southern Ocean. Field experiments were conducted with indigenous sea-ice bacterial communities to determine their ability to degrade oil. Three fertilizers, Inipol, fish meal, and soluble inorganic nutrients, were tested for stimulation of biodegradation. To test whether bioaugmentation can further enhance oil biodegradation processes and bridge the lag period of the development of an oil degrading population, bacteria were enriched from laboratory bioremediation experiments for reinoculation in oil contaminated sea-ice. The response of the bacterial sea-ice communities was assessed by the molecular methods denaturing gradient gel electrophoresis (DGGE) and fluorescent in situ hybridization (FISH). Hydrocarbon degradation efficiency of environmental samples treated with the fertilizers as well as the hydrocarbon degradation potential of single bacterial strains was determined with oil fingerprints obtained by means of GC-FID and radio tracer experiments of single hydrocarbon substances.

Oil treatments of field plots resulted in a highly reduced bacterial diversity with a shift from initial members of the *Bacteroidetes* phylum, *Gamma-*, and *Alphaproteobacteria*; as well as some *Actinobacteria* towards predominantly *Gammaproteobacteria* with frequently found members of the genera *Shewanella*, *Marinobacter*, *Pseudomonas*, and *Pseudoalteromonas*. Low FISH detection signals, indicating reduced activity, suggest a toxic effect of crude oil hydrocarbons on the sea-ice bacterial communities. Fertilizer treatments resulted in varying populations depending on the initial communities of the different sea-ice samples. However, oil plus fertilizer treatments also resulted in decreasing diversities over the course of incubation with a trend towards predominantly *Gammaproteobacteria* with the same dominating genera as in the oil but non-fertilized experiments. In experiments with gap water from Antarctic sea-ice, members of the *Alphaproteobacteria* were dominant and in experiments with water from Arctic melt pools the *Betaproteobacteria* genus *Ralstonia* was dominant. These genera occur only in low abundances in uncontaminated sea-ice and thus indicate the development of a specific population after oil contamination which is often predominated by the *Gammaproteobacteria*.

The experiments showed that under sea-ice conditions no significant hydrocarbon degradation occurred when treated with crude oil alone, whereas fertilization with organic as well as inorganic nutrients stimulated the hydrocarbon biodegradation even under sea-ice conditions. However, the effect on bacterial biomass production and hydrocarbon biodegradation was higher with fish meal than with the organic agent Inipol, probably due to its solidifying characteristics at temperatures around freezing.

Bioaugmentation with an enrichment of oil-degrading sea-ice bacteria was shown to enhance hydrocarbon biodegradation processes in experiments with gapwater from Antarctic sea-ice. Although growth by other bacteria in the inoculum as well as indigenous sea-ice bacteria exceeded that of the oil-degrading bacteria, the oil degraders appeared to have established themselves and thus bridged the 6 month lag period prior to the development of an in situ oil degrading population After six months of incubation, members of the genera *Oleispira*, *Pseudomonas*, and *Shewanella* of the *Gammaproteobacteria* as well as Rhodococci of the *Actinobacteria* survived and prevailed from the inoculum.

Bacterial isolates obtained from bioremediation experiments with Arctic and Antarctic sea-ice as well as with gap water from Antarctic sea-ice were tested for their hydrocarbonoclastic capabilities at low temperatures. Members of the genera *Oleispira* and *Pseudomonas* (*Gammaproteobacteria)* as well as *Dietzia* and *Rhodococcus* (*Actinobacteria)* were determined to be degraders of a broad range of short and long chain crude oil alkanes at temperatures as low as 0°C and -3°C. The *Pseudomonas* isolate as well as a *Marinobacter* strain were also able to grow on various aromatic compounds. Several *Shewanella* strains, one *Psychrobacter sp.* isolate and one *Marinomonas* sp. isolate (all *Gammaproteobacteria)* as well as a *Loktanella* sp. and a bacterium, related to the genera *Jannaschia* (both of the *Alphaproteobacteria)* were able to grow on short chain alkanes as well as on the 2-ring aromatic compounds naphthalene and acenaphthene.

PART I

INTRODUCTION

Introduction

There is a growing threat of oil contamination in the sea-ice covered areas of the polar regions, which requires the development of oil spill response methods as safety measures to protect this highly sensitive and vulnerable environment. This study deals with the biological aspects. Therefore it is important to understand the difficult and complex sea-ice environment.

Sea-ice environment

The seasonal sea-ice cover in polar regions and other seas constitutes one of the major habitats on our planet covering at its maximum extent about 13% of the Earth's surface. In the Arctic Ocean the minimum extent of the sea-ice cover is about 7 x 10^6 km^2 in summer and expands to 14 x 10^6 km^2 in winter (Maykut, 1985). In the Antarctic, sea-ice covers an area of 20 x 10^6 km^2 in winter and recedes to an area of 4 x 10^6 km^2 in summer. Apart from affecting ocean circulation and the world climate, sea-ice has a direct and indirect effect on polar ecosystems. It harbours a Sea-Ice Microbial COmmunity (SIMCO) made up of microalgae, mainly diatoms, bacteria, archaea, fungi, and proto- and metazoans (Horner, 1985; Krembs et al., 2000; Palmisano & Garrison, 1993).

There are several similarities between the Arctic and the Antarctic sea-ice systems; however, there are also substantial differences reflected in sea-ice development, thickness, structure, and seasonality. When the temperature of seawater (with a typical salinity of 34 psu) drops below -1.86°C, ice crystals, termed frazil ice, begin to form and rise to the water surface, where they gather to a layer of grease ice. The crystals vary in size, shape, and direction with time and water movement, the grease ice congeals into disc shaped floes, the so called pancake ice. The pancakes from 5-10 cm in diameter grow to 'super pancakes' of several meters in diameter which are about 10-50 cm thick (Thomas, 2004). During the thickening or growing process of ice in turbulent waters the layers can be subject to processes of breaking up, rafting on top of each other, deformations, and refreezing, causing an inhomogeneous ice body which can vary in structure and thickness from loose aggregations to large expanses of ice cover. Sea-ice, grown under quiet conditions, is made up primarily of columnar ice with vertically arranged crystals which grow slowly, layer upon layer, on the underside of the frazil ice surface. In the Arctic 60-80% of the pack ice is made up of columnar ice (Thomas, 2004). In contrast, 60-70% of the Antarctic sea-ice is composed of layers of frazil ice (Maykut, 1985) due to more turbulent conditions in the Southern Ocean.

In the Arctic Ocean the formation of sea-ice begins north of 70°N as land fast ice, which is attached to land. When the ice cover breaks up, the ice is released into the open ocean as pack ice. Most of the pack ice is then transported through the trans-Arctic drift stream over several (5-6) years to the Fram Strait where it exits the Arctic Ocean. Another major surface current, which transports sea-ice through the Arctic Ocean is the Beaufort Gyre, an anticyclonic whirl in the Canadian Basin, which can trap sea-ice for up to 7-10 years. The long drift of the Arctic sea-ice at high latitudes results in the fact that sea-ice lasts more than one season (multi-year ice), thus attaining thicknesses of 2-5m (Maykut, 1985). In the Southern hemisphere most of the sea-ice cover is formed annually (first-year ice) around the Antarctic continent between 55° and 70°S with ice thicknesses typically of 1m and rarely more than 2m. With warmer conditions the ice fields are pushed away from the continent to lower latitudes, resulting in the melting of about 80% of the pack ice each year. Furthermore, the snow cover on Arctic ice floes is generally thinner (average 30 cm) compared to the southern hemisphere (average 50 cm), resulting on the one hand in a decreasing albedo in the Arctic with increased surface melting and on the other hand in less insulation and thus a faster growth of Arctic sea-ice.

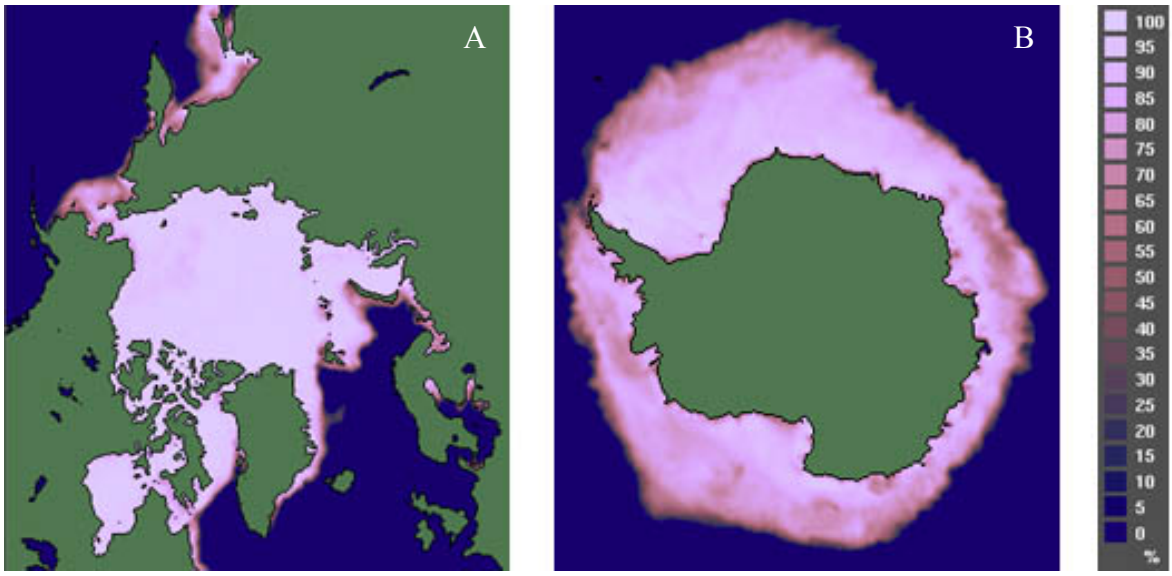

Figure 1. Extension of the sea-ice covers (shown in pink) during the winter season. (A) sea-ice cover in the northern hemisphere in February 2004 with a total area of 12.9 million sq km. (B) sea-ice cover in the southern hemisphere in August 2005 with a total area of 13.9 million sq km.

The surface layers of the Arctic Ocean are colder than those of the Southern Ocean. The Arctic Ocean is almost entirely surrounded by land masses and there is little contact to other oceans. There is a great input of low salinity water, estimated at 10% of the global run-off, into the Arctic Ocean, causing an effective barrier of heat fluxes from warmer water layers

below into the surface waters. The Antarctic continent, on the other hand, is covered with ice and snow throughout the year and thus averts a terrestrial input to the surrounding water masses (see Figure 1).

In spring and summer months, when the surfaces of ice floes begin to melt, shallow melt pools are a common occurrence, especially on Arctic ice floes, providing a habitat for surface assemblages (Brinkmeyer et al., 2004). In the Antarctic, however, the development of gap-layers is a typical feature of the perennial summer as a result of biophysical feedback processes at the onset of warming (Haas et al., 2001). An increase in temperature and solar radiation leads to algal blooms. The brownish colour of the algae enhances the absorption of radiation und thus leads to further melting. Gap-layers are found at, or just below the water level, overlain by a thin solid layer of ice (Haas et al., 2001) (see Figure 2).

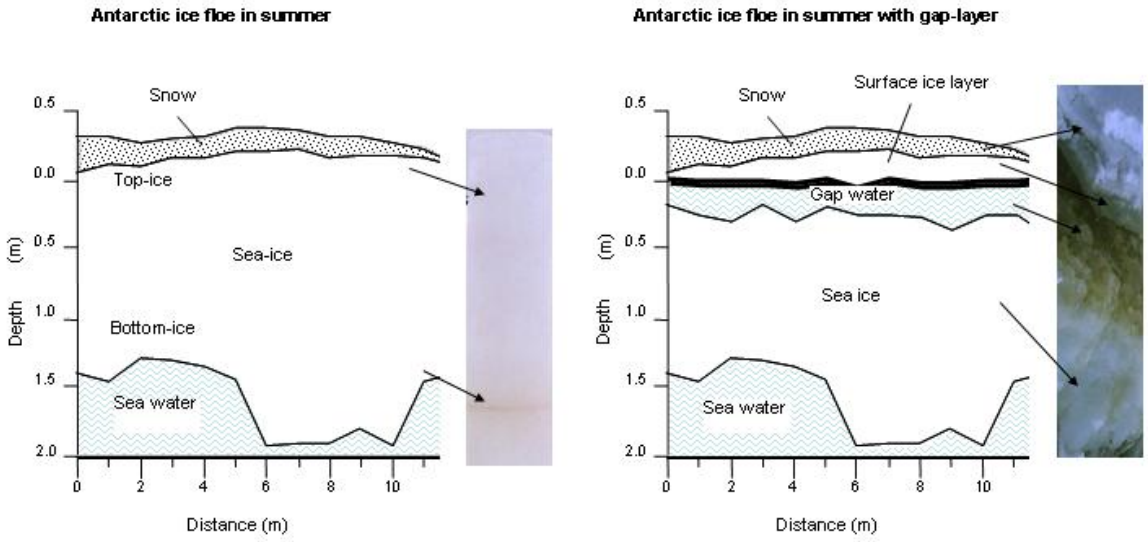

Figure 2. Schematic vertical cross-sections (modified from Kattner et al., 2004 and Haas et al., 2001) of two summer ice floes, the right one with a seawater filled gap-layer. Water level is 0 cm. The left photograph shows a vertical cross through the ice column with a typical algae stock layer at the bottom part and the right photograph a section of surface-ice layer from a refrozen gap-layer floe. The greenish bottom-layer is a refrozen gap-layer containing high algae standing stocks.

During the formation of sea-ice, dissolved salts, dissolved and particulate organic and inorganic matter, as well as organisms are excluded from the ice crystals and collected in a network of brine pockets and channels within the ice. Due to gravity and melting the brine drains to lower layers in the ice and some may be expelled from the ice. Salinity and volume

are strongly dependent on the temperature. The temperature at the ice-water interface is at the freezing point of about -2°C and quite stable, whereas the temperature on the surface of the ice varies considerably. The difference in temperature, which is especially strong in winter, causes a vertical gradient of temperature, salinity, and brine volume within the ice. Thus the width of the brine channels can vary from some micrometers to centimetres (Eiken, 1992; Weissenberger et al., 1992) and the salinity varies from freshwater to about 235 psu (Maykut, 1986). Furthermore, the absorbance of solar radiation accounts for a gradient of light intensities.

Sea-ice bacteria, often attached to surfaces of larger organisms, such as algae or detritus (Grossmann & Gleitz, 1993; Palmisano & Garrison, 1993), are enriched relative to those found in seawater (Helmke & Weyland, 1995; Sullivan & Palmisano, 1984), and show high production rates (Helmke & Weyland, 2004; Kottmeier & Sullivan, 1987). However, immediately after sea-ice formation, in young sea-ice, there is a reduction in bacterial activity compared with rates from the water body. In the course of time, when the ice floe grows, psychrophilic bacteria take over to dominate the communities and bacteria increase in number and activity rates (Grossmann & Dieckmann, 1994; Helmke & Weyland, 1995). High concentrations of dissolved organic matter (DOM), relative to those found in surface seawater have been determined for Arctic (Thomas et al., 1995) and Antarctic sea-ice (Herborg et al., 2001). DOM consists largely of carbohydrates, produced by excretion of organic polymers by algae and bacteria as well as by lysis of dead sea-ice organisms, which serve as food for the bacteria.

Bacterial communities in sea-ice are well adapted to their ambient, constantly cold environment with predominance of psychrophiles (Bowman et al., 1997b; Helmke & Weyland, 2004). In 1975 Morita (Morita, 1975) characterized psychrophilic and psychrotolerant microorganisms by their minimum, optimum, and maximum growth temperatures at or below 0°, 15°, and 20°C or 0°, 30°, and 35°C, respectively. Helmke and Weyland (Helmke & Weyland, 2004) found differences in the degree of cold adaptation between Antarctic and Arctic sea-ice bacteria. On average, the Antarctic isolates showed a narrow temperature range according to those defined by Morita, whereas the Arctic isolates had broader temperature ranges. The temperature maximum of more than 50% of the Arctic sea-ice bacteria touched or surpassed the defined line of 20°C. It is assumed that the higher occurrence of moderate psychrophiles is caused by the inflow of warm water masses as well as the terrestrial input. Also the different sea-ice structure and thickness of Arctic versus Antarctic sea-ice may cause a selective enrichment. Therefore the use of the term ‘moderate

psychrophiles' besides 'psychrophiles' and 'psychrotolerant' for a further group of cold adapted bacteria with a temperature range of ≤0°C to ≤25°C is recommended.

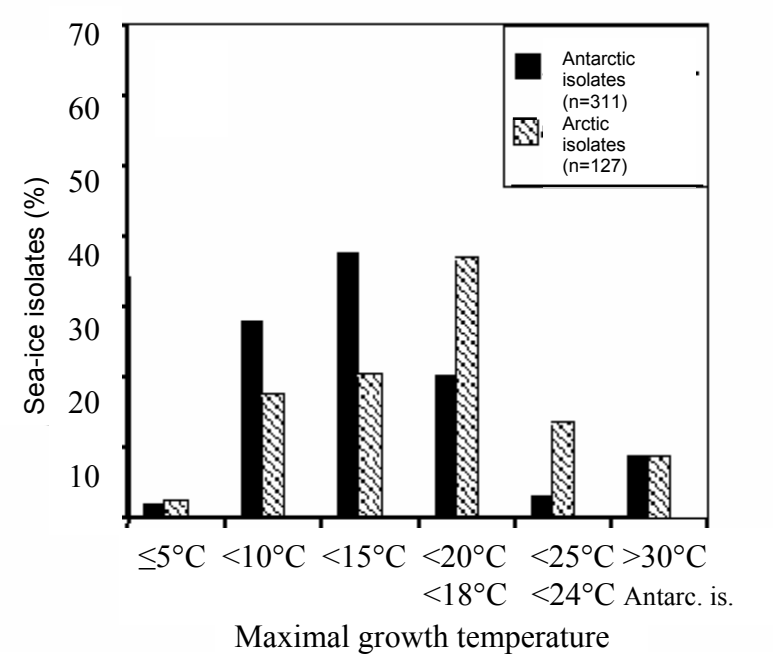

Figure 3. Maximal growth temperatures of bacteria isolated from sea-ice of the Southern Ocean and Arctic Ocean. Reprinted from Helmke and Weyland (2004).

Sea-ice bacterial diversity

Temperature, salinity, nutrients, ice structure, and light conditions vary considerably even within a small ice floe and induce the development of quite different SIMCO. The sea-ice community structure also varies considerably between the Arctic and Antarctic.

Phylogenetic diversity studies of different Arctic and Antarctic pack-ice and land-fast ice samples revealed that the bacterial sea-ice community consists of four major phylogenetic groups (Bowman et al., 1997b; Brinkmeyer et al., 2003; Brown & Bowman, 2001) of mainly heterotrophic organisms: The proteobacteria with members of the *Gamma-*, *Alpha-*, and *Betaproteobacteria* and the *Bacteroidetes* phylum (formerly *Cytophaga-Flavobacterium-Bacteroides* group) as the two major groups, as well as the low and high mole percent G+C gram positive bacteria (Bowman et al., 1997b; Brinkmeyer et al., 2003). Although the same phylotypes are found at both poles, there are differences in their quantitative distribution from pole to pole (Brinkmeyer et al., 2003). Only recently some new genera and several species from sea-ice (Bowman, 1998; Bowman et al., 1998a; Bowman et al., 1997c; Bowman et al., 1997d; Junge et al., 1998; McCammon & Bowman, 2000) have been described. The new genus *Octadecabacter* of the *Alphaproteobacteria* with psychrophilic gas-vacuolate strains (Gosink et al., 1997), *Polaromonas* of the *Betaproteobacteria* (Irgens et al., 1996), and from

the *Bacteroidetes* phylum the new genera *Polaribacter* (Gosink et al., 1998), *Psychroflexus* (Bowman et al., 1998b), and *Gelidibacter* (Bowman et al., 1997a) were described.

There is a high similarity in the bacterial genera found at both poles (Brinkmeyer et al., 2003; Brown & Bowman, 2001; Staley & Gosink, 1999a). On the other hand, some studies of biogeography investigated that species with high 16S rRNA similarity, which were isolated from both poles, were found not to be the same bipolar species (Staley & Gosink, 1999a). However, it has not yet been thoroughly explored whether these bacteria are endemic to the one or the other pole.

Due to primary production of numerous algae, oxygen is usually not a limiting factor for bacterial growth in sea-ice. Unlike most other environments, a large proportion of the sea-ice bacteria is cultivatable (Helmke & Weyland, 1995; Junge et al., 2002). However, pure cultures, selected for certain organisms, do not represent the natural diversity of bacterial communities in their environment (Amann et al., 1995).

Crude oil

It is generally agreed that crude oil arose from aquatic algae with some terrestrial organic material while terrestrial plants gave rise to coal reserves. The average age of crude oil is about 100 million years (71% between 180-85 million years) (Tissot & Welte, 1984). Crude oils are composed of the molecules carbon and hydrogen, with typical H/C ratios of 1.5-2, containing predominantly aliphatic compounds (linear chains namely ‘paraffins’ or rings namely ‘naphthenes’) and aromatic compounds. The proportions vary widely from oil to oil. In average, of 527 crude oils, saturates make up 58.2%, aromatics 28.6%, and polar compounds 14.2% (Tissot & Welte, 1984). Linear alkanes make up an average of 15% - 20% of undegraded crude oil. Branched alkanes, mainly occur in the range of C_6 to C_8, but pristane ($C_{19}H_{40}$) and phytane ($C_{20}H_{42}$). The sulfur content is typically 0.1% in light oils and sometimes more than 5% in heavy oils (Hunt 1996).

The vast majority of petroleum hydrocarbons is biodegradable under aerobic conditions (Prince & Atlas, 2005), except for some molecules such as hopanes (Prince & al., 1994). Moreover biodegradation of these compounds have been reported (Bost et al., 2001; Frontera-Suau et al., 2002), as well as of some oil resins and polar molecules.

Polycyclic aromatic hydrocarbons constitute only a minor proportion of crude oils. However, due to their toxicological properties, they are of ecological importance.

Low-level routine release represents 90% of the discharge volume (runoff, industrial effluents) of hydrocarbons. In the marine environment only about 18% arise from refineries, offshore operations, and tanker activities.

Relevance of oil degradation in polar regions

According to some estimates almost one fourth of the world's undiscovered petroleum resources may be located in the Arctic Ocean and there is a fast growing interest in the exploitation of these reservoirs. Eleven new hydrocarbon fields have been discovered in the Barents and Pechora Sea and three gas and condensate fields in the Kara Sea. Development and production of offshore projects in these ice-covered areas are ongoing and since 2002 there has been a dramatic increase in oil shipment in the Arctic Ocean. Ship traffic through research and tourism in the Southern Ocean is also increasing. Consequently, pollution through the release of petroleum hydrocarbons into the marine environment and the danger of an accidental major oil spill in the polar, ice-covered regions are increasing. In most cases, oil spills are detected too late and many oil spills will never be removed (Bambulyak & Frantzen, 2005). It is a political question how much oil contamination a society will tolerate. Experiences from oil cleaning operations showed that only 10-15% of the oil could be successfully removed under Arctic conditions (Bambulyak & Frantzen, 2005).

There is a growing interest to use the Northern Sea Route (NSR) as alternative transportation route for Arctic oil and gas. Since 2002 there has been a dramatic increase in oil shipment from the Russian part of the Barents Region along the Norwegian coast. In 2004 about 12 million tons of export oil and oil products were delivered to the western market. This volume may reach 50-150 million tons in the next decade (Bambulyak & Frantzen, 2005). Up to 20 million tons will come from Prirazlomnoye, the first offshore industrial oil field in the Russian part of the Barents Region, which is planned to begin in 2009 and will then operate all year round, most of the year in ice-covered waters (Bambulyak & Frantzen, 2005). In the European part of Russia there are three shipping possibilities for export oil. Only the route through the Barents Sea provides the possibility to ship large amounts directly to European and other major harbours. Before export oil reaches its final destination, it is not only transported by tankers, but also in pipelines and railways. Russia is the world's second largest crude oil producer with more than 1.5 million km of gas and oil pipelines, many of them dating from Soviet times, and are in poor condition (Philip et al., 2005). Due to the cold climate in winter and thawing in summer the steel of the pipelines is not durable and there are minor as well as major oil leaks every year in Arctic and sub-Arctic areas. But the

development of oil and gas wealth has recently seen large investments, domestic and foreign, into the Russian oil fields (Philip et al., 2005).

There are offshore projects for oil production in other parts of the Arctic Ocean as well. 'Snøhvit' ('Snow White'), located in the Barents Sea north of Norway, will be the largest sub-sea liquefied natural gas (LNG) project in the world, as well as the most northerly. In 2006, Statoil plans to begin supplying 250 Mmcf/d LNG [Mmcf: million cubic feet] (equal to 7075 litre) from Snøhvit until 2023.

In the central Beaufort Sea 'Northstar' and 'Liberty' are located offshore of the northern Alaskan coast. 'Northstar' lies west of Prudhoe Bay as an artificial island in a dynamic sea-ice environment. The oil is pumped through a pipeline which runs in permafrost beneath the sea floor, which itself is below the Arctic pack ice, for about 10 months per year. The pipeline is subject to harsh conditions and constitutes a potential risk of oil spills. The production facility itself is exposed to winter storms and rafting sea-ice. 'Northstar' began production in November 2001. 'Liberty' is located east of Prudhoe Bay and is more protected from pack-ice by barrier islands. The shipwreck of the supply ship 'Bahia Paraiso', which ran aground in January 1989 and subsequently sank near the Antarctic Peninsula, and former accidental oil spills in the Arctic, such as the one of the 'Exxon Valdez' in Prince William Sound in March 1989 and the 'Selendang Ayu' oil spill on the north side of Unalaska Island in 2004, highlighted the need of countermeasures for environmental safety and contingency strategies in polar regions , although the international safety rules demand a trend towards more advanced and safer vessels.

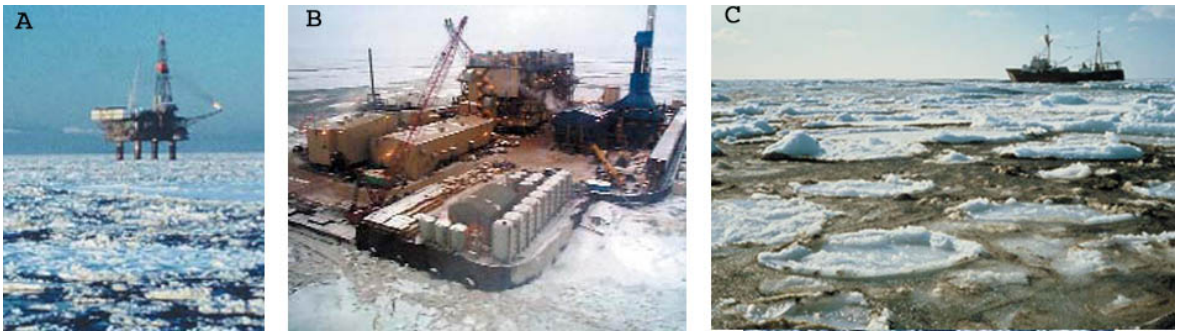

Figure 4. (A) Drilling platform Cook Inlet, Alaska. (B) Northstar offshore oil production facility, North Slope, Alaska. (C) Oil ice slush among pancake ice, Canadian East coast in 1986. Images reprinted from Dickins (2004).

Crude oil biodegradation

Many of the petroleum hydrocarbons are biodegradable by microorganisms. The biodegradability is dependent on the chemical structure of the molecule, the presence of viable microorganisms, able to degrade the substances, and environmental conditions (Philip et al., 2005). It is well established that microorganisms preferentially metabolize n-alkanes before iso-alkanes and low molecular weight hydrocarbons before the ones with high molecular weight. Therefore a decrease in the ratio of n-heptadecane to pristane and n-octadecane to phytane indicate biodegradation of crude oil in the early stage. After degradation of n-alkanes, the branched alkanes are attacked by bacteria. Pristane is readily degradable, so the ratio of pristane to phytane is also an indicator for the further biodegradation of the analyzed crude oil. To determine advanced biodegradation more resistant compounds, such as hopanes (Prince & al., 1994) or alkylated phenanthrenes (Douglas et al., 1992), have to be used as reference analytes.

Hydrocarbon degradation is limited due to a great insolubility of most hydrocarbons into water (Page et al., 2000; Yaws et al., 1990). Maier (Maier, 2000) defined bioavailability as the amount of contaminant present that can be readily taken up by living organisms, such as microbial cells. The bioavailability is influenced by many factors and is thus very complex. The solubility in the aqueous phase is one of the most important factors for bioavailability, which is in turn dependent on temperature. Non-polar, hydrophobic molecules have low water solubility. In general the solubility of hydrocarbons into the aqueous phase decrease with increasing size of molecules and thus bioavailability is limited.

For a long time it was considered that biodegradation of hydrophobic substances require prior transfer into the aqueous phase (Harms & Bosma, 1997; Ogram et al., 1985). However, several recent studies presented that hydrocarbons are degraded at rates which exceed their dissolution into the aqueous phase (Leahy et al., 1990; Thomas et al., 1986). Bioavailability is considered a dynamic process, determined by the rate of substrate mass-transfer to microbial cells relative to their specific catabolic activity (Johnsen et al., 2005). Microorganisms have developed strategies which enable them to efficiently utilize insoluble hydrocarbons. One of the strategies is increased cell affinity to hydrophobic surfaces, which enables the organisms to directly absorb the substrate (Maier, 2000). Another strategy is the release of biosurfactants, which are small, detergent like molecules with a hydrophilic head and a lipophilic tail (Johnsen et al., 2005).

Low molecular weight alkanes are relatively water soluble and act as solvents to the lipid membrane of bacteria. Therefore these alkanes are toxic to microorganisms (Sikkema et al.,

1995). Some substances can be more toxic or even carcinogenic when they are partially metabolized. Although progressed biodegradation may lead to an increased bioavailability of oil compounds through emulsification by surfactant production or partially oxidized intermediates to other organisms (Prince & Atlas, 2005), bioremediation studies have shown that nutrient addition leads to a decrease in oil toxicity (Mueller et al., 1997). Hydrophobic substances are also lipophilic and hence more likely to be biomagnified (Philip et al., 2005). Biomagnification begins with bioaccumulation, which is the uptake of a substance from the environment by a living organism that concentrates the chemical. Often, biomagnification starts at the top level of the food chain/web and chemical pollutants are thus passed to other organisms. Therefore decontamination of these substances is of great importance.

Degradation of oil under cold conditions

Oil biodegradation is reduced significantly at low temperatures (Leahy & Colwell, 1990). This is not only due to common deceleration of bacterial activity and growth but also due to a decreased bioavailability affected by an increase in oil viscosity under cold conditions. Furthermore, at low temperatures, reduced solubility of short chain alkanes (≤10) in the aqueous phase may enhance oil toxicity (Atlas, 1981; Leahy & Colwell, 1990; Whyte et al., 1998).

Nevertheless, biodegradation of petroleum hydrocarbons by indigenous microbial communities has been reported from various Arctic and Antarctic ecosystems of soil, freshwater, and seawater as well as from cold alpine sites (Aislabie et al., 2000; Atlas, 1981; Atlas, 1995; Bossert & Bartha, 1984; Braddock et al., 1997; Leahy & Colwell, 1990; Margesin & Schinner, 1997a; Margesin & Schinner, 1997b; Mohn et al., 2001; Mohn & Stewart, 2000; Whyte et al., 1999; Whyte et al., 1998). However, degradation rates are slow in cold climate and result in a long persistence of the contaminants in marine environments. A solution is to isolate and enrich indigenous cold-adapted microorganisms with hydrocarbonoclastic potentials and reinoculate them as part of the bioremediation strategy (bioaugmentation).

Behaviour of oil in ice

When oil is spilled into waters of the polar regions the spilled oil will spread to the edges of ice floes and under the ice where the buoyant oil will fill rough spaces on the underside. Oil between broken ice floes will tend to collect in leads, whereas the oil at the underside of ice floes will rise up through the brine channels and cracks within the ice and eventually pool on the ice surface (Owens et al., 1998). Possible distribution and interactions of oil in ice is

shown in Figure 5. Due to the dark colour of the oil, absorbance of solar radiation will increase and lead to rapid melting of the surrounding ice, causing the formation of melt pools on the ice surface. Oil will become encapsulated in the ice when new sea-ice formation occurs or during growth, when new layers of ice are formed at the ice-water interface and encapsulate the buoyant oil at the underside.

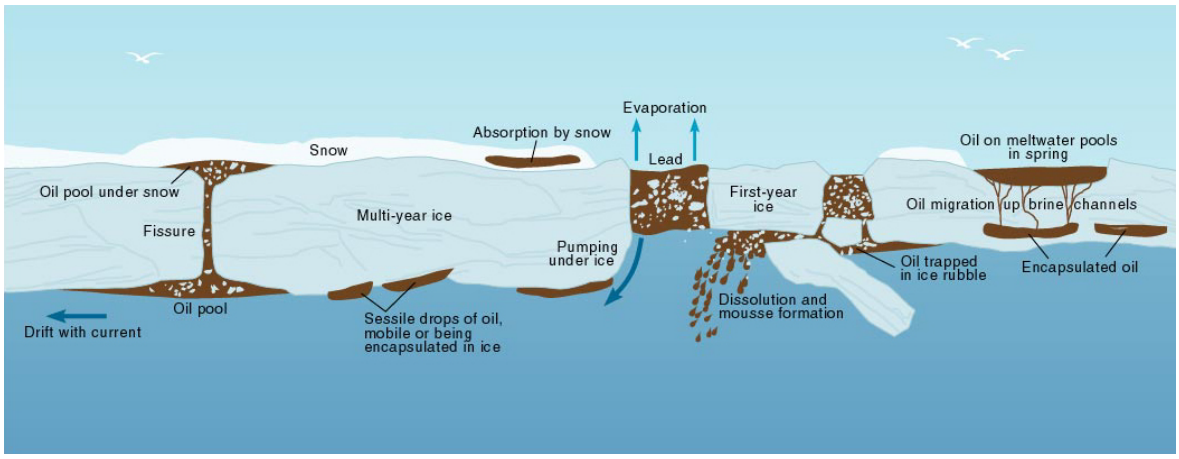

Figure 5. Possible distribution of oil in ice. Showing interactions of drops under the ice, growth of new ice below the oil, oil appearing on the surface in the spring, wind herding oil in melt pools, and the appearance of oil on top of the ice. Image reprinted from Arctic Monitoring Assessment Programme (AMAP) 1998.

Bioremediation of marine oil spills

Bioremediation or **biostimulation** is the acceleration of the natural degradation process through the addition of nutrients and/or oxygen. **Natural attenuation** is the natural degradation by the indigenous flora and **bioaugmentation** is the addition of oil-degrading exogenous microbes to enhance biodegradation of the contaminant.

Hydrocarbon-degrading microorganisms are ubiquitously found in all kinds of ecosystems (Margesin & Schinner, 2001), including Arctic and Antarctic marine sediments, but they represent in uncontaminated sites in general only a small percentage of the total community. However, after a contamination this proportion exceeds rapidly (Atlas, 1981; Margesin & Schinner, 1999). Bioremediation currently comprises only 10% to 15% of all remediation methods in the United States for the decontamination of groundwater and soil after the use of physical treatments. In Europe bioremediation of petroleum contaminated sites is the second largest application (Philip et al., 2005). Compared to physical treatments bioremediation is

inexpensive. For example, the cleanup of the Exxon Valdez oil spill expenses for the washing of oiled rocks were as high as $ 1 million per day, whereas bioremediation of several hundred miles shoreline was applied for less than $ 1 million (Philip et al., 2005).

Oxygen is typically not a limiting factor in most marine environments (Prince & Atlas, 2005), but bioavailable nitrogen, phosphorus and trace elements may be limiting in the case of large spills. This limitation is overcome by the careful application of fertilizers. Increased biodegradation, however, might cause anaerobic conditions. In these cases the supply of oxygen by aeration may be taken into consideration. The application of fertilizers was proven in the oil spill cleanup after the Exxon Valdez ran aground in 1989. Typical enhancements of biostimulation are two- to fivefold in shorelines (Bragg et al., 1994; Prince et al., 1999), this enhancement can add up to a difference of years for recovery (Prince & Atlas, 2005). The addition of iron, which is often limiting in marine environments, and low levels of vitamins have been shown to stimulate oil degradation (Dibble & Bartha, 1976; Radwan & Al-Muteirie, 2001).

Bioaugmentation, the seeding with exogenous microbes, has led to ambiguous results (Atlas & Bartha, 1992). Leahy and Colwell (Leahy & Colwell, 1990) found, in laboratory studies, that microbial seeding can enhance oil degradation in seawater but not in fresh-water environments. Some studies showed that seeding of PAH contaminated soils with PAH degrading microbes could enhance the degradation rate (Brodkorb & Legge, 1992; Grosser et al., 1991; Guerin & Boyd, 1992; Heitkamp & Cerniglia, 1989; Smith et al., 1997). Other authors reported that inoculation had no positive or only marginal effects on oil biodegradation rates (Dott et al., 1989; Margesin & Schinner, 1997a; Møller et al., 1995; Venosa et al., 1992). In many studies (Dott et al., 1989; Margesin & Schinner, 1997a; Møller et al., 1995; Venosa et al., 1992) improvement of bioaugmentation was observed in the early stage of the biodegradation process while later on the communities returned to the levels comparable with the non-bioaugmented samples.

Nevertheless, especially in high latitudes, where very short summer seasons do not permit long acclimatization periods for hydrocarbon-degradative populations (Whyte et al., 1998; Forsyth et al., 1995) bioaugmentation has to be taken into consideration as a bioremediation strategy. But rather than using commercial products, an enrichment of indigenous organisms from the contaminated site should be reinoculated as a bioremediation strategy (Prince & Atlas, 2005). Petroleum is a complex mixture of various hydrocarbons in which alkanes are the major constituents. Studies have shown that a single microbial species often degrades only one or two classes of hydrocarbons within a crude oil (Whyte et al., 1998). Whereas

mixcultures of microorganisms are not only superior to individual strains because they have a broader degradation spectrum, but also because a multiplex consortium responds to environmental changes more easily. A disadvantage with consortia, however, is the difficulty of standardization (Prince et al.).

Efficiency of bioremediation and bioaugmentation

It is complex and challenging to provide statistically significant evidence of bioremediation efficiency (Prince & Atlas, 2005), due to the heterogeneity of the amount of oil being spread in a certain section and the natural uneven distribution of microorganisms in environmental samples. The stimulation of microbial activity on hydrocarbon degradation can be more accurately determined in laboratory approaches, such as radiorespirometry. In these approaches a ^{14}C-labeled substrate is added to environmental samples or pure cultures in medium, and evolution of $^{14}CO_2$ is monitored.

Partially degraded hydrocarbons may sometimes be more toxic to organisms. However, bioremediation studies have shown that nutrient addition leads to a decrease in oil toxicity (Mueller et al., 1997). Due to the decrease in environmental impact of oil contamination through enhanced stimulation, bioremediation should be considered as alternative oil spill response method for oil cleanups, especially in areas, where application of other methods might be demanding. Remediation methodologies, used in temperate climates, have to be modified for application to polar regions. Furthermore, although there is an overlap in climate and sea-ice formation in the polar regions , there are several factors distinguishing the sea-ice conditions from pole to pole, as mentioned above. In the Antarctic most of the sea-ice cover is annual. Bioremediation strategies can thus only be applied during one season. After thawing of the ice cover, the remaining oil will end up in the seawater, where it can be removed by mechanical and/or physical treatments. However, this might be logistically challenging due to the remoteness of the Southern Ocean. Besides, the introduction of nonnative species in the marine Antarctic environment is not allowed. Therefore the cultivation of hydrocarbon utilizing Antarctic bacteria is of great interest for the development of an oil spill response method in these areas.

The long resistance of multi-year ice in the Arctic Ocean allows more time for recruitment of oil-degrading sea-ice bacteria for decontamination.

Aim and outline of the dissertation

Most of the bioremediation experiments in cold areas, including bioaugmentation experiments, have been conducted so far with soil, beaches, or seawater. In comparison, only a few studies were performed on sea-ice (Atlas, 1983; Atlas et al., 1978; Delille et al., 1997; Delille & Siron, 1993; Siron et al., 1995) at the beginning of this study. Therefore, little is known about the petroleum degradation potential of sea-ice bacteria and about the efficiency of nutrient amended bioremediation as well as about bioaugmentation strategies under sea-ice conditions. There is also very little information about the impact of these oil spill response methods on the indigenous bacterial communities as well as about the toxic effects of oil on sea-ice organisms. Furthermore, the introduction of nonnative species into the marine Antarctic environment is not allowed. Therefore the detection and cultivation of oil-degrading Antarctic bacteria is of great interest for the development of oil spill response strategies in these areas.
To gain further information on the effect of oil on sea-ice organisms, the major aims of this thesis were:

1. Determining the response of crude oil contamination to SIMCO.
2. To modify bioremediation approaches and test their application in laboratory as well as in the field.
3. Finding out whether members of the SIMCO do not only survive the oil contamination, but are able to degrade compounds of crude oil as well.
4. Development of an inoculum for bioaugmentation with cold-adapted, oil-degrading bacteria.
5. Assessing the degradation potential of aliphatic and aromatic compounds of crude oil.

The aim of this study was, first of all, to determine and assess the response of the indigenous sea-ice bacterial communities on crude oil contamination. In case of the development of an oil-degrading bacterial community, an enhancement of crude oil biodegradation by the indigenous sea-ice biota (bioremediation) was to be estimated at cold temperatures as well as under sea-ice conditions. Furthermore, the development of an inoculum consisting of different cold-adapted, oil-degrading bacteria, for the use of bioaugmentation, was to be compiled. The efficiency of the different bioremediation and bioaugmentation treatments was to be assessed and, concomitantly, the influence of the different treatments on the sea-ice bacterial communities was to be determined.

The response of the sea-ice microbial communities (SIMCO) to the different bioremediation treatments was determined by the molecular methods DGGE (Denaturing Gradient Gel Electrophoresis) and FISH (Fluorescent In Situ Hybridization). A combination of both methods was chosen to determine, on the one hand, quantitative data on the abundance of bacteria of the main groups and, on the other hand, qualitative data using DGGE to achieve phylogenetic information on the bacterial diversities. The efficiency of biodegradation through the different treatments was estimated by oil fingerprints obtained by the means of GC-FID.

PART II

OUTLINE OF

LABORATORY EXPERIMENTS

Outline of laboratory experiments

Preliminary bioremediation experiments were carried out to further complement the studies reported on in Manuscripts 1 – 4, Part II of this dissertation. They will be published within the scope of the EU-project ARCOP in the report 'Documentation of marine biological experiments' (D4.2.1.3). The pilot experiments served as basis for isolation of hydrocarbon utilizing bacteria and also for testing and improving considered methods for field and further experiments. Some bacterial strains were isolated and tested for their ability to degrade crude oil hydrocarbons at low temperatures. These degradation tests are published in Manuscript 3 and comprise a portion of the synthesis of this dissertation. A brief outline of these experiments is therefore provided here.

To assess whether bioremediation can enhance oil biodegradation in sea-ice by the indigenous bacteria, different fertilizers were tested to stimulate degradation of Southern Barents Sea crude oil containing high amounts of aliphatic compounds. Inipol, an oleophilic fertilizer, was successfully tested in Alaska for the Exxon Valdez oil spill cleanup (Bragg et al., 1994), containing urea as nitrogen source and tri(laureth-4)-phosphate as phosphorus source and surfactant, encapsulated within oleic acid. Due to its oleophilic formulation Inipol has the property of providing nutrients directly to oil-degrading microbes at the oil surface. The disadvantage of Inipol, however, is its toxicity and that it is itself a highly organic carbon source. In this study we used Inipol MS 3000, without 2 butoxy ethanol in the formulation, which is less toxic to the marine organisms. Ordinary fishmeal was also used as fertilizer, as a low cost alternative product, compared to the costly Inipol. Fishmeal contains a high amount of amino acids and is an excellent growth stimulant for many marine bacteria. Diversity studies (Bowman et al., 1997b 2003; Brown & Bowman, 2001) revealed that bacterial communities in Arctic and Antarctic sea-ice consist of mainly three dominant bacterial groups: bacteria of the *Bacterioidetes* phylum, *Gamma-*, *Alpha-*, as well as some *Betaproteobacteria*, and *Actinobacteria*. However, a high variability of the community compositions within different samples of sea-ice (Bowman et al., 1997b; Brinkmeyer et al., 2003; Brown & Bowman, 2001) as well as different communities in young and older (thicker) sea-ice (Grossmann & Gleitz, 1993; Helmke & Weyland, 1995) have been detected. Therefore, laboratory experiments were conducted with different kinds of sea-ice, mainly from the Arctic. Summer and winter sea-ice was incubated separately, but samples from different locations as well as samples from various parts of the sea-ice column were thoroughly mixed. In some experiments, samples of sea-ice from the Arctic as well as from

the Antarctic were mixed together, in order to obtain a pool of different bacterial species for the development of cold-adapted oil-degrading bacteria. Also in these experiments three different kinds of crude oil plus two different fertilizers (Inipol and fishmeal) were spread onto the ice to provide a broad range of various crude oil compounds and nutrient sources. The experiments served as a pool for the isolation of oil degrading bacteria and were not studied for changes in microbial communities.

Setup of bioremediation experiments for diversity studies

Bioremediation experiments were set up in aseptically treated glass tanks in a cold laboratory, at stable -3°C. The tanks were filled with 12 liters of sterile, prefiltered Arctic seawater. The ice cores (5 dm^3), taken during the Polarstern summer cruise ARK-XVIII/2 and the winter cruise ARK-XIX/1, were crushed into pieces of about 3 to 5 cm diameter in sterile plastic bags, then homogenized and afterwards added to the tanks. The tanks were then covered with glass lids which had been washed with ethanol. The experimental set up is shown in Figure 6. Three tanks were inoculated with 10 ml crude oil from the Southern Barents Sea; two of them were supplemented with additional fertilizers (Inipol MS 3000 and fishmeal). The spreading of the oil on the sea-ice is shown in Figure 7.

Figure 6 + 7. Experimental set up of laboratory bioremediation experiments

Control batches with no treatment and with fertilizers alone were set up later, and were sampled and incubated for the same periods.

The equilibrium between sea-ice and liquid water, which simulated the natural ice – seawater environment, was maintained for more than two years at -3°C. The tanks were aerated with an aquarium pump, equipped with a sterile filter to provide oxygen as electron acceptor.

Sub-samples of seawater (10 ml) and ice (3.5 cm hand corer) were taken from the bioremediation experiments at intervals of four weeks. To determine the bacterial diversity of the bioremediation experiments and to follow alterations in the composition of the bacterial communities, the samples were analyzed with the culture-independent finger print method DGGE. After one year of incubation oil samples were taken from the experiments and analyzed for biodegradation by GC-FID as described in Manuscript 2.

Results and discussion

The changes in bacterial sea-ice communities caused by crude oil contamination as well as oil plus fertilizer were analysed by DGGE. DGGE banding patterns of the oil contaminated summer and winter ice experiments are presented in Figure 8A and Figure 8B.

Sequence similarities of excised bands to their closest relative in the GenBank database are listed in Tables 1 and 2.

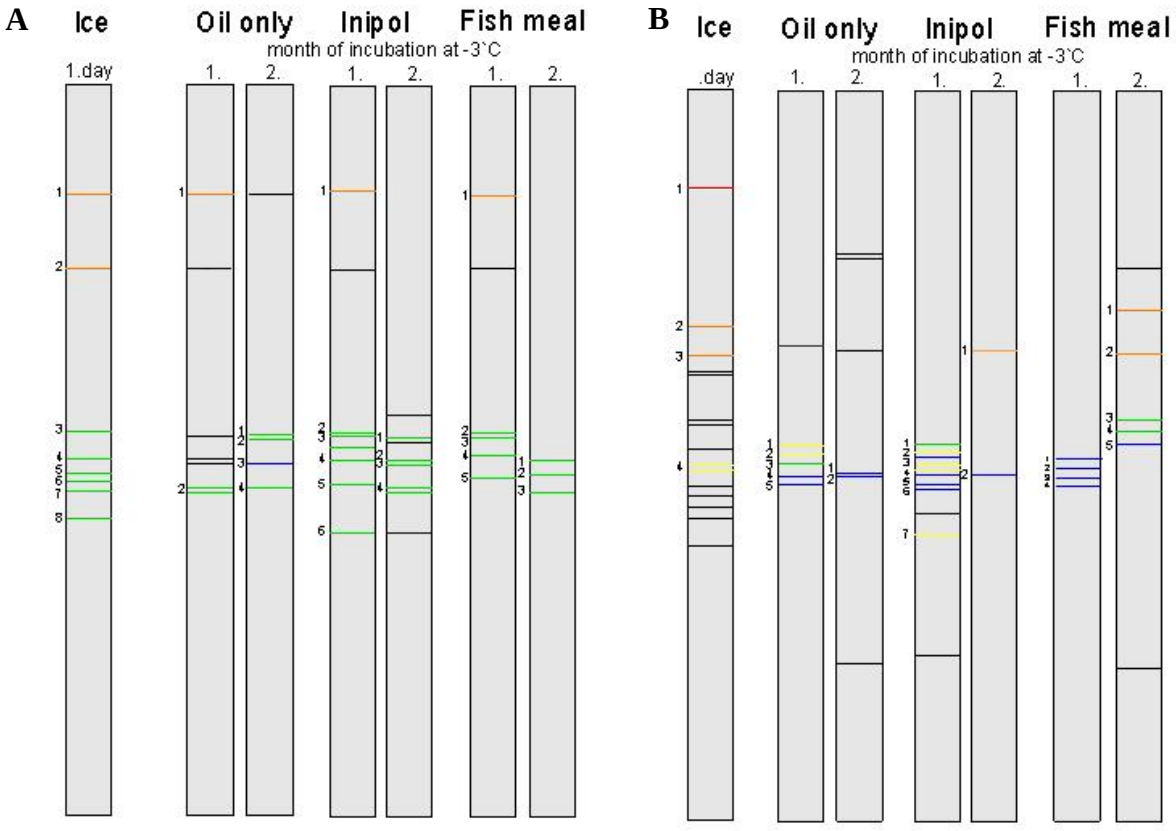

Figure 8. DGGE profiles of crude oil bioremediation experiment with Arctic summer (A) and Arctic winter (B) sea-ice at the beginning of the experiments and after one and after two months of incubation at -3°C

Table 1. Closest GenBank relatives from DGGE bands of the experiments with Arctic summer sea-ice

Treatment	Incubation time	Band#	Group	Genus	Closest GenBank relative	Acc. #	%-	Length
before	first day	1	*Bacteroidetes*	*Flavobacterium*	Arctic sea-ice bacterium ARK10287	AF4684	99,4	501
before	first day	2	*Bacteroidetes*	*Flavobacterium*	Flavobacterium frigoris LMG 21922	AJ5578	98,9	469
before	first day	3	*Gammaproteobacteria*	---	Uncult. Bacterium clone 156ds20	AY2126	98,5	468
before	first day	4	*Gammaproteobacteria*	*Pseudoalteromonas*	Pseudoalteromonas sp. Prot5	AF5134	98,3	476
before	first day	5	*Gammaproteobacteria*	*Pseudomonas*	Pseudomonas sp. ANT9287	AY1673	86,4	380
before	first day	6	*Gammaproteobacteria*	---	Unc. Arctic sea-ice bacterium	AY1655	96,85	508
before	first day	7	*Gammaproteobacteria*	*Shewanella*	Shewanella livingstonis	AJ3008	98,7	469
before	first day	8	*Gammaproteobacteria*	*Marinobacter*	*Marinobacter* sp. NK-1	AB0269	99,1	499
oil only	one month	1	*Bacteroidetes*	*Flavobacterium*	Arctic sea-ice bacterium ARK10287	AF4684	99,8	525
oil only	one month	2	*Gammaproteobacteria*	*Shewanella*	Uncultured bacterium clone ARKIA-124	AF4682	93,9	424
oil only	two months	1	*Gammaproteobacteria*	*Marinomonas*	Arctic sea-ice bacterium ARK10032	AF4683	98,8	485
oil only	two months	2	*Gammaproteobacteria*	*Glaciecola*	Uncultured bacterium clone ARKIA-34	AF4682	94,5	514
oil only	two months	3	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. 1999043680	AY2681	93,4	443
oil only	two months	4	*Gammaproteobacteria*	*Shewanella*	Shewanella livingstonis	AJ3008	99	503
oil + Inipol	one month	1	*Bacteroidetes*	*Flavobacterium*	Arctic sea-ice bacterium ARK10287	AF4684	99,6	534
oil + Inipol	one month	2	*Gammaproteobacteria*	*Pseudoalteromonas*	Pseudoalteromonas sp. 4-1-6-1	AY3830	99,4	501
oil + Inipol	one month	3	*Gammaproteobacteria*	*Oleispira*	Oleispira antarctica	AJ4264	95,1	518
oil + Inipol	one month	4	*Gammaproteobacteria*	*Glaciecola*	Uncultured bacterium clone ARKIA-34	AF4682	98,3	469
oil + Inipol	one month	5	*Gammaproteobacteria*	*Halomonas*	Halomonas sp. Ko501	AF5505	99,1	502
oil + Inipol	one month	6	*Gammaproteobacteria*	*Shewanella*	Shewanella livingstonis	AJ3008	99,8	498
oil + Inipol	two months	1	*Gammaproteobacteria*	*Glaciecola*	Uncultured bacterium clone ARKIA-34	AF4682	93,4	488
oil + Inipol	two months	2	*Gammaproteobacteria*	*Pseudomonas*	Gammaproteobacterium HTCC230	AY1020	90,1	517
oil + Inipol	two months	3	*Gammaproteobacteria*	*Pseudomonas*	Pseudomonas sp. ANT9287	AY1673	97,3	448
oil + Inipol	two months	4	*Gammaproteobacteria*	*Shewanella*	Shewanella livingstonis	AJ3008	98,8	522
oil + fish meal	one month	1	*Bacteroidetes*	*Flavobacterium*	Arctic sea-ice bacterium ARK10287	AF4684	99,8	526
oil + fish meal	one month	2	*Gammaproteobacteria*	*Shewanella*	Shewanella livingstonis	AJ3008	96,3	463
oil + fish meal	two months	1	*Gammaproteobacteria*	*Shewanella*	Shewanella livingstonis	AJ3008	98,8	495

Table 2. Closest GenBank relative from DGGE bands of the experiments with Arctic winter sea-ice

Treatment	Incubation	Band #	Group	Genus	Closest relative	Acc. #	%-similarity	Length
before	first day	1	*Bacteroidetes*	*Flavobacterium*	Flavobacterium sp. GOBB3-209	AF321038	93,2	467
before	first day	2	*Bacteroidetes*	*Flavobacterium*	Arctic sea-ice associated bacterium	AF468432	85,2	474
before	first day	3	*Bacteroidetes*	---	Uncultured Bacteroidetes	AY457134	93,1	259
before	first day	4	*Alphaproteobacteria*	*Octadecabacter*	Octadecabacter sp. ANT9190	AY167335	96,5	487
before	first day	5		*Cyanobacterium*	Uncultured cyanobacterium clone	AY323170	93,2	455
oil only	one month	1	*Alphaproteobacteria*	*Roseobacter*	Roseobacter sp.	X86469	88,6	158
oil only	one month	2	*Alphaproteobacteria*	*Sulfitobacter*	Sulfitobacter dubium strain KMM 3584T	AY180103	87,4	310
oil only	one month	3	*Gammaproteobacteria*	*Psychrobacter*	Psychrobacter glacincola strain LMG 21274	AJ430830	99,81	523
oil only	one month	4	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. Q3-8/14	AY216798	97,35	490
oil only	one month	5	*Betaproteobacteria*	*Ralstonia*	Betaproteobacterium PI_GH3.E7	AY162051	85,45	440
oil only	two months	1	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. 12I	AY191849	94,2	496
oil only	two months	2	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. Q3-8/15	AY216798	99,8	501
oil + Inipol	one month	1	*Gammaproteobacteria*	---	Uncultured bacterium isolate 6i08	AY177779	88,3	444
oil + Inipol	one month	?	*Alphaproteobacteria*	---	Uncultured Alphaproteobacterium	AY149734	87,7	171
oil + Inipol	one month	2	*Alphaproteobacteria*	*Shingomonas*	Arctic sea-ice bacterium ARK9996	AF468381	96,1	486
oil + Inipol	one month	3	*Alphaproteobacteria*	*Herbaspirillum*	Uncultured Alphaproteobacterium	AY043635	98,6	70
oil + Inipol	one month	4	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. Q3-8/14	AY216798	99,2	488
oil + Inipol	one month	5	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. 1999043680	AY268181	96,8	497
oil + Inipol	one month	6	*Betaproteobacteria*	*Ralstonia*	Ralstonia solanacearum GMI1000	AL646081	91,3	439
oil + Inipol	one month	7	*Alphaproteobacteria*	*Roseobacter*	Uncultured bacterium clone E6	AY268237	87,6	410
oil + Inipol	two months	1	*Bacteroidetes*	*Cytophaga*	Bacteroides bacterium ANT9285	AY167327	99,4	507
oil + Inipol	two months	2	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. Q3-8/15	AY216798	98,6	488
oil + fish meal	one month	1	*Bacteroidetes*	*Polaribacter*	Uncultured Polaribacter Arctic96B-11	AF354621	85,9	498
oil + fish meal	one month	2	*Betaproteobacteria*	*Burkholderia*	P. andropogonis	X67037	89,5	458
oil + fish meal	one month	3	*Betaproteobacteria*	*Herbaspirillum*	Herbaspirillum lusitanum	AF543312	86,3	454
oil + fish meal	one month	4	*Betaproteobacteria*	*Roseateles*	Roseateles depolymerans strain 61 A	AB003624	88,65	458
oil + fish meal	two months	1	*Bacteroidetes*	*CFB*	Abyssal strain AIII4	AF254114	98,4	494
oil + fish meal	two months	2	*Bacteroidetes*	*Cytophaga*	Bacteroides bacterium ANT9285	AY167327	93,4	485
oil + fish meal	two months	3	*Gammaproteobacteria*	*Psychrobacter*	Psychrobacter aff. glacinocola	AJ297439	96,9	522
oil + fish meal	two months	4	*Betaproteobacteria*	*Ralstonia*	Ralstonia sp. 13I	AY191853	99,2	529

The initial sample at the start of the summer multi-year sea-ice experiments harboured a bacterial community with members of the *Bacteroidetes* phylum and Gammaproteobacteria. The diversity was relatively low, compared to other studies of Arctic summer sea-ice communities, directly analysed after sampling (Brinkmeyer et al., 2003). Bacterial sea-ice communities of ice cores, taken in the same area of the Arctic Ocean and in the same season during the Polarstern cruise ARK-XVI/2, revealed community compositions with members of the *Bacteroidetes* phylum (Psychroflexus spp., *Cytophaga* spp.), Gammaproteobacteria (*Shewanella* sp., *Psychromonas* sp., *Colwellia* spp., *Glaciecola* spp., *Marinobacter* sp., and *Oceanospirillum* sp.), Alphaproteobacteria (*Roseobacter* spp.), and an Actinobacterium (Brinkmeyer et al., 2003). The bioremediation experiments were stored at -6°C for three weeks immediately after sampling before the experimental set up in a cold laboratory at the Alfred-Wegener-Institute in Bremerhaven. At -6°C, common sea-ice bacteria survive and are metabolically active, however, activity rates are highly reduced (Helmke, unpublished data). Therefore the samples were stored at -6°C in order to minimize changes or damage to the sea-ice inhabitants. However, the community composition at the beginning of the experiments leads to the assumption that a selection of specific bacteria might have occurred before the experiments were started. During the course of the incubation, a shift in community composition occurred from *Flavobacteria* of the *Bacteroidetes* phylum and *Pseudoalteromonas* sp., *Glaciecola* sp, *Pseudomonas* sp., *Shewanella* sp., and *Marinobacter* sp. of the Gammaproteobacteria towards predominantly Gammaproteobacteria, mainly comprising members of the genera *Shewanella*, *Glaciecola* and *Pseudomonas*. Members of the *Bacteroidetes* phylum were still detected after one month of incubation, but disappeared completely after two months in all oil treatments.

The bacterial diversity in the laboratory experiments with Arctic winter multi-year sea-ice (collected in April 2003) differed significantly from the summer sea-ice. The natural community at the beginning of the experiments included members of the *Bacteroidetes* and Gammaproteobacteria and, furthermore, a high abundance of Beta- and Alphaproteobacteria (Table 2). The occurrence of Betaproteobacteria is more characteristic for terrestrial ecosystems; however, a predominance of Betaproteobacteria in Arctic summer melt pools has recently been reported (Brinkmeyer et al., 2004). The samples of winter sea-ice also included refrozen melt pools, in which the high occurrence of Betaproteobacteria could be asserted. After oil contamination, the number of species decreased (represented by DGGE bands) with time. *Ralstonia* spp. of the Betaproteobacteria, however, could be detected in all oil

experiments after two months of incubation, whereas Alphaproteobacteria, still present after one month, disappeared completely. A new band in the lower part of the gel, presumably an Actinobacterium, could be detected in all oil experiments. Unfortunately, the *Actinobacterium* could not be sequenced and were thus not further identified.

The treatment with oil plus fishmeal first showed a high impact on bacterial diversity and after two months caused a strong shift in community composition from predominantly Betaproteobacteria towards mainly members of the Bacteroidetes phylum and *Gammaproteobacteria*.

The bacterial diversity decreased over the course of incubation time in both, the summer and winter sea-ice experiments treated with oil. A decrease in diversity has been observed in other bioremediation studies from various environments (Röling et al., 2002; Saul et al., 2005; Yakimov et al., 2005). A shift towards dominantly *Gammaproteobacteria* was observed in all summer sea-ice experiments as well as in the winter sea-ice, treated with oil plus fishmeal. Gammaproteobacteria are commonly detected in various samples of Arctic as well as Antarctic sea-ice and are one of the major groups inhabiting this ecosystem (Bowman et al., 1997b; Brinkmeyer et al., 2003; Brown & Bowman, 2001; Junge et al., 2002). However, the dominating genera of *Gammaproteobacteria* in the oil contaminated samples, occur in lower abundances in uncontaminated sea-ice. Many hydrocarbonoclastic marine bacteria, isolated from all over the world, also belong to this subclass of the Proteobacteria (Dyksterhouse et al., 1995; Gauthier et al., 1992; Golyshin et al., 2002; Hedlund et al., 1999; Yakimov et al., 1998; Yakimov et al., 2004b; Yakimov et al., 2003). However, in many characteristics they are distinct from other bacteria of this group (Yakimov et al., 2004b).

After two months of incubation, Shewanella spp. dominated the summer sea-ice communities, and was subsequently frequently isolated from these experiments (39.1 % of 425 strains). Growth of a psychrophilic Shewanella strain, isolated from Antarctic surface seawater on petroleum hydrocarbons, has recently been reported (Gentile et al., 2003), indicating the common occurrence and enrichment of *Shewanella* spp. after an oil contamination in polar marine ecosystems.

In the situation where oil and fertilizer occurred together, high bacterial biomass formed within a few weeks of incubation. However, after one year of incubation most of the crude oil was still highly viscous in lines or patches on the ice surface. Oil fingerprints by GC-FID revealed that, when whole oil patches were taken from the ice surface (as described in

Manuscript 2), no significant degradation occurred after one year of incubation at -3°C.

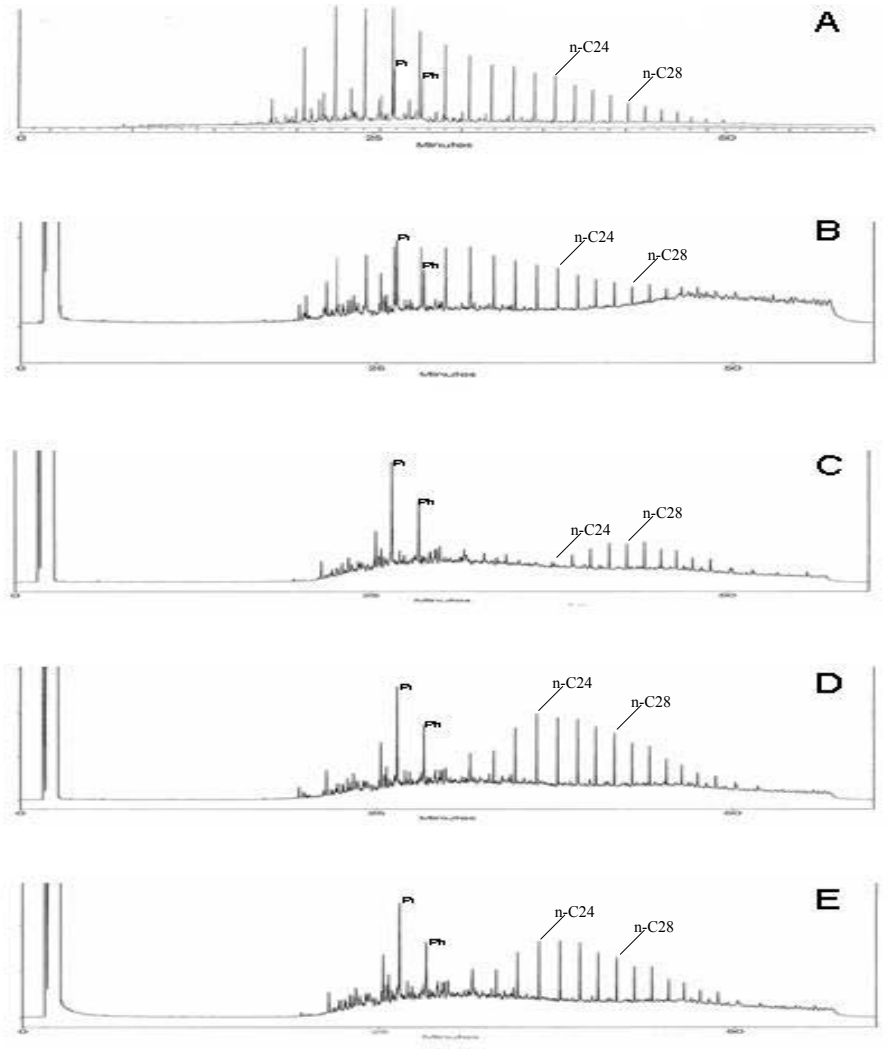

Figure 9. Oil fingerprints of the laboratory bioremediation experiments with Arctic sea-ice and crude oil from the Southern Barents Sea after two years of incubation at -3°C: (A) experiment without fertilization, (B) experiment with summer sea-ice fertilized with Inipol, (C) experiments with summer sea-ice fertilized with fish meal, (D) experiment with winter sea-ice fertilized with Inipol, (E) experiment with winter sea-ice fertilized with fish meal.

However, when oil samples were taken only at spots surrounded by bacterial biomass, oil fingerprints revealed ongoing biodegradation of n-alkanes when additionally treated with the fertilizers Inipol and fishmeal. Oil fingerprints of the initial oil experiment and after two years of incubation with Inipol and fishmeal are presented in Figure 9 for summer and winter sea-ice experiments. Oil fingerprints revealed that low molecular weight alkanes up to C_{14} disappeared from the experiments without biodegradation due to abiotic losses. The experiment with summer sea-ice and fishmeal showed the highest biodegradation. Normal

alkanes were almost completely biodegraded up to C24 and the alkanes C25-28 were also attacked by bacteria. However, the experiment with Inipol in summer sea-ice showed only initial biodegradation of short chain alkanes up to C20. In the experiments with winter sea-ice, both bioremediation treatments led to the same mineralization progress of petroleum hydrocarbons with low molecular weight n-alkanes up to C19 being completely degraded and alkanes in the range of C20 to C22 were only partly degraded. The results of the oil analysis indicated that biodegradation of crude oil hydrocarbons can be stimulated through bioremediation treatments even under sea-ice conditions at temperatures as low as -3°C. However, the rates of degradation are strongly reduced. Cold temperatures not only decrease bacterial metabolism but also significantly decrease the bioavailability of crude oil hydrocarbons. Only some organisms succeed in making the compounds bioavailable at these temperatures. Although bioremediation could successfully enhance the biodegradation, a considerable amount of oil was still present after two years of incubation. These biodegradation results indicate that decontamination of crude oil hydrocarbons in marine polar ecosystems at temperatures below freezing are expected to take longer than two years. Furthermore, one needs to take into consideration that, although the simulated temperatures of -3°C represent the average temperature of a polar summer month (Margesin & Schinner, 1999), the actual range commonly observed throughout the year is from about -30°C in the winter to 5°C in the summer months. This indicates that the biodegradation of hydrocarbons under natural conditions will take even longer. However, other factors, such as light radiation, may also influence the degradation processes under natural conditions. Rapid melting of sea-ice around oil contaminated areas would cause the formation of shallow melt pools on the ice surface and thus may even further enhance mineralization of oil compounds during the summer months.

A total of 634 bacterial strains were isolated from the laboratory experiments with Arctic summer and winter sea-ice as well as with mixed Arctic and Antarctic sea-ice, on minimal medium with crude oil or single hydrocarbon substrates (see Manuscript 3). Of these, 425 isolates were clustered into groups by means of ARDRA (see Manuscript 3). Results are discussed in the synthesis of this dissertation.
The control experiments, which were treated with fertilizer alone (no oil) have not yet been analyzed to compare the changes in diversity due to fertilizer treatment alone.

PART III

RESULTS AND DISCUSSION

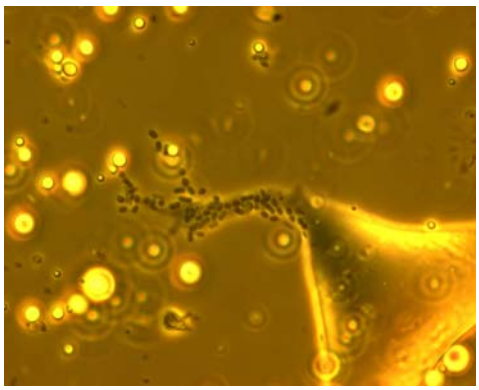

Table 3. Overview of the conducted experiments in chronological order. Objectives, applied methods, main results and conclusions.

	Type of experiment, conditions	Objectives and questions	Methods applied	Main results (pages in PART-III Results and discussion)	Conclusions
Manuscript 1	Laboratory experiments; without nutrient addition; Arctic summer sea-ice; 1 year, 1°C	o Influence of oil on SIMCO. o Does sea-ice harbour oil degrading bacteria?	FISH, DGGE, isolations, degradation potential evaluated by [^{14}C]-hexadecane	o Diversity of SIMCO was highly reduced after oil contamination. (p: 41) o Hexadecane degrading, cold adapted bacteria could be isolated. (p: 41) o Specific hydrocarbon degrading communities with dominantly *Gammaproteobacteria* enriched. (pp: 46-56)	Petroleum alkanes are degraded by sea-ice bacteria but degradation efficiency is low without additional nutrients. (pp: 56-58)
Outline of laboratory experiments	Laboratory bioremediation experiments; organic nutrients added in form of Inipol (successfully tested after Exxon Valdez accident) and fish meal (inexpensive alternative); Arctic summer and winter sea-ice; >2 years, -3°C	o Response of SIMCO to oil contamination and bioremediation treatments. o Does the addition of organic nutrients enhance oil degradation in sea-ice?	DGGE, isolations; hydrocarbon degradation determined by CO_2-formation (infrared) or by GC-FID	o Oil degrading communities, often with dominantly *Gammaproteobacteria* , developed similar to the experiments without organic nutrient addition. The composition was dependent on the initial communities. (pp: 29-34, 40-44) o Respiration activity and bacterial biomass increased significantly in fertilized samples (especially fish meal). (p: 43) o After 1 year no significant degradation was detected with GC-FID in homogenized sea-ice samples. After 2 years alkanes up to C_{24} were completely degraded at colonized spots. (pp: 33-34, 58-59)	Bioremediation enhanced the development of oil degrading sea-ice bacterial communities. The composition of the communities is quite similar to those developing without nutrient addition. (pp: 29-32, 40-44) CO_2 measurements turned out to be unfavourable if organic nutrients are used. GC-FID method appeared useful especially for qualitative estimations.

	Type of experiment, conditions	Objectives and questions	Methods applied	Main results (pages in PART-III Results and discussion)	Conclusions
Manuscript -4	Field bioremediation experiments; Inipol or fish meal added; annual winter sea-ice in Van Mijenfjorden, Svalbard February-April 2004, -30°C to –10°C, last two weeks –7°C to 0°C	o Response of SIMCO to bioremediation treatments in the field similarly as in the laboratory experiments? o Is oil bioremediation also a successful method in the field?	DGGE, FISH, total counts, GC-FID	o Changes in communities towards dominantly *Gammaproteobacteria* which occur in low abundances in common sea-ice. Results similar to laboratory experiments. (pp: 42-45, 46-56) o Degradation of crude oil not detectable with GC-FID. (pp: 56-58)	Oil degradation during winter is insignificant, but temperatures of –7°C to 0°C already induced in fertilized experiments a fast increase in biomass and shift in communities similar to those in the bioremediation laboratory experiments. (p: 43)
Manuscript 2	Bioremediation and bioaugmentation experiments (partly in the field); inorganic nutrients added; Antarctic summer sea-ice and gapwater; sea-ice: 7 months at –3°C gapwater: 6 months at 0°C	o Is oil bioremediation successful in summer sea-ice? o Comparison between different communities in different sea-ice types (bottom ice, top ice, gapwater). o Abbreviation of lag-phase prior to onset of degradation and enhancement of oil degradation through inoculation with oil-degrading sea-ice bacteria?	DGGE, FISH, isolations, GC-FID, degradation potential evaluated by [^{14}C]-hexadecane	o Changes in the composition of ice-matrix communities was relatively slow compared to gapwater communities but shifts in communities towards cold adapted oil degrading communities were obtained. (p: 40, 46-56) o The composition of the inoculum changed also but the inoculum was not completely out-competed by indigenous bacteria. (pp: 45-46) o Degradation of [^{14}C]-hexadecane was significant even in the sea-ice matrix if nutrients or inoculum was added. A significant (GC-FID) degradation of crude oil were obtained only in fertilized gapwater experiments. (pp: 56-58) o Degradation was significantly enhanced	Bioaugmentation successfully enhanced oil degradation. Inoculation with a complex oil degrading assemblage appeared to be superior to few selected species. But more knowledge is needed about the physiology of inoculated strains to compile inocula for specific sea-ice habitats.

Type of experiment, conditions	Objectives and questions	Methods applied	Main results (pages in PART-III Results and discussion)	Conclusions
			through inoculation. (pp: 56-58)	
Characterization of obtained isolates from the different experiments	o Gain more information about the degradation potential of sea-ice bacteria. o Physiological characterization to assess the role of the different species at contaminated sea-ice sites. o Defining strains for the use of bioaugmentation.	Substrate degradation patterns determined by means of GC-FID, Resazurin, Biolog; temperature and salinity tolerance tests	o Some isolates were shown to have high hydrocarbon degradation potentials concerning alkanes as well as some aromatic compounds. (pp: 59-63) o Hydrocarbon degradation capabilities can vary from species to species within one genus. (pp: 59-63) o Bacteria frequently detected at the onset of degradation turned out to be degraders of short chain alkanes. (pp: 60-61) o Good hydrocarbon degraders affiliated with *Gammaproteobacteria* and *Actinobacteria* (p: 59). One moderate degrader belonged to the *Alphaproteobacteria*. o Most isolates belonged to taxonomic groups with members well known for their capability to degrade hydrocarbons. (pp: 46-56) o Some strains have16S rRNA similarity < 97 % to described strains and can be considered new species. Description of isolate ice-oil-327 (16S rRNA similarity <94% to *Pseudomonas pertucinogena*) is in preparation.	Physiologically different cold adapted hydrocarbon degraders live in Arctic and Antarctic sea-ice. Detailed knowledge about the physiology will support the development of bioremediation strategies in contaminated sea-ice.

Results and discussion

The main conclusions of the experimental oil contamination and different bioremediation studies as well as those of the crude oil hydrocarbon degradation patterns are presented here, including an overview of data, which has not been published in one of the manuscripts of Part-II, to support the discussion. To begin with, the response of the indigenous sea-ice bacterial communities to crude oil contamination and to the different bioremediation treatments will be presented and discussed. Then the relationships of the bacterial isolates, which mainly represent the bacterial communities after several months of incubation, to other sea-ice bacteria or other bacteria from the polar regions as well as their relationship to other hydrocarbonoclastic genera is discussed and presented in Figures 10-13 (pp: 50-54). Subsequently, the effectiveness of the bioremediation treatments on the biodegradation processes as well as the hydrocarbon degrading abilities of some sea-ice isolates is discussed.

Changes in bacterial community composition

To sum up, the different experiments revealed that changes in community composition occurred more slowly at -3°C using the solid sea-ice matrix than in the experiments conducted using gapwater from Antarctic sea-ice at 0°C. Greater changes in community compositions were observed in the experiments using ice, collected from the ice-seawater interface. Due to the lower temperatures, biodegradation of petroleum hydrocarbons in the samples from the sea-ice matrix was expected to be reduced. Slight changes in SIMCO indicated a slow development of an oil degrading indigenous population caused by reduced bacterial metabolism, commonly observed at low temperatures, as well as by the severe bioavailability of crude oil compounds.

A lag phase prior to the onset of biodegradation after oil contamination has been observed, especially in cold environments (Margesin & Schinner, 1999).

Response of SIMCO to crude oil contamination

This study assessed the impact of sea-ice bacterial communities on crude oil contamination. Manuscript 1 addressed this topic on Arctic multi-year summer sea-ice. Manuscript 2 evaluated biodegradation in Antarctic summer sea-ice. Further results were also obtained from other laboratory experiments. Moreover, laboratory studies as well as the field experiments on Svalbard were conducted with Arctic winter sea-ice to early spring sea-ice.

All experiments showed a highly reduced diversity in the bacterial communities after oil contamination. In most sea-ice experiments, a shift in community composition occurred from initial members of the *Bacteroidetes* phylum, *Alpha*- and *Gamma*- as well as some of *Betaproteobacteria* towards predominantly *Gammaproteobacteria,* mainly with members of the genera *Shewanella*, *Marinobacter*, *Pseudomonas, Pseudoalteromonas* as well as some members of the genera *Glaciecola* and *Colwellia. Glaciecola* and *Colwellia,* however, only occurred in experiments showing a slow response of the bacterial communities to oil contamination.

The bacterial communities in the control experiments, without oil, also showed changes in community compositions and diversity with bacteria of the *Gammaproteobacteria* as well as with the *Bacteroidetes* phylum being dominant. However, DGGE and FISH analysis showed that the diversity in most untreated experiments was higher than in the oil treated experiments and comparable with the diversity of the initial samples (FISH analysis Manuscript 1 and Manuscript 2). In the untreated controls bacteria of the genera *Colwellia* were often found in Arctic as well as in the Antarctic ice experiments (Manuscript 2 and Manuscript 4). Also found in the Antarctic ice experiments were *Glaciecola*, *Marinomonas* and *Marinobacter* (Manuscript 2). In the Antarctic gapwater experiments *Roseobacter* of the *Alphaproteobacteria* and *Polaribacter*, *Bacteroides* and *Flavobacteria* of the *Bacteroidetes* phylum were detected in the untreated samples. This is substantiated by Saul et al. (Saul et al., 2005) and Röling et al. (Röling et al., 2002) who found less diverse populations dominated by *Gammaproteobacteria* after hydrocarbon contamination in Antarctic soil and in beach sediments respectively, compared to the uncontaminated control soil. Both studies, however, almost exclusively found bacteria of the *Bacteroidetes* phylum in the uncontaminated controls. Yakimov et al., (Yakimov et al., 2004a) showed a dramatic increase in the relative abundance of *Gammaproteobacteria* in crude oil contaminated seawater, sampled from 2 m below pack ice, near the Italian Station in the Ross Sea, Antarctica. Coinciding with findings of the sea-ice experiments of this study, *Colwellia*, *Halomonas*, *Marinobacter*, *Marinomonas*, *Oleispira*, *Pseudoalteromonas*, *Shewanella*, of the *Gammaproteobacteria* were dominant genera in the oil contaminated seawater. However, the initial composition of the bacterial community reflected a terrestrial community. Another seawater sample taken from a pristine site, consisting of a community with typically marine and sea-ice associated bacteria, showed no significant changes after oil contamination after a period of 30 days. These findings support the results obtained from the sea-ice experiments, where slow changes in the community composition were obtained during the first weeks of incubation, which then showed a shift

towards *Gammaproteobacteria* comprising mainly the same genera as in the seawater sample mentioned above.

FISH analysis, applied on samples from experiments treated with crude oil alone, taken from Arctic (Manuscript 1) and Antarctic sea-ice (Manuscript 2), as well as acridine orange counts of the samples from the Svalbard experiments, showed only a low abundance of small cells, compared to the untreated controls and bioremediated experiments. Furthermore, FISH analysis revealed very weak hybridization signals in the oil contaminated experiments compared to the untreated and all bioremediation treated experiments. Reduced cell size and decreased hybridization signals indicated a toxic effect of the crude oil on sea-ice microbial communities, when no additional nutrients were added.

Response of SIMCO to different bioremediation treatments

The response of SIMCO to the different bioremediation treatments were assessed in samples of Antarctic sea-ice (Manuscript 2) and Arctic sea-ice (laboratory experiments at -3°C and field experiments on Svalbard, Manuscript 4). As expected, over the course of time, the microbial communities changed in the oil contaminated plus fertilized batches as well as in the control batches with fertilizers only. The influence of the different treatments, however, did not show a response towards a selection of the same genera and species. Differences were obtained due to the varying initial community composition in the different ice samples as well as the kind of fertilizer used. There was a trend towards predominantly *Gammaproteobacteria* in all oil plus fertilizer treated experiments with summer sea-ice. Bacteria of the *Bacteroidetes* phylum as well as *Actinobacteria* were present in lower abundances. *Alphaproteobacteria* only occurred in the gapwater experiments. In all experiments with oil plus fertilizer, the bacterial diversity decreased in the course of incubation, compared to the initial samples as well as to the controls without oil. The only exception was the Svalbard experiment (Manuscript 4), where the initial bacterial diversity was highly decreased in the recently formed sea-ice. A typical sea-ice community (Bowman et al., 1997b; Brinkmeyer et al., 2003; Brown & Bowman, 2001; Junge et al., 2002; Staley & Gosink, 1999b) had developed in samples taken two months later, in April 2004.

A dramatic decrease in diversity has been observed in other bioremediation studies from marine environments (Röling et al., 2002; Saul et al., 2005; Yakimov et al., 2005). Yakimov et al. (2005) assumed that a decrease in diversity was due to the strong selection of marine hydrocarbonoclastic bacteria of the *Gammaproteobacteria*. A shift towards dominantly

Gammaproteobacteria has also been reported from an oiled beach on Svalbard (Grossman et al., 1999).

Fish meal, tested in various samples of Arctic sea-ice (laboratory experiments and field studies, Manuscript 4), led to the highest impact on the sea-ice microbial communities. In the field experiments fish meal did not only cause a strong decrease in bacterial diversity towards a bacterial community which was dominated by the genus *Pseudoalteromonas* in both, the oil contaminated and the control plots. But it also resulted in a considerable increase in bacterial biomass within a period of two weeks, when temperatures rose from a previous range of –30° to –10° to a range of –7° to 0°C. To overcome the general decreased oil degradation at low temperatures, it was attempted to initiate a rapid increase in bacterial biomass by addition of a readily available nutrient source. In combination with oil, it was expected that fish meal would favour the growth of mainly hydrocarbon utilizing bacteria. However, although the bacterial biomass rose significantly, the period with temperatures, allowing bacterial growth, was too short to obtain measurable biodegradation of crude oil hydrocarbons by means of GC-FID.

In the laboratory experiments, fish meal caused a shift towards predominantly *Shewanella* sp. in the summer sea-ice and towards a more diverse community with members of the genera *Shewanella*, *Ralstonia* and *Cytophaga* in the winter sea-ice. The genus *Ralstonia* is usually found in terrestrial environments and most probably originated from samples of the refrozen melt pool which were added to the experiments. *Betaproteobacteria* were shown to be abundant in surface melt pools on Arctic pack ice (Brinkmeyer et al., 2004).

The bioremediation agent Inipol EAP22 was shown to stimulate oil biodegradation on beaches after the Exxon Valdez oil spill in Alaska (Bragg et al., 1994; Pritchard & Costa, 1991). Fertilization with Inipol MS3000 did not seem to have a stronger effect on the bacterial sea-ice community during the field experiments on Svalbard than the control plot without any treatment, which harboured a similar assemblage. Nevertheless, bacterial biomass increased in the plots treated with oil plus Inipol as well as in the control plot with Inipol alone, consistent with findings of Delille et al. (Delille et al., 1997) who tested the effect of crude oil and Inipol EAP 22 in Antarctic land-fast ice on changes in bacterial abundances. Croft et al., (Croft et al., 1995) suggested that Inipol EAP22 stimulated the hydrocarbon-degrading microbial population in medium-fine sand and suggested that, after a lag phase, oil biodegradation was encouraged. In the laboratory experiments an increase in bacterial biomass occurred within a short period, however, by far not as much as obtained through the addition of fish meal. It was found that Inipol only had a small effect on the microbial communities during the first four

weeks of incubation at -3°C, but strongly decreased the diversity in the winter ice experiments after two months of incubation. The declined response of the pristine indigenous bacteria to oil and bioremediation treatments at temperatures below freezing indicated that Inipol was not as effective as expected. The viscous agent became solid when it was applied to the ice surface and thus did not deploy its positive properties to oil emulsification.

Soluble inorganic nutrients have been successfully used for many oil-bioremediation studies (Swannell et al., 1996; Venosa et al., 1996) and they have the advantage of delivering only the limiting nutrients such as nitrogen, phosphorus and iron but no additional carbon source. The disadvantage of soluble nutrients is, however, that they might be washed away from the hydrophobic contaminant (Zhu et al., 2004). Soluble nutrients were tested to stimulate crude oil biodegradation in Antarctic sea-ice (Manuscript 2). The treatment with oil plus inorganic nutrients led to an increase in abundance of *Gammaproteobacteria* in the bottom ice and in the gapwater experiments, whereas in the top layer of the ice only slight changes occurred, compared to the initial sample. The addition of inorganic nutrients alone to Antarctic top and bottom sea-ice resulted in highly decreased bacterial communities of dominantly *Gammaproteobacteria* with members of the genera *Colwellia* (bottom ice) and *Glaciecola* (top ice). In the gapwater experiments, on the other hand, the communities consisted of *Alphaproteobacteria* and members of the genus *Bacteroides* of the *Bacteroidetes* phylum. DeLong et al. (DeLong et al., 1993) found that most phylotypes from free-living bacterioplankton were distinct from macroaggregate-associated bacteria and were closely related to undescribed *Alphaproteobacteria*. This finding might explain the dominance of *Alphaproteobacteria* in the liquid gapwater experiments.

Bacteria of the genus *Octadecabacter* could only be detected by DGGE in uncontaminated sea-ice of the Arctic winter ice experiments and in Antarctic summer sea-ice, treated with nutrients only, after one month of incubation. However, later on they were no longer detectable, with the exception of one *Octadecabater* sp. isolate (gap-d-29; Figure 13) obtained from the gapwater experiments with oil plus nutrients. Brinkmeyer et al. (Brinkmeyer et al., 2003) found that *Octadecabacter* spp. is one of the most abundant groups of the *Alphaproteobacteria* in Arctic sea-ice, whereas in Antarctic sea-ice only a small percentage could be detected by FISH. However, the lack of *Octadecabacter* spp. in all Arctic sea-ice experiments, including the controls without any treatment, indicates their sensibility to changes in the natural ecosystem. *Colwellia* spp. and *Glaciecola* spp. are the most abundant groups of Gammaproteobacteria in Antarctic sea-ice and are also dominant in Arctic sea-ice

(Brinkmeyer et al., 2003). *Glaciecola* were only detected in the uninoculated Antarctic sea-ice experiments, where they disappeared or decreased in detection intensity in the oil contaminated bottom ice experiments during the course of incubation (Manuscript 2). *Colwellia* spp. were also detected in the Antarctic experiments and in the field experiments conducted on Svalbard. No significant oil biodegradation had occurred at the time when bacteria of the genera *Colwellia* and *Glaciecola* were detected. Furthermore, *Colwellia* spp. could not be isolated from any experiments on crude oil agar and only one strain of *Glaciecola* (gap-e-64) was obtained from the inoculated control, without oil. The *Glaciecola* isolate showed no hydrocarbonoclastic abilities in the degradation tests (Manuscript 3), suggesting that these two genera of the *Gammaproteobacteria* do not play a significant role in the biodegradation processes of crude oil hydrocarbons in the sea-ice environments of the polar regions .

Response of bacterial communities in bioaugmented experiments

In order to shorten the acclimatization period of the development of indigenous hydrocarbon degraders and to enhance the biodegradation processes, bioaugmentation, seeding with cold-adapted, oil degrading bacteria, was tested in combination with the addition of inorganic nutrients in the experiments with samples of Antarctic sea-ice. The initial inoculum was dominated by members of the genera *Psychrobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Shewanella*, and *Marinobacter*. The isolation of bacteria from the inoculum revealed that more phylotypes were present which, however, could not be detected by means of DGGE, probably due to their low abundance. An underestimation of low abundant bacteria has already been reported previously (Polz & Cavanaugh, 1998).

The addition of inorganic nutrients plus inoculation with oil degrading bacteria, isolated from Antarctic and Arctic sea-ice, showed almost no changes in community composition in the top layer of the ice matrix. After seven months, a shift of the genera within the *Gammaproteobacteria* occurred, as it did in the bottom ice already after one month of incubation. After seven months of incubation *Shewanella* and *Pseudoalteromonas* were the most abundant genera in both ice experiments and *Marinobacter* and *Colwellia* were present in the bottom ice experiments.

In the gapwater experiments only a few *Gammaproteobacteria* seemed to have survived from the inoculum after six months of incubation. *Alphaproteobacteria* and members of the *Bacteroidetes* phylum, which were assumed to be indigenous, were also present and seemed to have out-competed most of the bacteria from the inoculum. Although most of the

inoculated strains were apparently out-competed by dominantly indigenous *Alphaproteobacteria*, some bacteria were shown to originate from the inoculum by comparing 16S rRNA gene sequences obtained by means of DGGE and by cultivation of bacterial strains from inoculated and uninoculated experiments. The remaining strains of the inoculum, which apparently prevailed, belonged to the genera *Oleispira*, *Pseudomonas*, *Shewanella* of the *Gammaproteobacteria* (see Figure 10, p:50), and *Rhodococcus* of the *Actinobacteria* (see Figure 11, p:52).

Phylogenetic affiliation of isolates obtained from bioremediation experiments

Bacterial isolates, with the ability to grow on petroleum hydrocarbons, were obtained from laboratory experiments with Arctic summer and winter sea-ice. They were also obtained from batches which were set up with Arctic as well as with Antarctic sea-ice and three different kinds of fertilizers, in order to obtain a pool of different bacterial species for the development of cold-adapted, oil-degrading bacteria. The experiments served as pool for the isolation of oil degrading bacteria and were not studied for changes in microbial communities. Moreover, bacterial strains were isolated from bioremediation experiments with Antarctic gapwater (Manuscript 2).

Unlike other environments (Amann et al., 1995), a high percentage of bacteria from the bioremediation experiments, detected by DGGE, were cultivatable. This exceptional incidence has already been previously observed in various sea-ice samples (Brinkmeyer et al., 2003; Helmke & Weyland, 1995; Junge et al., 2002). A total of 634 bacterial strains were isolated from oil contaminated sea-ice samples and bioremediation experiments with sea-ice, of which 425 strains were grouped by means of ARDRA (see Manuscript 1 and Manuscript 3) and 16S rRNA genes, from at least one or more representative strains of each group, were sequenced. The closest GenBank relative and the number of isolates clustering into the same group are listed in Table 4. The %-coverage of all ARDRA screened isolates is shown in Table 5. The *Gammaproteobacteria* which consisted of nine different genera formed the largest group and harboured 71.5 % of all ARDRA screened isolates, consistent with findings of isolates obtained from uncontaminated sea-ice (Brinkmeyer et al., 2003). *Alphaproteobacteria* were represented by only three genera, which constituted 4.9 % of the isolates. The *Bacteroidetes* phylum was represented by seven genera; however, they only harboured 3.5 % of all ARDRA clustered strains. *Actinobacteria* were the second largest group of isolates. With only four representative genera the *Actinobacteria* constituted 20 % of

Table 4. Number of isolates obtained from the different phylotypes and their closest GenBank relatives

	Genus	#	Closest GenBank relative	Accession #	%-similarit
Bacteroidetes					
200	*Bacteroides*	1	*Bacteroides* bacterium Ko706	AF550590	98.3
Gap-48	*Cytophaga*	1	Uncultured *Cytophagales*	AJ535257	98.3
522	*Flavobacterium*	4	Arctic sea-ice bacterium ARK10287	AF468434	97.5
Gap-67	*Flexibacter*	1	Arctic sea-ice associated bacterium	AF468422	98.5
Gap-51	*Pibocella*	1	*Pibocella ponti* isolate S3-17	AY771726	98.5
Gap-25	*Psychroflexus*	1	Arctic sea-ice bacterium ARK10063	AF468410	99.8
214	*Winogradskyella*	3	Uncultured bacterium ARCTIC.123	AF277538	97.8
Gap-41	*Winogradskyella*	3	Uncultured *Bacteroides* bacterium	AY922252	99.2
Alphaproteobacteria					
226	*Loktanella*	1	Uncultured bacterium clone ARKDMS-62	AF468242	99.4
484	*Loktanella*	2	*Loktanella* salsilacus	AJ582229	98.1
Gap-29	*Octadecabacter*	4	*Octadecabacter* sp. ANT9202	AY167337	100.0
111a	*Roseobacter*	7	Arctic seawater bacterium R7967	AJ293823	99.8
Gap-54	*Roseobacter*	7	Arctic seawater bacterium R7967	AJ293823	99.3
Gammaproteobacteria					
50	*Halomonas*	5	*Halomonas* sp. Claire	AJ969936	98.6
203	*Halomonas*	7	*Halomonas* sp. M3-2A	AY730253	91.8
212	*Halomonas*	2	*Halomonas* variabilis ANT-3b	AY616755	97.5
232	*Halomonas*	9	*Halomonas* sp. Claire	AJ969933	97.9-98.5
256	*Halomonas*	2	*Halomonas* sp. T5301	AB183511	99.1
81	*Marinobacter*	5	*Marinobacter* sp. Splume2.1814c	AF212211	99.0
325	*Marinobacter*	4	Uncultured Arctic sea-ice bacterium clone ARKXV/1-	AY165590	98.0
1-Ca	*Marinobacter*	8	Arctic sea-ice bacterium ARK10244	AF468401	99.9
1-Ba	*Marinobacter*	4	*Marinobacter* sp. BSi20018	DQ060399	99.9
Gap-52	*Marinobacter*	1	*Marinobacter* sp. 1-Ca	AY770011	99.4
Gap-81	*Marinobacter*	12	Arctic sea-ice bacterium ARK10244	AF468401	99.7
472	*Marinomonas*	3	*Marinomonas* sp. BJK17	AJ717295	97.7
381	*Oleispira*	4	*Oleispira antarctica*	AJ426420	98.0
Gap-97	*Oleispira*	2	Uncultured bacterium clone ARKDMS-13	AF468253	100.0
412	*Pseudoalteromonas*	9	*Gammaproteobacterium* UMB10F	AF505740	96.8
374	*Pseudoalteromonas*	13	*Pseudoalteromonas* sp. ICO06	U85856	98.8
432	*Pseudoalteromonas*	3	*Pseudoalteromonas* sp. JL-54	AY745825	98.1
515	*Pseudoalteromonas*	1	*Pseudoalteromonas* sp. D32	AY576005	97.7
234	*Pseudomonas*	2	*Pseudomonas* sp. ARCTIC-P23	AY573032	99.5
327	*Pseudomonas*	1	Uncultured *Gammaproteobacterium* Arctic96B-9	AF354596	97.6
499	*Pseudomonas*	9	*Pseudomonas* sp. S23-11	AF456231	96.2-97.4
10-Eb	*Pseudomonas*	5	Arctic sea-ice bacterium Bsw20350	DQ064611	99.4
Gap-57	*Pseudomonas*	1	Arctic sea-ice bacterium R7078	AJ293824	99.1
Gap-39	*Pseudomonas*	4	Arctic sea-ice bacterium R7366	AJ293826	99.9
128	*Psychrobacter*	2	*Psychrobacter* sp. 215-51	AY444823	99.7
211	*Psychrobacter*	9	*Psychrobacter okhotskensis*	AB094794	97.1
471	*Psychrobacter*	10	*Psychrobacter glacialis*	AJ539102	97.9-98.6
Gap-82	*Psychromonas*	1	Marine psychrophilic bacterium	AJ308372	98.6
318	*Shewanella*	13	Antarctic seawater bacterium Bsw10155B	DQ064636	98.7
221	*Shewanella*	31	Uncultured *Shewanella* SIC.114	AF277467	98.7
485	*Shewanella*	5	Polar sea bacterium R7216	AJ295714	98.4
510	*Shewanella*	21	*Shewanella* sp. 10-Ea	AY770007	97.7
417	*Shewanella*	17	*Shewanella* sp. SC2A	AB003190	97.9-99.1
Gap-44	*Shewanella*	2	*Shewanella* sp. JL-56	AY745827	99.7
1-Ha	*Shewanella*	1	Uncultured bacterium clone ARKCH2B…	AF468231	98.9
1-Aa	*Shewanella*	3	*Shewanella frigidimarina* clone SE20	AY771750	99.1-99.5
369	*Shewanella*	12	*Shewanella frigidimarina*	AY771713	89.9
Gap-1	*Shewanella*	11	*Shewanella frigidimarina*	AY771750	99.7
Gap-53	*Shewanella*	3	*Shewanella frigidimarina*	AY771736	99.9
411	[*Shewanella*]	1	Uncultured bacterium clone ARKDMS-3	AF468260	96.8
424	*Shewanella*	5	Antarctic seawater bacterium Bsw10171	DQ064631	96.3
428	*Shewanella*	25	Antarctic seawater bacterium Bsw10155B	DQ064636	97.5-98.8
460	*Shewanella*	16	*Shewanella* sp. GA-22	AJ563805	99.2

Isolate	Genus	#	Closest GenBank relative	Accession #	%-similarity
Actinobacteria					
23	*Arthrobacter*	1	*Arthrobacter* sp. S23H2	AF041789	98.7
251	*Arthrobacter*	4	*Arthrobacter* sp. Tibet-IIVa3	DQ108397	97.9
48	*Dietzia*	1	Unidentified Actinomycetes bacterium isolate HTA218	AB002636	99.4
79	*Dietzia*	15	*Dietzia* sp. CR-3	AY205297	98.8
101	*Dietzia*	3	*Dietzia* sp. JTS6455-250	AB010905	99.4
125	*Dietzia*	20	*Dietzia natronolimnaea*	AJ717373	99.0
482	*Plantibacter*	9	*Plantibacter* sp. ARCTIC-P5	AY573051	99.8
Gap-10	*Plantibacter*	6	Arctic seawater bacterium ARK10062	AF468438	99.8
176	*Rhodococcus*	9	*Rhodococcus* sp. MBICO1430	AJ576249	98.9
227	*Rhodococcus*	5	*Rhodococcus luteus*	AJ576249	98.8
Gap-45	*Rhodococcus*	12	*Rhodococcus luteus*	AJ576249	99.8

Table 5. Number of total isolates from the four bacterial groups and the main genera as well as the %-coverage of all ARDRA screened isolates.

Group Phylotype	# of isolates	% of 425 ARDRA screened isolates
Bacteroidetes	15	3.5
Alphaproteobacteria	21	4.9
Gammaproteobacteria	304	71.5
Shewanella	166	39.1
Marinobacter	34	8.0
Halomonas	25	5.9
Pseudomonas	22	5.2
Pseudoalteromonas	26	6.1
Psychrobacter	21	4.9
Actinobacteria	85	20.0
Dietzia	39	9.2
Rhodococcus	26	6.1
Arthrobacter	5	1.2
Plantibacter	15	3.5

the isolates. The highest diversity of phylotypes was found within the *Gammaproteobacteria*. Twelve phylotypes belonged to the genera *Shewanella* and with 39.1 % formed the largest group. The genera Pseudomonas showed the second highest diversity with 6 phylotypes, which, however, comprised only 5.2 % of the isolates. Other genera with diverse phylotypes were *Halomonas* (5.9 %), *Marinobacter* (8.0 %) and *Pseudoalteromonas* (6.1 %) as well as *Dietzia* (9.2 %) of the *Actinobacteria*.

Bacteria of the genera *Shewanella* were often detected in the oil contaminated and bioremediation treated experiments, by the means of DGGE. FISH analysis with probe SF825, specific for *Shewanella frigidimarina*, which hybridized with most of the obtained *Shewanella* isolates, revealed detection values of up to 17 % of all DAPI stained cells. Some phylotypes of the *Shewanella* genus showed differences in the target sequence of probe SF825, suggesting an even higher number of *Shewanella* spp. in the samples. However, the

extremely high proportion of *Shewanella* spp. within the collection of isolates seemed to overestimate the abundance of bacteria of this genus.

Phylogenetic trees were reconstructed using the maximum likelihood algorithm in order to assign the relationship of isolates, grown on crude oil hydrocarbons, to other sea-ice bacteria and to other hydrocarbon degrading bacteria. Furthermore, the relationship of isolates obtained from the Antarctic gapwater experiments, to isolates, obtained from the inoculum is presented in the trees. Trees of the four phylogenetic groups: *Gammaproteobacteria*, *Alphaproteobacteria*, *Bacteroidetes*, and *Actinobacteria* are presented in Figures 10-13. The succession of the bacterial community from psychro-tolerant to dominantly psychrophilic has been observed during the growth of sea-ice from the time of its formation to thicker annual pack ice, (Grossmann & Gleitz, 1993; Helmke & Weyland, 1995). The temperature as well as the enrichment in nutrients, caused by high algae standing stocks, strongly influences the composition of the natural bacterial sea-ice community and leads to a dominance of specific bacterial groups, such as the *Colwellia* assemblage with mainly *Colwellia* spp., and *Glaciecola* spp. within the *Gammaproteobacteria*, the *Roseobacter* clade within the *Alphaproteobacteria*, and the *Cytophaga-Flavobacterium* group within the *Bacteroidetes* phylum (Brinkmeyer, 2003). These groups are all associated with algae or other surfaces in marine environments (Abell & Bowman, 2005; Acinas et al., 1999; DeLong et al., 1993; Eilers et al., 2001; Rath et al., 1998).

Although the ***Gammaproteobacteria*** are the most abundant group in natural sea-ice, the dominant phylotypes in the oil-contaminated experiments *Shewanella* spp., *Marinobacter* spp., *Pseudoalteromonas* spp., *Pseudomonas* spp., and *Halomonas* spp., were only detected in lower abundances in natural sea-ice, except for *Marinobacter* spp., which were also frequently found in samples of Arctic sea-ice (Brinkmeyer et al., 2003).

Most of the phylotypes, which grow on petroleum hydrocarbons, were closely related to bacteria previously found in sea-ice or seawater of the Arctic or Southern Ocean (Figures 10-13). However, most phylotypes of the genera *Pseudoalteromonas* and *Pseudomonas* appeared to be distinct from other sea-ice bacteria. The tree of the *Gammaproteobacteria* (Figure 10) shows that *Pseudomonas* isolates from uncontaminated sea-ice clustered in the *P. fluorescence* group, whereas isolates obtained from oil contaminated sea-ice were most closly related to Arctic seawater bacteria and clustered into the *Pseudomonas pertucinogena* group, which constituted an independent cluster of other authentic *sensu stricto Pseudomonas* species (Anzai et al., 2000). All isolates clustering into the *Pseudomonas pertucinogena* group showed hydrocarbonoclastic capabilities. Members of the genus *Pseudomonas* are,

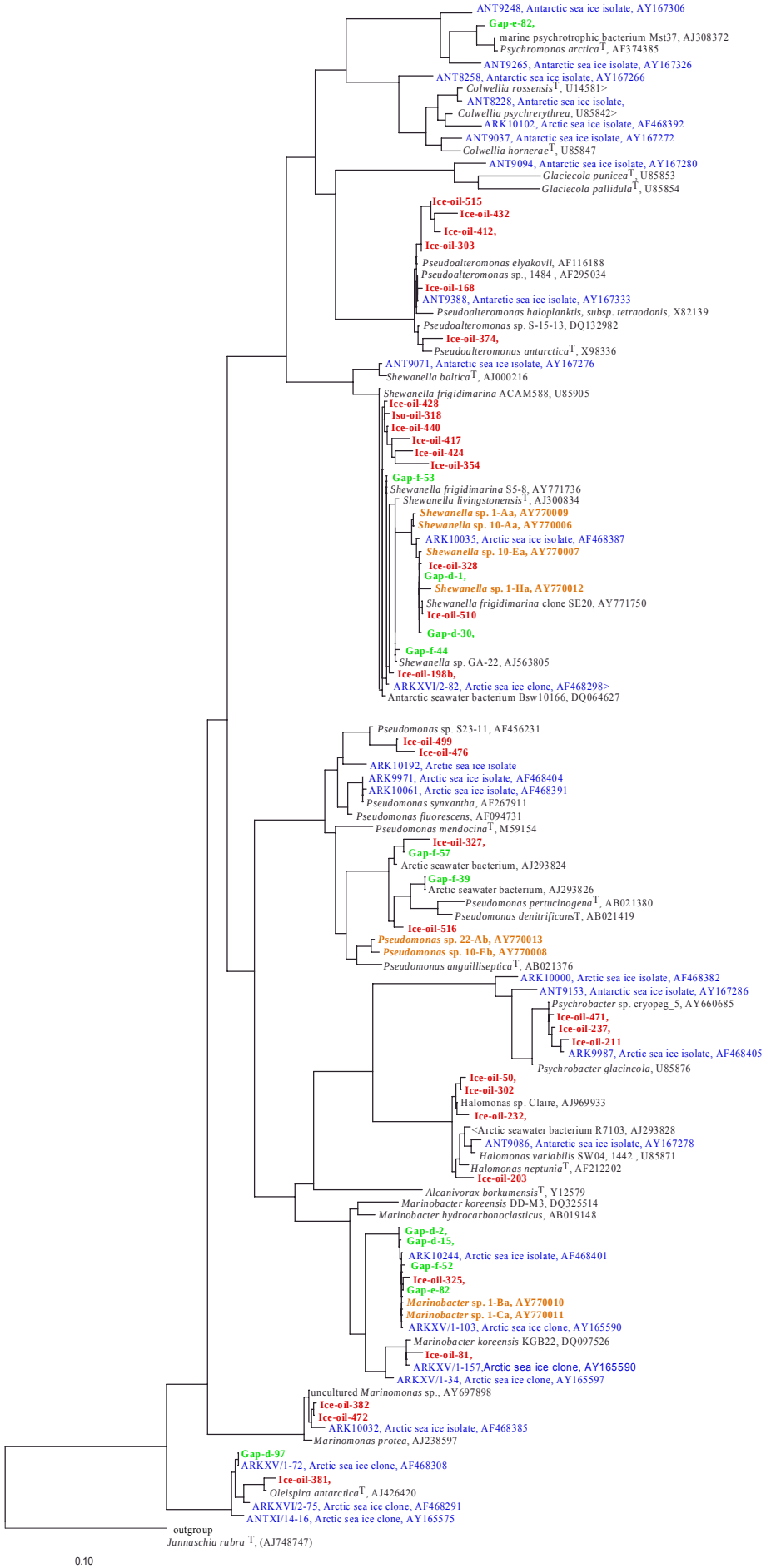
ANT9248, Antarctic sea ice isolate, AY167306
Gap-e-82,
marine psychrotrophic bacterium Mst37, AJ308372
Psychromonas arctica T, AF374385
ANT9265, Antarctic sea ice isolate, AY167326
ANT8258, Antarctic sea ice isolate, AY167266
Colwellia rossensis T, U14581>
ANT8228, Antarctic sea ice isolate,
Colwellia psychrerythrea, U85842>
ARK10102, Arctic sea ice isolate, AF468392
ANT9037, Antarctic sea ice isolate, AY167272
Colwellia hornerae T, U85847
ANT9094, Antarctic sea ice isolate, AY167280
Glaciecola punicea T, U85853
Glaciecola pallidula T, U85854
Ice-oil-515
Ice-oil-432
Ice-oil-412,
Ice-oil-303
Pseudoalteromonas elyakovii, AF116188
Pseudoalteromonas sp., 1484 , AF295034
Ice-oil-168
ANT9388, Antarctic sea ice isolate, AY167333
Pseudoalteromonas haloplanktis, subsp. tetraodonis, X82139
Pseudoalteromonas sp. S-15-13, DQ132982
Ice-oil-374,
Pseudoalteromonas antarctica T, X98336
ANT9071, Antarctic sea ice isolate, AY167276
Shewanella baltica T, AJ000216
Shewanella frigidimarina ACAM588, U85905
Ice-oil-428
Iso-oil-318
Ice-oil-440
Ice-oil-417
Ice-oil-424
Ice-oil-354
Gap-f-53
Shewanella frigidimarina S5-8, AY771736
Shewanella livingstonensis T, AJ300834
Shewanella sp. 1-Aa, AY770009
Shewanella sp. 10-Aa, AY770006
ARK10035, Arctic sea ice isolate, AF468387
Shewanella sp. 10-Ea, AY770007
Ice-oil-328
Gap-d-1,
Shewanella sp. 1-Ha, AY770012
Shewanella frigidimarina clone SE20, AY771750
Ice-oil-510
Gap-d-30,
Gap-f-44
Shewanella sp. GA-22, AJ563805
Ice-oil-198b,
ARKXVI/2-82, Arctic sea ice clone, AF468298>
Antarctic seawater bacterium Bsw10166, DQ064627
Pseudomonas sp. S23-11, AF456231
Ice-oil-499
Ice-oil-476
ARK10192, Arctic sea ice isolate
ARK9971, Arctic sea ice isolate, AF468404
ARK10061, Arctic sea ice isolate, AF468391
Pseudomonas synxantha, AF267911
Pseudomonas fluorescens, AF094731
Pseudomonas mendocina T, M59154
Ice-oil-327,
Gap-f-57
Arctic seawater bacterium, AJ293824
Gap-f-39
Arctic seawater bacterium, AJ293826
Pseudomonas pertucinogena T, AB021380
Pseudomonas denitrificans T, AB021419
Ice-oil-516
Pseudomonas sp. 22-Ab, AY770013
Pseudomonas sp. 10-Eb, AY770008
Pseudomonas anguilliseptica T, AB021376
ARK10000, Arctic sea ice isolate, AF468382
ANT9153, Antarctic sea ice isolate, AY167286
Psychrobacter sp. cryopeg_5, AY660685
Ice-oil-471,
Ice-oil-237,
Ice-oil-211
ARK9987, Arctic sea ice isolate, AF468405
Psychrobacter glacincola, U85876
Ice-oil-50,
Ice-oil-302
Halomonas sp. Claire, AJ969933
Ice-oil-232,
<Arctic seawater bacterium R7103, AJ293828
ANT9086, Antarctic sea ice isolate, AY167278
Halomonas variabilis SW04, 1442 , U85871
Halomonas neptunia T, AF212202
Ice-oil-203
Alcanivorax borkumensis T, Y12579
Marinobacter koreensis DD-M3, DQ325514
Marinobacter hydrocarbonoclasticus, AB019148
Gap-d-2,
Gap-d-15,
ARK10244, Arctic sea ice isolate, AF468401
Gap-f-52
Ice-oil-325,
Gap-e-82
Marinobacter sp. 1-Ba, AY770010
Marinobacter sp. 1-Ca, AY770011
ARKXV/1-103, Arctic sea ice clone, AY165590
Marinobacter koreensis KGB22, DQ097526
Ice-oil-81,
ARKXV/1-157,Arctic sea ice clone, AY165590
ARKXV/1-34, Arctic sea ice clone, AY165597
Juncultured Marinomonas sp., AY697898
Ice-oil-382
Ice-oil-472
ARK10032, Arctic sea ice isolate, AF468385
Marinomonas protea, AJ238597
Gap-d-97
ARKXV/1-72, Arctic sea ice clone, AF468308
Ice-oil-381,
Oleispira antarctica T, AJ426420
ARKXVI/2-75, Arctic sea ice clone, AF468291
ANTXI/14-16, Arctic sea ice clone, AY165575
outgroup
Jannaschia rubra T, (AJ748747)
0.10

Figure 10. Phylogenetic tree reconstructed by using the maximum likelihood (FastDNAml) algorithm for members of the *Gammaproteobacteria*. Outgroup *Jannaschia rubra* of the *Alphaproteobacteria*. The scale bar indicates 10% estimated sequence divergence. Isolates obtained from gapwater experiments are in green, from the laboratory bioremediation experiments in red and orange. Other sea-ice bacteria are highlighted in blue. Type strains are indicated with a ^{T}.

since many years, well known for their degradative properties of various hydrocarbons and have also been found in cold environments (Whyte et al., 1997). However, members with hydrocarbonoclastic abilities of the *Pseudomonas pertucinogena* group have not yet been reported.

Many marine hydrocarbonoclastic bacteria were found to belong to the *Gammaproteobacteria* within the genera *Alcanivorax* (Yakimov et al., 1998), *Cycloclasicus* (Dyksterhouse et al., 1995), *Marinobacter* (Gauthier et al., 1992), *Oleiphilus* (Golyshin et al., 2002), *Oleispira* (Yakimov et al., 2003), *Neptunomonas* (Hedlund et al., 1999), and *Thalassolitus* (Yakimov et al., 2004b). Growth on hydrocarbons by marine *Pseudoalteromonas* strains has also recently been reported (Hedlund & Staley, 2006). Bacteria, characterized for their ability to grow on petroleum hydrocarbons have recently been isolated from polar regions , belonging to the genus *Oleispira,* (Yakimov et al., 2003), *Pseudomonas* (Whyte et al., 1997), (Panicker et al., 2002), *Arthrobacter* (Eriksson et al., 2003), *Shewanella* (Gentile et al., 2003), *Halomonas* (Pepi et al., 2005). Of these organisms, the *Shewanella* strain GA-22 (AJ563805), *Oleispira antactica* (AJ426420), and *Halomonas* ANT-3b (AY616755) were relatively closely related to isolates of the bioremediation experiments (Figure 10).

Actinobacteria were shown to be the second largest group among the bioremediation isolates (Figure 11). Bacteria of the *Actinobacteriaceae* were repeatedly found in uncontaminated sea-ice of the Arctic and Southern Ocean, but in very low abundances (Bowman et al., 1997b; Brinkmeyer et al., 2003; Junge et al., 2002; Staley & Gosink, 1999b). However, in melt pools on the surface of Arctic sea-ice Actinobacteria accounted on average for 9 %, in some sample up to 20 %. Members of the *Arthrobacter* genus were found in sea-ice (Bowman et al., 1997b; Junge et al., 1998). However, bacteria of the genera *Rhodococcus* and *Dietzia* were more frequently isolated. They were more closely related to organisms found in terrestrial environments. Many members of the genus *Rhodococcus* are well known for their ability to utilize chemically diverse xenobiotics (Gurtler et al., 2004). Rhodococci occupy many niches, and their genes are widely distributed among other bacteria (Larkin et al., 2005).

Psychrotolerant hydrocarbon degrading *Rhodococcus* strains have also been found in oil-contaminated soil of the polar regions (Bej et al., 2000; Ruberto et al., 2005; Whyte et al., 1998). Members of the genus *Dietzia* were also found to degrade petroleum hydrocarbons (Zvyagintseva et al., 2001) and the terrestrial facultative psychrophile *Dietzia psychralcaliphila* has been found to grow on hydrocarbons (Yumoto et al., 2002).

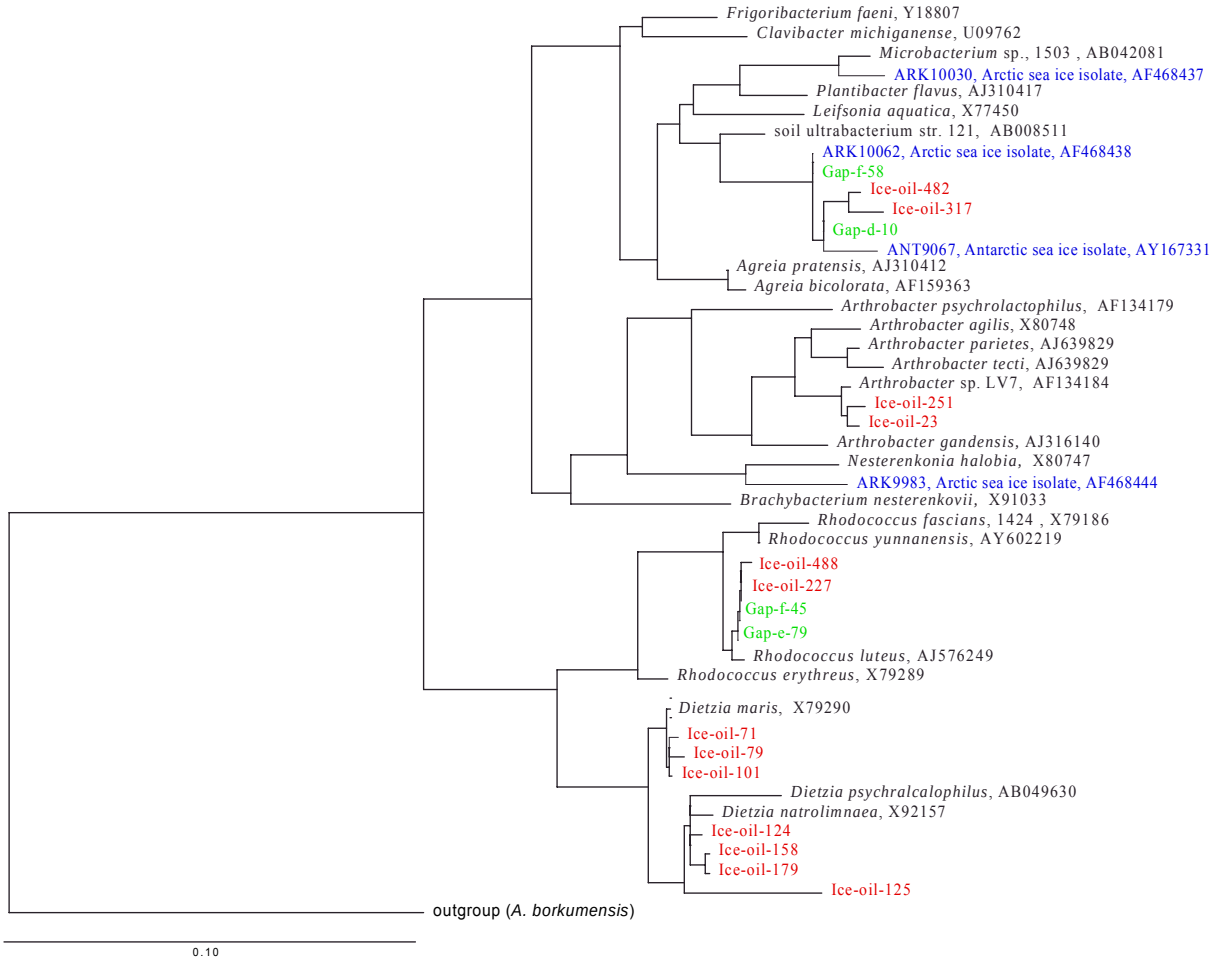

Figure 11. Phylogenetic tree reconstructed by using the maximum likelihood (FastDNAml) algorithm for members of the *Actinobacteria*. Outgroup *Alcanivorax borkumensis* of the *Gammaproteobacteria*. The scale bar indicates 10% estimated sequence divergence. Isolates obtained from gapwater experiments are in green, from the laboratory bioremediation experiments in red and orange. Other sea-ice bacteria are highlighted in blue.

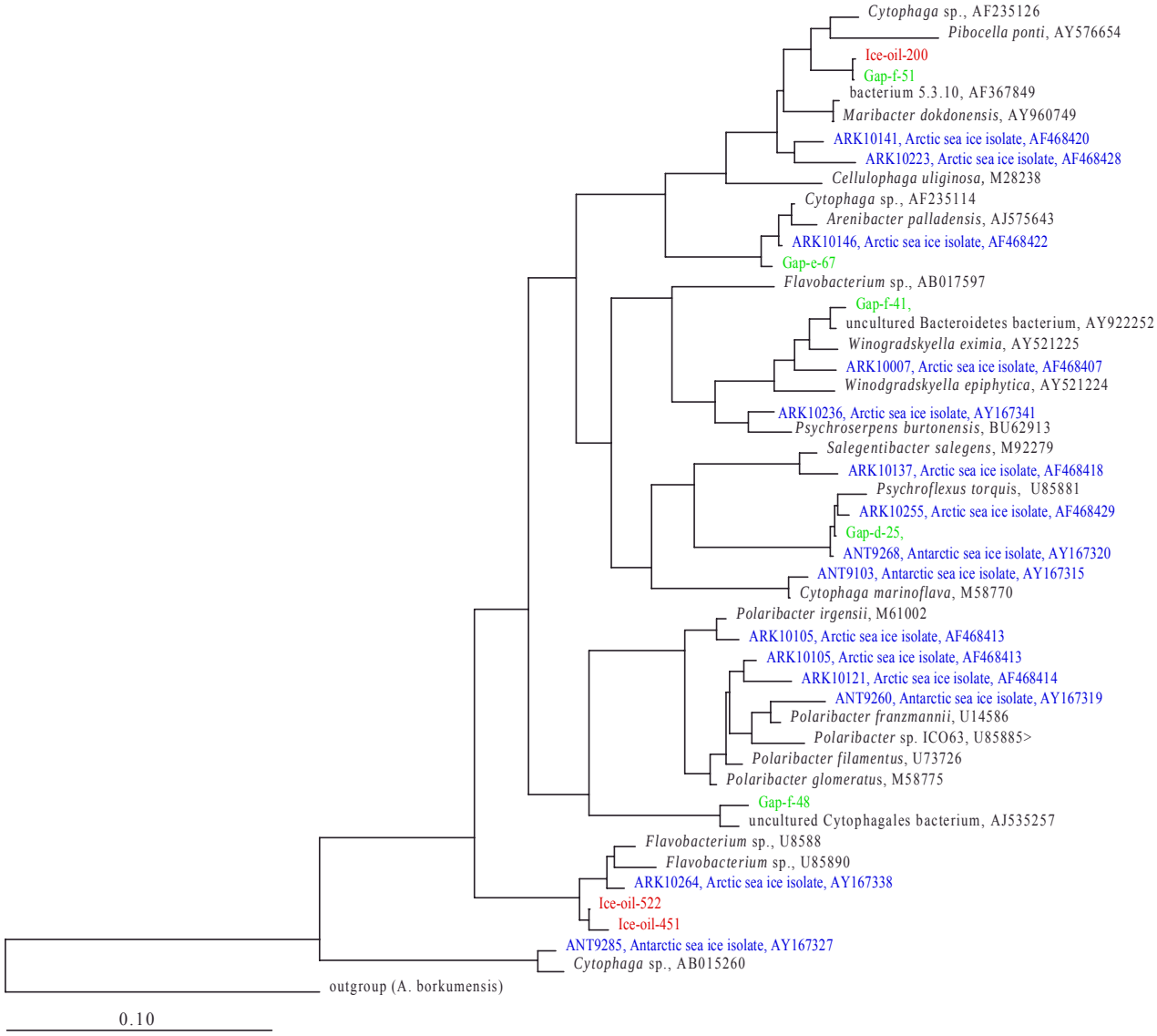

Figure 12. Phylogenetic tree reconstructed by using the maximum likelihood (FastDNAml) algorithm for members of the *Bacteroidetes* phylum. Outgroup Alcanivorax borkumensis of the *Gammaproteobacteria*. The scale bar indicates 10% estimated sequence divergence. Isolates obtained from gapwater experiments are in green, from the laboratory bioremediation experiments in red and orange. Other sea-ice bacteria are highlighted in blue.

Bacterial strains, which affiliated with genera of the ***Bacteroidetes* phylum** were all closely related to 16S rRNA genes of marine bacteria. Members of the *Bacteroidetes* phylum are known to be surface colonizers and are often associated with algae (Bowman et al., 1998b; Nedashkovskaya et al., 2005) and high nutrient concentrations. Bacteria of the genera *Flavobacterium*, *Flexibacter*, and *Psychroflexus* have been found in association with sea-ice, whereas the genera *Pibocella* and *Winogradskyella* were also found in Arctic marine environments. Many members of the family *Flavobacteriaceae* have been isolated from

seawater and are psychrophilic or psychro-tolerant. Some bacteria of this family are known for their hydrocarbonoclastic properties (Floodgate, 1984; Kwon et al., 2006).

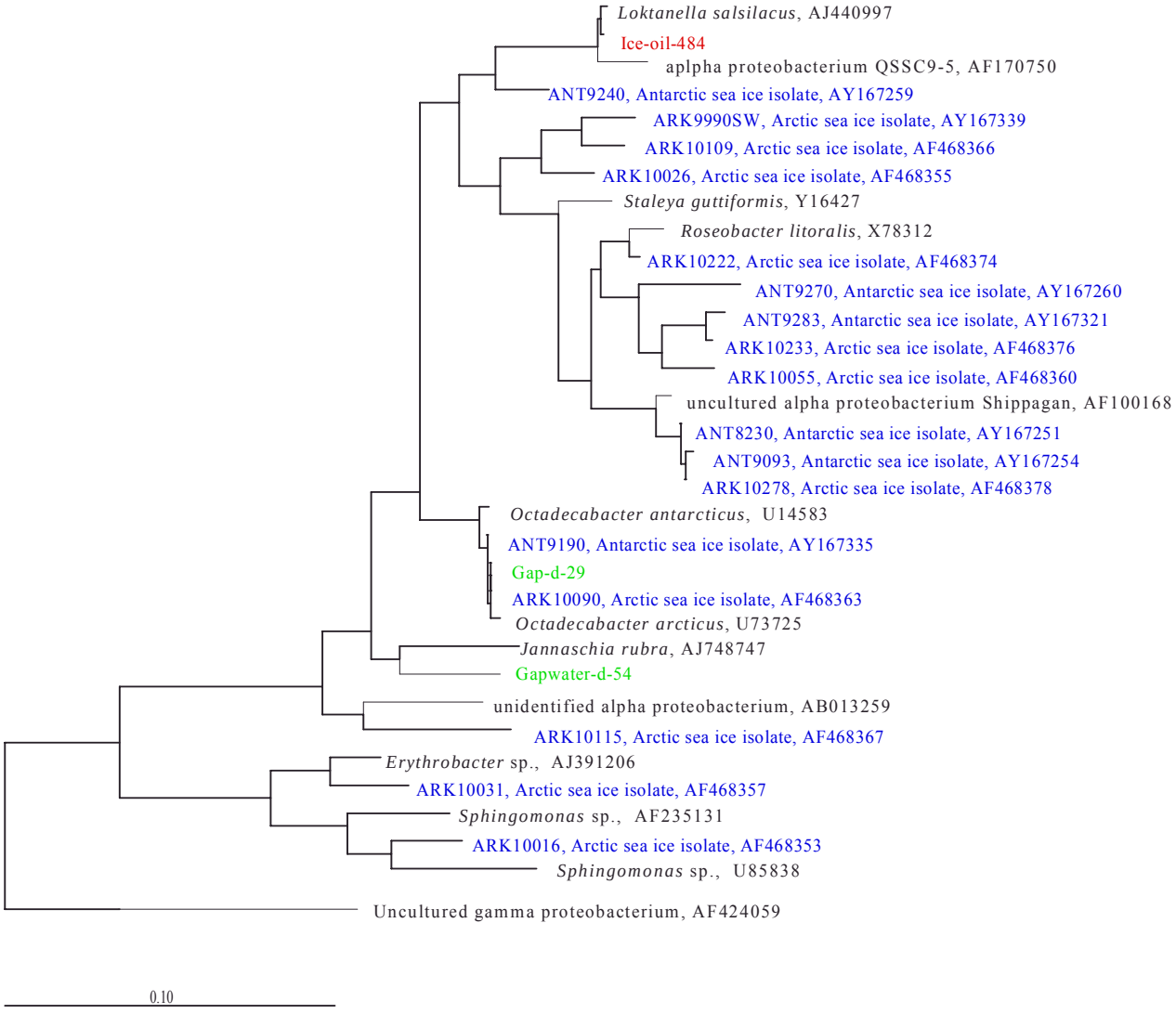

Figure 13. Phylogenetic tree reconstructed by using the maximum likelihood (FastDNAml) algorithm for members of the *Alphaproteobacteria* group. Outgroup is an uncultured Gammaproteobacterium. The scale bar indicates 10% estimated sequence divergence. Isolates obtained from gapwater experiments are in green, from the laboratory bioremediation experiments in red and orange. Other sea-ice bacteria are highlighted in blue.

Alphaproteobacteria were mainly detected in uncontaminated sea-ice and in experiments with Antarctic gapwater. Only one phylotype, represented by only one isolate Gap-d-29, of this group, was closely related to bacteria found in natural sea-ice (see Figure 13). This isolate is the only exception of *Octadecabacter* sp. found in an oil-contaminated sample. The other

three phylotypes are shown to be distinct from other sea-ice isolates and from other known hydrocarbon degrading bacteria (Figure 13). The group, represented by isolate gap-f-54, related to *Jannaschia rubra*, was frequently obtained from the gapwater bioaugmentation experiments with and without oil, suggesting that these isolates might originate from the inoculum, as they were not obtained by isolation from the uninoculated experiments. Isolate gap-f-54 was able to grow on some hydrocarbon compounds. In marine coastal areas the development of predominantly *Alphaproteobacteria* in oil bioremediation samples (Röling et al., 2002) and the occurrence in oil only plots (MacNaughton et al., 1999) has been reported. The detected genera *Shingomonas* and *Erythrobacter* have also been found in sea-ice (Bowman et al., 1997b; Brinkmeyer et al., 2003), but were not isolated or detected by DGGE in the experiments of this study (see Figure 13). Within the *Alphaproteobacteria* members of the genus *Shingomonas* have mainly been reported for their ability to utilize aromatic compounds (Baraniecki et al., 2002; Khan et al., 1996; Prak & Pritchard, 2002; Saul et al., 2005).

Bacteria of the genus *Ralstonia* within the ***Betaproteobacteria*** were detected by DGGE in experiments with Arctic winter sea-ice, but were not isolated in this study which may be due to NaCl concentrations of about 2 % in the media. *Betaproteobacteria* were found to be a predominant group in Arctic summer melt pools with salinities of 0% (Brinkmeyer et al., 2004). Hydrocarbonoclastic abilities by bacteria of the genus *Ralstonia* have recently been reported

Temperature tolerance tests of some isolates, grown on crude oil, showed that a great portion of the Arctic isolates of this study showed temperature maxima of about 25°C whereas some isolates, which made up a large proportion of the strains descending from the uninoculated Antarctic gapwater experiment-d responded psychrophilic (Manuscript 3, Table 1). These findings are in accordance with the results of Helmke and Weyland (2004) who found that a high percentage (up to 90 %) of isolates from Antarctic uncontaminated consolidated sea-ice were psychrophilic according to the definition of Morita (Morita, 1975), whereas isolates from Arctic sea-ice showed in general higher growth temperature maxima that exceed the defined line of 20°C by a few degrees. It is suggested that the higher occurrence of 'moderate psychrophiles' in the northern hemisphere is caused by the inflow of warm water masses as well as the terrestrial input. *Marinobacter* spp. and *Shewanella* spp., were isolated from oil contaminated Arctic sea-ice and uninoculated Antarctic gapwater experiments, were psychro-tolerant and psychrophilic, respectively. One strain (*Marinobacter* sp. 81) showed to be even

mesophilic (Manuscript 3) and did not only differ in physiological characteristics from isolates found in natural sea-ice, but is also phylogenetically distinct from other sea-ice *Marinobacter* strains (see Figure 10). Consistent with this study, many cold-adapted hydrocarbon oxidizing bacteria where found to be rather psychro-tolerant than psychrophilic (Eriksson et al., 2003; Whyte et al., 1996; Whyte et al., 1998). Most of the isolates, grown on crude oil hydrocarbons, are closely related to other bacteria previously found in sea-ice or in the polar regions. The observation, that they only occur in lower abundances in uncontaminated sea-ice, corresponds with findings in other marine environments (Atlas, 1981; Harayama et al., 1999). The development of a population with dominantly hydrocarbon oxidizing bacteria after oil contamination has also been reported (Margesin and Schinner 1999; Harayama 1999). The results indicate that the sea-ice communities with dominantly *Gammaproteobacteria* of the genera *Shewanella*, *Marinobacter, Pseudomonas*, *Pseudoalteromonas*, *Halomonas*, and *Oleispira*, as well as *Rhodococcus* and *Dietzia* of the *Actinobacteria* are hydrocarbon degrading bacteria which contribute to the decontamination processes and out-compete other indigenous sea-ice bacteria which frequently occur in pristine ice.

The efficiency of bioremediation and bioaugmentation

The efficiency of the application of the bioremediation and bioaugmentation method was assessed in the Antarctic experiments (Manuscript 2), for the field experiments on Svalbard (Manuscript 4) and in the laboratory batches as well. Furthermore, pure cultures of bacterial isolates were tested for their crude oil hydrocarbon degradation capacity at low temperatures in liquid medium.

To determine the efficiency of the different bioremediation treatments and which hydrocarbons were biodegraded, oil fingerprints were analyzed by means of GC/FID and GC/MS. An accurate qualification of the biodegradation rate is difficult to determine due to the high heterogeneity of the oil. In addition, the distribution of oil-degrading bacteria and thus the status of biodegradation within one experiment may vary dramatically within a range of millimetres. In order to obtain a representative sample, entire oil blobs were collected from all over the ice surface. For the laboratory experiments, samples from oiled spots, which were covered with bacterial biomass, were taken after two years of incubation at -3°C, in order to determine the process of hydrocarbon biodegradation within the biofilm. In contrast to this, in the pure culture experiments, the entire batch was used for oil analysis. To determine the rate of biodegradation, radiotracer experiments were conducted, using hexadecane as

representative substrate for alkanes, with environmental samples as well as with pure cultures (Manuscript 1 and Manuscript 2).

Oil fingerprints from all sea-ice experiments revealed that no significant degradation occurred in the experiments done with oil alone. Furthermore, the experiments with samples of Antarctic sea-ice showed that biodegradation of crude oil hydrocarbons was significantly higher in samples of melted ice, incubated at 0°C, than in the experiments with the solid sea-ice matrix, incubated at -3°C. This shows that temperature is the primary factor governing the process of oil degradation in sea-ice. This is consistent with the results obtained from the field experiments on Svalbard, in which no significant oil biodegradation occurred, when oil was supplied to the surface of the sea-ice matrix. However, the short period of only two weeks, with moderate temperatures for bacterial activity (-7°C to 0°C) was too short to achieve a measurable biodegradation of hydrocarbons.

However, the applied oil fingerprinting method showed that biodegradation of crude oil compounds occurs at -3°C. The degradation could be stimulated through the addition of nutrients in both, samples of melted ice and in bioremediation experiments conducted within the sea-ice matrix. In the experiments carried out with melted Antarctic ice samples, inorganic nutrients stimulated hydrocarbon biodegradation significantly after a period of six months of incubation at 0°C. In the laboratory experiments, Inipol and fish meal proved to enhance oil biodegradation in the sea-ice matrix, even at temperatures below the freezing point. However, at -3°C the rate of degradation in the ice matrix was considerably decreased, compared to the rates of degradation in the liquid samples at 0°C.

These results are in conformity with Hoff (Hoff, 1993)who found that environmental parameters such as temperature have an equivalent or perhaps larger role than nutrients in determining the rate of degradation at contaminated sites.

All tested fertilizers, Inipol, fish meal, and inorganic nutrients, proved to enhance the decontamination of highly persistent compounds. In this study, inorganic nutrients were found to stimulate biodegradation only in melted samples. However, this can be explained by the decreased onset of biodegradation as well as by the reduced oxidization of petroleum hydrocarbons in the ice matrix at temperatures below freezing. This suggests that inorganic nutrients stimulate the degradation processes to a measurable extent in the sea-ice matrix as well as when applied to extended exposure. The fertilization with fish meal led to a dramatic increase in bacterial biomass and seemed to enhance biodegradation more in some experiments carried out with Arctic summer sea-ice than through the addition of the oleophilic organic agent Inipol. Although a strong decrease in bacterial diversity was revealed

by the addition of fish meal, a larger number of different oil degrading bacterial isolates was obtained from the experiments fertilized with fish meal (Manuscript 3), compared to the isolates obtained from the experiments treated with Inipol. This leads to the conclusion that the treatment with oil plus fish meal favoured the abundance of the potential of cold-adapted oil degraders.

Bioaugmentation has only sometimes been reported to effectively enhance biodegradation in cold environments (Horowitz & Atlas, 1978; Margesin & Schinner, 1999). Nevertheless, it is argued that especially in regions with cold climates, seeding with hydrocarbon oxidizing microbes, which are adapted to their ambient environment, may shorten the lag period before the onset of biodegradation starts. This is mostly due to the relatively slow development of an oil degrading indigenous population. The application of bioaugmentation was tested in the Antarctic experiments (Manuscript 2) by seeding the samples with oil degrading sea-ice bacteria. Again, in conformity with the results achieved by the addition of fertilizers, the only method applied in melted ice samples resulted in an enhanced oxidation of petroleum hydrocarbons. Bioaugmentation was shown to further stimulate the biodegradation processes. The fact that additional nutrients were used to supplement the inoculated experiments by adding saw dust itself, compared to the experiments which were fertilized with inorganic nutrients alone, is assumed not to have significantly contributed to the degradation processes of crude oil hydrocarbons, as the addition of inorganic nutrients supplied nitrogen and phosphorus concentrations in the range of 50 to 200 μM, which were suggested to be an optimal concentration for biodegradation in beaches (Bragg et al., 1994).

Why biodegradation may not be effective in the solid sea-ice system

The reasons for decreased oxidation rates of crude oil hydrocarbons in the sea-ice matrix are complex and it is assumed that several factors influence the degradation processes, some of which are reported on below:

Temperatures below freezing cause a decrease in bacterial metabolism, although cold-loving bacteria are able to grow and multiply at ≤ 0°C and are characterized by enzymes with high catalytic efficiencies at low temperatures. The optimal growth temperature for psychrophilic organisms is ≤15°C and for psychrotolerant organisms is >15°C (Morita, 1975). Furthermore, the viscosity of oil increases with decreasing temperatures, resulting in a reduced spreading of the oil and thus in a decreased surface area for microbial colonization and attack, and hence to a higher concentration of the contaminant in the settled spots. Moreover, evaporation of short chain alkanes, which are toxic to many organisms, is reduced, whereas the water solubility is

also reduced with decreasing temperatures and increasing chain lengths. The result is decreased bioavailability of these compounds and thus a delay of the onset of biodegradation.
Another factor, influencing the biodegradation of hydrocarbons in sea-ice, is the sea-ice matrix itself. Sea-ice bacteria live in the microhabitats of brine channels within the ice matrix, which provides an increased availability of surfaces and nutrients (Bowman et al., 1998b). Many of them are epiphytic or live in close association with microalgae or other surfaces (DeLong et al., 1993; Grossmann & Gleitz, 1993). The sea-ice bacteria attached to surfaces may not move and even free-living bacteria may not have been able to move through the labyrinth of brine channels and get to the contaminant at the ice surface. Furthermore, high concentrations of available dissolved organic matter (DOM) have often been reported to occur in sea-ice, which are assumed to be preferentially consumed. Therefore it is suggested that an oil degrading population first has to develop around the crude oil spots. During the course of contamination, when crude oil hydrocarbons are assumed to penetrate to deeper layers in the ice matrix, bacteria within the ice channels are also affected by the contaminant.
It is assumed that the slow response of bacterial communities to crude oil contamination and bioremediation treatment reflect these constraints.

The potential of using bacterial isolates from oil contaminated sea-ice for the degradation of crude oil

Bacterial strains, isolated from oil contamination as well as bioremediation experiments, were tested for their petroleum hydrocarbon degradation potential, with the long-term objective of optimising the development of an inoculum for the bioaugmentation of crude oil contamination in sea-ice (Manuscript 3). Growth characteristics were also determined in order to understand their role in crude oil degradation processes and function in the sea-ice ecosystem.
Most tested isolates preferentially degraded n-alkanes rather than branched alkanes and polycyclic aromatic hydrocarbons (PAHs) at low temperatures, which has been observed previously in crude oil biodegradation studies (Prince et al., 2002; Wang et al., 1995). Short chain alkanes were more readily degraded than the longer ones, which is also a common feature of many other alkane degrading organisms (van Beilen et al., 1994). Six isolates, belonging to the genera *Pseudomonas* and *Oleispira* of the *Gammaproteobacteria* as well as to *Dietzia* and *Rhodococcus* of the *Actinobacteria*, emerged as potential crude oil alkane degraders. Oil fingerprints of representative isolates of these genera as well as of sterile controls and at three different temperatures are shown in Figures 14 and 15. Short chain

alkanes up to C_{14} disappeared due to weathering and were not taken into account for the degradation characterization of the isolates. At 15°C, all n-alkanes (up to C_{34}) were completely mineralized by five of these strains and partially by the sixth strain *Dietzia* sp. isolate-101. At 0°C and -3°C the longer chain n-alkanes C_{25} to C_{34} were only partially degraded, whereas the short chain alkanes up to C_{24} were readily degraded after three months growth in pure culture. These data suggest that n-alkanes $\geq C_{24}$ are a barrier for biodegradation due to their severely restricted bioavailability at temperatures near or below freezing. The incubation time of about three months corresponds to a summer season in the polar regions . The average temperature for January was found to be -3°C in the Antarctic (Margesin & Schinner, 1999), indicating that bioremediation of crude oil contamination during one season would decontaminate short chain alkanes only, whereas the longer ones would persist through the winter season.

Growth of bacterial sea-ice isolates by applying the resazurin method as well as microscopic analysis was used to estimate their capability to degrade single crude oil hydrocarbon compounds, including various PAHs. Growth tests revealed that the isolate-327, affiliated with the genus *Pseudomonas*, which was shown to potentially degrade alkanes in crude oil, was able to grow on various 2- and 3-ring aromatic compounds as well as on the long chain n-alkane C_{36} at 15°C. It seemed odd that this strain was not able to grow on phytane as single substrate, although phytane in crude oil was degraded at 15°C. Another strain, *Marinobacter* sp. isolate-81, also showed a broad growth spectrum on various short and long chain alkanes as well as on various PAHs, whereas other isolates of this genus did not show hydrocarbonoclastic abilities. In addition, isolate-81 was shown to be mesophilic, whereas the other *Marinobacter* strains were psychrophilic. The phylogenetic tree (Figure 10) confirmed these observations by showing that they were distinct within the *Marinobacter* group. These findings indicate that not all *Marinobacter* spp. belonging to sea-ice assemblages, have hydrocarbon degrading abilities. The *Oleispira* isolate-381 showed a high degradation potential of alkanes in crude oil. However, growth on single hydrocarbon compounds was only determined for some compounds. *Shewanella* spp., most frequently isolated from the bioremediation experiments, showed no significant hydrocarbon degradation in crude oil (C_{14} to C_{34}), however, growth tests on single substrates revealed that they only grew on short chain alkanes up to C_{16}, but also on the highly concentrated hexadecane (4%). *Shewanella* spp. further showed high growth rates at -3°C. It can be assumed that their preferential oxidation

of readily degradable hydrocarbons and their ability to grow fast at low temperatures makes *Shewanella* spp. prevail in high abundances within the collection of isolates as well as in many sea-ice communities of the bioremediation experiments.

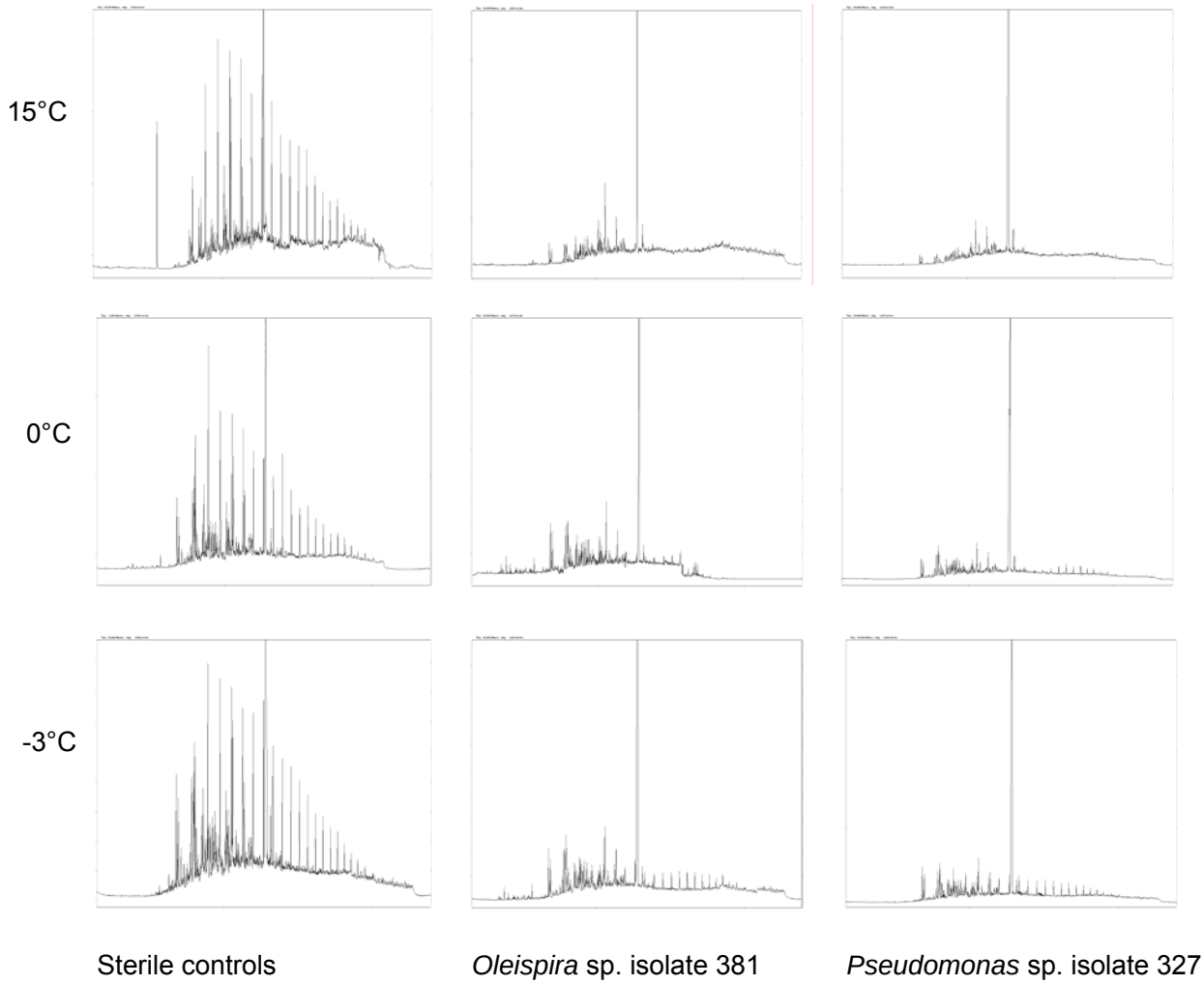

Figure 14. Oil fingerprints of degradation experiments at low temperatures, containing 0.2% crude oil from the Southern Barents Sea. Presented are the sterile controls and two bacterial isolates of the *Gammaproteobacteria*, grown in pure culture in liquid medium at 15°C for 90 days, at 0°C for 95 days, and at -3°C for 102 days.

Members of the two genera *Dietzia* and *Rhodococcus* of the *Actinobacteria* were shown to degrade a broad range of alkanes when grown on crude oil (0.2 %). Members of *Rhodococcus* were furthermore able to grow on 2-ring aromatic compounds, some also on 3- and 4-ring PAHs. However, growth tests on single hydrocarbons revealed that all tested *Rhodococcus* strains did not grow on hexadecane, when added at a high concentration of 4%. Therefore it is assumed that, although Rhodococci are potential degraders of a broad range of various

petroleum hydrocarbons at low temperatures, they may not prevail when large amounts of oil are spilled into the marine ice-covered environments. Some bacteria of the genus *Dietzia* also covered a broad degradation range of crude oil alkanes, whereas another strain (isolate-124) only grew on short chain alkanes up to C_{16}. However, temperature characteristics showed that their growth was insignificant when temperatures dropped below freezing, suggesting that these phylotypes might easily be out-competed in the sea-ice environment.

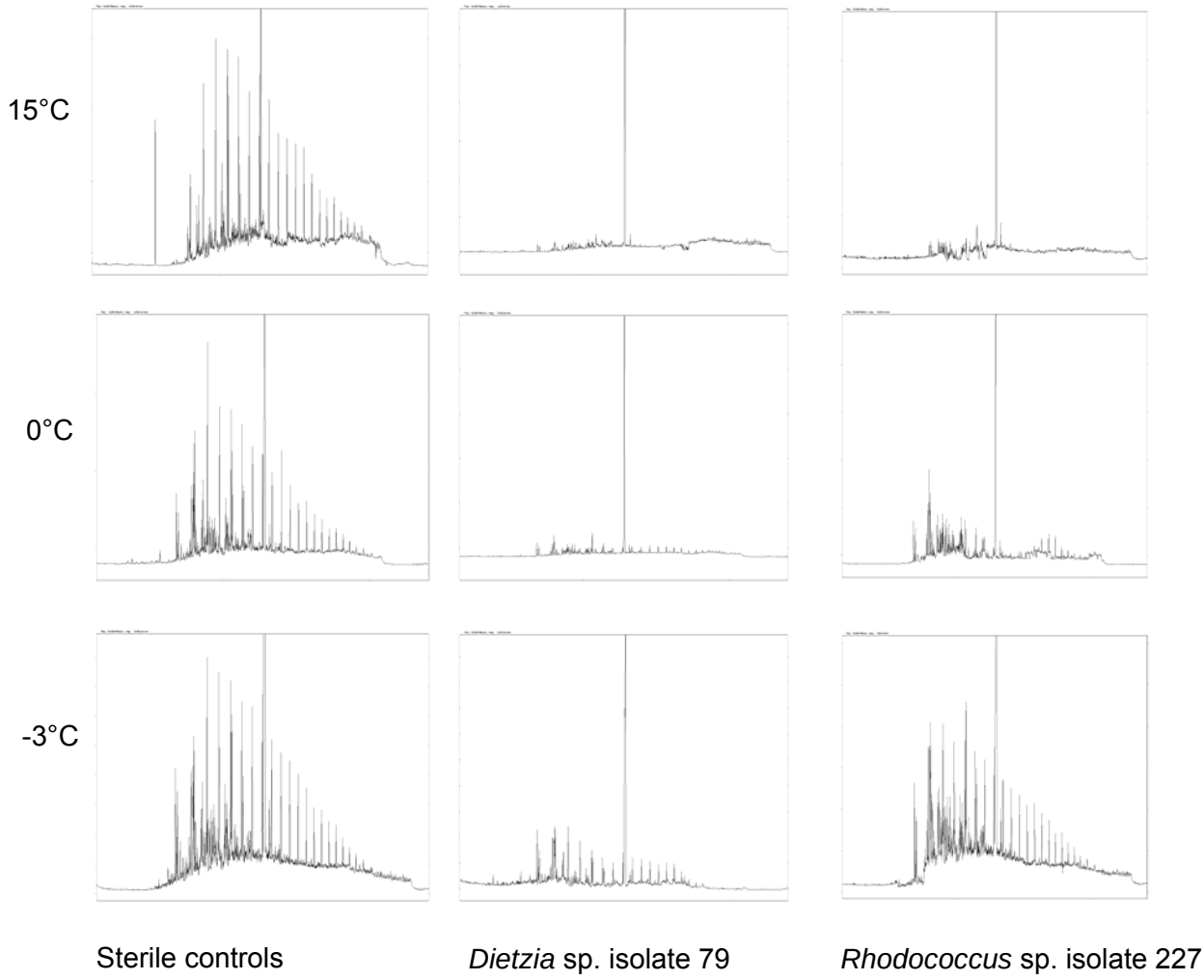

Figure 15. Oil fingerprints of degradation experiments at low temperatures, containing 0.2% crude oil from the Southern Barents Sea. Presented are the sterile controls and two bacterial isolates of the *Actinobacteria*, grown in pure culture in liquid medium at 15°C for 90 days, at 0°C for 95 days, and at -3°C for 102 days.

The *Loktanella* sp. isolate-484 of the *Bacteroidetes* phylum showed slight growth on short chain hydrocarbons. Hydrocarbonoclastic capabilities have been reported for members of the family *Flavobacteriaceae* (Floodgate, 1984; Kwon et al., 2006), however, the *Flavobacterium* strain 522 did not show any growth on single hydrocarbons.

Only one strain of the *Alphaproteobacteria*, which was related to *Jannaschia* sp., namely isolate gap-f-54, showed to be able to grow on alkanes up to C_{20} and on the 2-ring PAHs naphthalene and acenaphthene.

In total, more isolates where able to degrade crude oil alkanes rather than PAHs, coinciding with findings of Eriksson et al. (Eriksson et al., 2003), who suggested that psychro-tolerant PAH degraders are less ubiquitous than mesophilic PAH degraders and may require a very long time to enrich.

Some bacterial strains did not appear to have a hydrocarbon degrading ability when grown in pure culture, although they were isolated either on crude oil agar or on single hydrocarbons as sole carbon source. Furthermore, they were repeatedly detected in bioremediation experiments by means of DGGE, which suggests that they may play a role in the petroleum hydrocarbon oxidizing processes in the natural sea-ice environment, when interchanges between other organisms occur. It is well known that many bacteria take up only dissolved hydrocarbons (Bouchez et al., 1995; Philip et al., 2005; Wodzinski & Bertolini, 1972). It is therefore assumed that some organisms may not be able to increase the bioavailability of hydrocarbons at low temperature, but rather profit from other organisms which dissolve these compounds. Alternatively, they may degrade by-products and metabolites produced by a partial degradation of hydrocarbons by other organisms. Mixed cultures may be superior to pure cultures and may even degrade a broader spectrum of xenobiotic compounds (Pelz et al., 1999). Moreover, only some representative strains have been tested for their hydrocarbon degradation capacity, assuming that some more strains of the collection of 634 isolates might have hydrocarbonoclastic capabilities. However, hydrocarbonoclastic abilities may vary within species of one phylotype.

Conclusions

Bioremediation experiments revealed that fertilization stimulated the biodegradation of crude oil hydrocarbons under sea-ice conditions and that bioaugmentation further enhanced the degradation processes. However, at temperatures below freezing, in the sea-ice matrix, the degradation processes decreased considerably. Nevertheless, it is assumed that during the summer months, when irradiation promotes a rapid melting of the ice surrounding the oil, surface melt pools will develop on the ice and thus significantly speed up bioremediation, which will thus further enhance decontamination processes of accidentally spilled petroleum hydrocarbons. However, it has to be considered that biodegradation of high amounts of oil will take several years. The combination of bioremediation methods with the use of dispersants may be considered profitable at cold temperatures, due to the unfavourable characteristic of hydrocarbons at low temperatures. However, the effectiveness and toxicity of dispersants showed that in most cases the chemically dispersed oil was more toxic than the physically dispersed oil. Moreover, the legislation for the use of dispersants varies from country to country and is not allowed in most European countries. Furthermore, no applications in cold water are described in the literature (Fingas, 2002). Therefore, inoculation with indigenous oil-degrading sea-ice bacteria may be a powerful tool to enhance decontamination processes of hydrocarbon contaminations in the marine polar environments.
Small amounts of petroleum hydrocarbons can successfully be removed from the polar sea-ice environment through the application of bioremediation during the summer seasons. A recommendation would therefore be to apply the bioremediation method for the cleanup of low contaminations, such as small losses of petroleum hydrocarbons due to shipping operations. Furthermore, if major oil spills occur, bioremediation should be taken into consideration as a second step in the oil spill contingency strategy after mechanical cleanup, when low amounts of hydrocarbons will remain.

PART IV

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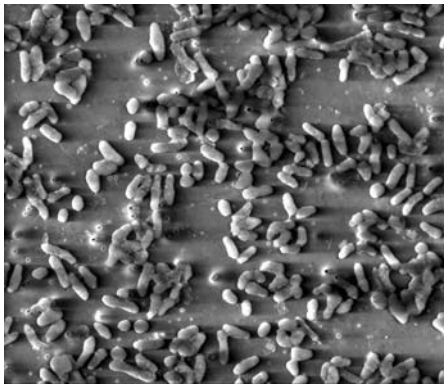

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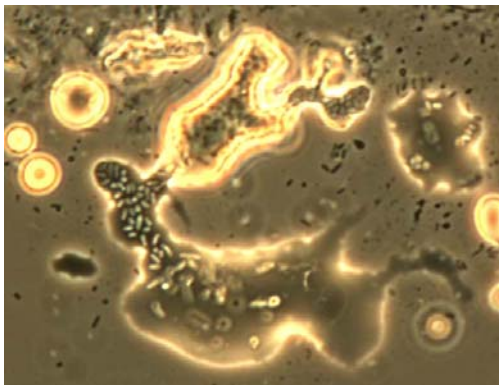
PART V

MANUSCRIPTS



List of Publications and Manuscripts

Manuscript 1

Birte Gerdes, Robin Brinkmeyer, Gerhard Dieckmann, and Elisabeth Helmke.
Influence of crude oil on changes of bacterial communities in Arctic sea-ice.
FEMS Microbiology Ecology. (2005), 53: 129-139

Manuscript 2

Birte Gerdes, Christiane Uhlig, Gerhard Dahlmann, Gerhard Dieckmann, Friedrich Widdel, and Elisabeth Helmke. Efficiency of bioremediation in crude oil contaminated Antarctic sea-ice and gap-layer water. *Manuscript in preparation*.

Manuscript 3

Birte Gerdes, Gerhard Dieckmann, and Elisabeth Helmke.
Degradation of petroleum hydrocarbons by cold-adapted bacteria from Arctic and Antarctic sea-ice at low temperatures. *Manuscript in preparation*.

Manuscript 4

Birte Gerdes, Gerhard Dieckmann, and Elisabeth Helmke. Bioremediation field experiments with crude oil in sea-ice conducted in Van Mijenfjorden, Svalbard (2004). *Manuscript in preparation*.

Manuscript 5

Johanna Ikävalko, Birte Gerdes, and Gerhard Dieckmann. An experimental study of the effects of Statfjord crude oil, and application of Inipol and fish meal on the sea-ice biota in Svalbard in February-April 2004. *Proceedings of the twenty-eighth Arctic and Marine Oilspill program* (AMOP) Technical Seminar. (2005), Volume 2: 993-1003.

Manuscript 1

INFLUENCE OF CRUDE OIL ON CHANGES OF BACTERIAL COMMUNITIES IN ARCTIC SEA-ICE

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

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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

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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%,

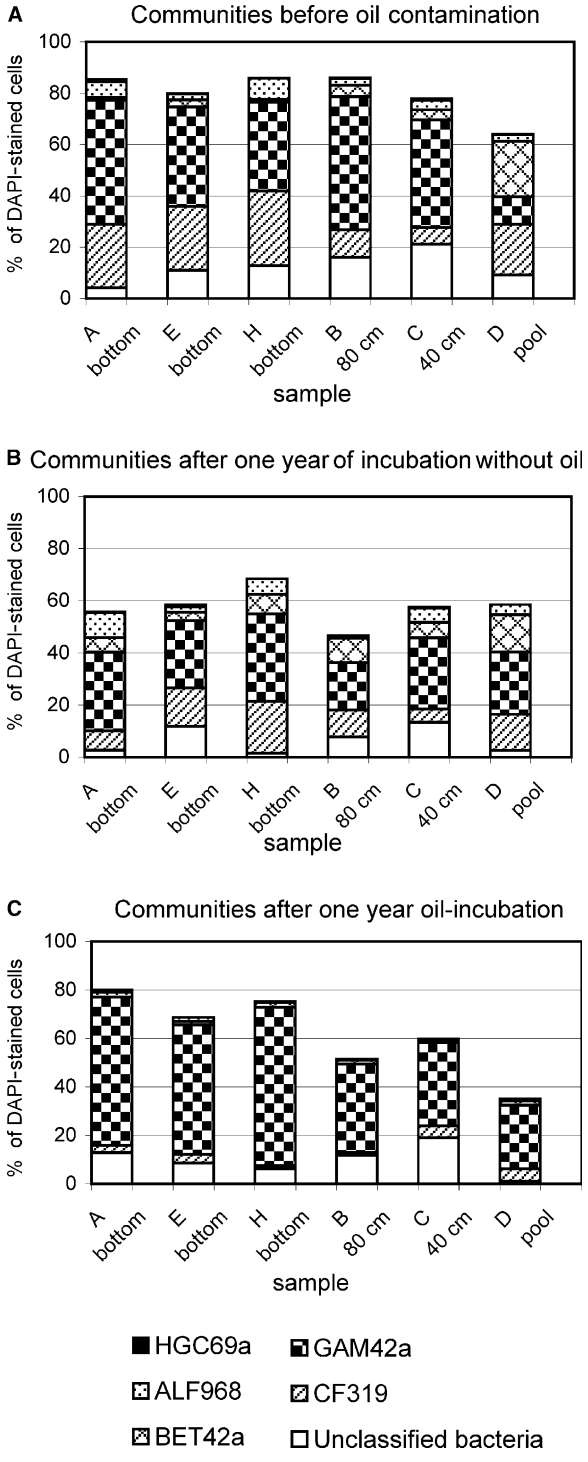

Fig. 1. Percentages of DAPI-stained bacteria detected by FISH using the probes HGC69a for gram-positive (*Actinobacteria*), ALF968 for α-proteobacteria, BET42a for β-proteobacteria, GAM42a for γ-proteobacteria, and CF319a for the *Cytophaga Flavobacteria* of the *Bacteroides* group. The percentage of 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.

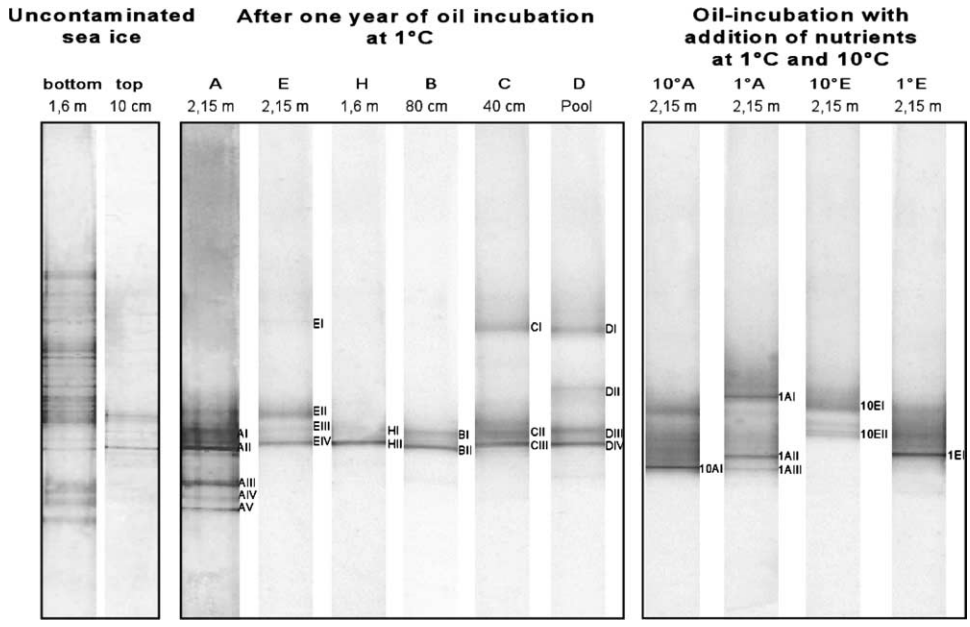

Fig. 2. DGGE profile of 16S rRNA gene fragments of non-contaminated sea-ice samples, samples from oil-treated microcosm experiments after one year of incubation at 1 °C, and samples from oil-treated microcosm experiments after 6 months of incubation at 1 and 10 °C. Numbering on the right hand side of 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.

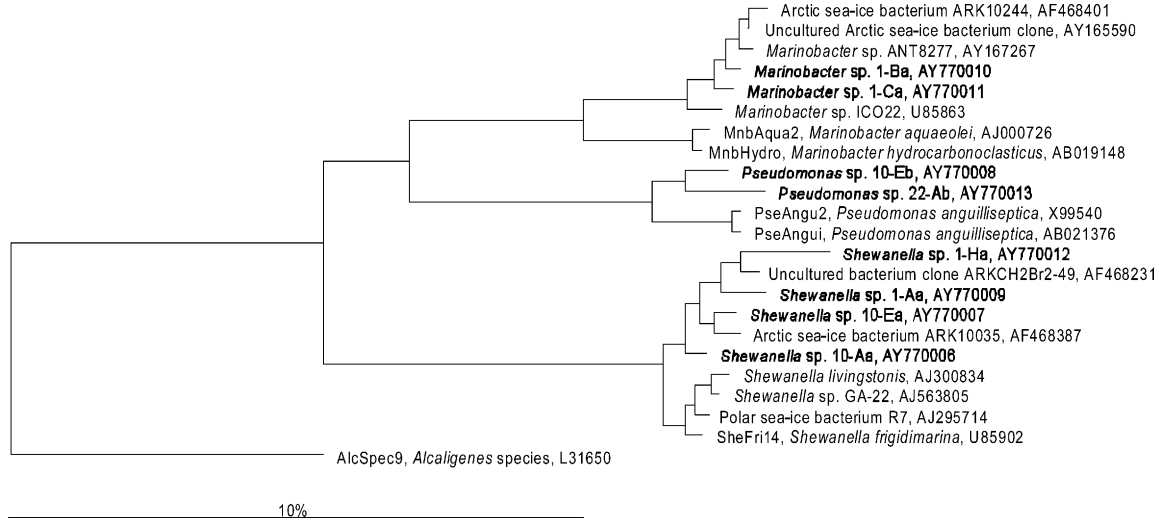

Fig. 3. 16S rRNA gene-based phylogenetic tree showing the relation of eight isolates cultivated from oil-contaminated Arctic sea-ice samples to their closest relatives in the GenBank as well as their phylogenetic distance to *M. hydrocarbonoclasticus* and *M. aquaeolei*. The tree was reconstructed using 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.

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Manuscript 2

EFFICIENCY OF BIOREMEDIATION AND BIOAUGMENTATION IN CRUDE OIL CONTAMINATED ANTARCTIC SEA-ICE

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Keywords: bioremediation, bioaugmentation, biodegradation, sea-ice, hydrocarbons, low temperature

Manuscript in preparation

Abstract

Oil bioremediation experiments were conducted on Antarctic sea-ice samples from the top (surface) and bottom (water interface) of the ice column and water from gap-layers which are typically formed in the Antarctic summer months at or just below the water level. The efficiency to stimulate petroleum hydrocarbon degradation through fertilization with inorganic nutrients as well as through additional inoculation with cold-adapted, oil-degrading sea-ice bacteria (bioaugmentation) was tested. In addition, we tested the influence of the different bioremediation treatments on sea-ice bacterial communities by the means of DGGE and FISH. In the gap-layers experiments, fertilization enhanced biodegradation of n-alkanes significantly up to the range of C_{24}, and bioaugmentation substantially increased the biodegradation to an almost complete degradation of n-alkanes up to the range of C_{28}. After six months only two bands of the gapwater bioaugmentation experiments, which affiliated with the genera *Marinobacter, Oleispira, Pseudomonas* and *Rhodococcus*, could be retraced to the original inoculum. The experiments with solid sea-ice showed accelerated utilization of [^{14C}C]-hexadecane with both treatments at -3°C. However, oil-degradation in the experiments incubated for one month on the ice floe and subsequently six months at -3°C was not significantly stimulated. DGGE-patterns revealed only small changes in the bacterial sea-ice community composition compared to the controls. Cell size and hybridization signals with probe EUB338 indicated a negative effect on the indigenous community when treated with crude oil only.

Introduction

The seasonal sea-ice cover in Polar Regions and other seas constitutes one of the major, albeit ephemeral, habitats on our planet, covering at its maximum extent about 13% of the Earth's

surface. In the Antarctic, sea-ice covers an area of 20 x 10^6 km^2 in winter and recedes to an area of 4 x 10^6 km^2 in summer. Apart from affecting ocean circulation and world climate, sea-ice also has a direct and indirect effect on polar ecosystems. Sea-ice microbial communities (SIMCO) are dwelling in a network of channels and pockets filled with liquid brine, which arise during the formation of sea-ice. The physico-chemical parameters, such as volume and salinity of these microhabitats, vary in relation to temperature. The development of gap-layers (see Figure 1) is a typical feature of the perennial Antarctic sea-ice in summer as a result of biophysical feedback processes at the onset of warming (22). Increase in temperature and solar radiation lead to algal blooms within the sea-ice. The brownish colour of the algae enhances the absorption of radiation and thus leads to further melting. Gap-layers are found at, or just below the water level, overlain by a thin solid layer of ice (22). Microbial communities of surface sea-ice and gap-layers are highly productive (27).

Increasing human activities in the polar regions, particularly the quest for oil but also tourism, fishing and even research, increase the risk of oils spills at these high latitudes (14). The wreck of the supply ship “Bahia Paraiso”, which ran aground and subsequently sank near the Antarctic Peninsula, highlighted the need for research on hydrocarbon degradation in the Southern Ocean ecosystems (15). Biodegradation of petroleum hydrocarbons by indigenous microbial communities has been reported from various Arctic and Antarctic ecosystems of soil, freshwater, and sea water as well as from cold alpine sites (1, 4, 5, 7, 9, 28, 31, 32, 35, 36, 50, 51). However, bioaugmentation led to ambiguous results. In many studies (16, 31, 37, 46) improvement of bioaugmentation was observed in the early stage of the biodegradation process, while later on the communities returned to the levels comparable with the non-bioaugmented samples. Nevertheless, especially in high latitudes, where very short summer seasons do not permit long acclimatization periods for hydrocarbon-degradative populations (51), bioaugmentation has to be taken into consideration as bioremediation strategy.

Little is known about biodegradation of oil in sea-ice as well as about bioremediation effects on SIMCO (15). In this study we assessed the efficiency of bioremediation treatments with inorganic nutrients and inoculation with a consortium of oil-degrading bacteria from Arctic- and Antarctic sea-ice and considered the impact of these treatments on the development of the microbial communities.

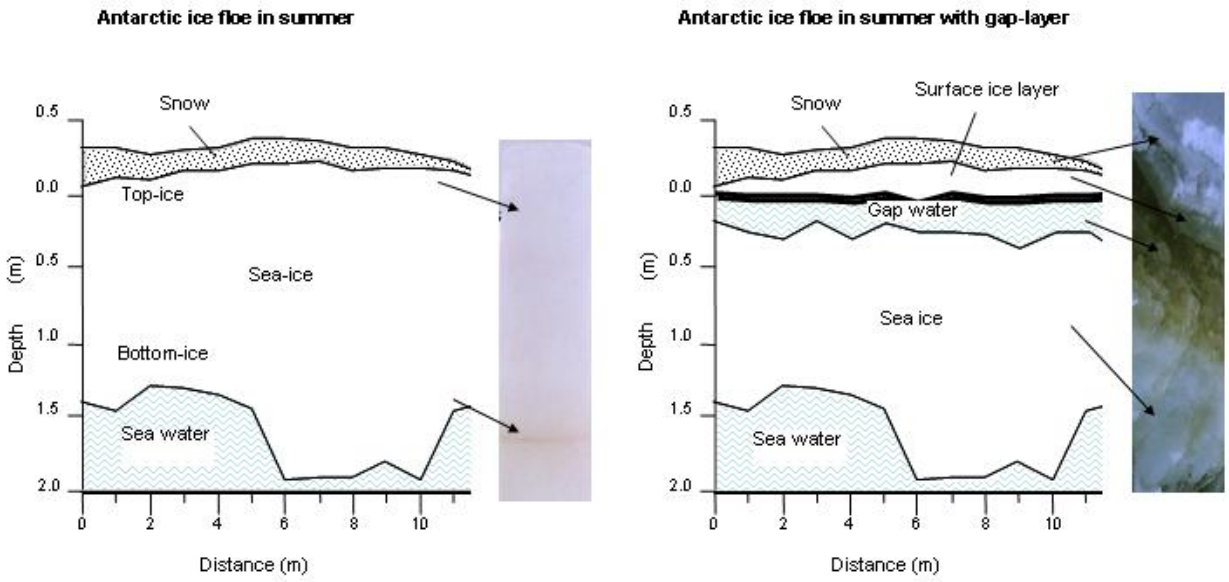

Figure 1. Schematic vertical cross-sections (modified from Kattner et al., 2004 and Haas et al., 2001) of two summer ice floes, the lower one with a seawater filled gap-layer. Water level is 0 cm. The left photograph shows a vertical cross through the ice column with a typical algae stock layer at the bottom part and the right photograph a section of surface-ice layer from a refrozen gap-layer floe. The greenish bottom-layer is a refrozen gap-layer containing high algae standing stocks.

Material and Methods

Sample site and sampling

Sea-ice- and gap-water samples were obtained from one ice flow (68° S, 55° W) during the ISPOL cruise (Ice Station POLarstern) ANTXXII/2 (November 2004 to January 2005) with the R.V. "Polarstern". Sea-ice samples were obtained by drilling ice cores (9 cm in diameter) using a Austin Kovacs Enterprises (AKE) Mark III corer, and gap-layer water was pumped

with an ethanol rinsed hand pump into an autoclaved 20 l container (Nalgene). For molecular analysis samples were processed as described by Helmke and Weyland (24). Ice cores were immediately cut into 10 cm segments on the ice floe and transferred into sterile plastic bags. The segments were crushed and allowed to melt in sterile plastic containers with an equal volume of sterile filtered seawater to avoid disruption of cells during the melting process. Sea-ice samples for the bioremediation trials were not melted.

Inoculum preparation

To provide a broad variety of indigenous hydrocarbon degraders from sea-ice, we compiled an inoculum for our bioaugmentation experiments from different oil-contaminated long-term microcosm batches with Arctic and Antarctic sea-ice, which were fertilized with different organic (Inipol and fish meal) and inorganic (Na_2 HPO4 0.065 gl^{-1}, $NaNO_3$ 0.75 gl^{-1}, traces of $FeCl_3$) nutrients and 0.1% Statfjord crude oil as carbon source. Most phylotypes of SIMCO are found at both poles (11, 12), although with different quantitative distribution in Arctic and Antarctic pack ice communities (11). Furthermore, mixtures of microorganisms have a broader degradation spectrum than individual strains and respond more easily to environmental changes (41). For the immobilization enrichment cultures from the different experiments were mixed in equal volumes. Thereafter, one gram of sterilized and dried sawdust was added to 50 ml of the mixture and shaken at 150 rpm for four days at 4°C to allow the cells to attach to the sawdust surface. The sawdust was then allowed to settle down and most of the supernatant was removed until only 10 ml remained. The sawdust suspensions were immediately used to inoculate the bioremediation experiments of the sea-ice samples. The remaining bacterial mixtures were stored at 4°C and inocula were later freshly prepared and immobilized for the gapwater experiments.

The diversity of the inoculum was analysed by DGGE and FISH. Furthermore, psycho-tolerant and psychrophilic strains of 14 bacterial genera were isolated on Minimal-Agar

(KNO_3 0. 75 gl^{-1}, NH_4Cl 0.75 gl^{-1}, yeast extract 0.1 mgl^{-1}, and traces of $FePO_4$) with 0.5% [v/v] Statfjord crude oil. The collection encompasses 14 bacterial genera with *Bacteroides* and *Winogradskyella* of the phylum *Bacteroidetes* (formerly *Cytophaga-Flavobacterium-Bacteroides* phylum), *Shewanella*, *Marinobacter*, *Pseudomonas*, *Pseudoalteromonas*, *Alteromonas*, *Halomonas*, *Psychrobacter*, *Oleispira*, and *Marinomonas* of the *Gammaproteobacteria*, and *Dietzia*, *Rhodococcus* and *Arthrobacter* of the *Actinobacteria*.

Set up of bioremediation experiments

Bioremediation experiments with top and bottom sea-ice samples were set up in 2 l autoclaved polycarbonate beakers on the 3rd December 2004. The beakers were filled up to the 1800 ml line with previously crushed and homogenized sea-ice (2-5 cm in diameter). Experiments with gap-layer water (800 ml) were set up in 1l autoclaved Schott flasks on the 5th January 2005, shortly after development of the gap-layer.

Six different batches were set up for bottom and top sea-ice as well as for the gap-layer water. The experiments were labelled as follows: a, no additional treatment (control 1); b, addition of inorganic nutrients (control 2); c, oil only; d, oil and inorganic nutrients; e, inorganic nutrients and inoculum (control 3); f, oil, inorganic nutrients, and inoculum. Inorganic nutrients were added in concentrations of Na_2HPO_4 0.065 gl^{-1}, $NaNO_3$ 0.75 gl^{-1}, and traces of $FeCl_3$. The inoculum suspension (preparation see above) was added to the experiments e and f in a volume of 10 ml. To estimate weathering of Statfjord crude oil during the experiments, two sterile controls, killed with $HgCl_2$ (0.0072% end concentration), were incubated under the same conditions.

The batches with the top and bottom sea-ice samples were incubated in the field for 30 days in a depression on the ice floe. To avoid rapid melting of the ice due to an increased absorption of sun radiation by the dark colour of oil, the experiments were covered with sterile linen and a thin layer of snow. The temperatures at the ice surface ranged from -4.3°C to -0.1°C with an

average temperature of -2.9°C. At the end of the ISPOL-experiment the beakers were retrieved from the ice floe and transferred to an incubator which was run at stable –3°C. Gap-layer experiments were incubated in a cold room at 0°C right from the beginning.

Oil analysis by GC-FID and GC-MS

All experiments with crude oil, including the two sterile controls, were analysed for oil biodegradation by means of GC-FID. Those samples, which showed biodegradation, were also analysed by GC-MS to assess possible effects especially on polyaromatic hydrocarbons and alkylated homologues.

Crude oil from the sea-ice experiments was scraped from the entire surface to obtain a representative sample. At least 200 ml water from the gap-layer batches were transferred into glass bottles. Immediately after sampling, bacterial activity was stopped by the addition of formalin (4% final concentration) and stored at 4°C until further processing.

Extraction: Oil-samples (60 mg) from sea-ice experiments were transferred in solvent rinsed glass flasks and extracted with 10 ml hexane/dichloromethane (9:1 v/v). Samples from gapwater experiments (200ml) were extracted with 10 ml hexane/dichloromethane (9:1 v/v) in solvent rinsed separatory funnels on a rotary shaker for 20 min. After separation of the organic phase from the gapwater all extracts were sonicated (50/60 Hz, 150W) for 10 min. If samples contained moisture, a spatula of sodium sulphate was added.

Cleanup: Hexane/dichloromethane extracts were cleaned on silica columns (1ml, Bond Elut). Columns were conditioned two times with 10 ml dichloromethane and two times with 10 ml hexane (UniSolv, Merck), then 500 µl of the extract were transferred onto the column. To elute hydrocarbons from the column, 500 µl hexane/dichloromethane (4:1, [v/v]) was used.

GC-FID analyses were done using a HP 5890 Series II (Hewlett Packard) chromatograph equipped with a FID (Hewlett Packard) and a capillary column (10m x 0.1 mm x 0.1 µm; HP5, Hewlett Packard) under following conditions: initial temperature 40°C for 0.5 min, rate

30°C/min, final temperature 300°C, run time 15 min, equilibration time 3 min; detector: 320°C; injector: 280°C; carrier gas H_2 (208 kPa) 0,6 ml/min.

GC-MS analyses were done using a HP 6890 plus gas chromatograph (Hewlett Packard) equipped with a capillary column ZB5 (30 m x 0.25 mm x 0.25 µm) and a mass detector HP 5973A (Hewlett Packard) under the following conditions: initial temperature 40°C for 1 min, rate 6°C/min, final temperature 310°C, run time 79 min, equilibration time 2 min; detector: EM voltage 200V, time 20min; injection volume 3 µl; carrier gas He (70kPa) 0.6 ml/min.

Biodegradation removes preferentially normal alkanes over branched compounds, resulting in a decrease of C_{17}/pristane and C_{18}/phytane ratio. As long as n-alkanes are present in large quantities, pristane and phytane are almost not touched by bacteria (48). Therefore, to estimate the initial qualitative rate of degradation changes, the ratios of straight chain alkanes to branched alkanes such as n-C_{17}/ pristane and n-C_{18}/phytane (5) were measured.

[^{14}C]-hexadecane incubation experiments

Bacterial utilization of hexadecane was determined on top and bottom sea-ice by measuring the respiration of [^{14}C]-hexadecane to $^{14}CO_2$. Three replicates of each treatment and one control (killed with 0.0072% $HgCl_2$) were prepared in autoclaved Erlenmeyer flasks each containing 100 ml of crushed sea-ice in 10 ml sea-ice brine. One series of top- and bottom-ice samples was treated with 10 ml nutrients (KNO_3 0. 75 gl^{-1}, NH_4Cl 0.75 gl^{-1}, yeast extract 0.1 mgl^{-1}, and traces of $FePO_4$) and another series of both ice samples with nutrients plus inoculum. All experiments were then charged with 10µl [^{14}C]-hexadecane ([$^{14}C_6H_5CH_3$]; 58 mCi $mmol^{-1}$; Sigma), sealed with silicon septa, and incubated at -3°C in the dark. Each experiment was equipped with a glass vial containing 2 ml 1 M KOH to trap the $^{14}CO_2$ released into the headspace. At sampling times, 500 µl KOH of each experiment were taken with a syringe and transferred into a CO_2 trapping 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 1 M KOH. The dilution effect was taken into account when calculating the formation of $^{14}CO_2$. All counts were corrected for background values which were measured with 500 µl 1M KOH in 18 ml scintillation cocktail (LumasafeTM Plus, Lumac; Groningen,Netherlands). The incorporation of ^{14}C into biomass was not determined due to a high residue of non-incorporated hexadecane on the polycarbonate filters after filtration in spite of several washing steps.

The gap-layer water was not analysed for the utilization of [^{14}C]-hexadecane due to the late development of the layer at the end of the cruise.

DNA extraction PCR amplification and DGGE analysis

Before extraction of bacterial DNA from the sea-ice bioremediation experiments, samples were melted and cells collected on polycarbonate filters (pore size, 0.2 µm). Bacteria of the gap-layer experiments were also collected on filters and all filters stored at – 20° until further processing. Total community nucleic acids were extracted from filters using the Ultra Clean Soil DNA-Kit (MoBio, USA) with additional lysozyme (final concentration 1 mg/ml) pre-incubations at 37°C. To increase the sensitivity of DGGE analysis, a nested PCR technique was applied as described by Gerdes et al. 2005.

PCR-products were analysed by DGGE, based on the protocol of Muyzer et al. (38) using a gradient-chamber. Approximately 25-30 µl of the PCR products were loaded onto polyarcylamide gels prepared and further processed as described by Gerdes et al. (19).

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 28 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min.

Fluorescence in situ hybridization (FISH)

FISH analysis was applied to examine the community structures of bacteria in all gapwater experiments after six months of incubation at 0°C as well as in the initial gapwater and the inoculum. Samples of the sea-ice experiments were tested for hybridization with probe EUB338 I-III. All samples were fixed for 3-4 h at 4°C with formalin (37% formaldehyde solution, final concentration 1% [v/v]) subsequently bacteria were collected on white polycarbonate filters (diameter, 47 mm; pore size, 0.2 µm; Nucleopore), and then rinsed with 10 ml particle free, distilled water. Filters were air dried and stored at –20°C until further processing. For FISH analysis filters were cut into small pieces and vortexed roughly for 10 min in a 15 ml cup (Falcon) with 4 ml 50% ethanol. Hexane (1 ml) was added to samples containing crude oil and mixed for about 15 min to remove residue oil compounds which cause a high autofluorescence under UV excitation. The organic phase, containing oil residues was then removed from the sample. Subsamples (volume depending on cell concentration) were immobilized on white polycarbonate filters (diameter, 25 mm; pore size, 0.2 µm).

FISH analysis was conducted according to the method of Glöckner et al. (20)using Cy3-labled oligonucleotide probes (final concentration 5 ng/µl; Thermo Hybaid, Ulm, Germany) specific for large phylogenetic groups and characteristic for sea-ice communities (11). The specificity of the probes used ranged from domain to species level. The probe Non338 (47) was used to test for non-specific probe binding.

For counterstaining, air-dried hybridized samples were mounted in a mixture of 4 parts Citifluor AF1 (Citifluor Ltd., London, United Kingdom) and 1 part Vecta Shield (Vector Laboratories, Burlingame, California) 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 800 and 1000 DAPI-stained objects were counted per probe and sample.

Isolation

Bacteria were isolated from gapwater experiments d and f, which showed significant biodegradation of the Statfjord crude oil, as well as from the control experiment e to estimate the fate of the inoculated strains. Hundred µl of a serial dilution (10^0 - 10^6) was plated on Marine Agar (Difco 2216) and on minimal agar (KNO_3 0. 75 gl^{-1}, NH_4Cl 0.75 gl^{-1}, yeast extract 0.1 mgl^{-1}, and traces of $FePO_4$) with 20µl Statfjord crude oil evenly distributed on the agar surface. The plates were incubated at 1°C until colonies became visible. Single colonies were picked with an inoculating needle and restreaked twice to obtain pure cultures.

PCR amplification for 16S rRNA gene analysis

DNA from isolates was extracted by boiling for 3 min, followed by a freeze-thaw cycle. The extracted DNA was then purified using a PCR-Purification kit (Qiagen, Hilden, Germany).
Nearly full-length 16S rRNA gene sequences were amplified from nucleic acid extracts of the isolates by PCR with a thermal cycler (Eppendorf, Hamburg, Germany). The Bacteria-specific primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. PCR was performed as described by Gerdes et al. (19). Amplified ribosomal DNA restriction analysis (ARDRA) (33) was used to characterize the 16S rRNA gene diversity of the obtained isolates as described by Gerdes et al. (19). Representative strains of ARDRA pattern groups were selected for sequencing. Almost full length sequence data were compared to those deposited in the GenBank using BLAST algorithm (2) and aligned using multiple sequence alignment by MAFFT ver.5.667 (http://timpani.genome.ad.jp/%7Emafft/server). Phylogenetic trees with sequences of the

gapwater isolates and isolates from the inoculum as well as their closest relatives of the GenBank database were reconstructed using the Neighbour Joining analysis.

Results

Effectiveness of bioremediation treatments on oil degradation

The quantification of hydrocarbon biodegradation is difficult due to the patchy distribution of spilled oil and bacteria as well as to the loss of hydrocarbons through evaporation and dissolution. Therefore the initial qualitative rate of degradation was determined with the ratio of straight chain alkanes to branched alkanes such as n-C_{17}/ pristane and n-C_{18}/phytane. Biodegradation preferentially removes normal alkanes over branched compounds resulting in a decrease of the C_{17}/pristane and C_{18}/phytane ratio. As long as n-alkanes are present in large quantities, pristane and phytane are almost not touched by bacteria (48). Ratios of n-C_{17}/ pristane, n-C_{18}/phytane, pristane/phytane are presented in Table 1. These results revealed that in all three oil bioremediation experiments with samples of top- and bottom-sea-ice no significant biodegradation occurred. The chromatograms (not shown) showed similar patterns as the sterile control. Also the oil analyses of experiment c (oil alone) with gapwater (Table 1 and Figure 2) showed no significant difference compared to the sterile control. However, the chromatograms of the experiments d (oil and nutrients) and f (oil and nutrients plus inoculum) revealed considerable oil-biodegradation. In sample d, which was treated with oil and nutrients, n-alkanes of low molecular weight and medium chain length were biodegraded (Table 1, Figure 2). In sample f, treated with oil, nutrients, and inoculum, n-alkanes were almost completely biodegraded. Only a small amount of high molecular weight alkanes (n-C_{27} to n-C_{32}) remained in the sample. Aromatic hydrocarbons did not seem to be affected.

Degradation of [^{14}C]-hexadecane

The degradation potential of [^{14}C]-hexadecane was determined for top and bottom sea-ice samples without additional treatment, with nutrient addition, and with nutrient plus inoculum. The amounts of $^{14}CO_2$ evolved from 10 µl [^{14}C]-hexadecane are presented in Figure 3. Although oil analysis by GC-MS revealed no significant degradation of oil hydrocarbons in the sea-ice samples, Figure 2 shows a clear mineralization of [^{14}C]-hexadecane for all treatments, after two weeks. Moreover, the addition of inorganic nutrients as well as of nutrients plus inoculum stimulated the utilization of [^{14}C]-hexadecane significantly. After 47 days of incubation at -3°C all experiments had reached a stationary phase. In the two sea-ice experiments without additional nutrients only 12.2% (bottom ice) and 15.4% (top ice) of the total ^{14}C evolved to $^{14}CO_2$, while the addition of inorganic nutrients induced an increase of up to 58.8% (bottom ice) and 46.7% (top ice). The experiment treated with nutrients plus inoculum led to an increase of up to 61.5% (bottom ice) and 46.9% (top ice) $^{14}CO_2$, respectively.

Table 1. Ratios of n-alkanes to pristane and phytane

sample	treatment	time point	n-C17/ pristane	n-C18/phytane	pristane/phytane
Statfjord oil	original, fresh	start	2.32	2.29	1.23
Sterile control		after seven months	2.41	2.25	1.18
gapwater	(a) oil only	after six months at 0°C	2.06	2.18	1.12
gapwater	(d) oil and nutrients	after six months at 0°C	0.60	0.71	1.17
gapwater	(f) oil, nutrients and inoculum	after six months at 0°C			1.18
top sea-ice	(a) oil only	after seven months at -3°C	2.09	2.36	1.21
top sea-ice	(d) oil and nutrients	after seven months at -3°C	2.28	2.41	1.15
top sea-ice	(f) oil, nutrients and inoculum	after seven months at -3°C	2.2	2.35	1.00
bottom sea-ice	(a) oil only	after seven months at -3°C	2.62	2.38	1.12
bottom sea-ice	(d) oil and nutrients	after seven months at -3°C	2.39	2.52	1.16
bottom sea-ice	(f) oil, nutrients and inoculum	after seven months at -3°C	2.31	2.29	1.09

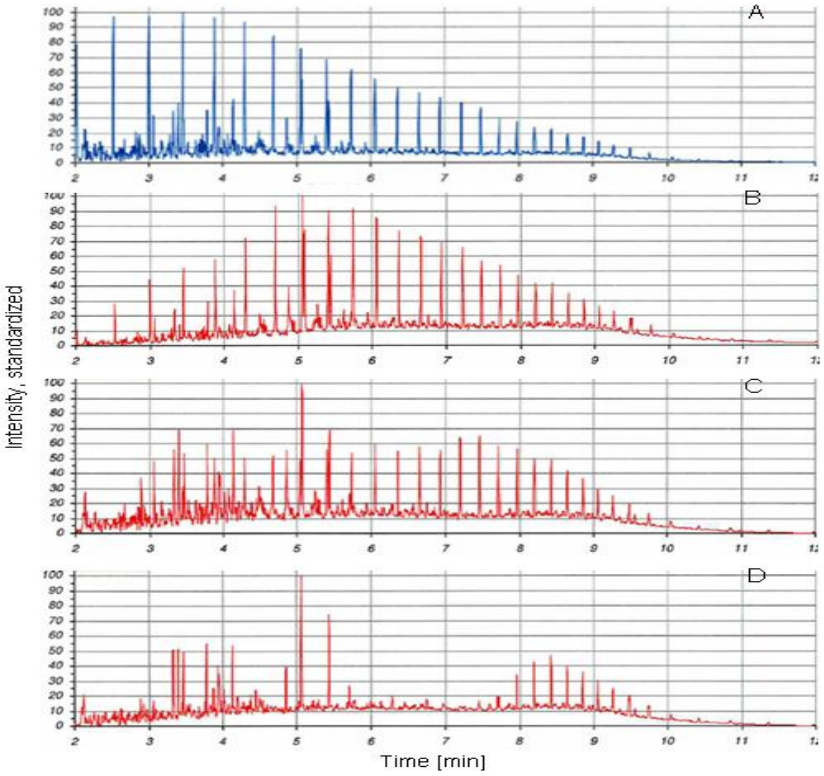

Figure 2: GC fingerprints of Statfjord crude oil (A) at the beginning of the experiments, and of the gapwater experiments after six months of incubation at 0°C with crude oil alone (B), with inorganic nutrients (C), with inorganic nutrients plus inoculum of oil-degrading sea-ice bacteria (D). Pristane and phytane as well as n-alkanes C_{17} and C_{18}, identified by retention time, are indicated.

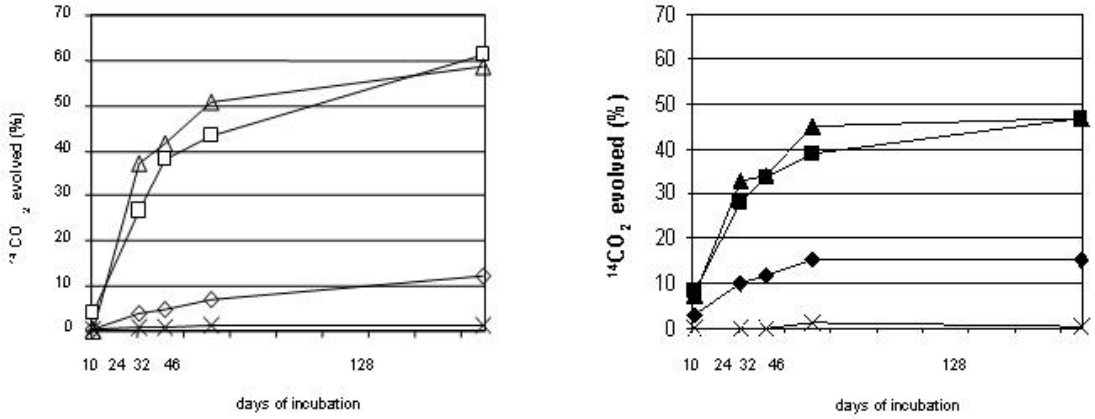

Figure 3. . Production of $^{14}CO_2$ from [^{14}C]-hexadecane in experiments with different treatments and Antarctic sea-ice from top (filled symbols) and bottom (bordered symbols) part of the sea-ice column. Symbols: ♦◊, ice only; ▲Δ, fertilized with inorganic nutrients; ■Í, fertilized with inorganic nutrients plus inoculum; x, sterile control

Influence of bioremediation treatments on microbial communities detected by DGGE

The impact of the different bioremediation treatments on the bacterial community structure was assessed by PCR-DGGE based on 16S rRNA gene fragments. Figure 4 shows DGGE-patterns of the inoculum, gapwater, and both sea-ice samples at the beginning of the experiments and of all treatments including their controls (a, b, e) at the end of the experiments (gapwater after 6 six months of incubation at 0°C, bottom ice and top ice after one month's incubation on the ice floe and another six months of incubation at -3°C). The dominant bands of the different DGGE-patterns were excised and sequenced and their closest relatives in the GenBank database are compiled in Table 2.

Experiments with samples of the sea-ice matrix:

The bacterial communities of both sea-ice samples (Figure 4B+C) consisted mainly of members of *Bacteroidetes* (*Polaribacter* spp.) and *Gammaproteobacteria* (*Glaciecola* spp., *Psychrobacter* sp., and *Heliobacter* sp.) at the beginning of the experiments. After one month most of the communities still resembled the initial sea-ice communities. After seven months, the communities shifted slightly more in the bottom ice experiments, mainly with changes on the genus and species level. The number of bacteria affiliated with the *Bacteroidetes* phylum decreased in the nutrient amended experiments b, d, e, and f, with exception of the top ice experiment d. The control, with inorganic nutrients alone, caused a dramatic decrease in bacterial diversity in both sea-ice samples. *Alphaproteobacteria*, affiliating with ~ 94 % similarity to 16S rRNA genes of the genus *Octadecabacter* (Figure and Table 2, band A6) only developed in experiment b (nutrient addition alone) of the bottom as well as top ice after the first month, but disappeared during later incubation. Most bacterial strains of the inoculum could be detected in the sea-ice bioaugmentation experiments after one month of incubation on the ice floe, although some bands disappeared or decreased in intensity in the bottom ice experiment f (only bacteria affiliating with the genera of *Shewanella* (band G3d) and

Pseudomonas (band G1a) could be detected by DGGE). After an additional six month's incubation at -3°C there was an apparent shift in both sea-ice samples with oil and nutrients plus inoculum (f) predominantly towards bacteria of the genera *Pseudoalteromonas* and *Shewanella*, as well as *Colwellia* and *Marinobacter* in the bottom ice.

DGGE of the gapwater experiments (Figure 4A) showed that the initial community was composed mainly of members of the *Bacteroidetes* and of *Gammaproteobacteria*. After six months of incubation, bacteria of the *Bacteroidetes* still constituted a considerable part of the community, whereas the Gammaproteobacterium had disappeared. Instead, *Gammaproteobacteria* of the genus *Shewanella* spp. appeared in sample d, e, and f. *Alphaproteobacteria,* affiliating with the genera of *Rhodobacteraceae* and *Roseobacter*, appeared to be a dominant group in the communities of all treatments, except the ones with oil and nutrients. In the control without treatment (a) as well as in the sample treated with oil only (c), new bands appeared in the lower region of the gel, representing bacteria with high GC-content. The inoculum of oil-degrading bacteria consisted mainly of *Gammaproteobacteria* as well as of some members of the *Bacteroidetes* phylum and an Actinobacterium (see also preparation of inoculum). After six months in sample e and f, which were treated with nutrients plus inoculum most of the inoculated bacteria disappeared even though the *Gammaproteobacteria* appeared to remain as a dominant group next to *Alphaproteobacteria* and *Bacteroidetes*. Bacteria affiliating with 99.3 % 16S rRNA-similarity to the genus *Rhodococcus* occurring in the inoculum were also still present in samples e and f after an incubation of six months.

Effect of oil on sea-ice bacteria and changes of community structure in the gap-water experiments by FISH-analysis

The experiments with top and bottom sea-ice samples were only used for probe EUB338I-III in order to observe the impact of the oil and bioremediation treatment on the rRNA content of

the cells. In the environmental samples from the beginning of the experiments, cells accounted for 87.5 ± 5 % and 93.5 ± 14 % in top – and in bottom sea-ice with probe EUB338I-III (see Table 3). After seven months of incubation at -3°C these fractions declined remarkably (Table 4) in both samples d, treated with oil and nutrients, to 18 ± 2% (bottom ice) and 42± 4% (top ice) in both samples c, treated with oil only the cells were of small size and hybridization signals could hardly be detected (1 ± 4%). In contrast, in samples e and f (with nutrients plus inoculum) the percentage of detectable cells was as high as in the initial samples for the other treatments.

Gapwater experiments: FISH analysis was applied to the initial gapwater sample as well as to all samples after the bioremediation experiments to determine the natural community structure and subsequent changes due to the different bioremediation treatments with oil and nutrients. Microscopic fields with remaining oil particles were not taken into account due to a strong autofluorescence. Counts with domain-, group-, and genera specific Cy3-labeled probes relative to DAPI-stained cells are presented in Table 2. All samples examined except sample c, showed cells of relatively large size (4 μm) and with bright hybridization signals. The cells in sample c, treated with oil only, were quite small and the hybridization signal less bright. Yields for the domain-specific probes EUB338I-III ranged from 55% in the sample c (oil only) to 95% in the sample e treated with nutrients and inoculum. The community composition was further analysed with probes specific for phylogenetic-groups within the domain *Bacteria,* which are typical for sea-ice communities (8, 11, 12). With our set of group-specific probes we could assign nearly all of the cells detected with probes EUB338I-III. Counts with CF319a were as high as 69% in the gapwater at the beginning of the experiments. The shape of most cells detected with this probe were very long and filamentous, often four to five cells in a line. After six months of incubation at 0°C the abundance of CF319a-positive cells decreased in all samples to values of 54% in sample b, treated with nutrients only, down to 18% in sample a with no additional treatment.

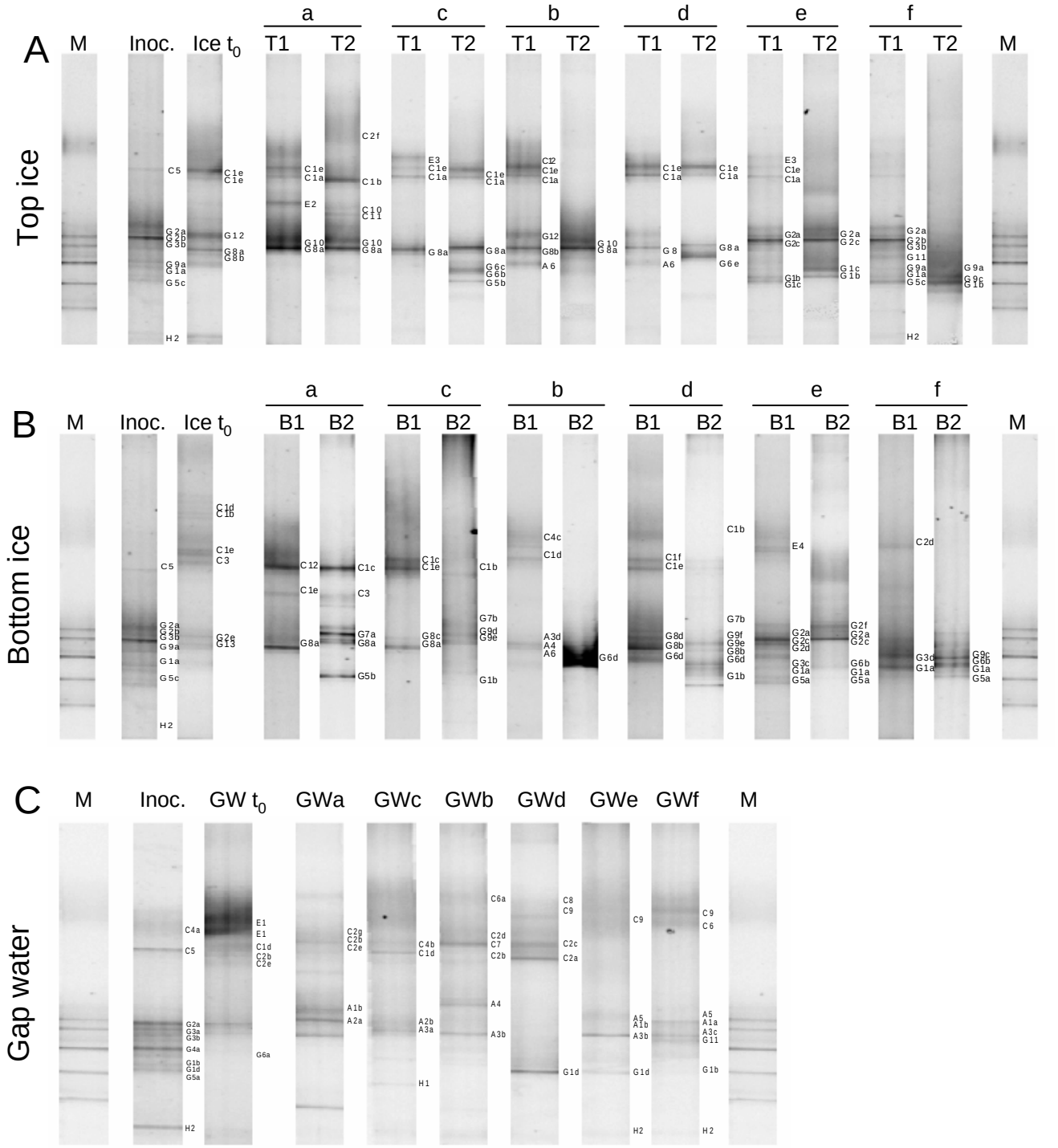

Figure 4. DGGE profiles of 16S rRNA gene fragments of oil bioremediation experiments. Experiments with top ice (A) and with bottom ice (B) are showing on the left side the inoculum as well as the ice community at the beginning of the experiments (t_0) and the treatments a-f after one month of incubation in the field (indicated with 1 on top of the lanes) and after another six months at -3°C (indicated with 2 on top of the lanes). Gapwater experiments (C) at the beginning (t_0) and after six months of incubation for the treatments a-f, indicated at the top of each lane. Marker (M), containing from top to bottom of the gel: *Cytophaga* sp. strain ARK 668, *Psychrobacter frigidicola* (ATCC 700361), *Sulfitobacter brevis* (DSMZ 11443), *Herbaspirillum* sp. (DSMZ 12597), *Roseovarius tolerans* (DSMZ 11457), *Clavibacter michiganensis* (DSMZ 20134).

Table 2. Closest GenBank relative of excised DGGE bands.

Band name	Closest relative in GenBank database	Accession. no.
Eukarya:		
E1	Uncul. phototrophic eukaryote	AY672825
E2	*Micromonas* sp. RCC299	AY702149
E3	*Isochrysidales* sp. SIC.42345	AF277481
E4	Uncul. diatom clone R8C-B8	AY678496
Bacteroidetes phylum:		
C1a	*Polaribacter franzmannii* strain ANT9260	AY167319
C1b	*Polaribacter irigensii* strain ANT9210	AY167317
C1c	*Polaribacter* sp. ANT9167	AY167324
C1d	Uncul. *Polaribacter* SIC.B9029	AF277564
C1e	Uncul. *Polaribacter* Arctic97A-15	AF354620
C1f	Uncul. *Polaribacter* sp. clone SBI04_16	DQ186943
C2a	*Bacteroides* bacterium ANT9285	AY167327
C2b	Uncul. *Bacteroides* bacterium clone PI_4z8g	AY580713
C2c	Uncul. *Bacteroides* bac. clone 131677	AY922211
C2d	Uncul. *Bacteroides* bac. clone CD204F10	DQ200466
C2e	Uncul. *Bacteroidetes* clone PLY-P2-60	AY354740
C2f	Uncul. *Bacteroides* bacterium clone 131854	AY922250
C2g	Uncul. *Bacteroidetes* bacterium isolate DGGE band GWS-c10-PA	DQ080958
C3	Uncul. *Lawinella* ARCTIC 161	AF277544
C4a	Arctic sea-ice bacterium AWS-4M6	AF283866
C4b	Arctic sea ice bacterium ARK10267	AF468430
C4c	Arctic sea ice bacterium ARK10217	AF468427
C5	*Psychroflexus torquis* ANT9232b	AY167318
C6a	*Flavobacteriaceae* bacterium 14III/A01/012	AY576695
C6b	*Flavobacterium gelidilacus* strain LMG 21619	AJ507151
C7	Uncul. *Owenweeksia* sp. Clone F1C07	AY697869
C8	*Tenacibaculum* sp. MPG-74/AN6	AF530150
C9	*Psychroserpens* sp. A622	AY781191
C10	Uncul. Antarctic sea ice bac. cl. ANT/4_14-37	AY165580
C11	*Bruminimicrobium glaciale*	AF521195
C12	Marine psychrochile ICO76	U85882
Alphaproteobacteria:		
A1a	*Rhodobacteraceae* bacterium D4	AY841771
A1b	Uncul. *Rhodobacteraceae* clone F3C01a	AY794150
A2a	*Roseobacter* sp. ANT9115	AY167254
A2b	*Roseobacter* sp. ANT9274	AY167261
A3a	Uncul. alpha proteobacterium cl. JL-ECS D113	AY663927
A3b	Uncul. alpha bacterium clone 131720	AY922224
A3c	Uncul. bacterium clone D101	AY375134
A3d	Uncul. Antarctica sea ice bac. cl. ANT/4_14-13	AY165563
A4	Alpha proteobacterium MGP-7	AF53013
A5	Arctic seawater bacterium R7967	AJ293823
A6	*Octadecabacter* sp. ANT9190	AY167335
A7	Arctic sea ice associated bacterium ARK10207	AF468373

Band name	Closest relative in GenBank database	Accession. no.
Gammaproteobacteria:		
G1a	*Shewanella frigidimarina* ACAM 588	U85905
G1b	*Shewanella* sp. SW2.4	AY536552
G1c	*Shewanella livingstonis*	AJ300834
G1d	*Shewanella* sp. 10-Ea	AY770007
G1e	*Shewanella* sp. BSi20586	DQ007443
G2a	*Psychrobacter arcticus* 273-4	CP000082
G2b	*Psychrobacter fozii*	AY771717
G2c	*Psychrobacter okhotskensis*	AB094794
G2d	*Psychrobacter luti,* strain LMG 21276	AJ430828
G2e	*Psychrobacter* sp. Nj-16	AJ842217
G2f	*Psychrobacter* sp. 63	AY656801
G3a	*Pseudomonas fluorescens* strain PC38	DQ178235
G3b	*Pseudomonas* sp. A1SH	DQ011930
G3c	*Pseudomonas* sp. BWDY-5	DQ200856
G3d	*Pseudomonas* sp. Isolate R-20805	AM114534
G4a	Uncul. bacterium DGGE band 2.2T	AY831479
G4b	Uncul. bacterium clone ARKDMS-52	AF468251
G5a	*Marinobacter* sp. 1-Ca	AY770011
G5b	*Marinobacter* sp. ANT8277	AY167267
G5c	*Marinobacter* sp. NK-1gene	AB026946
G6a	*Colwellia rossensis* strain ANT9279	AY167311
G6b	*Colwellia aestuarii*	DQ055844
G6c	*Colwellia* sp. WED7.6	AY536569
G6d	*Colwellia* sp. ANT9187	AY167295
G6e	*Colwellia* sp. ANT8258	AY167266
G7a	*Marinomonas arctica* strain 29-1	*DQ103888*
G7b	*Marinomonas* sp. BJK17	*AJ717295*
G8a	*Glaciecola* sp. ANT9081	*AY167334*
G8b	*Glaciecola* sp. ANT9166	AY167288
G8c	*Glaciecola* sp. ANT9154	AY167287
G8d	*Glaciecola* sp. ANT9055	AY167274
G9a	*Pseudoalteromonas* sp. ARCTIC-P22 1	*AY573037*
G9b	*Pseudoalteromonas* sp. Prot2	*AF513411*
G9c	*Pseudoalteromonas* sp. EBD	*DQ218321*
G9d	*Pseudoalteromonas* sp. H12	*AY277259*
G9e	*Pseudoalteromonas* sp. WED2.1	*AY536554*
G9f	*Pseudoalteromonas* sp. 3025	*AM110980*
G10	*Uncul. Antarctic sea ice bacterium*	*AY165570*
G11	*Oleispira antarctica*	*AJ426420*
G12	*Psychromonas* sp. ANT9265	AY167326
G13	*Heliobacter pylori* 26695	AE000511
H1	*Agreia* sp. 37-4	AF513393
H2	*Rhodococcus luteus*	AJ576249

Abbreviations

Uncul.: Uncultured, cl.: clone, bac.: bacterium

Diversity studies of sea-ice communities performed by Brinkmeyer (11) and Bowman (8) showed that Gammaproteobacteria are the most abundant group. Most bacteria of the inoculum also belonged to the Gammaproteobacteria with Shewanella, Pseudoalteromonas, and Marinobacter being the dominating genera. To follow up the development of these types,

genera-specific or species-specific probes were applied for these three members of the *Gammaproteobacteria*. At the beginning of the experiments only 5% of DAPI stained cells could be detected in the gapwater with probe GAM42a, while after six months of incubation the detection yields increased in most samples to values ranging from 62% in the sample f treated with oil, nutrients, plus inoculum to 13% in the sample treated with oil alone. Yields of the three species-specific probes (Table 4) indicated that in most experiments (a - e) a large proportion of *Gammaproteobacteria* belonged to the *Shewanella frigidimarina* (SF825) group, *Marinobacter*-ICO22 (MB-ICO22) group and *Pseudoalteromonas-Colwellia* (PSA184) group. However, in sample f which showed the highest oil-biodegradation, obviously only a small percentage of these types remained. Only lower numbers of Actinobacteria could be detected with probe HGC69a in the inoculum and after seven months in samples e and f which were inoculated. The relative abundance of *Betaproteobacteria* detected with probe BET42a was usually low (0 - 7%). However in sample a and in the inoculum, BET42a-positive cells accounted for 21% and 14%, of all cells respectively, although they were not detected by sequencing of DGGE bands.

Table 3. Abundances (± standard deviation) of bacterial cells detected with probe EUB338I-III in oil bioremediation experiments of sea-ice experiments at the beginning and after seven months of incubation FISH. Numbers have been corrected by subtracting counts detected with probe NON338. Mean and standard deviation (SD) were calculated from counts of 10 to 20 randomly chosen fields.

Abundance (%) of total cells (mean + SD) detected with probe:		
	Top-ice	Bottom-ice
Sea-ice and brine(to)	87.5 ± 5	93.5 ± 14
after seven months at -3°C		
a) ice only	84 ± 15	75 ± 10
b) ice and nutrients	92 ± 16	119 ± 13
c) ice and oil	1 ± 4	2 ± 4
d) ice, oil and nutrients	42 ± 4	18 ± 2
e) ice, nutrients and inoculum	110 ± 15	87 ± 12
f) ice, oil, nutrients, and inoculum	101 ± 30	111 ± 35

Table 4. Abundances (± standard deviation) of bacterial phylotypes in gapwater oil bioremediation experiments at the beginning and after six months of incubation at 0°C observed by FISH. Numbers have been corrected by subtracting counts detected with probe NON338. Mean and standard deviation (SD) were calculated from counts of 10 to 20 randomly chosen fields.

Gapwater experiemnts	Abundance (%) of total cells (mean + SD) detected with probe:								
Treatment	EUB I-III	CF319a	HGC69a	ALF968	BET42a	GAM42a	SF825	PS184	MB-ICO22
Start point (to)	88 ± 5	69 ± 9	0 ± 0	22 ± 11	2 ± 1	5 ± 3	1 ± 1	15 ± 4	0 ± 0
Inoculum (to)	78 ± 4	11 ± 4	1 ± 1	15 ± 4	14 ± 4	38 ± 3	11 ± 3	10 ± 4	11 ± 4
a) gapwater only	90 ± 6	18 ± 5	0 ± 0	28 ± 8	21 ± 5	43 ± 8	1 ± 1	4 ± 2	0 ± 0
b) nutrients only	74 ± 11	54 ± 8	0 ± 0	14 ± 2	7 ± 4	2 ± 1	2 ± 2	2 ± 1	0 ± 0
c) oil only	55 ± 9	34 ± 5	0 ± 0	1 ± 1	1 ± 1	13 ± 5	0 ± 0	8 ± 4	0 ± 0
d) oil plus nutrients	68 ± 7	21 ± 5	0 ± 0	8 ± 6	0 ± 0	40 ± 3	17 ± 5	5 ± 1	2 ± 1
e) nutrients plus inoculum	95 ± 5	38 ± 11	3 ± 1	17 ± 4	4 ± 2	19 ± 3	4 ± 2	6 ± 2	3 ± 1
f)oil, nutrients, inoculum	89 ± 7	22 ± 3	2 ± 2	20 ± 4	0 ± 0	62 ± 7	5 ± 2	1 ± 1	3 ± 2

Phylogenetic analysis of bacterial isolates from gapwater experiments

From the gapwater experiments d, e, and f, 77 representatives of the cultivatable part of the communities were isolated. Their taxonomic relationship was determined on the basis of the almost full length 16S rRNA gene. Nineteen different ARDRA-pattern groups were found covering 14 different genera. In Table 5 representative isolates of these phylogenetic groups are listed with their closest GenBank relative. Further, the number and origin of the isolates belonging to the specific ADRA-groups are given. The diversity of the isolates from experiment d was reduced compared to the inoculated experiments e and f. About 90% of the isolates from experiment d belonged to only two phylotypes of the genera *Shewanella* and *Marinobacter*. The *Marinobacter* phylotype differs slightly from the two other *Marinobacter* spp. found in sample f in the target region of probe MB-ICO22. Thus the FISH results probably underestimated the abundance of the *Marinobacter p*hylotype in sample d. To asses, which bacteria in sample f originated from the inoculum, a phylogenetic tree was reconstructed (data not shown) with almost full length 16S rRNA gene sequences of the 19 gapwater isolates of experiments e and f, their closest GenBank relative, and 56 bacterial isolates from the inoculum. The analysis revealed that four of eleven sequences (Gap-f-45,

Gap-f-51 and Gap-f-52) from sample f affiliated closer to bacterial strains of the inoculum than to any other sequence of the GenBank database.

Table 5. Closest relative in GenBank database to bacterial isolates obtained from gapwater experiments d (oil and inorganinic nutrients) and f (oil, inorganic nutrients, and inoculum)

Isolat	Origin	GenBank Relative	Accession #	%-similarity	Length	Genus	Group
Iso#1	GW-d	*Shewanella frigidimarina* clone	AY771750	99.72	1431	*Shewanella*	Gammaproteobacteria
Iso#2	GW-d	Arctic sea ice bacterium ARK10244	AF468401	99.58	1434	*Martnobacter*	Gammaproteobacteria
Iso#10	GW-d	Arctic sea ice bacterium ARK10062	AF468438	99.79	1415	*Planttbacter*	Actinobacteria
Iso#13	GW-d	*Shewanella frigidimarina* clone	AY771750	99.86	1438	*Shewanella*	Gammaproteobacteria
Iso#15	GW-d	Arctic sea ice bacterium ARK10244	AF468401	99.72	1427	*Martnobacter*	Gammaproteobacteria
Iso#19	GW-d	*Shewanella frigidimarina* clone	AY771750	99.79	1439	*Shewanella*	Gammaproteobacteria
Iso#25	GW-d	Arctic sea ice bacterium ARK10063	AF468410	99.79	1420	*Psychroflexus*	Bacteroidetes
Iso#29	GW-d	*Octadecabacter* sp. ANT9202	AY167337	100.00	1371	*Octadecabacter*	Alphaproteobacteria
Iso#30	GW-d	*Shewanella frigidimarina* clone	AY771750	99.72	1437	*Shewanella*	Gammaproteobacteria
Iso#39	GW-f	Arctic seawater bacterium R7366	AJ293826	99.86	1433	*Pseudomonas*	Gammaproteobacteria
Iso#41	GW-f	Uncultured Bacteroidetes bacterium	AY922252	99.18	1345	*Winogradskyella*	Bacteroidetes
Iso#44	GW-f	*Shewanella* sp. JL-56	AY745827	99.65	1440	*Shewanella*	Gammaproteobacteria
Iso#45	GW-f	*Rhodococcus luteus*	AJ576249	99.79	1401	*Rhodococcus*	Actinobacteria
Iso#48	GW-f	Uncultured Cytophagales	AJ535257	98.32	1426	*Cytophaga*	Bacteroidetes
Iso#51	GW-f	*Pibocella ponti* isolate S3-17	AY771726	98.53	1429	*Pibocella*	Bacteroidetes
Iso#52	GW-f	*Martnobacter* sp. 1-Ca	AY770011	99.43	699	*Martnobacter*	Gammaproteobacteria
Iso#53	GW-f	*Shewanella frigidimarina* isolate	AY771736	99.86	1437	*Shewanella*	Gammaproteobacteria
Iso#54	GW-f	Arctic sea ice bacterium R7967	AJ293823	99.26	1346	*Roseobacter*	Alphaproteobacteria
Iso#57	GW-f	Arctic sea ice bacterium R7078	AJ293824	99.09	1432	*Pseudomonas*	Gammaproteobacteria
Iso#58	GW-f	Arctic sea ice bacterium ARK10062	AF468438	99.93	1422	*Planttbacter*	Actinobacteria

Discussion

Oil biodegradation is reduced significantly at low temperatures (28). This is not only due to common deceleration of bacterial activity and growth, but also due to a decreased bioavailability affected by an increase in oil viscosity under cold conditions. Furthermore, at low temperatures reduced solubility of short chain alkanes (≤10) in the aqueous phase may enhance oil toxicity (5, 28).

Influence of crude oil and bioremediation treatments on changes in bacterial community structures

DGGE analyses with the solid sea-ice experiments revealed that changes in bacterial communities due to the different bioremediation treatments, were relatively small for most treatments compared to the controls and the initial sea-ice samples, with the exception of the

controls with nutrients only. Other studies reported less diverse communities as a result of oil contamination compared to the pristine samples (19, 42, 43, 52). The slow succession in the bacterial communities, indicated by DGGE, led to the assumption that the response of the bacteria to the different treatments is decelerated in the solid ice system.

In the sea-ice experiments f inoculated bacteria of the genus *Pseudoalteromonas*, *Shewanella* and *Colwellia* could still be detected by DGGE after seven months of incubation. Immobilized on saw dust they resisted competition with the indigenous sea-ice biota, but did not utilize oil hydrocarbons, although some bacteria of these genera are well-known hydrocarbon-degraders (18, 23, 34). Apparently they may have preferred other organic compounds or were unable to make the crude oil bioavailable. We assume that the sea-ice habitat, the small cavities which form the network of brine channels, may not provide optimal conditions (such as the availability of oxygen) for aerobic hydrocarbon degradation or that the bacteria were not able to move within the ice matrix and thus to the contaminant at the ice surface.

In contrast, DGGE as well as FISH analyses of the gapwater experiments revealed that the indigenous community changed during the six months incubation at 0°C in almost all treatments. A high number (more than 20%) of *Betaproteobacteria* were detected with probe BET42a in experiment a, without additional treatment. *Betaproteobacteria* are predominantly represented in freshwater and soil communities and the percentage of marine bacterioplankton clone libraries is typically less than 10% (13). However, they have also been reported to be predominant in summer melt pools on Arctic pack ice (10) and more than 30% of clones from an Antarctic pristine marine site affiliated with *Betaproteobacteria* (53). *Betaproteobacteria* were not detected by DGGE, probably due to problems with DNA isolation. DNA extractions from various *Betaproteobacteria*, which were isolated from Arctic and Antarctic sea-ice, were difficult and required additional treatments with lysozyme and lysostaphin (unpublished data).

Our DGGE data showed that most of the bacteria introduced in the gapwater experiments were no longer detectable after six months of incubation. Just a few bands could be retraced to our inoculum, consistent with Atlas (6) and Lee (29), who found that allochthonous microorganisms do not necessarily stay active and alive in contaminated ecosystems. Margesin and Schinner (32) suggested that the failure of their bioaugmentation study was due to the fact that the introduced microorganisms could not compete with the indigenous microorganisms and that they may have been replaced. However, the remaining organisms obviously successfully enhanced the decontamination process.

Effectiveness of bioremediation

The two different bioremediation treatments applied in this study, fertilization with inorganic nutrients alone as well as fertilization plus inoculation with a consortium of cold-adapted oil degrading bacteria, resulted in two different outcomes. As indicated by the oil fingerprints in both experiments with solid sea-ice (top and bottom ice), fertilization with inorganic nutrients as well as nutrients plus inoculation had no stimulating effect on the degradation of petroleum hydrocarbons in comparison to the sterile control (data not shown). However, mineralization experiments with [^{14C}C]-hexadecane presented conspicuous stimulation through the addition of inorganic nutrients as well as through the addition of nutrients plus inoculum. Both bioremediation treatments (fertilized and inoculated) resulted in high respiration rates with more than 50% of the total ^{14}C content being respired. Presumably the positive effect of bioremediation is easier to detect because hexadecane is only a single compound added in a low concentration, whereas oil, which was added in higher amounts, contains various compounds besides hexadecane and degradation only becomes visible at a later stage. Furthermore, some compounds of the oil may have been toxic to the sea-ice organisms as indicated by the low hybridization signals with probe EUB338I-III in the sea-ice experiments c and d. Coinciding with the hexadecane experiments, both bioremediation treatments induced

a significant stimulation of petroleum hydrocarbon degradation in the liquid gapwater experiments. Fertilization with inorganic nutrients alone already led to an increased degradation of low molecular weight n-alkanes up to C_{24}. Bioaugmentation (inorganic nutrients plus inoculum) even enhanced this effect to a complete degradation of n-alkanes up to the range of C_{28} and the isoprenoids pristane and phytane were attacked as well.

Biodegradation of oil has been studied extensively, at least under aerobic conditions, and the preferential degradation of linear before branched alkanes, and smaller before larger aromatic compounds is well established (40, 45). The advanced degradation in our bioaugmentation experiment suggests that some of the introduced strains survived competition with the indigenous flora. Phylogenetic analyses by DGGE corroborated our findings, only two bands of the seeded samples e and f apparently originated from the inoculum, which can be affiliated to the genus of *Oleispira* and *Rhodococcus*. A degradation potential of a broad range of alkanes by bacteria of these genera is well known (39, 44, 51, 54). Unlike to most other marine environments (3), studies showed that there is a strong overlap in the fraction of cultivatable bacteria compared to the PCR-detected fraction (8, 11) in sea ice. Congruently, isolation and phylogenetic analysis of bacterial strains also indicated that only a part of the bacteria, cultivatable on oil-agar, originated from the inoculum. These were the bacteria affiliated with the genera *Rhodococcus*, *Marinobacter*, *Pseudomonas* and *Pibocella*. Bacteria of the genera *Marinobacter* and *Pseudomonas* are also known for their ability to degrade petroleum hydrocarbons (17, 30, 49, 55). Furthermore, oil-biodegradation tests of the bacterial isolates from the inoculum revealed that all four strains were able to degrade n-alkanes up to the range of C_{14} and the *Rhodococcus* and *Pseudomonas* strains even to C_{24} and C_{28}, respectively, at low temperatures of 0°C (data not shown) in liquid mineral medium. This indicates that these bacteria, which apparently asserted themselves against competition with the indigenous biota, mutually stimulated the degradation of n-alkanes in experiment f. Furthermore, representatives of the *Shewanella* and *Marinobacter* phylotypes (ARDRA-

group I and IV), occurring frequently in experiment d, were neither isolated from experiment e nor f, indicating that the non-inoculated experiments developed differently from the inoculated ones. The experiments with the sea-ice matrix were treated with the same (quality and quantity) inorganic nutrients and inoculated with the same consortium of oil-degrading bacteria as the gapwater experiments. However, both incubation temperatures as well as the structure of the contaminated sample in one case liquid (gapwater) in the other case solid, differed considerably. A temperature below freezing, as applied in the sea-ice matrix experiments, may explain the contradictory results, consistent with Hoff (25) who found that environmental parameters such as temperature have an equivalent or perhaps larger role than nutrients in determining the rate of degradation at contaminated sites. However, indigenous sea-ice microbial communities are well adapted to the ambient cold temperatures. Most sea-ice bacteria can grow down to –5°C (8, 21, 24). Junge et al. (26) even showed bacterial activity at -20°C in Arctic winter sea-ice. Mineral oil degradation at temperatures below 0°C has been reported previously by ZoBell (56). Oil degradation tests with the isolates from the inoculum showed degradation of aliphatic hydrocarbons at -3°C in liquid medium (Gerdes et al., unpublished data). In contrast to the gapwater experiments, the sea-ice experiments were incubated on an ice floe, but were covered with snow to avoid melting due to irradiation, in the first month. The exposure of the oil to the atmosphere caused weathering of the oil with low molecular hydrocarbons up to C_{12} being evaporated. The weathered oil is less bioavailable and thus might have contributed to the failure of oil bioremediation in the sea-ice experiments. On the other hand, when an accidental oil spill occurs under natural sea-ice conditions, the adjacent snow and sea-ice will melt rapidly due to the dark colour of the oil. Increased absorbance of irradiation by the oil will lead to higher temperatures at the contaminated sites attributed to decreased surface albedo and thus leading to the formation of melt pools on the ice surface. Degradation of petroleum hydrocarbons in the melt-pools

stimulated by bioremediation and bioaugmentation will probably result in similar degradation rates as the ones obtained with the gapwater experiments.

Conclusions

Nutrient amended bioremediation successfully stimulated degradation of petroleum hydrocarbons in gap-layer water at 0°C and bioaugmentation enhanced this degradation process. Therefore, nutrient amended bioaugmentation should be taken into account as an appropriate tool in the design of bioremediation processes in polar sea-ice environments. As the introduction of nonindigenous species is not accepted at present in Antarctica, indigenous oil-degrading strains are needed to apply the bioaugmentation technique in the field. In our experiments, long-chain alkanes as well as PAH compounds resisted degradation; therefore an optimized inoculum has to be supplemented with degraders of these compounds or should be deployed in the following spring season when n-alkanes are already degraded. To remediate an oil spill on a large scale, bioremediation methods could be accompanied by the application of cold-active solubilizing agents (51) or be applied after mechanical treatments. In consideration of the application of different response methods and the reduced metabolism as well as the unfavourable characteristics of oil at freezing temperatures, cleaning processes in sea-ice will certainly take more than one season.

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Manuscript 3

DEGRADATION OF PETROLEUM HYDROCARBONS BY COLD-ADAPTED BACTERIA FROM ARCTIC AND ANTARCTIC SEA-ICE AT LOW TEMPERATURES

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Keywords: low temperature, hydrocarbons, biodegradation, sea-ice, bioremediation, bioaugmentation

Manuscript in preparation

Abstract

Bacterial isolates, obtained from long-term bioremediation experiments with crude oil contaminated samples of Arctic and Antarctic sea-ice as well as with Antarctic gapwater, were tested for their taxonomic affiliation as well as for their physiological potentials, but especially for their capability to degrade petroleum hydrocarbons. ARDRA screening of 425 pure cultures revealed 63 phylotypes distributed among the *Gammaproteobacteria, Actinobacteria, Alphaproteobacteria,* and the *Bacteroidetes* phylum. The majority of the phylotypes were closely related to bacteria commonly found in sea-ice which, however, represent only low abundant groups. Isolates of the genera *Rhodococcus* and *Dietzia* within the *Actinobacteria* as well as isolates of *Oleispira* and of a *Pseudomonas* affiliated group within the *Gammaproteobacteria* were shown to be potential crude oil alkane degraders at temperatures as low as -3°C and 0°C. Growth tests on various single hydrocarbons as sole carbon source revealed that several *Gammaproteobacteria* of the genera *Shewanella*, *Pseudomonas*, *Psychrobacter,* and *Marinomonas* grew on short chain alkanes as well as on 2-ring and 3-ring aromatic compounds. *Alphaproteobacteria* isolates, affiliated to the genera *Jannaschia* and *Loktanella,* were also able to use different hydrocarbons while strains belonging to the *Bacteroidetes* phylum were unable to mineralize any of these compounds. Most of the isolates grew at temperatures above 20°C. Mainly *Marinobacter* spp. and *Shewanella* spp. isolates, deriving from Antarctic sea-ice, responded psychrophilic. The results showed that after oil contamination in sea-ice a very specific bacterial community developed, which is able to degrade petroleum hydrocarbons at low temperatures.

Introduction

The Arctic Ocean is thought to hold at least a quarter of the world's undiscovered oil and gas reservoirs and there is a growing interest in the exploitation of these hydrocarbon fields. Development and production of offshore projects in these ice-covered areas is ongoing (Snow White, Northstar, Liberty, Prirazlomnoye) and since 2002 there has been a dramatic increase in oil shipment in the Arctic Ocean as well as ship traffic related to research and tourism in the Southern Ocean. Consequently, the threat of petroleum hydrocarbon pollution of the marine environment and the danger of an accidental major oil spill in the polar, ice-covered regions is increasing. Former accidental oil spills, such as the one of the 'Exxon Valdez' in Alaska and the 'Bahio Paraiso' in Antarctica, highlighted the need to gain more information on counter measures particularly on the hydrocarbon degradation potential of cold-adapted microorganisms at low temperatures. In high latitude regions, bacterial growth, activity and thus degradation rates are commonly reduced. Consequently, contaminants may remain in the ecosystems for long periods. Bioremediation is a potential tool for oil spill response, even in cold climates (Margesin & Schinner, 1997; Margesin & Schinner, 2001; Whyte et al., 1998). However, previous studies showed that only in the summer months bioremediation can be applied in sea-ice. The short period of a summer season does thus not allow a long acclimatization time for the cold-adapted hydrocarbon-degrading sea-ice bacteria to develop. Therefore, bioaugmentation, seeding with adapted exogenous microbes, should be taken into consideration as an additional potential bioremediation tool. Biodegradation of petroleum hydrocarbons has been reported for numerous bacterial strains from various environments. However, the degradation capacity of oil-hydrocarbons by cold-adapted bacteria is poorly known (Delille et al., 1997; Deppe et al., 2005), especially at sub-zero temperatures. Bioaugmentation can only be successfully applied in the marine polar environment if we have a substantial knowledge about the physiology and ecology of indigenous bacteria from Arctic and Antarctic sea-ice before and after oil contamination as well as of the bacteria provided for inoculation. During the last 10 years our knowledge about

sea-ice microbial communities in general has widened considerably(Deming, 2002; Helmke & Weyland, 1995; Junge et al., 2002; Staley & Gosink, 1999; Staley et al., 2002). We learned a lot about temperature adaptation (Helmke & Weyland, 2004), about taxonomic composition (Bowman et al., 1997; Brinkmeyer et al., 2003; Brown & Bowman, 2001), and about the activity status of the different sea-ice communities(Grossmann & Dieckmann, 1994; Helmke & Weyland, 1995; Huston et al., 2000; Junge et al., 2004). Unlike other environments (Amann et al., 1995), in samples of sea-ice high viable to total count ratios were found (Helmke & Weyland, 1995; Junge et al., 2002) as well as a strong agreement of molecular analyses and cultural methods (Brinkmeyer et al., 2003). These facts and the necessity to cultivate isolates for investigations on physiology and degradation capacity, which can subsequently be applied for bioaugmentation induced us to focus on bacterial strains obtained from bioremediation experiments with crude oil and different samples of Arctic and Antarctic sea-ice and gapwater. The strains were characterized for their ability to degrade crude oil alkanes at 15°C, 0°C and -3°C and whether they can grow on different hydrocarbons as sole carbon source. To gain an initial understanding of their functional role in the ecosystem, additional temperature and salinity tolerances were determined and substrate utilization examined.

Materials and Methods

Samples and bioremediation experiments

Samples of sea-ice were collected by drilling ice cores (9 cm in diameter) in multi-year Arctic pack ice during the R.V. Polarstern cruises ARKXVIII/2 (September-October 2002) and ARKXIX/1b (March-April 2003) in an area northeast of Svalbard (79° - 81°N, 5°-12°E) and the cruise ANTXVIII/5b (April-Mai 2001) in the Bellinghausen Sea (66 - 71°S, 275 - 290°W). Ice cores were immediately cut into 20 cm segments, transferred into sterile plastic bags and stored at -6°C until set up of bioremediation experiments in the home laboratory. Microcosm bioremediation experiments were set up with different samples of sea-ice (about 5 l) in 10 l

sterile Arctic seawater supplemented with artificially weathered and non-weathered crude oil from the Barents Sea, and different fertilizers (Inipol; fish meal; inorganic nutrients KNO_3 0.75 gl^{-1}, NH_4Cl 0.75 gl^{-1}, traces of $FePO_4$). The experiments were incubated at -3°C to provide temperature conditions at which the system of frozen sea-ice and liquid seawater was in equilibrium. Experiments with melted samples of sea-ice were also incubated at 4°C.

Isolation of hydrocarbon degrading strains

Bacterial strains were isolated from the different crude oil bioremediation experiments with different samples of Arctic and Antarctic sea-ice, incubated at -3°C, 0°C or 4°C for several months. Hundred µl of a serial dilution (10^0 - 10^6) was plated on minimal agar (KNO_3 0. 75 gl^{-1}, NH_4Cl 0.75 gl^{-1}, yeast extract 0.1 mgl^{-1}, and traces of $FePO_4$) supplemented with either 20µl crude oil from the Barents Sea (weathered and non-weathered), eicosane (5 mg in hexane), tetracosane (5 mg in hexane), phenanthrene (5 mg in hexane) or anthracene (5 mg in toluene) evenly distributed on the agar surface. The plates were incubated at 20°C and 4°C until colonies became visible. Single colonies were picked with an inoculating needle and restreaked twice on minimal agar plates, with crude oil as small droplets on the agar surface, to obtain pure cultures.

PCR amplification for 16S rRNA gene analysis

DNA was extracted from all isolates by 3 min boiling in a water bath followed by two freeze-thaw cycles. The extracted DNA was then purified using 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'-AGAGTTTGATCCTGGCTCAG-3') and the universal primer 1492R (5'-GGTTACCTTGTTACGACTT-3') were used. PCR was performed as described by Gerdes et al. (Gerdes et al., 2005).

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) (Massol-Deya et al., 1999) was used to characterize the 16S rRNA gene diversity of 425 isolates within the collection of 634 strains obtained from the microcosm experiments. After simultaneous digestion (3 h, 37°C) with the restriction enzymes *RSAI* and *HAEIII* (5U of each) (Promega), according to the manufacturer's instructions, digestion products were separated on a 3.5 % Metaphor gel, and the resulting restriction pattern was photographed under UV light. The ARDRA pattern was clustered with the Bionumerics Gelcompare software (Applied Maths, Sint-Martens-Latern, Belgium) using the Ward and Pearson correlation method.

A total of 68 bacterial isolates of different ARDRA pattern groups were selected for sequencing. Almost full-length sequences were compared to those deposited in the GenBank database by using the BLAST algorithm (Altschul et al., 1990). Sequence data were analyzed with the ARB software package (http://www.mikro.biologie.tu-muenchen.de). A phylogenetic tree was reconstructed by using the maximum likelihood algorithm.

Oil analyses by GC-FID

To investigate the range of alkane utilization by a selection of 25 different isolates (Ice-oil isolates listed in Table 2) at three different temperatures of 15°C, 0°C, and -3°C, degradation tests were conducted with non-weathered crude oil from the Southern Barents Sea, which consists of mainly linear and branched alkanes. Erlenmeyer flasks with 50 ml medium (KNO_3 0. 75 gl^{-1}, NH_4Cl 0.75 gl^{-1}, peptone 0.5 gl^{-1}, yeast extract 0.1 mgl^{-1}, and traces of $FePO_4$) were autoclaved and afterwards supplemented with 100µl Barents Sea crude oil (0.2% vol/vol) and inoculated in parallel for each temperature with 2 ml (OD_{600} of 0.2) of a pre-culture of each isolate, grown at 4 °C on crude oil. Formalin killed controls (2% final concentration) were incubated under the same conditions at each temperature to assess abiotic losses of hydrocarbons during incubation and extraction. The flasks were sealed with screw caps and incubated, on a

rotary shaker at 150 rpm, for 90 days at 15°C, for 95 days at 0°C, and for 102 days at -3°C. Immediately after incubation the degradation process was stopped by adding 2 ml formalin and an internal standard of 2.5 mg fluoranthene was added to each sample.

Extraction: Samples were transferred into solvent rinsed separatory funnels and Erlenmeyer flasks were rinsed three times with 10 ml hexane/dichloromethane (9:1 v/v). Hexane/dichloromethane was added to the sample and the suspension was extracted on a rotary shaker at 20°C overnight. After separation of the organic phase from the suspension, a spatula of sodium sulphate was added to remove residual moisture, then all extracts were sonicated (50/60 Hz, 150W) for 10 min.

Cleanup: Hexane/dichloromethane extracts were cleaned on silica columns (1ml, Bond Elut). Columns were conditioned two times with 10 ml dichloromethane and two times with 10 ml hexane (UniSolv, Merck), then 500 µl of the extract were transferred onto the column. To elute hydrocarbons from the column, 500 µl hexane/dichloromethane (4:1, vol/vol) was used.

The samples were analyzed by GC-FID using a HP 5890 Series II (Hewlett Packard) chromatograph equipped with a FID (Hewlett Packard) and a capillary column (30 m x 0.25 mm x 0.25 µm; SPB-1, Supelco) under the following conditions: initial temperature 40°C for 2 min, rate 5°Cmin^{-1}, final temperature 300°C, run time 10 min, equilibration time 3 min; detector: 300°C; injector: 280°C; carrier gas He 0,6 ml/min.

Growth test on single petroleum aliphatic and aromatic compounds

Thirty-eight isolates were tested for their capability to degrade a variety of hydrocarbons including PAH on the basis of growth. As an indicator for growth, resazurin (0.3% final concentration) was added to the culture medium. Resazurin undergoes reduction in growing cultures changing from blue to pink to colourless. The colour change is reliable and can be examined visually.

The utilization tests were performed in microtiter plates (250 µl per well). Most of the hydrocarbons were applied in a final concentration of 0.1% except for phytane, which was used

in a final concentration of 0.05%, tetradecane was added in a final concentration of 2%, and hexadecane in a final concentration of 4%. The mineral medium employed consisted of $(NH_4)_2HPO_4$ 1 gl^{-1}, KCL 0.1 gl^{-1}, $MgSO_4x7\ H_2O$ 0.1 gl^{-1}, $CaCl_2$ 0.05 gl^{-1}, NaCl 27.5 gl^{-1} and Nitrogen-Base without amino acids (Difco 291940) 67 mgl^{-1} in distilled water. The medium was sterile filtered and adjusted to a pH of 7.5. The mineral medium was then inoculated with a suspension of bacteria cells that were grown on agar plates (ZoBell agar 2214E) for about one week at 2°C. The cells were harvested from the surface of the agar and washed twice with mineral medium before application. Two hundred and fifty µl of the inoculated mineral medium was dispensed in each well containing the hydrocarbon. Each test was performed in triplicate and incubation was performed at 15°C, 0°C and 4°C. Colour changes were visually scored at different time intervals.

Physiological and biochemical characteristics

In order to assess physiological profiles with different organic substrates, which the isolates are able to use as sole carbon source, Biolog microtiter plates (Biolog, California, USA) SF-N2 were applied. The microtiter plates contain 95 different substrates (Table 5) and were prepared following the manufacturers instructions but conditions were adapted to marine bacteria, using a minimal medium with $(NH_4)_2HPO_4$ 1g l^{-1}, $MgSO_4$ x $7H_2O$ 0.2 g l^{-1}, KCl 0.2 g l^{-1}, NaCl 25 g l^{-1}, yeast nitrogen-base without amino acids (Difco 291940) 0.67 mg l^{-1}. Incubation was performed at 15°C, 0°C and -3°C. Bacterial growth was assayed by measuring the optical density at 590 nm with a Multiscan plus MKII photometer (Flow Laboratories, Meckenheim, Germany) for microtiter plates as well as by visual examination.

Temperature and salinity characteristics

To assess the minimum, maximum, and optimal growth temperature, the isolates were incubated in microtiter plates with modified marine broth (peptone 5 g l^{-1}, yeast extract 1 g l^{-1}, $FePO_4$ 10 mg l^{-1}, 75% seawater) as well as with modified marine broth plus hexadecane (final

concentration 2%) at 37°C, 30°C, 25°C, 20°C, 15°C, 10°C, 5°C, 1°C, and -3°C. The ability of organisms to grow at the different temperatures was examined daily over a period of 11 days by measuring the optical density at 590 nm using a Multiscan plus MKII photometer (Flow Laboratories, Meckenheim, Germany).

The salinity tolerance was determined for salinity concentrations of: 130, 100, 70, 35, 20, 10, and 0 g l^{-1} . The dependence on specific seawater ions of some strains was taken into account by applying a four salt mixture consistent of: NaCl 24g l^{-1}, $MgCl_2$ x $6H_2O$ 10.6g l^{-1}, $CaCl_2$ 1.5g l^{-1}, KCl 0.7g l^{-1} for the 3.5psu level. The other salinities were prepared by diluting or concentrating the mixture. Tests were run in microtiter plates with modified marine broth (peptone 5 g l^{-1}, yeast extract 1 g l^{-1}, $FePO_4$ 10 mg l^{-1}). The plates were incubated at 25°C, 15°C, 0°C and -3°C, and bacterial growth was determined daily for a period of 10 days and also after an additional week of incubation by measuring the optical density at 590 nm using a Multiscan plus MKII photometer (Flow Laboratories, Meckenheim, Germany).

Environmental scanning electron microscopy (ESEM)

Principles and operation of ESEM have been described elsewhere (Little et al., 1991). The wet mode of the ESEM (Electroscan Corp., Wilmington, MA) enables direct observations of uncoated wet material samples.

Samples of bacterial isolates grown in liquid medium (KNO_3 0. 75 gl^{-1}, NH_4Cl 0.75 gl^{-1}, yeast extract 0.1 mgl^{-1}, and traces of $FePO_4$) with crude oil or aromatic substances (naphthalene, acenaphthene, phenanthrene, flouranthene, and, pyrene) were placed on glass slides and the medium was removed by placing a dry tissue at the edge of the droplet. The cells and oil or aromatic compounds were then washed twice with distilled water. One droplet of the rinsed samples was placed directly into a counterbore type (0.1 cm x 0.1 cm) specimen mount of a Peltier cooling device in the ESEM chamber. Samples were imaged at 20 keV at a temperature of 2°C and a chamber vapour pressur of 3.6 torr.

Nucleotide sequence GenBank accession numbers

Almost full-length 16S rRNA sequences of isolates from this study were deposited in the GenBank under the accession numbers DQ530454 to DQ530482, DQ521378 to DQ521398, and DQ533957 to DQ533972.

Results

Phylogenetic diversity of isolates

A total of 634 bacterial strains were isolated from crude oil contaminated sea-ice as well as from bioremediation experiments with Arctic and Antarctic sea-ice and gapwater from an Antarctic sea-ice floe. Of this collection 425 strains were grouped by means of ARDRA. From each group at least one or more representative strains were selected and their 16S rRNA genes sequenced. The different genera with the number of obtained phylotypes and isolates are listed in Table 1. Of these, 38 isolates were selected and characterized for their growth abilities at different temperature and salinity ranges. The temperature and salinity growth characteristics together with their sources and accession numbers are listed in Table 2. Their phylogenetic relation to other sea-ice bacteria, type strains, closest relatives, as well as known hydrocarbon degraders is shown in Figure 1. With a few exceptions representatives of these phylotypes commonly occur in Arctic and Antarctic sea-ice, however, in uncontaminated ice in much lower abundances (Brinkmeyer et al., 2003). On the other hand, phylogenetic groups which are frequently found in sea-ice such as *Glaciecola*, *Roseobacter,* and *Polaribacter* in samples of Arctic sea-ice and *Colwellia* and *Octadecabacter* in Antarctic sea-ice (Brinkmeyer et al., 2003), were not or only in very low numbers among these isolates. The 63 phylotypes distributed among the *Gammaproteobacteria, Alphaproteobacteria*, *Actinobacteria* and the *Bacteroidetes* phylum. The highest diversity of phylotypes was found within the *Gammaproteobacteria*. *Actinobacteria* are rare in common sea-ice but accounted for the second highest fraction of the isolates with members of *Arthrobacter*, *Dietzia*, *Rhodococcus* and strains affiliated to *Leifsonia*. The

Bacteroidetes phylum was represented by just a few strains associated with the genera *Arenibacter*, *Cytophaga*, *Flavobacterium*, *Maribacter*, *Psychroflexus*, and *Winogradskyella*. Only four phylotypes distributed among the *Alphaproteobacteria*. The phylotype represented by the isolate gap-f-54 was abundant in different bioremediation experiments with Antarctic gapwater and it was <97 % identical to *Jannaschia rubra*.

Table 1. Bacterial groups and genera of the obtained isolates. Percent coverage of all ARDRA screened isolates, number of isolates and phylotypes.

Group/genera	# of phylotypes	# of isolates	% of 425 ARDRA screened isolates
Gammaproteobacteria	**41**	**304**	**71.5**
Halomonas	5	25	5.9
Marinobacter	5	34	8.0
Marinomonas	1	3	0.7
Oleispira	2	6	1.4
Pseudoalteromonas	4	26	6.1
Pseudomonas	6	22	5.2
Psychrobacter	3	21	4.9
Psychromonas	1	1	0.2
Shewanella	14	166	39.1
Actinobacteria	**10**	**85**	**20.0**
Arthrobacter	2	5	1.2
Dietzia	4	39	9.2
[*Leifsonia*]	2	15	3.5
Rhodococcus	2	26	6.1
Alphaproteobacteria	**4**	**21**	**4.9**
Loktanella	2	3	0.7
[Jannaschia]	1	14	3.3
Octadecabacter	1	4	0.9
***Bacteroidetes* phylum**	**8**	**15**	**3.5**
Arenibacter	1	1	0.2
[*Cytophaga*]	1	1	0.2
[*Flavobacterium*]	1	4	0.9
Maribacter	2	2	0.4
Psychroflexus	1	1	0.2
Winogradskyella	2	6	1.4
	63	**425**	**100**

Table 2. Description of bacterial isolates. isolate origin, and growth characteristics in response of temperature [°C] and salinity [psu].

							growth characteristics			
		original sample		experiment		isolation on	temperature [°C]			salinity
Isolate	Accession #	type	region/season	nutrients		medium plus	-3°C/min.	optimum	max.	[psu]at
Dietzia sp. Ice-oil-79	DQ521378	sea-ice	Arctic winter	Inipol	4°C	crude oil	0	15	≥37	ng
*Dietzia*sp. Ice-oil-101	DQ521380	sea-ice	Arctic winter	Inipol	4°C	crude oil	0	25 - 37	≥37	ng
Dietzia sp. Ice-oil-124	DQ521381	sea-ice	Arctic winter	Inipol	4°C	crude oil	++	20 -37	≥37	0 - 10
Rhodococcus sp. Ice-oil-227	DQ521384	sea-ice	Arctic summer	fish meal	-3°C	anthracene	+	20 - 30	30	0 - 7
Rhodococcus sp. Ice-oil-488	DQ521396	sea-ice	Arctic summer	fish meal	-3°C	eicosane	+	10 - 20	30	0 - 3.5
Rhodococcus. sp. gap-f-45	DQ530468	gapwater	mixed	inorganic	0°C	crude oil	0	20	25	0 - 3.5
Rhodococcus sp.gap-e-79	DQ530478	gapwater	mixed	inorganic.	0°C	crude oil	+	15 - 20	30	ng
[*Leifsonia*] sp. Ice-oil-482	DQ521394	sea-ice	mixed	fish meal	-3°C	anthracene	+	10 - 20	25	0 - 7
[*Leifsonia*] sp.gap-d-10	DQ530457	gapwater	Antarctic summer	inorganic	0°C	crude oil	0	10 - 15	20	1 - 7
[*Leifsonia*] sp.gap-f-58	DQ530475	gapwater	Antarctic summer	inorganic	0°C	crude oil	0	20	25	ng
Arthrobacter sp. Ice-oil-251	DQ521385	sea-ice	Arctic summer	fish meal	-3°C	phenanthrene	++	20 - 25	≥37	ng
Shewanella sp. Ice-oil-318	DQ533961	sea-ice	Arctic summer	fish meal	-3°C	crude oil	+++	15 - 20	25	1 - 7
Shewanella sp. Ice-oil-428	DQ521391	sea-ice	mixed	mixed	-3°C	crude oil	+++	15 - 20	30	0 - 10
Shewanella sp. Ice-oil-198b	DQ521382	sea-ice	mixed	mixed	-3°C	crude oil	+++	10	25	1 – 3.5
Shewanella sp. gap-f-53	DQ530472	gapwater	Antarctic summer	inorganic	0°C	crude oil	+++	15 - 20	25	0 - 10
Shewanella sp. gap-d-19	DQ530460	gapwater	Antarctic summer	inorganic	0°C	crude oil	+++	15	20	1 - 10
Shewanella sp. gap-f-44	DQ530467	gapwater	Antarctic summer	inorganic	0°C	crude oil	+++	5-10	25	n.d
Pseudoalt. sp. Ice-oil-374	DQ521389	sea-ice	Arctic summer	fish meal	-3°C	crude oil	++	15 - 30	30	1 - 13
Pseudoalt. sp. Ice-oil-303	DQ521386	sea-ice	Arctic summer	Inipol	-3°C	tetracosane	++	15 - 37	≥37	1 - 10
Pseudoalt. sp. Ice-oil-412	DQ533962	sea-ice	Arctic winter	Inipol	-3°C	crude oil	+++	15 - 20	30	1 - 13
Marinobacter sp. Ice-oil-81	DQ521379	sea-ice	Arctic winter	Inipol	4°C	crude oil	5	37	≥37	ng
Marinobacter sp. Ice-oil-325	DQ521387	sea-ice	Arctic summer	fish meal	-3°C	crude oil	++	5 - 15	20	1 - 3.5
Marinobacter sp.gap-d-2	DQ530455	gapwater	Antarctic summer	inorganic	0°C	crude oil	++	10	15	1 - 7
Marinobacter sp.gap-d-15	DQ530459	gapwater	Antarctic summer	inorganic	0°C	crude oil	+	5-10	10	n.d.
Marinobacter sp.gap-f-52	n.d.	gapwater	Antarctic summer	inorganic.	0°C	crude oil	++	5 - 15	20	n.d.
Marinobacter sp.gap-e-81	DQ530479	gapwater	Antarctic summer	inorganic	0°C	crude oil	+	20 - 25	≥37	n.d.
Oleispira sp. Ice-oil-381	DQ521390	sea-ice	Arctic winter	fish meal	4°C	crude oil	ng	ng	ng	ng
Oleispira sp.gap-d-97	DQ530482	gapwater	Antarctic summer	inorganic.	0°C	crude oil	ng	ng	ng	ng
Marinomonas sp. Ice-oil-472	DQ521393	sea-ice	mixed	oil only	-3°C	anthracene	+++	5 - 10	30	1 - 7
Psychrobacter sp. Ice-oil-	DQ521392	sea-ice	Arctic summer	Inipol	-3°C	anthracene	+++	15 - 25	30	0 - 13
Psychromonas sp.gap-e-82	DQ530480	gap-water	Antarctic summer	inorganic	0°C	crude oil	+++	20	25	1 - 10
Pseudomonas sp. Ice-oil-327	DQ521388	sea-ice	Arctic summer	fish meal	-3°C	crude oil	+	10 - 20	30	0 - 10
Pseudomonas sp. Ice-oil-499	DQ521397	sea-ice	mixed	fish meal	-3°C	eicosane	+	10 - 15	20	0 - 7
Pseudomonas sp.gap -f-39	DQ530464	gapwater	Antarctic summer	inorganic	0°C	crude oil	++	10 - 15	20	0 - 10
Pseudomonas sp.gap-f-57	DQ530474	gapwater	Antarctic summer	inorganic	0°C	crude oil	+	15	25	1 - 7
Halomonas sp. Ice-oil-232	n.d.	sea-ice	Arctic summer	Inipol	-3°C	anthracene	++	20 - 37	≥37	1 - 10
Halomonas sp. Ice-oil-302	DQ533958	sea-ice	Arctic summer	Inipol	-3°C	tetracosane	+++	20	37	1-13
Loktanella sp. Ice-oil-484	DQ521395	sea ice	Arctic summer	fish meal	-3°C	eicosane	+	15	25	1-7
[*Jannaschia*] sp.gap-f-54	DQ530473	gapwater	Antarctic summer	inorganic	0°C	crude oil	0	15 - 20	30	3.5
Maribacter sp. Ice-oil-200	DQ521383	sea-ice	mixed	mixed	-3°C	crude oil	+++	15 - 20	25	1 - 7
Maribacter sp.gap-f-51	DQ530471	gapwater	Antarctic summer	inorganic	0°C	crude oil	+	20	30	1-7
Flavobacterium sp. 522	DQ521398	sea-ice	Arctic summer	Inipol	-3°C	tetracosane	+++	5 - 20	30	0 -7
Arenibacter sp.gap-e-67	DQ530476	gapwater	Antarctic summer	inorganic	0°C	crude oil	1	10	25	1-7
Psychroflexus sp.gap-d-25	DQ530461	gapwater	Antarctic summer	inorganic	0°C	crude oil	+	5	10	1-3,5
	DQ530465	gapwater	Antarctic summer	inorganic	0°C	crude oil	+++	15-25	≥37	1-10

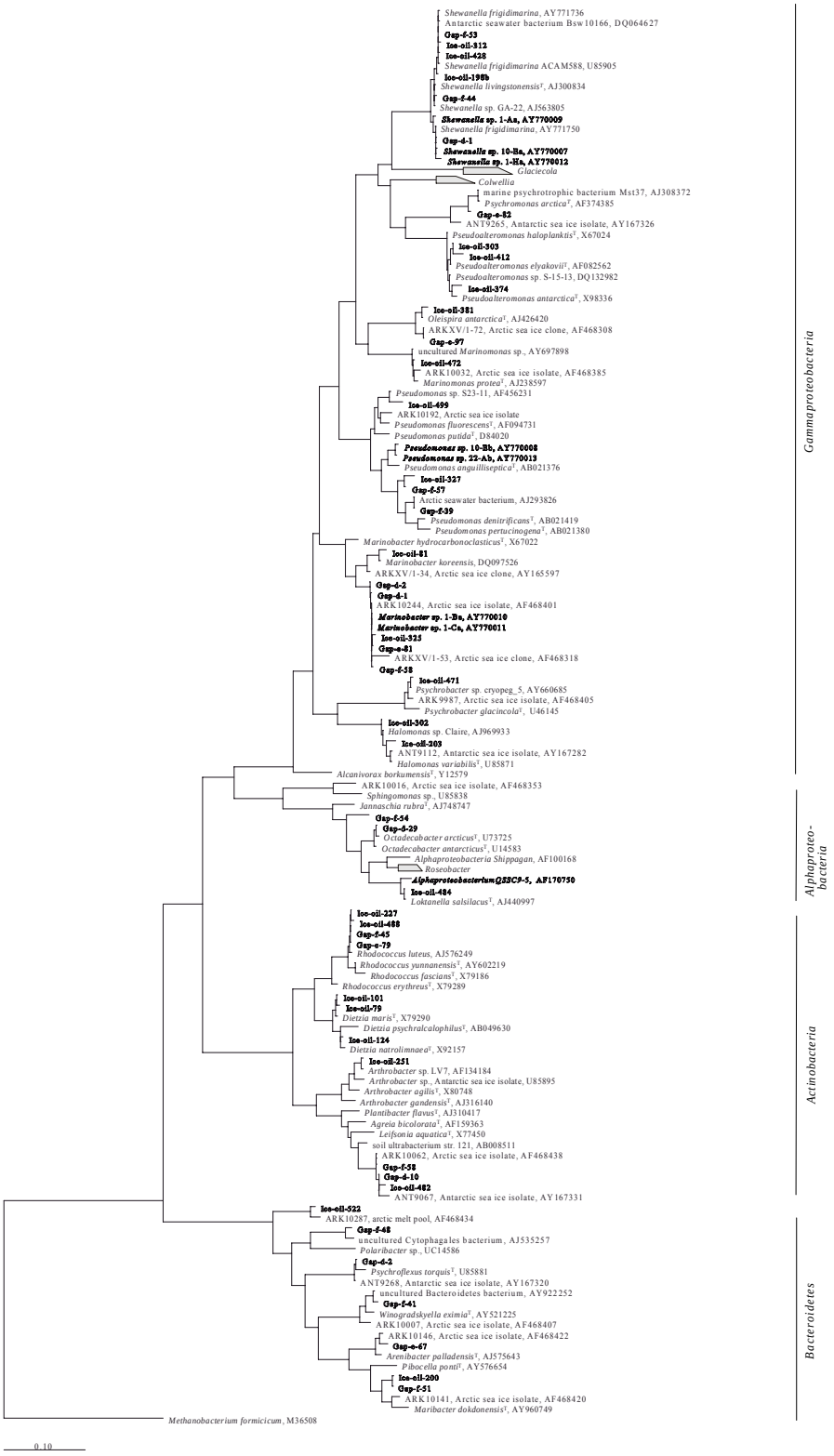

Figure 1. Phylogenetic tree, based on 16S rRNA genes, reconstructed by using the maximum likelihood algorithm, showing the relation of the isolates obtained from crude oil bioremediation experiments to their closest GenBank relatives as well as to other sea-ice bacteria and hydrocarbonoclastic bacteria.

Degradation of crude oil alkanes

A collection of 25 strains was chosen from the isolate collection for testing their hydrocarbon degradative capabilities (Ice-oil isolates listed in Table 2). The criteria for the selection was their ability to grow well on crude oil from the Southern Barents Sea, with a high amount of n-alkanes, or of single hydrocarbon compounds (tetradecane, eicosane, phenanthrene, anthracene) as well as to cover a broad phylogenetic diversity. In order not to run into oxygen limitation, the experiments were aerated so that short chain alkanes up to n-C_{13} evaporated. Previous studies with Antarctic sea-ice showed that these alkanes also disappeared through weathering under natural conditions. Therefore, the degradation of alkanes longer than n-C_{14} as well as the branched alkanes pristane and phytane was in the focus of our interest. The extent of biodegradation was graded into four categories compared to the control, incubated at the same temperatures: (s) slightly degraded (decreased peak area up to 15 %), (p) partly degraded (decreased peak area up to 50 %), (ac) almost completely degraded (decrease peak area up to 85 %), and (c) completely degraded (no peak). The range of oxidized n-alkanes at the different temperatures, as well as the degradation of the branched alkanes are given in Table 3. At 15°C six isolates were able to degrade the whole spectrum of n-alkanes, five of them degraded all n-alkanes completely, and isolate 101 also oxidized a wide range completely, some almost completely.

The six strains, affiliated with the genera *Dietzia*, *Rhodococcus*, *Pseudomonas*, and *Oleispira*. At the lower temperatures of 0°C and -3°C, n-alkanes were only mineralized by these six strains, although to a lower extent as at 15°C as expected. The degradation range of alkanes decreased more at the lower temperatures by isolate Ice-oil-101 as by isolate Ice-oil-79, indicating a lower adaptation to cold temperatures, coinciding with the temperature growth characteristic in nutrient medium (see Table 2). The branched alkanes pristane and phytane were degraded at 15°C by the six Ice-oil isolates: 79, 101, 227, 327, 381, and 488 to different

extents, but not, or only slightly at the lower temperatures. Furthermore, all six strains colonized the entire surface of the oil droplets during incubation at all three temperatures.

Table 3. Degradation of aliphatic compounds of Statfjord crude oil (0.2%) in liquid medium.

	15°C (90 days)		0°C (95 days)		- 3°C (102 days)	
Isolate	n-alkane	pristane, phytane	n-alkane	pristane, phytane	n-alkane	pristane, phytane
D. sp. 79	C_{14}-C_{34} (c)	pr ph (ac)	C_{14}-C_{19} (ac), C_{20}-C_{30} (p)	pr, ph (s)	C_{14}-C_{15} (s), C_{16}-C_{19} (p), C_{20}-C_{24} (s)	not degraded
D. sp. 101	C_{15} (ac), C_{16}-C_{18} (c), C_{19}-C_{24} (ac), C_{25}-C_{34} (p)	pr ph (s)	C_{14}-C_{18} (p)	not degraded	C_{15}-C_{17} (s)	not degraded
R. sp. 227	C_{14}-C_{34} (c)	pr (c), ph (ac)	C_{15}-C_{16} (p), C_{17}-C_{25} (ac)	pr, ph (s)	C_{15}-C_{19} (p), C_{20}-C_{30} (s)	not degraded
R. sp. 488	C_{14}-C_{34} (c)	not degraded	C_{14}-C_{23} (p), C_{24}-C_{25} (s)	not degraded	C_{14}-C_{19} (p) C_{20}-C_{26} (s)	not degraded
P. sp. 327	C_{14}-C_{34} (c)	pr ph (ac)	C_{14}-C_{24} (ac), C_{24}-C_{25} (p)	not degraded	C_{14}-C_{25} (p), C_{25}-C_{30} (s)	not degraded
O. sp. 381	C_{14}-C_{34} (c)	pr ph (p)	C_{14}-C_{24} (p), C_{24}-C_{30} (s)	not degraded	C_{14}-C_{24} (p), C_{24}-C_{30} (s)	not degraded

Categories of degradation: c completely, ac almost completely, p partly, s slightly

ESEM microscopic image

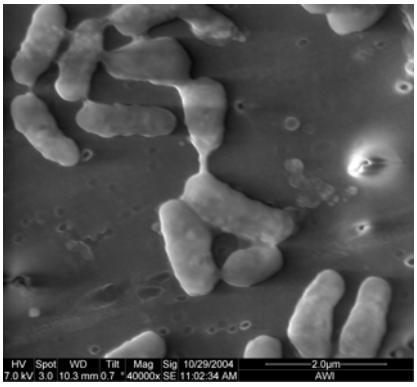

Figure 2. ESEM microscopic images showing extracellular substances producing cells of *Dietzia* sp. isolate Ice-oil-101 (DQ521380), colonizing on crude oil droplets during growth on hydrocarbons at 0°C.

Table 4. Growth of isolates on single hydrocarbon substrates as sole carbon source

Isolate	Incubation temperature [°C]	Peptone medium	Tetradecane	Hexadecane	Eicosane	Tetracosane	Triacontane	Tetratriacont.	Hexatriacont.	Phytane	Naphtalene	Acenaphthene	Phenantrene	Anthracene	Fluoranthene	Pyrene
Dietzia sp. Ice-oil-101	15	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	+	+	n.d.	n.d.	n.d.	n.d.
Dietzia sp. Ice-oil-124	15	+	+	+	(+)	(+)	(+)	-	-	-	-	-	-	(+)	-	-
Rhodococcus sp. Ice-oil-227	15	+	+	-	+	+	+	+	+	(+)	+	+	-	-	-	+
Rhodococcus sp. Ice-oil-488	15	+	+	-	+	+	+	+	-	-	+	+	(+)	-	-	+
Rhodococcus sp. Gap-f-45	4	+	+	-	+	+	+	+	-	-	+	+	+	-	+	-
Rhodococcus sp. Gap-e-79	15	+	+	-	+	+	+	+	+	-	(+)	(+)	+	+	-	+
[*Leifsonia*] sp. Gap-f-58	10	+	(+)	+	-	-	-	-	-	-	-	-	+	-	-	-
[*Leifsonia*]sp. Ice-oil-482	15	+	-	-	(+)	-	-	-	-	-	-	-	-	-	-	-
Arthrobacter sp. Ice-oil-251	15	+	(+)	-	-	-	-	-	-	-	-	-	-	-	-	-
Shewanella sp. Ice-oil-318	15	+	+	+	+	(+)	+	+	-	-	-	-	-	-	-	-
Shewanella sp. Ice-oil-428	15	+	+	+	-	-	-	-	-	-	s	s	-	-	-	-
Shewanella sp. Gap-d-19	0	+	+	+	+	-	+	-	-	-	n.d.	n.d.	+	-	-	-
Shewanella sp. Gap-f-53	15	+	+	+	-	-	s	-	-	-	-	-	-	-	-	-
Pseudoalteromonas sp. Ice-oil-303	15	+	+	+	s	s	s	s	-	-	-	-	+	s	s	-
Pseudoalteromonas sp. Ice-oil-374	15	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
Marinobacter sp. Ice-oil-81	15	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+	+
Marinobacter sp. Ice-oil-325	15	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
Marinobacter sp. Gap-d-2	0	+	+	+	s	s	+	s	-	-	-	-	+	-	-	-
Marinobacter sp. Gap-d-52	10	+	+	+	s	s	s	-	-	-	-	(+)	-	-	-	-
Oleispira sp. Ice-oil-381	15	+	+	-	-	-	-	-	-	-	+	+	-	+	+	-
Marinomonas sp. Ice-oil-472	15	+	+	-	s	s	s	-	-	-	+	+	-	-	-	-
Psychrobacter sp. Ice-oil-471	15	+	+	+	+	+	+	-	-	-	+	+	+	-	+	-
Psychromonas sp. Gap-d-82	4	+	s	+	-	-	-	-	-	-	+	+	-	-	-	-
Pseudomonas sp. Ice-oil-499	0	+	+	(+)	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp. Ice-oil-327	15	+	+	+	+	+	+	+	+	(+)	+	+	+	+	+	+
Pseudomonas sp. Gap-f-39	4	+	+	+	-	-	+	-	-	-	-	-	+	-	-	-
Pseudomonas sp. Gap-f-43	4	+	+	+	-	-	-	-	-	-	+	+	-	-	-	-
Pseudomonas sp. Gap-f-46	4	+	+	+	-	-	s	-	-	-	-	-	-	-	-	-
Pseudomonas sp. Gap-f-57	4	+	+	+	s	+	+	-	-	-	+	+	+	-	-	-
Pseudomonas sp. Gap-f-76	4	+	+	+	-	-	-	-	-	-	+	+	-	-	+	-
Loktanella sp. Ice-oil-484	4	+	+	+	-	-	-	-	-	-	(+)	s	-	-	-	-
[*Jannaschia*]sp. Gap-f-54	15	+	+	+	+	+	+	+	s	-	(+)	+	+	+	+	-
Maribacter sp. Ice-oil-200	15	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Flavobacterium sp. Ice-oil-522	15	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arenibacter sp. Gap-e-67	4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Psychroflexus sp. Gap-d-25	4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Winogradskyella sp. Gap-d-41	4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Growth on single hydrocarbon substrates as sole carbon source

To estimate the capability of the sea-ice isolates to use single crude oil hydrocarbon compounds, including various PAH, for bacterial growth the resazurin method as well as

microscopic analysis were applied to 37 isolates (Table 4). Growth tests revealed that the isolate-327, affiliating with the genus *Pseudomonas*, which was shown to potentially degrade alkanes in crude oil, was also able to grow on various 2- and 3-ring aromatic compounds as well as n-alkanes up to the range of C_{36} at 15°C. Oddly, this strain was not able to grow on phytane (0.05%) as single substrate, although phytane was degraded in crude oil at 15°C. Other *Pseudomonas* isolates, namely Gap-f-39 and Gap-f-57 grew on short chain alkanes up to C_{16} and the 2-ring aromatic compounds. The *Marinobacter* sp. isolate-81 also showed a broad growth spectrum on various short and long chain alkanes as well as on various PAH, whereas other isolates of this genus did not show hydrocarbonoclastic abilities. The different *Shewanella* spp. isolates , most frequently isolated from the bioremediation experiments (data not shown), showed no significant hydrocarbon degradation in crude oil (C_{14} to C_{34}), however, growth tests on single substrates revealed that they grew on short chain alkanes up to C_{16}, also on the higher concentrated hexadecane (4%). Strains of *Psychrobacter* and *Marinomonas* showed no degradation of crude oil n-alkanes in the range from C_{14} to $C_{34,}$ but were able to grow on short chain aliphatic and aromatic compounds. Members of the genus *Rhodococcus* were not only able to degrade n-alkanes of crude oil, but were also able to grow on 2-ring aromatic compounds and some strains grew even on 3- and 4-ring PAH. Surprisingly, the growth test with hexadecane as single hydrocarbon source revealed that none of the tested *Rhodococcus* strains was able to grow on hexadecane, when added in a high concentration of 4%. The two *Dietzia* isolates (Ice-oil-79 and Ice-oil-101) also showed a broad degradation range of n-alkanes, whereas another *Dietzia* strain (Ice-oil-124) grew only on short chain alkanes up to C_{16}. None of the isolates of the *Bacteroidetes* phylum was able to use any of the applied hydrocarbons. Among the *Alphaproteobacteria* representatives of two genera *Loktanella* and *Jannaschia* could utilize different alkanes and some 2-ring and 3-ring PAH. Several bacterial strains did not appear to have hydrocarbon degrading abilities when grown

in pure culture, despite the fact that they were isolated either on crude oil agar or on single hydrocarbons as sole carbon source.

Growth characteristics

Bacterial growth at a temperature range from -3°C to 37°C showed that 33 of 45 tested isolates were psychro-tolerant and 9 strains were psychrophilic, according to the definition of Morita et al. (Morita, 1975). Only one strain, namely the *Marinobacter* sp. isolate Ice-oil-81 showed mesophilic growth characteristics. The two *Oleispira* strains did not grow in the used medium. All isolates, which affiliated with the genus *Dietzia* (Ice-oil-79, 101, and 124), also showed an adaptation to higher temperatures, compared to the other strains.

Sea-ice bacterial communities live in the brine channels within the sea-ice column. The salinity within these brine channels is dependent on the temperature and can vary from 0 to more than 150 psu. The formation of melt pools on the ice surface is typical for the Arctic summer season, with nearly freshwater conditions. Therefore, the isolates were tested for their growth at a salinity range from 0 to 13 psu at the three different temperatures of 25°C, 15°C and 0°C (Table 2).The gapwater from an Antarctic sea-ice has higher salinities. Of the tested isolates 20 strains originated from gapwater which had a salinity of 26 psu. Most isolates showed a broad halo-tolerance at all three temperatures. Several strains were able to grow at 0 psu salinity indicating a terrestrial origin. Isolates of the genera *Pseudoalteromonas* tolerated salinities as high as 13 psu at 0°C but were, as members of a typical marine group, not able to grow under freshwater conditions.

Substrate utilization patterns

The Biolog system was used to obtain further information on the capacity of the different strains to utilize carbon sources other than hydrocarbons. Table 5 shows the utilization pattern of most of the tested sea-ice isolates. The four strains: *Rhodococcus* sp. Ice-oil-227, *Pseudoalteromonas* sp. Ice-oil-303, *Pseudoalteromonas* sp. Ice-oil-374, *Halomonas* sp. Ice-oil-232 are not included in the table as these strains could grow on most of the offered carbon

sources (92%, 87%, 90%, 80% respectively). *Rhodococcus* sp. Ice-oil-227, an excellent hydrocarbon degrader, is among these four strains. The two other potential petroleum hydrocarbon degrading strains *Marinobacter* sp. 81 and *Pseudomonas* sp. Ice-oil-327 also exhibited a very complex degradation pattern. While *Marinobacte*r sp. 81 preferred carbon acids and amino acids, *Pseudomonas* sp. Ice-oil-327 utilized a variety of carbohydrates. The oil degrading strains *Oleispira* sp. Ice-oil-381 and *Oleispira* sp. Gap-d-97 did not grow in the synthetic medium applied. A very narrow utilization spectrum was obtained by two *Marinobacter* strains, one *Shewanella* and one *Maribacter* strain. All four strains did not belong to the group of potential hydrocarbon degraders. They utilized only tween40 (Polyoxyethylene sorbitan monopalmitate) a well-known emulsifier that was also used by all other strains.

Discussion

To get an idea about the community compositions of sea-ice bacteria after an oil contamination, a representative collection of bacteria was isolated from different oil contaminated and bioremediated experiments with Arctic and Antarctic sea-ice as well as with gapwater from Antarctic ice floes. This collection was studied with respect to their phylogeny as well as to their ability to degrade aliphatic crude oil hydrocarbons at low temperatures, in order to gain knowledge about the function of the diverse members of the communities from oil contaminated and bioremediated sites. Further, the results are expected to give evidence concerning a selection of potential strains for bioaugmentation in sea-ice, which appears to be a promising tool for contaminated sea-ice as recent pilot studies indicated (Gerdes et al., in preparation).

22 different genera were isolated from different oil contaminated experiments. Most of the obtained isolates were closely related to bacteria previously found in sea-ice (see Figure 1). Nevertheless, under the influence of crude oil hydrocarbons, the compositions of the

Table 5. Substrate utilization profiles of some isolates at 15°C, 0°C, and -3°C.

substrates	D. sp. 79	D. sp. 124	D. sp.155	R. sp. 488	L. sp. 482	A. sp. 251	S. sp. 318	S. sp. 428	S. sp.198b	M. sp. 325	M sp. 412	M. sp. 81	P. sp. 471	Ps. sp.327	Ma. sp 200
carbon sources utilized (%)	6	22	9	22	40	13	1	20	17	1	1	29	23	22	1
alpha-cyclodextrin		+						+	+						
de+trin		+						+	+			+			
glycogen						+						+			
tween 40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
tween 80		+	+	+				+	+			+	+	+	
D-arabitol				+										+	
cellobiose		+			+			+	+						
D-fructose	+		+	+	+							+			
L-fructose					+										
D-galactose		+		+	+			+							
gentiobiose		+			+	+		+							
alpha-D-glucose		+			+	+		+				+		+	
maltose		+			+			+				+			
D-mannitol		+		+	+							+		+	
D-mannose				+											
D-melibiose					+										
beta-methyl-D-glucoside			+												
D-psicose			+	+										+	
D-raffinose					+										
D-sorbitol				+										+	
sucrose		+		+	+	+		+	+					+	
D-trehalose				+	+									+	
turanose					+										
+ylitol				+										+	
methylpyruvate		+						+	+						
mono-methylsuccinate	+			+										+	
acetic acid	+		+	+	+								+	+	
cis-acinitic acid												+			
citric acid												+	+		
D-galactonic acid lactone					+								+		
D-galacturonic acid					+							+	+		
D-gluconic acid					+							+	+		
D-glucosaminic acid					+								+		
D-glucuronic acid													+		
alpha-hydro+ybutyric acid					+										
beta-hydro+ybutyric acid	+	+	+	+	+			+	+			+	+	+	
gamma-hydro+ybutyric acid					+										
itaconic acid														+	
alpha-ketoglutaric acid		+		+		+						+	+	+	
alpha-ketovaleric acid															
D,L-lactic acid					+	+						+			
propionic acid			+	+	+							+	+	+	
quinic acid									+						
sebacic acid				+								+		+	
succinic acid				+										+	
bromo succinic acid		+		+								+		+	
D-alanine													+		
L-alanine		+			+	+			+			+	+		
L-alanyl-glycine		+			+				+			+			
L-asparagine					+							+	+		
L-aspartic acid					+										
L-glutamic acid	+		+	+	+							+	+	+	
glycil-L-aspartic acid												+			
glycil-L-glutamic acid		+						+	+						
L-histidine													+		
hydro+y L-proline					+								+		
L-leucine		+						+	+			+			
L-ornithine												+			
L-proline		+			+	+		+	+			+	+		
L-pyroglutamic acid						+						+			
L-serine								+					+		
L-threonine					+										
gamma-amino butyric acid													+		
urocanic acid					+								+		
inosine					+			+	+						
uridine					+										
thymidine					+										
putrescine		+			+	+		+	+						
2-aminoethanol				+										+	
2,3-butanediol					+										
glycerol						+		+				+			

communities changed considerably compared to those found in uncontaminated sea-ice (Bowman et al., 1997; Brinkmeyer et al., 2003; Brown & Bowman, 2001). Similar to uncontaminated sea–ice, isolates of the *Gammaproteobacteria* accounted for the highest fraction, but, in contrast, frequently obtained phylotypes, such as members of the genera *Shewanella*, *Halomonas*, and *Pseudomonas* only occur in low abundances in common sea–ice, whereas members of the genera *Glaciecola* and *Colwellia,* which account for a high proportion in sea-ice, were not isolated. Further, *Actinobacteria* made up the second highest number of isolates and phylotypes in contaminated sea-ice samples, whereas their occurrence in common sea-ice is almost near the detection level, with the exception of Arctic melt pools (Brinkmeyer et al., 2004). Isolates of the *Alphaproteobacteria* and of the *Bacteroidetes* phylum account each for about 20 % to 30 % of the common ice communities of the Polar Regions (Brinkmeyer et al., 2003), but decreased to minor groups in our isolate collection from oil contaminated ice. These data as well as the fact that most of the isolates were closely related to well-known hydrocarbon degraders indicated that the indigenous sea-ice bacterial communities develop towards an active oil degrading community after an oil contamination and bioremediation treatment.

Our experiments with crude oil at low temperatures confirmed that sea-ice isolates of the genera *Dietzia*, *Rhodococcus*, *Oleispira,* and *Pseudomonas* are able to degrade crude oil alkanes at 0°C as well as at –3°C, although longer chain alkanes are less readily degraded than shorter ones, coinciding with earlier findings (van Beilen et al., 1994; Whyte et al., 1998). Only n-alkanes up to n-C_{23} - n-C_{25} were significantly degraded in the lower temperature range. This indicates that n-alkanes $\geq C_{26}$ are a barrier for biodegradation due to the severely restricted bioavailability of these substrates at temperatures at or below freezing. It is well-known that the bioavailability of hydrocarbons decreases with increasing chain length due to reduced solubility in the aqueous phase (Page et al., 2000; Whyte et al., 1999a; Whyte et al., 1998; Yaws et al., 1993). The solubility decreases further at lower temperatures and thus

leads to lower bioavailability (Maier, 2000) of hydrocarbons with increasing chain length. Microorganisms obviously develop strategies to take up substrates that are poorly bioavailable (Leahy & Colwell, 1990; Maier, 2000; Thomas et al., 1986). One uptake mechanism is increased cell affinity for hydrophobic substances, allowing micro-organisms to directly absorb the substrate which has a low water solubility. Another strategy is the production and release of surface active biosurfactants. The isolates of the genera *Dietzia*, *Rhodococcus*, *Oleispira*, and *Pseudomonas*, which degraded crude oil alkanes at temperatures at and below 0°C, grew on the oil surface and covered entire droplets with a biofilm. This incidence indicated their ability to form cell wall hydrophobic substances even at temperatures as low as -3°C (see Figure 2), which apparently enabled them to efficiently take up insoluble crude oil hydrocarbons. In addition, many of the sea-ice bacteria can produce extracellular polymeric substances (EPS). When growing under cold temperature conditions, these may also interact with hydrophobic substrates. However, it is still an open question how cold- adapted hydrocarbon degrading bacteria modulate their membran fluidity in response to the counteracting influences of low temperature and hydrocarbon toxicity (Heipieper et al., 1992; Whyte et al., 1999b).

The isolate *Pseudomonas* sp. Ice-oil-327 was the most efficient among our alkane degrading strains considering degradation at -3°C and the broad spectrum of utilized single hydrocarbons including aromatic compounds. These capabilities make this strain advance to a potential candidate for use in bioaugmentation approaches under ice conditions. For many years members of the genus *Pseudomonas* have been well-known for their degradative properties of various hydrocarbons and have also been found in cold environments (Whyte et al., 1997). The 16S rRNA gene analysis revealed that this strain affiliated with the *Pseudomonas pertucinogena* group, one of seven subclusters of the genus *Pseudomonas*, defined by Anzai et al. (Anzai et al., 2000) Members of this group with hydrocarbonoclastic abilities have not yet been reported.

The *Oleispira* isolate Ice-oil-381 also showed a high degradation potential but this strain as well as the *Oleispira* sp. gap-d-97 hardly grew in complex liquid media and on single hydrocarbon sources. An enrichment of biomass for bioaugmentation might therefore be difficult. Nevertheless, *Oleispira* spp. has to be considered as potential bioaugmentation candidates as they occur regularly in contaminated sea-ice, indicating their excellent capability to adapt to the very specific conditions of contaminated sea-ice and to succeed under field conditions.

Rhodococci have been found to be dominant alkane degraders in polar soils (Eriksson et al., 2001; Saul et al., 2005; Whyte et al., 2002). They are also very frequent among our sea-ice isolates although these strains showed a less pronounced cold adaptation than for example *Oleispira* spp.. Our data indicated that the Rhodococci are potential alkane degraders among our isolates from sea-ice, suggesting that these organisms are ubiquitously distributed in contaminated polar environments. Growth tests on single hydrocarbons revealed that all tested *Rhodococcus* spp. did not grow on hexadecane, when added in a rather high concentration of 4%. This may explain why Rhodococci are mainly observed in a later status of an oil spill when high amounts of readily degradable hydrocarbon compounds have already vanished.

Shewanella spp., were the most frequently isolated bacteria in our experiments in spite of a limited hydrocarbon degradation capability. Growth tests on single substrates revealed that they oxidize preponderantly more easily degradable short chain alkanes up to C_{16}. All of our *Shewanella* spp. excel in a rapid growth at -3°C. This and their preferential oxidization of short chain hydrocarbons are obviously the reason for their predominance in the early status of oil contaminated sites. Recently, Gentile et al. (Gentile et al., 2003) isolated from Antarctic seawater the psychrophilic *Shewanella* strain GA-22. This strain is closely related to our *Shewanella* sp. 198b while the other *Shewanella* isolates were more distinct, but showed similar hydrocarbonoclastic abilities. The conformity with the Antarctic seawater strain might

be a further indication that *Shewanella* spp. are also ubiquitous in marine oil contaminated polar environments.

The *Marinobacter* spp. made up the second highest group of isolates among the *Gammaproteobacteria*. The *Marinobacter* group includes several well-known hydrocarbonoclastic degraders (Gauthier et al., 1992). Representatives of this genus have also repeatedly been found in crude oil bioremediation experiments with sea-ice and were dominating in oil-contaminated samples of Arctic sea-ice (Gerdes et al., 2005). Among our isolates there was a broad variety with respect to hydrocarbon degradation capabilities as well as temperature adaptation. For example the mesophilic strain *Marinobacter* sp. 81 could grow on various aliphatic and aromatic compounds while other cold adapted *Marinobacter* spp. showed only a limited hydrocarbon degradation spectrum. In spite of the limited degradation capability the cold adapted types are more common in sea ice, indicating a specific yet unknown role within the community of oil contaminated sea ice.

In contrast to the *Gammaproteobacteria* and the *Actinobacteria*, the *Alphaproteobacteria* represented only a small group among our isolates. Within the *Alphaproteobacteria* members of the genus *Shingomonas* are mainly known for their hydrocarbonoclastic abilities (Baraniecki et al., 2001; Khan et al., 1996; Prak & Pritchard, 2002). In our collection of isolates *Loktanella* spp. as well as strains affiliating with the *Jannaschia* genus turned out to utilize several hydrocarbons. Moreover, the frequent appearance of the *Jannaschia* relatives in hydrocarbon contaminated Antarctic gapwater suggested that these *Alphaproteobacteria* might play a significant role at least in some contaminated sea-ice locations.

Although all our strains were isolated from crude oil media, several isolates like those of the Bacteroidetes phylum were not able to significantly degrade petroleum-hydrocarbons at low temperatures when grown in pure culture. The higher abundance of some of these organisms in oil contaminated sea-ice indicates however a functional role in crude oil oxidization in combination with other organisms. They may thus provide hydrocarbons to other organisms

due to their ability to dissolve hydrophobic substances in the aqueous phase, or they may produce metabolites which are more easily available for these organisms. Mixed cultures were sometimes found to degrade an even broader spectrum of hydrocarbons than the individual cultures alone (Pelz et al., 1999).

Growths response on different temperatures by the tested isolates showed that a great proportion of the Arctic isolates were psychrotolerant with temperature maxima of about 25°C to 30°C, whereas most of the psychrophilic strains originated from Antarctic gapwater experiments. These findings are in accordance with results of Helmke and Weyland (Helmke & Weyland, 2004) who found that a high percentage (up to 90%) of isolates from uncontaminated Antarctic sea-ice were psychrophilic, according to the definition of Morita (Morita, 1975), whereas isolates from Arctic sea-ice showed in general higher growth temperature maxima that exceed the defined line of 20°C by a few degrees. It is suggested that the higher occurrence of 'moderate psychrophiles' in the northern hemisphere is caused by the inflow of warm water masses as well as the terrestrial input. With respect to hydrocarbon degradation our psychrotolerant strains were shown to degrade crude oil hydrocarbons more efficiently at low temperatures than the psychrophilic strains. In other cold environments, hydrocarbonoclastic strains were often found to be psychro-tolerant (Bej et al., 2000; Whyte et al., 1997) and Whyte et al. (Whyte et al., 1998) argued that psychro-tolerant microorganisms might even be better for the application in contaminated sites in cold climate than psychrophilic strains, whose growth is already inhibited at temperatures at or above 15°C to 20°C.

Our degradation experiments with different isolates indicated that even under optimised conditions during a three-month period, corresponding to a Polar summer season, aliphatic compounds, which are the major constituents of crude oil, can be removed from the environment by bioremediation. However, other compounds, such as PAH and high molecular weight hydrocarbons, will remain through the winter season. PAH occur in lower

abundances in crude oil but, due to their toxicity, decontamination of these hydrocarbons is of high importance. It is still an open question whether high molecular weight hydrocarbons, including PAH, can be removed by bioremediation during the subsequent spring and summer seasons.

The development of an oil-degrading population might take a long time due to the commonly reduced bacterial activities at low temperatures. Therefore, especially in the Polar Regions, where only the short summer season provides moderate temperatures, bioaugmentation may bridge the lag period prior to the onset of biodegradation and thus can be recommended as a powerful tool as an oil spill response method in sea-ice environments. First studies showed a progressed degradation of crude oil hydrocarbons due to the application of bioaugmentation with an inoculum of cold-adapted oil degrading bacteria in artificially oil contaminated samples of Antarctic gapwater (Gerdes et al., in preparation). This study has shown that bacterial strains of the *Gammaproteobacteria , Actinobacteria,* and *Alphaproteobacteria* obtained from the sea-ice environment, are excellent degraders of crude oil hydrocarbons even at low temperatures, and are therefore promising candidates for a consortium of oil-degrading bacteria to clean-up hydrocarbon contaminations in sea-ice.

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Manuscript 4

NOTE

(IN PREPARATION)

BIOREMEDIATION FIELD EXPERIMENTS WITH CRUDE OIL IN SEA-ICE CONDUCTED IN VAN MIJENFJORDEN, SVALBARD (2004)

Birte Gerdes, Gerhard Dieckmann , and Elisabeth Helmke

Keywords: bioremediation, bioaugmentation, biodegradation, sea-ice, hydrocarbons, low temperature

Abstract

The threat of crude oil contamination in the ice-covered areas of the Arctic Ocean is increasing due to a growing interest in exploiting and shipping of Arctic oil and gas.

However, little is known about the impact of oil on sea-ice microbial communities (SIMCO). This study investigated the influence of crude oil contamination alone and with the addition of two organic fertilizers (Inipol MS3000 and fish meal) on changes in bacterial community composition as well as the effect on oil biodegradation during a field experiment conducted in Van Mijenfjorden from February to April 2004. In situ temperatures from February to April within the ice ranged from -30.8 ºC to 0.5°C at the surface and to -1.9 ºC at the ice-water interface. At the start of the experiment SIMCO, consisted mainly of diatoms (*Eukarya*) (Ikävalko et al., 2005) and bacterial abundance was low (1.62 x 10^4 cells/ml). After 63 days, the bacterial diversity shifted towards a predominance of bacterial groups, commonly found in sea-ice, including members of *Polaribacter* sp. and *Flavobacteria* of the *Bacteroidetes* phylum and *Colwellia* sp. within the *Gammaproteobacteria*. Crude oil alone as well as oil plus Inipol had little to no effect on the bacterial community compositions, which is probably due to solidification at the cold temperatures. The treatment with oil plus fish meal strongly influenced bacterial community compositions resulting in a predominance of *Pseudoalteromonas* sp. of the *Gammaproteobacteria*. Two weeks before the end of the experiments, the in situ ice temperatures rose to temperatures above -7°C, causing melting of the ice and resulting in an early termination of the experiments. Significant microbial biodegradation of the crude oil was not observed during this short incubation period.

Introduction

Due to the exploration of oil 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, the danger of hydrocarbon contamination in the Arctic is increasing. However, little is known about the potential impact of accidental oil spills on this environment. The ice conditions along the Northern Sea Route (NSR) are subject to seasonal and regional fluctuations. In the winter months, November to April, the entire region is covered by very dense drifting pack ice. Large ice fields, containing significant concentrations of multi-year ice, have been discovered in regions of the southern Kara- and Laptev Sea in summer (Lovas and Vefsnmo, 1998).

Sea-ice constitutes an important and extreme ecosystem harbouring highly active sea-ice microbial communities (SIMCO) (Grossmann and Dieckmann, 1994; Helmke and Weyland, 1995; Junge et al., 2004), which play a significant role in the marine food webs of Polar regions (Brown and Bowman, 2001; Smith and Clement, 1990; Staley et al., 2002). It is therefore important to assess the influence of crude oil contamination on bacterial communities inhabiting Arctic sea-ice as well as to elucidate their potential to degrade petroleum hydrocarbons at cold temperatures. SIMCO live mainly in brine channels and in pockets in the ice matrix which arise during its formation (Thomas and Dieckmann, 2003). This microhabitat is an extreme environment with salinities ranging from 0 up to 150 and temperatures from -1°C to -50°C in winter. The volume of the brine channels and pockets is directly dependent on the in situ temperature and varies from 1% to 30%. Most sea-ice bacteria are psychrophilic (Bowman et al., 1997; Gosink and Staley, 1995; Helmke and Weyland, 1995) and differ in size, activity and taxonomy from free living bacteria in the underlying sea water (Bowman et al., 1997; Brinkmeyer et al., 2003; Helmke and Weyland, 1995).

The diversity of SIMCO has been studied mainly in the Antarctic (Bowman et al., 1997; Brinkmeyer et al., 2003; Brown and Bowman, 2001; Sullivan and Palmisano, 1984). Only recently the diversity of Artic sea-ice bacteria has been investigated (Brinkmeyer et al., 2003 ; Brown and Bowman, 2001). Bioremediation studies in cold marine environments have mainly been conducted mainly on seawater, sediments or beaches in the Arctic Ocean (Atlas, 1977; Atlas and Budosh, 1976; Bragg et al., 1994; Floodgate, 1984; Grossman et al., 1999; Prince and Bragg, 1997; Sendstad et al., 1982; Sergy et al., 1998; Sveum and Ladousse, 1989; Swannell et al., 1994) and in the Southern Ocean (Cavanagh et al., 1998; Delille et al., 1998; Delille and Vaillant, 1990; Yakimov et al., 2004). Only a limited number of studies has been performed on sea-ice (Atlas, 1983; Atlas et al., 1978; Delille et al., 1998; Siron et al., 1995; Siron et al., 1993).

This study assessed the effectiveness of two different organic fertilizers, Inipol and fish meal, to stimulate crude oil bioremediation of Arctic sea-ice during winter and early spring (February to April 2004) and investigated the influence of oil as well as the bioremediation treatments on the bacterial sea-ice community.

Materials and Methods

Sample site and set up of bioremediation experiments

The bioremediation study was conducted in Van Mijenfjorden on Svalbard from 22nd February 2004 to 25th April 2004, with permission of the Sysselmannen organisation. In Van Mijenforden the sea-ice conditions were quite stable in the period from January to April. The field site included ten experimental areas (65 x 45 cm) (Figure 2) and was located near Svea (Figure 1), a small village with an operating coal mine. To avoid spreading of the oil to the adjacent environment, the experimental areas were enclosed by white plastic walls, which were dug about 60 cm into the ice, using a chain saw. Inipol, an oleophilic fertilizer, successfully tested in Alaska for the Exxon Valdez oil spill clean up (Bragg et al., 1994),

contains urea as nitrogen source and tri(laureth-4)-phosphate as a phosphorus source, and a surfactant, encapsulated within oleic acid. Due to its oleophilic formulation Inipol has the benefit to supply nutrients directly to oil-degrading microbes at the oil surface. The disadvantage of Inipol, however, is its toxicity and that is it itself a high organic carbon source. In this study we used Inipol MS 3000, without 2 butoxy ethanol in the formulation, which is less toxic to marine organisms. Ordinary fishmeal was also used as fertilizer, and as low cost alternative product, compared to the expensive Inipol. Fishmeal contains a high amount of amino acids and is an excellent growth stimulant for many marine bacteria. The average C:N:P ratio (62:5:1) of the used fish meal was determined to be about the same as in Inipol. To study the influence of crude oil and bioremediation treatments, plots with oil alone (150 ml), with oil plus Inipol (15 ml), and oil plus autoclaved fish meal (15 g) were set up in duplicates. Three plots without oil treatment served as controls (see Figure 2). The oil on the experimental areas was then covered with snow to avoid attraction to and contact with birds and other animals. The experimental site was marked with flags. The temperature within the ice was measured directly adjacent to the field site, from the ice surface in 10 cm segments through the ice column to the ice-seawater interface, continuously every hour during the first month of the experiments, (Figure 3A). For the second month, air temperature data, measured at the nearby airport in Svea, were used for experimental verification (see Figure 3B). After final sampling in April 2004, the boxes were removed from the ice and the remaining oil was collected in a container and disposed of at the University of Svalbard (UNIS).

Sampling and sample preparation

Oil samples were collected in 5 ml sterile glass vials by scraping residue oil from the ice surface using a sterile metallic spatula and stored at -25°C until further processing. Samples of sea-ice were obtained by drilling ice cores (9 cm in diameter) using a Austin Kovacs Enterprises (AKE) Mark III corer. Using sterile techniques described previously by Helmke and Weyland (Helmke and Weyland, 1995), ice cores were cut into 20 cm segments with an

ethanol rinsed hand saw, and immediately transferred into sterile plastic bags, for further processing in the field laboratory of SINTEF (Foundation for Scientific and Industrial Research at the Norwegian Institute of Technology (NTH)) in Svea. For molecular analysis, the samples were processed as described by Helmke and Weyland (Helmke and Weyland, 1995). The ice segments were crushed and allowed to melt in sterile plastic containers with an equal volume of sterile filtered seawater to avoid disruption of cells during the melting process. Sea-ice microbial communities (SIMCO) were collected on polycarbonate filters (pore size 0.2 µm) and stored at -25°C until later extraction of nucleic acids.

Figure 1. Location of the experimental field site in Van Mijenfjorden, Svalbard

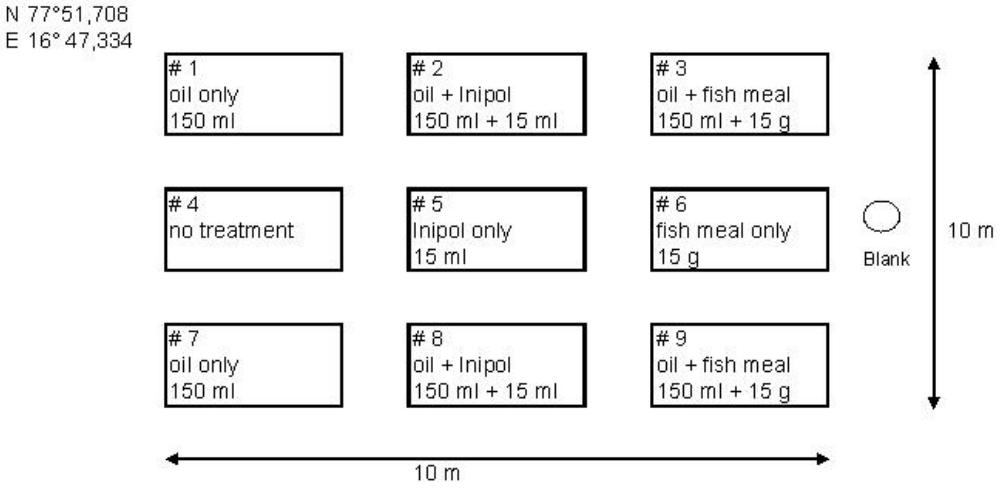

Figure 2. Arrangement of experimental field site, including nine study plots (Drawing not to scale)

Total bacterial counts

Samples from uncontaminated sea-ice in February and of each experimental plot in April were fixed with formaldehyde (2% final concentration) and stored at 4°C prior to enumeration. Bacterial cells were enumerated by epifluorescence microscopy according to the acridine orange direct count method (AODC) of Hobbie (Hobbie et al., 1977). The microscopic counting was carried out in the home laboratory one month after sampling.

PCR and DGGE

Total community nucleic acids were extracted from filters using the Ultra Clean Soil DNA-Kit (MoBio, USA) with additional lysozyme (final concentration 1 mg/ml) pre-incubations at 37°C. To increase the sensitivity of DGGE analysis, a nested PCR technique was applied as described by Gerdes et al. 2005. PCR-products were analysed by DGGE, based on the protocol of Muyzer et al. (Muyzer et al., 1993) using a gradient-chamber. Approximately 25-30 µl of the PCR products were loaded onto prepared polyarcylamide gels and processed further as described by Gerdes et al. (Gerdes et al., 2005). 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 28 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72°C for 3 min.

Oil analysis

Total petroleum hydrocarbons (TPH), aliphatic hydrocarbons and aromatic hydrocarbons of the oil samples were analyzed according to Texas Commission on Environmental Quality methods 1005 and 1006 (www.tnrcc.state.tx.us). Samples were extracted with hexane and aliquots of the extract were used to determine TPH content of the extract. Aliphatic and aromatic hydrocarbons were separated using silica column (Merck 60 mesh). One millilitre of the extract was added on top of the column. Aliphatic hydrocarbons were eluted from the

column with hexane and aromatic hydrocarbons with dichloromethane. Finally, a 1:1 mixture of dichloromethane and acetone was used to elute heavier aromatic hydrocarbons from the column. The samples were analysed in a Hewlett Packard 5890 gas chromatograph with flame ionization (FID) detection, On-Column injection port and BPX-5 (15 m, 0.32 mm, 1.0 µm) column.

Results and discussion

Temperature profiles for the first month of the study were obtained throughout the ice column from the ice-atmosphere interface, on which oil and fertilizers were spread down to the ice-seawater interface (Figure 3A). For the second month the air temperature was measured at the airport in Svea, and is shown in Figure 3B. The profile shows that the temperature at the ice surface was below -7°C for most of the time during the first month. Studies of sea-ice bacteria indicated, however, that they tolerate such low temperatures,

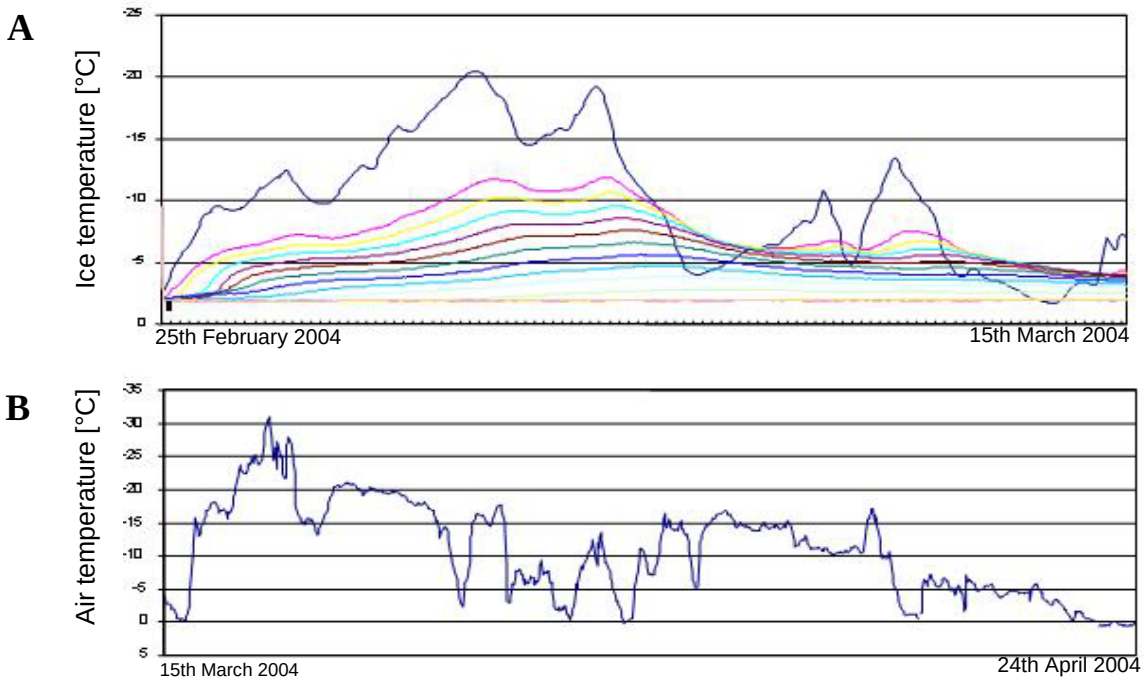

Figure 3. (A) Temperature profile through the sea-ice column near the study site in Van Mijenfjorden during the time of setup until 15th March 2004. The temperature was recorded continuously from the ice-atmosphere interface every 10 cm to the ice-seawater interface. (B) Air temperature during the time from 14th March to 24th April.

activity and growth is negligible {Helmke, unpublished data}. During the second month of the experiments the temperature ranged from -31°C to ~ 0°C. The temperature during the last two weeks was unusually high compared to recorded data from the last twenty years and caused melting of the sea-ice cover as early as the end of April. Therefore the experiments were terminated two months earlier than planned and removed from the fjord.

Changes in bacterial communities

At the start of the field experiments on Svalbard in February, conducted on newly formed sea-ice, the samples had a limited number of bacterial cells (see Table 2) and consisted of mainly diatoms (Ikävalko et al., 2005). After two months the bacterial diversity and abundance increased in both the untreated ice as well as in the samples treated with oil only. Both communities consisted predominantly of *Flavobacteria* and *Polaribacter* spp. within the *Bacteroidetes* phylum as well as *Colwellia* spp. of the *Gammaproteobacteria*. A similar community was found in the plots treated with oil plus Inipol. The treatment with oil plus fish meal, however, resulted in a strong reduction in diversity towards mainly *Gammaproteobacteria* with members of *Pseudoalteromonas* sp.. A study on the eukaryotic sea-ice biota of these experiments revealed a decrease in protist diversity and abundance in all plots treated with oil. However, decreases in protist diversity and abundance were less marked in the oil plus fertilizer plots versus the oil only plots (Ikävalko et al., 2005). The oleophilic fertilizer Inipol, which became solid when spread onto the cold ice surface, as well as the highly viscous oil appeared to have no effect on the bacterial sea-ice communities over the course of the 63-day experiment. A succession of sea-ice microbial communities from psychro-tolerant bacteria with low bacterial biomass and low heterotrophic activity, comparable to those of the water column, to dominantly psychrophilic bacteria, with higher activity rates, has previously been reported (Helmke and Weyland, 1995) during sea-ice formation. *Colwellia* spp. within the Gammaproteobacteria were found to be one of the most

abundant phylotypes in sea-ice and *Polaribacter* spp. of the *Bacteroidetes* phylum were also repeatedly found in sea-ice (Brinkmeyer et al., 2003). Members of *Pseudoalteromonas* are also commonly detected in sea-ice, however, at lower abundances than *Colwellia* spp.. Bowman et al. (Bowman et al.) found that isolates of *Colwellia* spp. and *Pseudoalteromonas* spp., including isolates within the *Bacteroidetes* phylum were all psychrophilic. Compositions of bacteria in the experimental plots were typical of those found in maturing sea-ice, however, the high abundance of mainly *Pseudoalteromonas* sp. in the oil plus fish meal treated plots showed a strong influence of the supplied nutrients on the bacterial community. Members of *Pseudoalteromonas* spp. were recently isolated from oil-contaminated sea-ice experiments with Arctic sea-ice and hydrocarbon degradation tests showed that some members were able to degrade oil-hydrocarbons at low temperatures {Gerdes et al., unpublished}. Members of *Pseudoalteromonas*, able to grow on polycyclic aromatic hydrocarbons (PAH), have also been isolated from other marine environments (Hedlund and Staley, 2006); (Melcher et al., 2002).

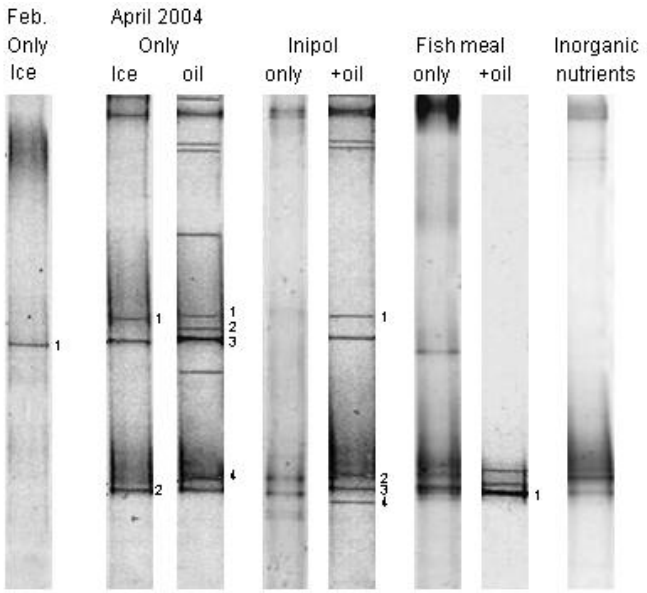

Figure 4. DGGE profiles of 16S rRNA gene fragments of a sea-ice sample in February 2004 at the beginning of the experiments and after 63 days from the different experimental plots

Table 1. Sequence similarities of excised DGGE bands of Figure 5

Treatment	Band	Closest GenBank Relative	Access. No.	Identity [%]	Length [bp]	Group
Ice Feb.	Feb. -1	Uncultured diatom clone HT2A8 16S ribosomal RNA gene	AF418973	97.74	486	*Eukarya*
Ice only	April - 1	Arctic sea ice associated bacterium ARK10272	AF468432	99.8	496	*Bacteroidetes*
Ice only	April - 2	Uncultured bacterium clone ARKTICE-87	AF468313	95.12	533	Gammaproteobacteria
Ice + oil	April - 1	Uncultured *Polaribacter* SIC.B9029	AF277564	98.87	530	*Bacteroidetes*
Ice + oil	April - 2	Uncultured Antarctic sea ice bacterium clone ANTXI/4_15-37	AY165580	95.18	498	*Bacteroidetes*
Ice + oil	April - 3	Uncultured Antarctic sea ice bacterium clone ANTXI/4_15-37	AY165580	99.63	534	*Bacteroidetes*
Ice + oil	April - 4	Uncultured bacterium clone ARKTICE-87	AF468313	98.34	543	Gammaproteobacteria
Oil + Inipol	April - 1	Arctic sea ice associated bacterium ARK10272	AF468432	90.17	458	*Bacteroidetes*
Oil + Inipol	April - 2	Gamma proteobacterium UMB3E	AF505723	87.5	152	Gammaproteobacteria
Oil + Inipol	April - 3	Uncultured bacterium clone BN_37	AY550844	88.78	401	Gammaproteobacteria
Oil + Inipol	April - 4	Gamma proteobacterium UMB7C	AF505732	84.16	385	Gammaproteobacteria
Oil + FM	April - 1	*Pseudoalteromonas* sp. KMM 520	AY040230	99.82	542	Gammaproteobacteria

Table 2. Total bacterial counts determined by AODC.

Treatment	Sampling	Cell number	± SD	Description
Ice only	February	1.62×10^4	0.5×10^4	Small cells
Ice only	April	0.96×10^4	0.16×10^4	Small cells
Oil only	April	5.61×10^4	0.62×10^4	Small cells
Inipol only	April	60×10^4	11×10^4	Small and medium sized cells
Oil + Inipol	April	199.1×10^4	52.4×10^4	Small cells, some big cells
Fish meal only	April	377×10^4	86×10^4	Aggregates, medium sized cells
Oil + fish meal	April	5680×10^4	1500×10^4	Many comparable small cells
Oil + inorganic	April	130×10^4	48×10^4	Small cells

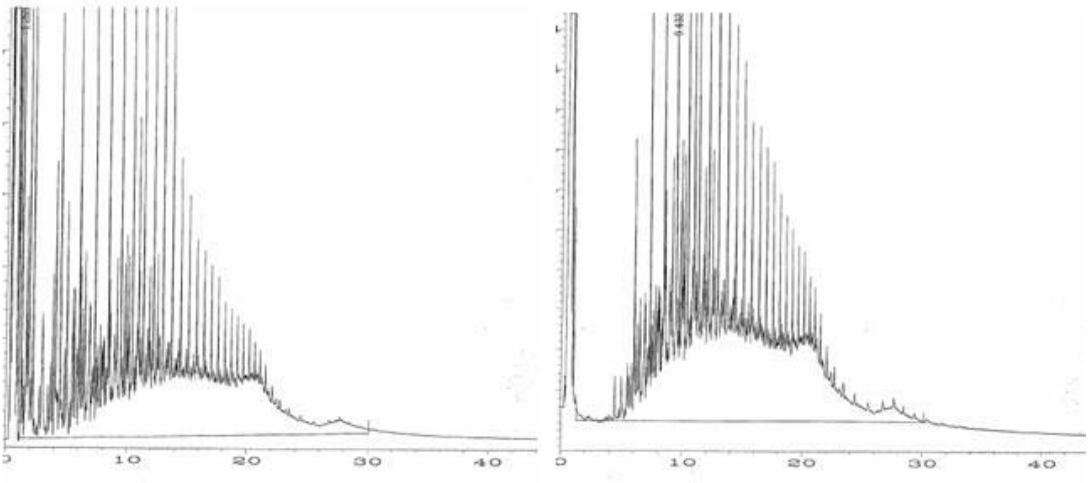

Figure 5. Oil fingerprints, analysed by GC/FID. (A) Statfjord crude oil at the beginning of the experiments (20th February 2004). (B) Statfjord crude oil after 63 days from plot #1 (oil only) (25th April 2004).

Oil analysis by GC/FID revealed that no significant oil degradation occurred during the time from 22nd February 2004 to 25th April 2004 in all oil treated plots. Oil fingerprints showed that only low molecular weight hydrocarbons disappeared (see Figure 5) which is most likely due to weathering (abiotic losses). Negligible degradation was expected in the oil only areas and it was not surprising to obtain the same results for the areas treated with oil plus Inipol, as it had no effect on the bacterial communities. It seems odd, that the oil plus fish meal treated plots also showed no significant oil biodegradation. Fish meal is rich in organic nutrients which were apparently more readily available than Inipol and were preferentially degraded before oil-hydrocarbons, which have limited bioavailability at low temperatures. Samples from these plots contained considerable amounts of bacterial biomass and extracellular substances, although moderate temperatures only occurred for a short period at the end of April. Moreover, the period with temperatures between -7°C and 0°C was expected to last longer and did not permit enough time to achieve a visible degradation of the relatively high amount (150 ml) of added oil. It is therefore possible that biodegradation of crude oil hydrocarbons occurred at the ice-oil interface and was not detected due to homogenised sampling of the crude oil from the entire experimental plot. The excessive secondary production indicates potentially high degradation rates at temperatures below freezing which are promising for the degradation of petroleum hydrocarbons as soon as a cold-adapted, oil degrading sea-ice biota develops.

Conclusion

The ice-cover in Van Mijenfjorden is annual and permits only short periods for bioremediation. Large proportions in Arctic Ocean are, however, covered with multi-year ice which allows more time for recruitment of oil-degrading sea-ice bacteria even during summer and thus for decontamination of oil pollution. The use of inorganic nutrients rather than organic nutrients, rich in carbon content, is recommended to shorten the acclimatisation

period of the indigenous sea-ice bacteria on hydrocarbon mineralization. However, the use of inorganic nutrients for crude oil bioremediation in sea-ice needs to be investigated. Furthermore, the application of bioaugmentation should be considered as bioremediation tool, to further shorten the time for the development of an oil-degrading bacterial community. This means that oil-degrading bacteria from sea-ice need to be isolated and compiled to a potential consortium of cold-adapted, hydrocarbon oxidising bacteria.

Acknowledgements

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Manuscript 5

AN EXPERIMENTAL STUDY OF THE EFFECTS OF STATFJORD CRUDE OIL, AND APPLICATION OF INIPOL AND FISH MEAL ON THE SEA-ICE BIOTA ON SVALBARD IN FEBRUARY-APRIL 2004

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Abstract

For the study of crude oil and nutrient addition effects on arctic sea-ice biota, a field experiment was carried out in Van Mijenfjorden, Svalbard, during 22.2.-25.4.2004. During the 63 day long experiment, three different compounds (Statfjord crude oil, Inipol and fish meal) were added in different combinations onto snow-free sea-ice surface. In February, the sea-ice biota consisted solely of diatoms. At the end of the experiment the natural sea-ice community consisted of both diatoms and euglenid flagellates (abundance ≤3400 cells 100 ml^{-1} melted sea-ice). The treatment with oil only, oil+Inipol, and oil+fish meal led to a general decrease in protist diversity and abundance. The most dramatic decrease in the abundance of all protists was caused by the addition of oil only, and throughout the ice cover, while the addition of oil+Inipol, and oil+fish meal led to the disappearance of all other protist groups than diatoms. The negative effects of Inipol and fish meal were most severe in the ice surface, while the interior and the bottom parts of the ice cover were less impacted by the treatments. Heterotrophic flagellates seemed to be able to migrate downward from the ice surface when only oil was added onto the ice. Also, the use of oil only, and oil+Inipol induced the formation of diatom resting spores. In control samples, the addition of fish meal only caused a notable increase of heterotrophic flagellates (≤25 000 cells 100 ml^{-1} melted ice).

1 Introduction

The shipping of crude oil and oil products is expected to increase in the Arctic, in particular in the Barents Sea, in the near future (UNEP 2004). In addition to increasing transportation, the risk of an oil spill or an accident is rising by e.g. the lack of experience in large-tonnage tanker navigation under arctic conditions, and insufficient potential of emergency services (UNEP 2004). Environmental Risk Analysis (ERA) and Environmental Impact Assessments (EIA) are made for areas at risk (e.g. UNEP 2004, www.arctic-council.org). While birds and mammals mostly suffer from mechanical damages, like oiling of the plumage or skin, which cause problems in particular with thermoregulation (Leighton et al. 1985, Engelhardt 1985), most underwater nature is threatened also by chemical effects of hydrocarbons. Oil can direct damage marine organisms on several systematic levels (e.g. Atlas 1985, Rice 1985, Wells & Percy 1985, Bunch 1987, Cross & Martin 1987, Mageau et al. 1987, Neff et al. 1987, Aunaas et al. 1991, Sakshaug et al. 1994). Eggs, as well as larval and juvenile stages of organisms are particularly sensitive to hydrocarbons. Sessile and filtrating organisms in the open water, littoral and benthos, such as macroalgae, crabs, sea stars, mussels and zooplankton, may become covered by oil, their filtration and/or swimming apparatuses can be blocked by oil particles, or oil may be stored in lipid storages. This, in turn, will lead to biomagnification in the arctic foodweb, as a consumer may engulf large quantities of oil together with its prey organism (Sakshaug et al. 1994).

The knowledge of the consequences of oil contamination on unicellular organisms, in particular primary producers which are the foundation of sea-ice covered arctic marine ecosystems is sparse and patchy (e.g. Vandermeulen & Ahren 1976, Cross 1987). It is assumed, that algae remain unaffected or that the effects of oil on phytoplankton are limited to short term effects, such as temporary decrease of photosynthetic rates and species diversity, but that the recovery would be relatively fast (Cross 1987, Patin 2001). It is imperative that the matter be further investigated.

Our experiment was an effort to resolve some of the remaining questions regarding the effects of oil and added nutrients (Inipol, fish meal) on arctic sea-ice biota. We

exposed sea-ice biota to Statfjord crude oil (provided by SINTEF, Norway), Inipol, and nutrient rich fish meal (the two latter are thought to improve oil bioremediation). Inipol was a commercial product (no longer manufactured due to its toxicity, www.epa.gov/oilspill/ncp/inipolea.html) with concentrations of phosphorous (tri(laureth-4) -phosphate, 0,7%) and nitrogen (urea, 7,4%) to accelerate bioremediation (e.g. www.atofinachemicals.com). It had been used to accelerate oil biodegradation (e.g. Bergueiro-López et al. 1997). In experiments with oil only, the ice biota was affected the most, as both the species diversity and abundance decreased notably. Oil also caused the downward migration of heterotrophic flagellates within the ice cover, and the formation of diatom resting stages. The addition of fish meal alone led to a dramatic increase of heterotrophic organisms.

2 Material and Methods

The experiment was made during 22.2.-25.4.2004 (63 days). The total experimental area on ice was 10m x 10 m, and was divided into 12 subareas (each 64cm x 45cm), and sealed from the surrounding ice by plastic boxes (Fig. 1). Blank ice core samples were taken prior to experimental treatments on 22.2.2004. Statfjord oil (150 ml), oil and Inipol (150 ml + 15 ml, treatment is called oil+inipol from here on), and oil and fish meal (150 ml + 15 g, treatment is called oil+fish meal from here on) was distributed onto snow-free sea-ice. Snow was showelled back onto the ice after the addition of the agents. Also control areas with 1) no treatment, 2) only Inipol (15 ml), and 3) only fish meal (15 g) were established.

All ice core samples were taken with a SIPRE type auger and cut into subsamples: blank samples into 0-16, 16-33, 33-56, 56-83 and 83-111 cm, experiment samples into top part 0-25 cm, interior part 50-75 cm, and bottom part (95-120, 98-123 or 103-128 cm). Control samples with no treatment, only Inipol, or fish meal were taken only from the uppermost 25 cm of the ice cover. Each subsample was melted in 200 ml sterile sea water with added sea salts corresponding to the salinity of sea water (34). Melted samples were preserved either with formalin (22.2.2004) or Lugol's (25.4.2004) solution (5% final concentration).

Samples for the analysis of the species composition of sea-ice biota, and the abundance of protists in different experiment fields were concentrated according to the Utermöhl technique (Utermöhl 1958) and examined with an inverted Leitz DMIL microscope, 250-500x final magnification.

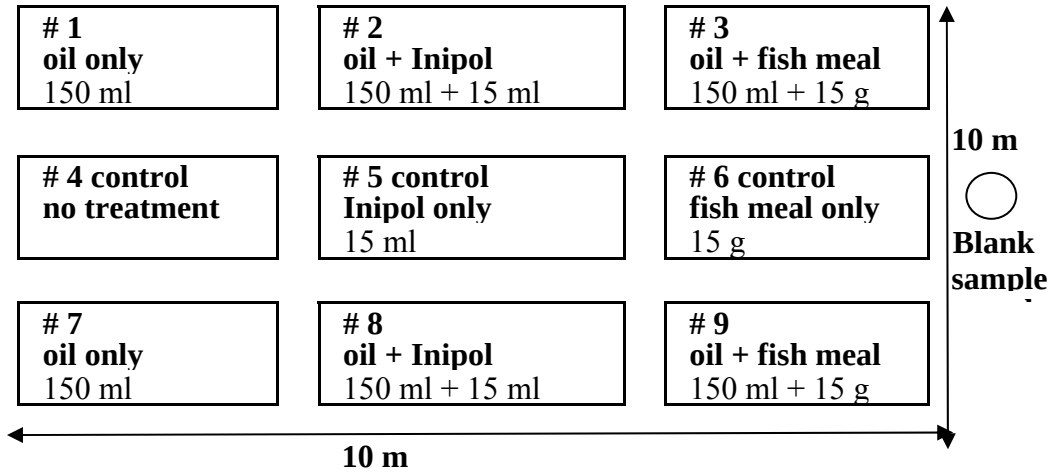

Figure 1. Experimental design (drawing not to scale) for the study of effects of Statfjord crude oil, Inipol and fish meal on the arctic sea-ice biota.

3 Results

Tables 1 shows protist taxa that we recorded in samples in February (controls prior to the experiment) and in April.

Table 1. Species composition of sea-ice biota on 22.2.2004

	0-16 cm	**16-33 cm**	**33-56 cm**	**56-83 cm**	**83-111 cm**
Bacillariophyceae					
Bacillaria paxillifera		x		x	
Banquisia belgicae	x	x	x	x	x
Coscinodiscus oculus-iridis		x			
Cylindrotheca closterium	x	x	x	x	x
cf. *Haslea wawrikae*		x	x	x	x
Navicula spp.	x	x			x
Nitzschia frigida	x	x	x	x	x
Pseudonitzschia sp.		x			x

In samples from 22.2.2004, the ice biota consisted solely of diatoms (Table 1 , Fig. 2). The dominant species were *Banquisia belgicae*, *Cylindrotheca closterium*, cf. *Haslea wawrikae* and *Nitzschia frigida*. Cell abundance of diatoms was generally, however, low (<1000 cells 100 ml melted ice^{-1}). The ice surface was very dry (no brine seeping), thus most organisms were concentrated in the lower layers of the ice sheet (56-111 cm). After 63 days (25.4.2004), the ice biota was more diverse (Table 2, Fig. 3), comprising of some dinoflagellates (phototrophic *Gymnodinium* spp., heterotrophic *Katodinium* sp.), and some heterotrophic flagellates, in particular *Thaumatomastix* sp. (Protista Incertae Sedis) in the upper 0-25 cm. Diatoms were distributed evenly in the ice cover, and their abundance had increased 2-10x within 63 days (≤3400 cells 100 ml^{-1} melted sea-ice). The diatom community comprised of e.g. *Bacillaria paxillifer*, *Banquisia belgicae*, *Thalassionema nitzschioides, Nitszchia frigida*, and *Cylindrotheca closterium*.

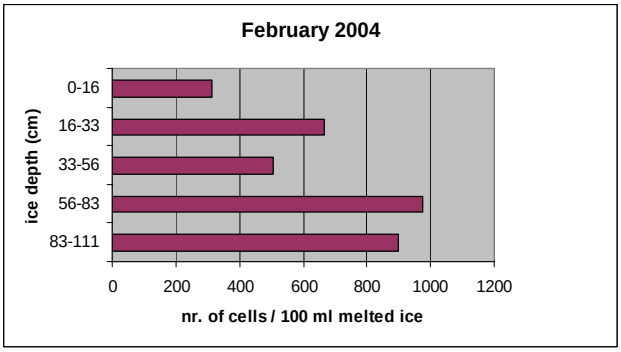

Figure 2. Vertical stratification of organisms in sea-ice on 22.2.2004

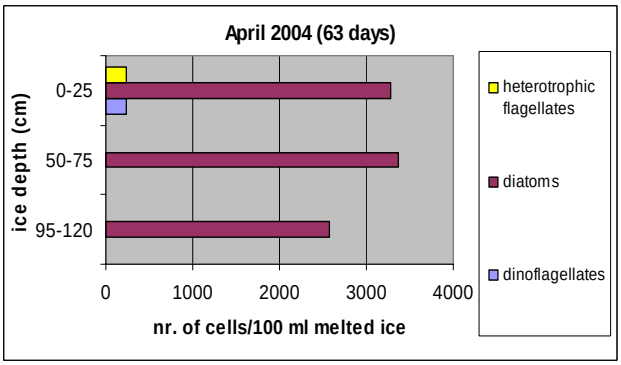

Figure 3. Vertical stratification of organisms in sea-ice on 25.4.2004

After the addition of Inipol only (Fig. 4), both the diversity and the abundance of algae crashed. The abundance of diatoms (mainly *Nitzschia frigida)* was <1000 cells 100 ml^{-1} melted sea-ice. The addition of fish meal only (Fig. 4) caused a remarkable increase of heterotrophic flagellates, in particular *Protaspis* sp. and *Telonema subtile* (Protista Incertae Sedis; abundance ≤25 000 cells 100 ml^{-1} melted ice), and the colourless *Polytoma papillata* (Chlorophyceae; abundance nearly 6000 cells 100 ml^{-1} melted ice). Also with this treatment, the diatom abundance decreased to <1500 cells per 100 ml melted ice.

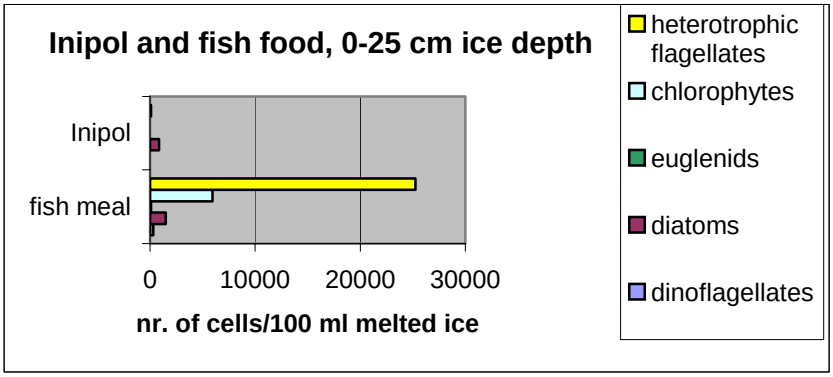

Figure 4. Vertical stratification of organisms in the upper 25 cm of sea-ice after treatments with Inipol only and fish meal only.

The addition of oil only (Fig. 5) caused a dramatic decrease of diatoms (<500 cells 100 ml^{-1} melted sea-ice) throughout the ice cover (total thickness 128 cm), the formation of diatom resting spores (<100 cells/spores 100 ml^{-1} melted sea-ice), and the migration of heterotrophic flagellates from the ice surface into the ice interior.

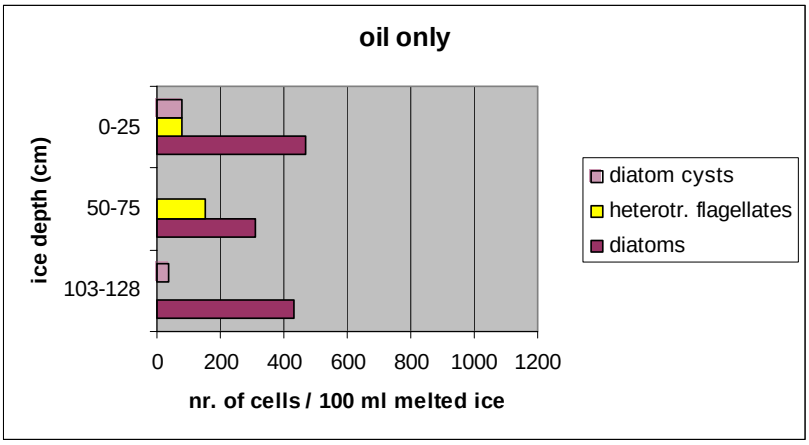

Figure 5. Vertical stratification of organisms in sea-ice when only oil was added.

The negative effects of oil+Inipol (Fig. 6) were more restricted to the ice surface (0-25 cm), where diatoms decreased dramatically (abundance <200 cells 100 ml^{-1} melted sea-ice). Live, healthy looking diatom cells and diatom resting spores were present only in the lower ice layers (95-120 cm). In the ice interior (50-75 cm), the use of oil+Inipol caused a notable decrease in diatom abundance (≤ 3400 cells 100 ml^{-1} melted sea-ice in 50-75 cm in the blank samples (Fig. 3) to ≤ 1000 cells 100 ml^{-1} melted sea-ice; Fig. 6).

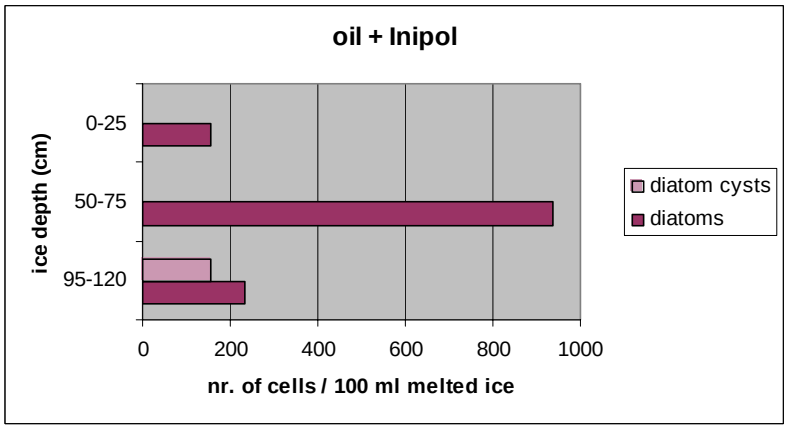

Figure 6. Vertical startification of organisms in sea-ice when oil+Inipol were added.

When oil and fish meal was added (Fig. 7), no live organisms were present in the surface ice (0-25 cm). In the ice interior and bottom layers only diatoms were present but in low abundance (≤ 1100 cells 100 ml^{-1} melted sea-ice).

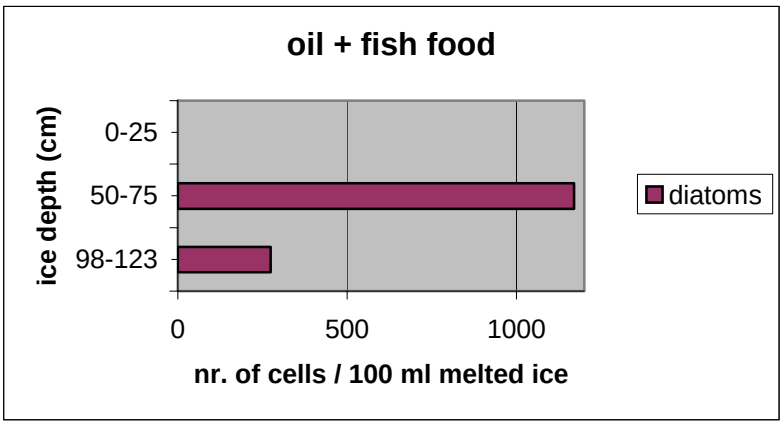

Figure 7. Vertical stratification of organisms in sea-ice when oil and fish meal were added.

4 Discussion

Once oil is released into the arctic marine environment in the presence of ice cover, several processes may take place, depending e.g. on the season, the site of the oil spill (below or on the ice cover), and the state of ice growth or melting (e.g. Jordan & Payne 1980, Payne et al. 1991). During the pack ice season, an under-ice spill will lead to the formation of oil lenses (uneven ice underside) or large sheets (even ice underside) beneath the ice sheet. The arctic pack ice underside has an irregular profile, and oil will fill ice cracks and other depressions. During the ice growth (new ice formation and rafting of ice floes), oil may become sealed in. At this stage, no evaporation occurs. It may take even 7 years for an oil lense within the ice to migrate to the top of the arctic multi-year ice (Nelson-Smith 1982, Payne et al. 1991).

Oil weathering is possible only when oil is in contact with moving water (i.e. fresh melt water on sea-ice surface in summer, or water currents in the underlying water column). Therefore, hydrocarbon concentrations increase notably during ice break-up (Nelson-Smith 1982, Payne et al. 1991). When oil is spilled onto sea-ice, as in our experiment, oil forms a hydrophilic sheet onto ice and/or becomes trapped in brine channels within ice. As long as the ice is not melting, oil weathering processes are very low or non existent, thus the exposure time on the ice biota is longer. During the melting of the ice surface in summer, fresh meltwater migrates downwards, and finally through the multi-year arctic ice. Meltwater migration facilitates the release of ice associated organisms into the underlying water column, where they may act as a "seed" for vernal phytoplankton bloom. Thus, meltwater migration would also promote 1) oil weathering as the oil becomes in contact with moving water, and 2) the penetration of oil into the ice and the water column. Acute toxic effects of an oil spill on the arctic ice biota and the planktonic communities in the water column would thus not appear sooner than during the surface melting or break-up of the ice. Spring is probably the most critical period of the growth season in the Arctic, as that is when organisms' metabolism - and thus hydrocarbon intake - is accelerated due to improved light climate and increased water temperature. These processes are concentrated in the marginal ice zone (MIZ) which makes this region particularly vulnerable to oil spills.

Petroleum and its products may also have a mechanical effect on marine organisms (Nelson-Smith 1982) and within the ice. It can penetrate into brine channels and cling to its surfaces. As a substantial part of the ice, associated organisms live attached to brine

channel surfaces (either attached or e.g. gliding), oil films in brine channels have a direct mechanical effect on unicellular organisms. Furthermore, the presence of oil decreases albedo, thus causing heating, accelerating the melting of the ice and ice break-up, and preventing the gas exchange between the sea-ice surface and the atmosphere.

The effect of PAH compounds on unicellular, in particular sea-ice-associated organisms is to a large extent unstudied. Cross (1987) has conducted studies of the effects of oil spills on ice algae. No adverse effects could be detected for neither algal density, biomass, nor productivity after a moderate predisposition of crude oil (Cross 1987). Also, systematically higher organisms, such as marine invertebates remained unaffected by crude oil application (Cross & Martin 1987, Killie & Gulliksen 1994), but also severe effects have been recorded in laboratory and field experiments (e.g. Wells & Percy 1985, and references therein). It is suggested, that multicellular invertebrate organisms with more developed anatomy and physiology (e.g. crustaceans) have a better PAH metabolism than the less developed ones, such as unicellular protozoans (Robertson 1998). PAH compounds affect cell functions in two ways (Robertson 1998). Firstly, they can interfere with several cellular processes by binding reversibly to lipophilic sites in the cell. Secondly, PAHs' hydrophilic metabolic products can interact with DNA, and lead to the formation of so called adducts, which, in turn, are thought to play a role in the tumour induction caused by the carcinogenic PAH compounds.

Low concentrations (1-20 µg/l) of PAH compounds can inhibit the growth of multicellular algae (Anderson & Gossett 1986). Patin (2001) identified potential effects of oil spills on marine organisms in the Barents Sea ecosystem. In phytoplankton these were changes in the photosynthesis, species composition which disappeared after the elimination of oil (within hours or days) (Patin 2001). The sea-ice community, however, lives trapped within the ice, and thus the elimination of hydrocarbon compounds is notably slower than in the open water ecosystems. Our experiment gave an indication that diatoms may be more tolerant to oil than other, in particular "naked" protists (organisms with no cell wall). The diatom silica frustule may, at least in moderate predisposition, protect the cells from acute lethal effects of oil. Diatoms are also capable of producing asexual resting stages (i.e. spores or cysts) when environmental conditions deteriorate. Later, after the improvement of environmental conditions, such as the disappearance of PAHs through biodegradation, migration or other pathways, a new, vegetative diatom cell will germinate from the spore. How long diatom resting spores can remain viable under a PAH exposure is not known and should be studied experimentally using algal cultures. Generally, laboratory studies are required to reveal the sensitivity of different algal groups (diatoms, blue-greens, chlorophytes etc.) and even species to oil.

Our experiment indicates also that the application of any of the compounds (crude oil, Inipol, fish meal) in any combination (solely or in mixtures) onto ice will cause notable acute damage to the sea-ice community. The consequences of relatively long exposure of crude oil, Inipol or fish meal are indicated as a decline in the species diversity and the abundance of organisms. The use of fish meal only favoured heterotrophic flagellates that are capable of using organic compounds as energy source. In all treatments, apart from the application of oil only, the effects of a treatment on ice biota were pronounced in the ice surface layers, and less in ice interior and the bottom layers. Later in spring, if the experiment would have been continued, also the ice interior and bottom would assumably have become affected by the ice surface melting processes, meltwater migration and thus effects of oil weathering, and also the presence of Inipol and fish meal.

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6 Disclaimer

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Vermerk über den Umfang der Überarbeitung dieser Dissertation nach PromO § 11 (2):

Im Einvernehmen mit dem Prüfungsausschuss wurde eine Tabelle mit der chronologischen Übersicht der durchgeführten Experimente (Table 3. Overview of the conducted experiments in chronological order. Objectives, applied methods, main results and conclusions.) auf den Seiten 37 - 39 dieser Dissertation nachträglich zugefügt.

Eidesstattliche Erklärung

Gem. §6(5) Nr. 1-3 PromoO

Hiermit erkläre ich, dass ich die vorliegende Arbeit ohne unerlaubte, fremde Hilfe angefertigt habe und keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt habe. Die in den benutzen Werken wörtlich oder inhaltlich entnommenen Stellen sind als solche kenntlich gemacht.

Bremerhaven, den 04.05.2006

Birte Gerdes