Physiological investigations of aerobic petroleum degradation in marine sediment microcosms

Florin Musat

Physiological investigations of aerobic petroleum degradation in marine sediment microcosms

Dissertation

zur

Erlangung des Grades eines

Doktors der Naturwissenschaften

– Dr. rer. nat. –

dem Fachbereich Biologie/Chemie der

Universität Bremen vorgelegt von

Florin Musat

aus

Bukarest

Bremen 2005

Die Untersuchungen zur vorliegenden Doktorarbeit wurden am Max-Planck-Institut für Marine Mikrobiologie in Bremen durchgeführt.

1. Gutachter: Prof. Dr. Friedrich Widdel, Universität Bremen
2. Gutachter: Prof. Dr. Heribert Cypionka, Universität Oldenburg

Table of contents

List of abbreviations

(Very common abreviations and units are not listed)

AR	Acetylene Reduction
BSA	Bovine Serum Albumin
DAPI	4´,6-diamidino-2-phenylindole
DCM	Dichloromethane
FID	Flame Ionization Detector
FISH	Fluorescent *in situ* Hybridization
GC / MS	Gas Chromatography / Mass Spectrometry
HPLC	High Performance Liquid Chromatography
nifH	Structural gene of dinitrogenase reductase
OD	Optical Density
PAH	Polycyclic Aromatic Hydrocarbons
PBS	Phosphate Saline Buffer
Phy	Phytane
Pri	Pristane
RT-PCR	Reverse Transcription PCR
SOB	Sulfur-Oxidizing Bacteria
SRB	Sulfate-Reducing Bacteria
TCD	Thermal Conductivity Detector

Summary

Crude oil contamination of tidal areas causes significant environmental and economic damage. The most important factor contributing to petroleum removal from the environment is biodegradation by microorganisms. Even though microbial oil degradation has been studied over decades, there is little microbiological knowledge about degradation in the relatively compact, particle associated oil layers ("oil mats") as they are formed on sediments in coastal regions. In this study, (1) stratified microbial communities were allowed to develop on oil-contaminated marine sediments; (2) the extent of petroleum mineralization under conditions of supplementation with inorganic nutrients and optimum distribution on solid matrices was investigated; and (3) fixation of atmospheric nitrogen as an important inorganic nutrient limiting biodegradation of crude oil was examined by measurement of nitrogenase activity and molecular study of the underlying gene (*nifH*) and involved microorganisms.

1. Establishment of stratified microbial communities on oil-contaminated marine sediments in experimental microcosms

Contamination of tidal sediment with crude oil and the development of microbial populations associated with oil were simulated in aquaria in the laboratory. Controllable addition of oil was achieved by even distribution of oil over pristine sediment and further addition of dried sediment as a carrier, followed by seawater addition. The oil layers gradually led to the development of stratified microbial communities (phototrophic, aerobic, anaerobic). The phototrophic microbial community was dominated by filamentous heterocystous and non-heterocystous cyanobacteria. There was no apparent stimulation of the growth of cyanobacteria by the added oil. Within one month the surface of the oiled sediment regained its initial, olive-gray color indicating aerobic biodegradation of oil. However, below ~1 mm depth the oil-sediment layers were still present even after incubation for one year, and the color of the underlying sediment turned black. The color changes and microsensor measurements showed that addition of oil significantly promoted subsurface oxygen consumption and microbial sulfate reduction. Sediment microcosms as established in the present study are promising systems for the future study of the long-term fate of oil on and in marine sediments and of the significance of bacterial guilds and environmental parameters (light, oxygen, fertilization, etc.) for biodegradation of oil.

2. Quantification of the biodegradation of oil associated with surfaces and particles

The extent of the biodegradability of crude oil spread on different inert surfaces and allowed to age by evaporation of the volatile fraction was investigated. Biodegradation was quantified by measurement of formed CO_2 and comparison of the data to the amount of total carbon in crude oil. Mineralization was determined with and without supplementation by inorganic nutrients (ammonium, phosphate and iron(III)) which are known as important limiting factors of crude oil biodegradation in marine habitats. Optimal degradation occurred if oil was finely distributed on sand. However, even under such optimal conditions, mineralization of crude oil to CO_2 was rather incomplete, ranging between 15 and 20% of the initially added oil-carbon. In comparison, defined compounds (naphthalene, glucose) in parallel control experiments were mineralized to large extent (ca. 65 and 77%, respectively). Assuming assimilation of 50% of the degraded organic carbon in oil (based on published reports), between 42 and 62% of the original (non-weathered) oil was removed by the combined processes of evaporation, mineralization and assimilation. Accordingly, between 38 and 58% are likely to persist for a long, indefinite period of time. Gas-chromatographic analysis of the residual fraction showed that normal and branched alkanes were almost completely degraded.

3. Nitrogen fixation in microbial communities growing on oil-contaminated marine sediments

The potential of microbial guilds growing on oil-contaminated and pristine marine sediments to fix atmospheric nitrogen was studied. Molecular investigation based on amplification, reverse-transcription PCR and sequencing of the dinitrogenase reductase structural gene (*nifH*) showed the presence and expression of phylotypes similar with those of cyanobacteria and sulfate-reducing bacteria. For measurements of nitrogenase activity, stratified microbial communities were newly established on oil-contaminated and pristine sediments in bottles, similar as in the aquaria. The bottles were incubated under light/dark conditions or permanently in the dark. Nitrogenase activity was measured using the acetylene reduction (AR) assay. For the microbial communities developed under light/dark conditions on the oil-contaminated and pristine sediments, highest AR activity was determined in the light, suggesting that heterocystous cyanobacteria were the dominant diazotrophs. Nitrogenase activity in the experiments incubated permanently in the dark was very low. The results demonstrated that cyanobacteria were responsible for the bulk nitrogen fixation in the experimental oil-contaminated and pristine marine sediments.

A.1. Oil pollution and degradation – a brief overview

A.1.1. Composition of crude oil

Crude oils are very complex mixtures of hydrophobic compounds, formed by long-term geochemical reactions of buried, aged biomass especially in marine sediments. The main constituents of crude oil are hydrocarbons, comprising a mixture of alkanes (straight-chain and branched alkanes), cycloalkanes, and aromatic hydrocarbons (Tissot and Welte, 1984).

The *n*-alkanes in crude oil usually range from methane to compounds with 40 carbon atoms and more. The largest *n*-alkane reported in crude oil is $C_{79}H_{160}$ (Prince, 2002). Branched alkanes with up to 30 carbon atoms have been identified in crude oil, but the most abundant are pristane ($C_{19}H_{40}$) and phytane ($C_{20}H_{42}$), derived from the phytol chains of chlorophylls (Tissot and Welte, 1984). Cyclic alkanes (also named naphthenes) include cyclopentane, cyclohexane and decalin along with their alkylated derivatives (Fig. 1). Hopanes, which represent alkylated polycyclic aliphatic hydrocarbons of bacterial origin, are among the most stable compounds in crude oils, and have been used since recently as conserved biomarkers relative to which petroleum biodegradation can be estimated (Prince *et al*., 1994). All saturated hydrocarbons represent on average 55% of crude oils (Tissot and Welte, 1984).

The simplest aromatic compound in crude oil is benzene. Higher molecular mass aromatics present in crude oil are polycyclic aromatic hydrocarbons (PAH), for instance with two (naphthalene), three (phenanthrene, anthracene) or four rings (chrysene) (Fig. 1). Even more abundant than the unsubstituted parent compounds are their alkylated derivatives. PAH with more than four or five rings are present but not abundant in petroleum. The aromatic hydrocarbons represent about 30% of crude oils (Tissot and Welte, 1984).

Crude oils also contain small amounts of bound oxygen, nitrogen and higher amounts of sulfur, i.e. contain polar molecules. They are conventionally divided into asphaltenes (phenols, fatty acids, naphthenic acids, ketones, esters and porphyrins) and resins (pyridines, quinolines, carbazoles, sulfoxides and amides). An example of an asphaltene and a naphthenic acid are depicted in Fig. 1. The polar compounds represent about 15% of crude oils. In particular these compounds cause the color of crude oil.

Depending on the chemical composition, crude oils are generally classified into light and heavy ones. The light crude oils have low viscosity and a preponderance of low-molecular mass molecules, while the heavy crude oils are more viscous and enriched in high-molecular mass compounds, such as PAH and asphaltenes (Tissot and Welte, 1984).

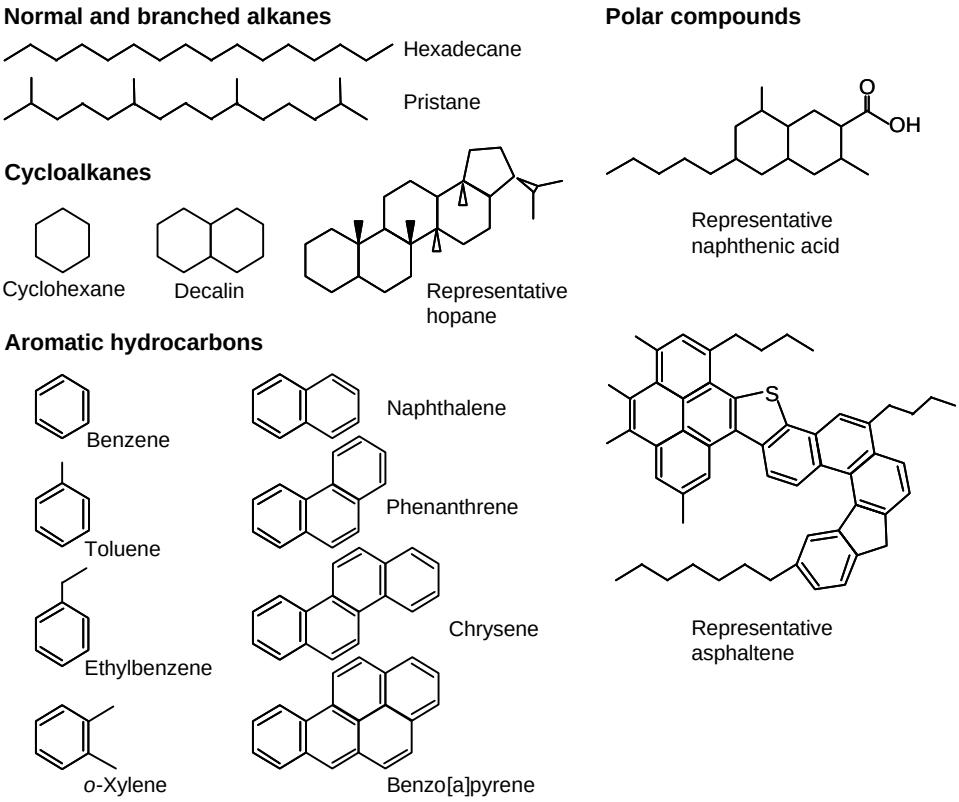


Fig. 1. Examples of main classes of compounds found in crude oil.

Petroleum may have been present in the biosphere probably since its formation, due to escape through natural seeps on land and in the sea (Wilson *et al*., 1974; Prince, 2002). The very recent anthropogenic activity with respect to petroleum exploitation significantly increased local inputs of petroleum hydrocarbons into the biosphere. On a global scale, however, petroleum is not the major source of hydrocarbons in the environment. Biogenic hydrocarbons constitute more than 98% of the total hydrocarbons in the environment (Bragg *et al*., 1992) (Fig. 2). Higher plants produce large amounts of aliphatic hydrocarbons, including isoprenes and monoterpenes (Tissot and Welte, 1984). Some bacteria produce toluene (Fischer-Romero *et al*., 1996) and termites have been reported to produce naphthalene (Chen *et al*., 1998).

Nevertheless, large accidental oil spills are of great concern, due to the high local load of the sea and shorelines with hydrocarbons and other petroleum compounds with partly significant toxicity (Floodgate, 1984; Swannell, 1996; Harayama *et al*., 1999) (Fig. 3). For example the wreckage of *Amocco Cadiz* in 1978 off the coast of Brittany spilled more than 200×10^3 t of crude oil, affecting approximately 350 km of coastline (Swannell, 1996). Another example, well known mainly for the large scale application of bioremediation

methods during the cleanup effort, is the *Exxon Valdez* spill in 1989 that discharged 40×10^3 t of crude oil into Prince William Sound, Alaska, (Bragg *et al*., 1992).

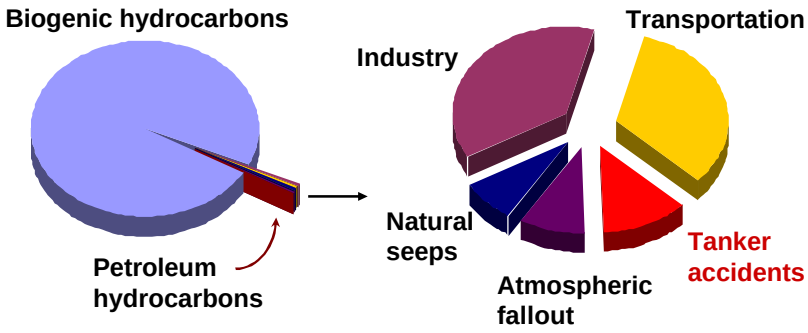


Fig. 2. On a global scale, most hydrocarbons that enter the environment are biogenic, produced for example by higher plants or phytoplankton. Tanker accidents, however, although representing a minor global contribution, have serious consequences on the environments where they occur. Adapted from Bragg *et al*., 1992.

Although not among the top spills with respect to the amount of oil released, the *Exxon Valdez* affected more than 2000 km of coastline (Fig. 3B) and led to the hitherto largest application of bioremediation techniques (Pritchard and Costa, 1991; Bragg *et al*., 1994; Prince and Bragg, 1997).

Fig. 3. Examples of major oil spills in the marine environment. **A.** Oil spilled in 1978 from the tanker *Amocco Cadiz* off the coast of Brittany affected 350 km of coastline. **B.** More than 2000 km of Alaskan coastline were affected in 1989 by the oil spilled from *Exxon Valdez*. **C.** The IXTOC I well blowout in 1979 released 600×10^3 t of crude oil into the open waters of the Gulf of Mexico. **D.** Oil spilled in the Arabian Gulf in 1991 affected more than 700 km of coastline. Pictures are from National Oceanic and Atmospheric Administration (www.noaa.gov).

Other examples of major oil spills include the IXTOC I well blowout in 1979 in the Gulf of Mexico (600×10^3 t of petroleum spilled) and the release of crude oil in the Arabian Gulf and Kuwaiti desert in 1991 (more than 800×10^3 t), the last one representing the largest single oil spill ever recorded (Fig. 3).

Such and other events intensified studies concerning the fate of the spilled oil, biodegradation of hydrocarbons by microorganisms, and the application of different technologies that accelerate the natural biodegradation (bioremediation) processes, in particular by overcoming the growth-limiting factors of the hydrocarbon-degrading bacteria (Atlas, 1995a; Atlas, 1995b; Swannell *et al*., 1995; Lee and Merlin, 1999; Prince *et al*., 1999).

A.1.2. The fate of crude oil spilled into the environment

Petroleum spilled in open waters has a trend to spread horizontally and to form a continuously thinning layer. The speed of such spreading depends especially on the viscosity of the crude oil and the ambient temperature. The spilled hydrocarbons are subject to a variety of physical, chemical and biological processes. Compounds with less than about 15 carbon atoms per molecule evaporate (Prince *et al*., 2003) and are either degraded photochemically (Hurley *et al*., 2001), or are scavenged from the atmosphere by rain and then biodegraded in water and soil (Arzayus *et al*., 2001). Exposure to intense sunlight can result in photooxidation of the aromatic fraction of crude oil. Laboratory experiments under exposure to UV light showed that the saturated hydrocarbons are resistant but the aromatic compounds are sensitive to photooxidation. The sensitivity to photooxidation increases with the size of the compounds and with increasing alkyl substitution. For instance, trimethyl substituted chrysene is more sensitive than chrysene, and chrysene and methyl-substituted chrysenes are more sensitive than phenanthrene and methyl-substituted phenanthrenes (Garrett *et al*., 1998, Prince *et al*., 2003). The products of photooxidation of aromatic hydrocarbons are alcohols, aldehydes, ketones and acids, and they appear in the polar fraction of crude oil (Garrett *et al*., 1998).

Further processes immediately upon oil spills are dissolution of the more water-soluble components (polar compounds, low molecular-mass aromatic hydrocarbons, such as benzene, toluene, ethylbenzene, xylenes) into the sea, and formation of emulsions of oil in water, or water in oil (the latter known as mousses) mainly as a result of wave action (Floodgate, 1984; Tkalich and Chan, 2002).

The result of the processes described above is principally dilution and partial oxygenation, but not a removal of the spilled oil. The major process for oil removal from the environment is biodegradation which, therefore, has received most attention over the past decades in the study and treatment of oil pollution (Atlas, 1981; Leahy and Colwell, 1990; Atlas and Bartha, 1992; Prince, 1993; Atlas, 1995; van Hamme *et al*., 2003) and will be described in the following section in more detail.

A.1.3. Biodegradation of crude oil

Hydrocarbons have been part of the biosphere for a long time during the history of life, and it is not surprising that the ability for hydrocarbon degradation is common among microorganisms. Hydrocarbon-degrading organisms have been isolated from numerous oxic and anoxic environments, including deep subsurface oil reservoirs (Aitken *et al*., 2004), deep-sea (Bazylinski *et al*., 1989), open or coastal seawater (Atlas and Bartha, 1972; Kasai *et al*., 2002), marine sediments (Braddock *et al*., 1995; Geiselbrecht *et al*., 1996), soils (Bossert and Bartha, 1984), freshwater (Cooney, 1984) and mountains (Margesin and Schinner, 1997). Although anaerobic biodegradation of hydrocarbons has been recently demonstrated (Heider *et al*., 1999; Widdel and Rabus, 2001), it is generally accepted that crude oil and individual hydrocarbons are much faster degraded under oxic than anoxic conditions. Moreover, it seems that aerobic microorganisms are able to degrade a wider range of hydrocarbons than anaerobic microorganisms (Prince, 2002).

A.1.3.1. Aerobic hydrocarbon-degrading bacteria

A vast number of taxonomically diverse hydrocarbon-degrading bacteria have been isolated from aerobic environments (Table 1.). Species of *Pseudomonas*, *Flavobacterium*, *Acinetobacter*, and *Vibrio* have been consistently isolated from oil-contaminated marine environments (Walker and Colwell, 1976; Austin *et al*., 1977; Shiaris and Cooney, 1983). New marine hydrocarbon-degrading bacteria have been recently isolated, including *Alcanivorax* (Yakimov *et al*., 1998), *Cycloclasticus* (Dyksterhouse *et al*., 1995), *Marinobacter* (Gauthier *et al*., 1992), *Neptunomonas* (Hedlund *et al*., 1999), *Oleiphilus* (Golyshin *et al*., 2002), and *Oleispira* (Yakimov *et al*, 2003). Many of the newly isolated bacteria use a limited range of carbon compounds as substrates, with a preference for hydrocarbons.

In unpolluted environments, the hydrocarbon-degrading bacteria constitute usually less than 0.1% of the microbial community as estimated from MPN counting of viable cells on

defined culture media (Atlas, 1981; Floodgate, 1984). Following contamination with oil, the hydrocarbon degraders are rapidly enriched and can reach up to 100% of the viable microorganisms (Walker and Colwell, 1976; Roubal and Atlas, 1978; Atlas, 1981; Lindstrom *et al*., 1991; Braddock *et al*., 1995). Using 16S rRNA-targeted oligonucleotide probes, it has been shown that the newly described *Alcanivorax* is numerically dominant in oil-contaminated seawater, where it is involved in the biodegradation of normal- and branched-alkanes (Syutsubo *et al*., 2001; Hara *et al*., 2003).

Table 1. Bacterial genera that contain aerobic hydrocarbon-degrading species (adapted from Prince, 2002)

Taxonomical group	Genera
Alphaproteobacteria	*Agrobacterium, Azospirillum, Sphingomonas, Xanthobacter, Brevundimonas*
Betaproteobacteria	*Achromobacter, Acidovorax, Alcaligenes, Comamonas, Spirillum*
Gammaproteobacteria	*Acinetobacter, Aeromonas, Alcanivorax, Alteromonas, Azotobacter, Enterobacter, Cycloclasticus, Erwinia, Escherichia, Klebsiella,Marinobacter, Moraxella, Oleiphilus, Oleispira, Pasteurella, Proteus, Pseudomonas, Serratia, Vibrio, Xanthomonas*
High-GC Gram positives	*Actinomyces, Arthrobacter, Aureobacterium*
Firmicutes	*Bacillus, Lactobacillus, Peptococcus, Sarcina*
Actinobacteria	*Brevibacterium,Corynebacterium, Micrococcus, Mycobacterium, Nocardia, Rhodococcus, Streptomyces, Thermoleophilum*
Bacteroidetes	*Cytophaga, Flavobacterium*

Marine PAH degraders include species of the genera *Cycloclasticus*, *Flavobacterium*, *Marinobacter*, *Moraxella*, *Pseudomonas*, and *Vibrio* (Geiselbrecht *et al*., 1996). Most probable number studies indicate *Vibrio* and *Cycloclasticus* as predominant PAH degraders in marine environments (Geiselbrecht *et al*., 1996; Kasai *et al*., 2002). Nevertheless, more studies are required to determine the dominant oil-degrading bacteria in marine environments, in particular species that degrade aromatic hydrocarbons. It is known for example, that *Marinobacter*, which has been consistently enriched from oil-contaminated marine environments including oil-contaminated microbial mats (McGowan *et al*., 2004), has the ability to degrade both aliphatic and aromatic hydrocarbons (Gauthier *et al*., 1992;

Hedlund *et al*., 2001). However, the abundance of *Marinobacter* in oil-contaminated marine environments is unknown.

A.1.3.2. Pathways of aerobic hydrocarbon biodegradation

Aerobic biodegradation of hydrocarbons has been known since the beginning of the 20th century, and considerable progress has been made towards the understanding of the involved biodegradation pathways. The aerobic hydrocarbon-degrading bacteria use molecular oxygen as both reactant for the activation of hydrocarbons and terminal electron acceptor. Biodegradation of alkanes has been revealed in much detail, particularly in *Pseudomonas putida* strain GPo1 (formerly *Ps. oleovorans* strain GPo1) (van Beilen *et al*., 1994). This strain oxidizes alkanes with 5 to 12 carbon atoms, but similar pathways are involved in strains that grow on alkanes with up to 20 carbon atoms (Prince, 2002). Alkanes are activated by terminal oxidation to an alcohol, a reaction catalyzed by an alkane monooxygense (Fig. 4). Monooxygenases are enzymes that incorporate one oxygen atom directly from O_2 into the substrate. The primary *n*-alkanol is further oxidized to the corresponding aldehyde and carboxylic acid which undergoes β-oxidation to acetyl-CoA that enters the citric acid cycle.

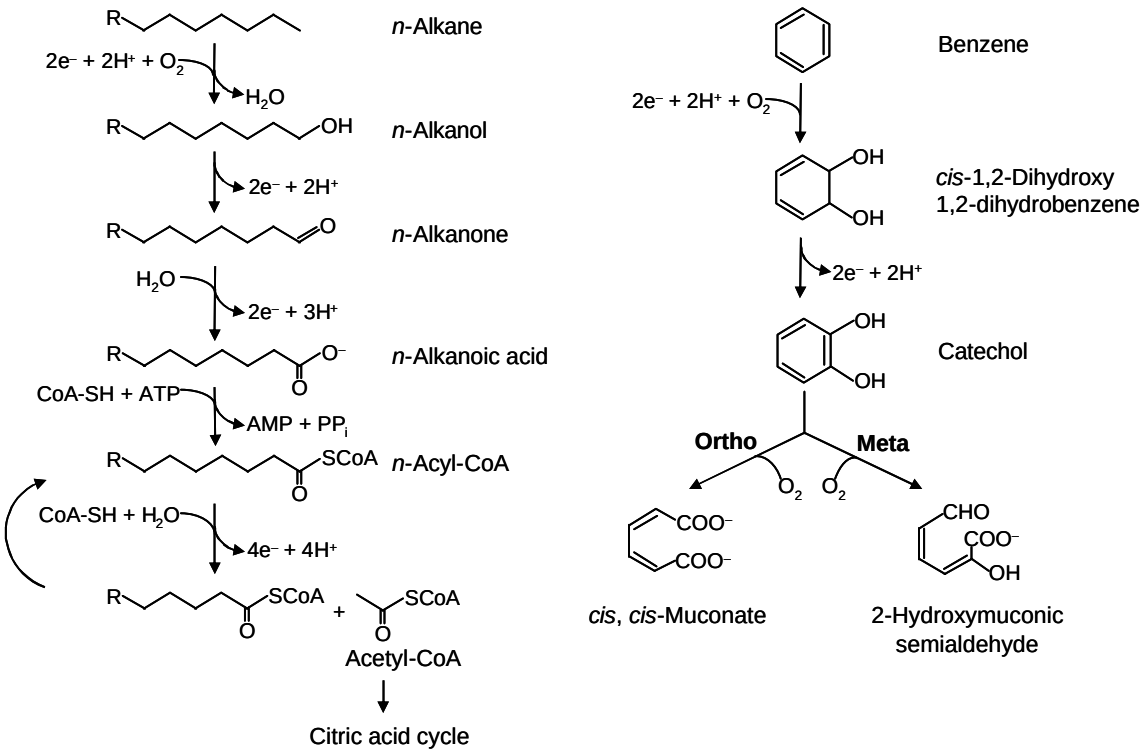


Fig. 4. General pathways of degradation of aliphatic and aromatic hydrocarbons. Adapted from Prince, 2002 and Cerniglia, 1984.

Bacteria activate aromatic hydrocarbons by oxidation to unsaturated non-aromatic *cis*-dihydrodiols (Cerniglia, 1984; Heitkamp *et al*., 1988; Kelly *et al*., 1991). The reaction is catalyzed by dioxygenases, enzymes that incorporate both atoms of molecular oxygen into the ring (Gibson and Parales, 2000). Formed dihydrodiols are first rearomatized by dehydrogenation, which in the case of benzene (and other substrates or intermediates) yields catechol, a central intermediate in the degradation pathway of aromatic hydrocarbons (Gibson, 1984; Juhasz and Naidu, 2000). Catechol can be further oxidized via two pathways, both catalyzed by ring-cleavage dioxygenases. The *ortho*-pathway involves cleavage of the bond between the carbon atoms with hydroxyl groups, yielding *cis,cis*-muconic acid. In the *meta*-pathway the bond between a carbon atom with a hydroxyl group and an adjacent carbon without a hydroxyl group is cleaved, leading to 2-hydroxymuconic semialdehyde (Fig. 4). Both ring-cleavage products are further degraded to succinate, pyruvate, acetate and fumarate, which are utilized for respiratory energy conservation and synthesis of cellular constituents (Cerniglia, 1984).

Alkyl-substituted aromatic hydrocarbons are activated either by a dioxygenase attack at the aromatic ring or by a monooxygenase attack targeting the alkyl side chain. For toluene, for example, as many as five activation pathways have been described, targeting either the aromatic ring or the methyl group (Shields *et al*., 1989; Assinder and Williams, 1990).

A.1.3.3. The possible role of cyanobacteria in petroleum-degrading microbial communities

Cyanobacteria are a very diverse group of prokaryotes with oxygenic photosynthesis similar to that encountered in higher plants (Rippka, 1979, 1988; Cohen and Gurevitz, 1992). Some strains are also capable of typical bacterial anoxygenic photosynthesis (Cohen and Gurevitz, 1992; Stal, 2000). Under low grazing pressure, cyanobacteria may accumulate together with other, functionally different groups of microorganisms, giving rise to laminated structures known as cyanobacterial (microbial) mats (Stal, 2000). More recently, growth of cyanobacterial layers on oil-contaminated sediments in coastal areas of the Arabian Gulf suggested that cyanobacteria are involved in biodegradation of petroleum hydrocarbons (Sorkhoh *et al*., 1992; Al-Hasan *et al*., 1994, 1998, 2001; Hoffman, 1996). In previous studies, it has been reported that cyanobacteria are able to partially oxidize aromatic hydrocarbons such as naphthalene, 1- and 2-methylnaphthalenes, biphenyl or phenanthrene (Cerniglia *et al*., 1980a, 1980b, 1980c; Cerniglia *et al*., 1983; Narro *et al*., 1992). However, such reports have been viewed critically because cyanobacteria are typical photoautotrophic

rather than biodegradative organisms, and a direct participation in the oxidation of oil hydrocarbons is unlikely. Moreover, there is no clear indication that cyanobacterial strains used in the reported studies were axenic. Cyanobacteria usually grow in close associations with heterotrophic bacteria, and there are indications that actually these aerobic heterotrophs are directly involved in the degradation of hydrocarbons (Abed and Köster, 2005).

Nevertheless, it has been suggested that cyanobacteria can participate indirectly to the biodegradation of hydrocarbons (Cohen, 2002). Growth of cyanobacterial layers in close contact with crude oil could provide physical support for the oil-degrading bacteria, preventing them from being washed out by waves or tides. In addition, cyanobacteria could also provide oxygen and fixed nitrogen for the aerobic oil-degrading bacteria.

A.1.3.4. Biodegradation of hydrocarbons by anaerobic bacteria

Until relatively recently it has been assumed that hydrocarbon biodegradation can only occur under oxic conditions. This view was favored because in aerobic hydrocarbon-degrading bacteria oxygen serves both as reactant in the activation mechanism and as terminal electron acceptor (Gibson, 1984). However, during the last decade it has been clearly demonstrated that hydrocarbons are oxidized under strictly anoxic conditions by novel bacteria (Heider *et al*., 1999; Widdel and Rabus, 2001; Harayama *et al*., 2004). Anaerobic, phototrophic, nitrate-, sulfate- or Fe(III)-reducing bacteria capable of growth with hydrocarbons as sole electron donor and organic carbon source have been isolated from aquatic terrestrial or marine environments (Table 2).

Utilization of *n*-alkanes was demonstrated under sulfate-reducing (Aeckersberg *et al*., 1991; So and Young, 1999) or nitrate-reducing (Ehrenreich *et al*., 2000) conditions. Alkanes are also degraded by complex microbial communities under methanogenic conditions (Zengler *et al*., 1999). Even the branched alkane, pristane, has been shown to be degraded by nitrate-reducing enrichment cultures (Bregnard *et al*, 1997). Novel anaerobic, nitrate- or sulfate-reducing bacteria that can degrade aromatic hydrocarbons such as toluene, ethylbenzene and naphthalene have been isolated (Evans *et al*., 1991; Rabus and Widdel, 1995; Galushko *et al*., 1999; Meckenstock *et al*., 2000). Considerable progress has been made toward elucidating the anaerobic activation mechanisms of hydrocarbons, and examples are known for aliphatic, aromatic and alkyl-substituted aromatic hydrocarbons.

Table 2. Diversity of anaerobic bacteria with the ability to degrade saturated and aromatic hydrocarbons.

Taxonomical group	Organisms	Hydrocarbons utilized	References
Alphaproteobacteria	*Blastochloris sulfoviridis*	Toluene	Zengler *et al*., 1999
Betaproteobacteria	*Thauera aromatica*	Toluene	Evans *et al*., 1991
	Azoarcus sp. strain EbN1	Ethylbenzene, toluene	Rabus and Widdel, 1995
	Azoarcus sp. strain HxN1	Alkanes (C_6–C_8)	Ehrenreich *et al*., 2000
Gammaproteobacteria	*Vibrio* sp. NAP-4 *Pseudomonas* sp. NAP-3	Naphthalene	Rockne *et al*., 2000
Deltaproteobacteria	Strain NaphS2	Naphthalene	Galushko *et al*., 1999
	Strain mXyS1	*m*-Xylene, *m*-ethyltoluene, toluene	Harms *et al*., 1999
	Desulfobacula toluolica	Toluene	Rabus *et al*., 1993
	Strain Hxd3	Alkanes (C_{12}–C_{20})	Aeckersberg *et al*., 1991
	Geobacter metallireducens		Lovely *et al*., 1993

The alkanes are anaerobically activated by addition to fumarate, yielding (1-methylalkyl)succinate (Rabus *et al*., 2001). The reaction proceeds via a radical mechanism involving carbon-2 of the alkane (Fig. 5.). The biodegradation pathway is supposed to continue by conversion of (1-methylakyl)succinate to the CoA-thioester, rearrangement of the C-skeleton, decarboxylation and further β-oxidation (Wilkes *et al*., 2003).

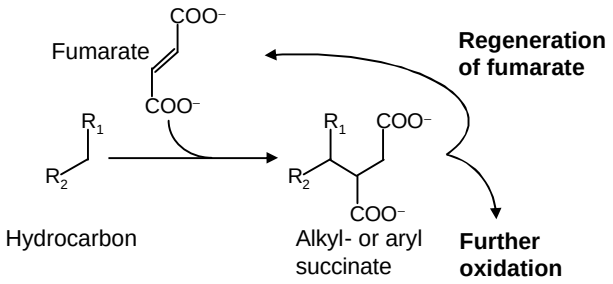


Fig. 5. Saturated and alkyl-substituted aromatic hydrocarbons are anaerobically activated by addition to fumarate, yielding alkyl- or arylsuccinate derivatives. Fumarate is regenerated through further degradation.

Aromatic hydrocarbons with alkyl substituents such as toluene, ethylbenzene, or 2-methylnaphthalene are also activated by addition to fumarate, yielding benzylsuccinate (Evans *et al*., 1992), (1-phenyethyl)succinate (Kniemeyer *et al*., 2003), or naphthyl-2-

methyl-succinate (Meckenstock *et al.*, 2004), respectively (Fig. 5.). However, the denitrifying strain EbN1 activates ethylbenzene by a different mechanism: ethylbenzene is oxidized by ethylbenzene dehydrogenase to 1-phenylethanol, which is further transformed to benzoyl-CoA (Rabus *et al.*, 2002).

There are no data yet of how benzene, the simplest aromatic hydrocarbon, is activated anaerobically. The proposed pathway for the activation of the unsubstituted polycyclic aromatic hydrocarbons naphthalene and phenanthrene includes an initial carboxylation yielding 2-naphthoic acid and a phenanthrene carboxylic acid, respectively (Zhang and Young, 1997).

A.1.4. Factors that influence biodegradation of crude oil in the environment

Biodegradation of petroleum hydrocarbons in the marine environment is influenced by the oil composition and load, and by environmental factors such as temperature, the availability of oxygen and inorganic nutrients (nitrogen, phosphorus, and iron), pH, salinity, and pressure (Atlas, 1981; Leahy and Colwell, 1990). Essentially, any factor influencing the activity of the hydrocarbon-degrading bacteria will ultimately influence the biodegradation rates.

The chemical composition, physical state and local quantity of the oil or component hydrocarbons influence both the rate and extent of degradation. Different oil fractions are degraded at different rates; as a general rule, biodegradability decreases in the following order: *n*-alkanes > branched alkanes > low-molecular-mass aromatics > cycloalkanes > PAH > complex polar compounds (Floodgate, 1984; Leahy and Colwell, 1990). The different degradation rates are the result of several factors, most notably the bioavailability of the hydrocarbons for the hydrocarbon-degrading bacteria, the enzyme activity, and the degree of toxicity of some hydrocarbons.

It is generally accepted that hydrocarbons interact with microorganisms non-specifically and move passively into the cell (Bateman *et al.*, 1986). Therefore, hydrocarbon uptake can only occur if the hydrocarbon-degrading bacteria come in direct contact with their substrates. Bacteria may access the substrate via the (usually very small) fraction dissolved in water, via direct adherence to oil droplets or layers, or by interaction with oil pseudosolubilized by biosurfactants (Bouchez-Naitali *et al.*, 1999). The resulting bioavailability may significantly influence the rate and extent of biodegradation of crude oil. Direct contact with the water-solubilized hydrocarbons is limited by the solubility of hydrocarbons in water, which decreases with increasing molecular mass and with the degree of saturation (Sikkema *et al.*,

1995, Table 3). An increase in the surface area of spilled oil as a result of wave action for example (physical emulsification) may increase the dissolution rate and improve substrate bioavailability.

Aerobic hydrocarbon-degrading bacteria have been frequently shown to produce biosurfactants, e.g. lipopolysaccharides and rhamnolipids, which form hydrocarbon emulsions in aqueous systems (Hommel, 1990) or, as cell surface-associated compounds, allow adhesion of the cells to the hydrophobic substrate (Zhang and Miller, 1992, 1994). Bacterial strains are known that grow directly on crude oil droplets and can only be removed by addition of an exogenous surfactant (Van Hamme and Ward, 2001). The result is a reduction of the distance between the hydrocarbon phase and the cell and increased exchange surface such that mass transfer is enhanced. But this also implies that the adherence of bacteria to oil droplets or layers is in turn limited by the surface of the oil layers. Uptake of hydrocarbons is also increased by formation of hydrocarbon emulsions in water by biosurfactants. As a result of emulsification, the exchange surface is increased, which facilitates higher dissolution rates and prevents mass transfer limitation. For instance, growth of *Pseudomonas* strains with long-chain alkanes was enhanced by pseudosolubilization of hydrocarbon with biosurfactants (Sekelsy and Shreve, 1999) or by encapsulating solid alkanes in liposomes (Miller and Bartha, 1989). Also, biodegradation of aromatic compounds in crude oil by *Rhodococcus rhodochrous* was enhanced by production of biosurfactants (Iwabuchi *et al*., 2002).

Table 3. Data for some aromatic hydrocarbons, showing the decrease in solubility with increasing molecular mass (adapted from Sikkema *et al*., 1995)

Compound	Formula	M_r	Solubility (mmol/l at 25 ^oC)
Benzene	C_6H_6	78.11	22.900
Toluene	C_7H_8	92.14	6.280
Ethylbenzene	C_8H_{10}	106.17	1.270
o-xylene	C_8H_{10}	106.17	2.020
Naphthalene	$C_{10}H_8$	128.17	0.797
Phenanthrene	$C_{14}H_{10}$	178.23	0.025

The toxic effects of hydrocarbons are highest in case of the volatile hydrocarbons, such as the monoaromatics benzene, toluene, ethylbenzene, and xylenes (BTEX). Accordingly, refined oil, which is rich in volatile hydrocarbons, is more toxic to microorganisms than crude oil (Sikkema, 1995). Alkanes are usually regarded as having little or no toxic effects.

A stimulation of the microbial uptake of compounds with low dissolution rates (e.g. naphthalene and phenanthrene), for example by addition with a solvent (Smith *et al*., 1991), could also result in an increased toxic effect (Sikkema *et al*., 1995).

As long as petroleum is in the liquid form, it floats on water and forms a continuously thinning layer with increasing surface contact for potential contact with hydrocarbon-degrading bacteria. However, gradual evaporation and adsorption to sediments can lead to the formation of highly viscous to solid layers (asphalt) or tarballs, which are very resistant to degradation and can persist for many years in the environment (Leahy and Colwell, 1990; Barth, 2003).

Temperature has a strong influence on both physical nature and chemical composition of the oil, and the rate of hydrocarbon metabolism by microorganisms. At low temperatures the viscosity of crude oils increases and the evaporation of toxic, low-molecular mass compounds is reduced, thus delaying the onset of biodegradation. The metabolic activity is also reduced at low temperatures. Nevertheless, biodegradation of petroleum at low temperatures has been described (Cundell and Traxler, 1973; Juck *et al*., 2000; Yakimov *et al*., 2004). Higher temperatures lead to faster evaporation of the low-molecular mass compounds, thus reducing the toxicity of crude oil. The rates of biodegradation by mesophilic species typically increase with the temperature, reaching a maximum around 30 ^{o}C, above which the membrane toxicity of hydrocarbons is increased, thus leading to decreased biodegradation rates (Leahy and Colwell, 1990). But also degradation of hydrocarbons at temperatures as high as 70 ^{o}C has been reported (Klug and Markowetz, 1967; Mateles *et al*., 1967). Thermophilic alkane-degrading bacteria have been isolated from high-temperature environments such as a deep-sea hydrothermal vent (Bazylinski *et al*., 1989).

One of the most important factors that often limit biodegradation is the availability of of oxygen, and there are indeed significant differences in the extent of biodegradation in oxic and anoxic environments (Prince, 2003). In the presence of adequate amounts of oxygen, availability of inorganic nutrients, such as bound nitrogen, phosphorus and iron, is likely to be the most important limiting factor, because these elements are not provided by crude oil (Pritchard *et al*., 1992). Studies on oil degradation in the marine environment have indeed shown that growth and activity of hydrocarbon-degrading microorganisms is strongly limited by bound nitrogen and phosphorus, followed by iron (Prince, 2002; Floodgate, 1984; Leahy and Colwell, 1990). As a result, hydrocarbon biodegradation was strongly stimulated in both laboratory experiments (Atlas and Bartha, 1972a, 1972b; Oh *et al*., 2001; Olivieri *et al*.,

1976) and field applications (Bragg *et al*., 1994; Prince *et al*., 2003; Prince and Bragg, 1997) by fertilization with combined nitrogen and phosphorus. But also iron has been recognized as a growth-limiting element in the marine environment, and supplementation with iron has been proven to increase the rate of crude oil degradation (Dibble and Bartha, 1976).

However, in order to estimate the importance of these factors, accurate determinations of the extent of petroleum biodegradation under different environmental or laboratory conditions are needed. The methods currently used to quantify the extent of biodegradation, with their particular advantages and limitations, will be discussed in the following section.

A.1.5. Quantification of oil biodegradation

Since crude oils are complex mixtures of thousands of compounds, estimation of the extent of crude oil biodegradation is not an easy task. Several methods are currently employed to estimate the extent of petroleum biodegradation: determination of qualitative and quantitative changes in oil composition (Bragg *et al*., 1994), measurement of bulk oil-carbon mineralization via CO_2 determination (Atlas and Bartha, 1972), gravimetric determination of the residual oil fraction (Atlas and Bartha, 1992), and spiking of crude oil with ^{14}C-labelled hydrocarbons and quantification of formed $^{14}CO_2$ (Walker and Colwell, 1976).

Biodegradation of crude oil is commonly quantified by determination of quantitative and qualitative changes in oil composition by gas chromatography (GC) and mass spectrometry (MS) analyses (Atlas and Bartha, 1992; Swannell *et al*., 1996). These methods allow the determination of the extent of biodegradation of individual hydrocarbons and classes of hydrocarbons. However, a main limitation is that not all compounds in crude oil can be resolved by GC, and biodegradation of the usually large unresolved fraction cannot be estimated. Also, high-molecular mass alkanes and PAH with 30 and more atoms of carbon cannot be analyzed by this method since they lack sufficient volatility (Atlas and Bartha, 1992). Nevertheless, because the other methods require controlled laboratory conditions (see below), GC-MS has been used to estimate biodegradation of crude oil *in situ* (Atlas and Bartha, 1992). Since distribution of crude oil in the environment has a considerable degree of patchiness and estimation of the initial amount of oil for a certain sample is difficult, a large number of replicate samples must be analyzed in order to obtain statistically valid results (Atlas and Bartha, 1992; Bragg *et al*, 1994). For this reason, the degree of biodegradation has been more often estimated by comparing the molecular ratios of GC-resolvable hydrocarbons within the biodegraded sample with those determined for the original oil. The approach is based on the assumption that branched alkanes (e.g. pristane and phytane) are

biodegraded at a much lower rate than normal alkanes (Atlas, 1981; Swannel, 1996). Therefore, an increase of the ratio branched/normal alkanes in the biodegraded sample versus the original oil may permit to estimate the extent to which the hydrocarbons have been degraded (Pritchard and Costa, 1991). These molecular ratios are not normally affected by evaporation or dissolution, and are useful indicators of biodegradation, as long as the internal marker is not itself biodegraded. Under conditions where the branched alkanes are also degraded (Dible and Bartha, 1976; Atlas and Bartha, 1992), this assumption is invalid, and alternative natural internal standards, such as hopanes (polycyclic saturated hydrocarbons), are needed (Prince *et al*., 1994; Bragg *et al*., 1994). Nevertheless, even hopanes have been proven to be biodegraded by crude oil-degrading enrichment cultures isolated from hydrocarbon-contaminated soils (Frontera-Suau *et al*., 2002).

Alternatively, measurements of bulk oil-carbon mineralization by quantification of the CO_2 formed during biodegradation have been used (Atlas and Bartha, 1972a, b). Such experiments require closed experimental systems, the presence of defined amounts of petroleum and sufficient volume of air such that biodegradation is not limited by availability of oxygen. The measured CO_2 is related to the amount of carbon present in the crude oil. By this approach, the extent of complete mineralization of the oil-carbon can be accurately determined. However, conversion of oil to biomass and partial oxidation of some hydrocarbons may not be estimated by this method (Atlas and Bartha, 1992).

The extent of biodegradation may also be quantified by extraction of residual oil with solvents and evaporation of the solvent followed by gravimetric determinations. Gravimetric determinations may be used in closed, experimental systems and require the use of defined amounts of petroleum in the experiment. However, such determinations may overestimate biodegradation if volatile compounds are lost during evaporation of the extracting solvent. Conversely, biodegradation may be underestimated if compounds not found in crude oil or intermediates of biodegradation that have incorporated oxygen are also extracted and increase the residual weight (Atlas and Bartha, 1992).

Biodegradation of petroleum has been also estimated by spiking crude oil with ^{14}C-hydrocarbons and measurement of the formed $^{14}CO_2$ (Walker and Colwell, 1976b). It assumes that the biodegradation of the labeled compounds is representative for all other components of crude oil, even though this is rather unlikely (see p. 14). Therefore, quantifications using this method are limited to single substrates.

In conclusion, there is no single method to accurately determine the extent of oil biodegradation, and only the application of complementary methods under defined conditions may lead to unbiased results.

A.2. Nitrogen fixation – a brief literature overview

It is generally accepted that nitrogen availability is a major factor regulating primary production in shallow marine environments (Capone, 2001). Biological nitrogen fixation, the reduction of atmospheric N_2 to biologically available ammonium, has been recently recognized as an important source of nitrogen in the marine environment (Capone, 2001; Zehr *et al*., 2001; Zehr *et al*., 1998). Biological nitrogen fixation is exclusively a property of prokaryotes. All major groups of cyanobacteria have representatives able to fix nitrogen, including unicellular and non-heterocystous species (Bergman *et al*., 1997). The ability to fix nitrogen is also common among members of anoxygenic phototrophic bacteria (Chromatiaceae, Chlorobiaceae, Chloroflexaceae and Rhodospirillaceae), chemoautotrophic bacteria, and heterotrophic bacteria, including aerobes, microaerophiles, facultative and strict anaerobes (Zehr *et al*., 2003).

Nitrogen fixation is catalyzed by nitrogenase, a two component protein system with a molecular mass of about 245 kDa, which reduces N_2 to NH_3 in an energy-demanding process according to:

$$N_2 + 8\ H^+ + 8\ e^- + 16\ ATP \quad ? \quad 2\ NH_3 + H_2 + 16\ ADP + 16\ P_i \qquad (1)$$

Nitrogenase consists of two different metalloproteins, the molybdenum-iron (MoFe-) protein (or dinitrogenase) and the iron (Fe-) protein (or dinitrogenase reductase). The Fe-protein is a homodimer and contains an [4Fe:4S] cluster. The Fe-protein can bind two molecules of ATP per dimer and couples ATP hydrolysis with electron transfer to the FeMo-protein, which contains the substrate reduction site. The MoFe-protein is a heterotetramer with the general structure $\alpha_2\beta_2$; each $\alpha\beta$ heterodimer contains 2 metal centers, the P-cluster (two [4Fe:4S] clusters) that participates in the electron transfer from the Fe-protein, and the FeMo-cofactor (7Fe:9S:Mo), which is the site of nitrogen reduction (Yoo *et al*., 2000; Rees and Howard, 2000). Alternative nitrogenases with vanadium instead of molybdenum or only iron have also been described (Eady, 1996), but the molybdenum-containing nitrogenases (often referred to as "conventional" nitrogenases) are the most widespread in nature, and the most intensively studied to date.

Nitrogenase components are encoded by the genes *nifH*, *nifD* and *nifK*; *nifH* codes for the Fe-protein, and *nifD* and *nifK* code for the α- and β-subunits, respectively, of the MoFe-protein. In addition to the structural genes, a number of other gene products are required for regulation and assembly of the functional proteins. Nitrogenase gene expression is highly regulated at the transcriptional (Chen *et al*., 1998) and post-translational levels (Kim *et al*., 1999). Due to the high energy requirement of nitrogen fixation, the *nifHDK* operon is strictly regulated in response to factors that control nitrogen fixation, such as oxygen or availability of fixed nitrogen (ammonia, nitrate).

The structural *nif* genes are well conserved and have been used to investigate the diversity and activity of the nitrogen-fixing microorganisms in many different habitats. The studies of nitrogenase diversity have been largely based on the phylogenetic analysis of *nifH* gene sequences (Zehr and McReynolds, 1989). Nitrogenase *nifH* genes fall into four basic groups designated as Clusters I – IV (Zehr *et al*., 2003). Cluster I contains conventional Mo-containing nitrogenases from Proteobacteria (Alpha, Beta, Epsilon and Gamma), Cyanobacteria and Firmicutes, and some alternative vanadium-containing nitrogenases. The sequences from non-Mo, non-V nitrogenases, together with some archaeal sequences form Cluster II. Cluster III contains sequences from distantly related mocroorganisms, many of which are strict anaerobes (Deltaproteobacteria, Firmicutes, Spirochetes, Green sulphur bacteria). Cluster IV is a divergent group of non-functional *nif*-like sequences from Archaea. The *nifH* database is one of the largest non-ribosomal gene libraries, with more than 2000 sequences, most of them from environmental samples. Culture-independent methods have revealed a great diversity of *nifH* sequences in habitats ranging from invertebrate guts, plants, soils, lakes, rivers and microbial mats, to the open ocean (Ueda *et al*., 1995; Okhuma *et al*., 1999; Steppe *et al*., 1996, 2001; Zehr *et al*., 1998; Lovell *et al*, 2000; Hurek *et al*., 2002; Omoregie *et al*., 2004).

The ability of cyanobacteria to fix nitrogen and the recent observation that cyanobacterial layers develop on oil-contaminated sediments suggested that these organisms may indirectly stimulate biodegradation of petroleum by providing fixed nitrogen for the oil-degrading heterotrophs. Given the high bioenergetic requirements of nitrogen fixation, photoautotrophic microorganisms such as cyanobacteria have a significant advantage over heterotrophic microorganisms, whose nitrogen fixing ability is limited by the availability of organic energy substrates. Even if crude oil is a very rich source of carbon and energy and contains only marginal amounts of bound nitrogen, the ability to fix nitrogen is not common among oil-degraders, and there are only a limited number of reports indicating nitrogen

fixation by hydrocarbon-degrading bacteria (Table 4). Moreover, heterotrophic nitrogen fixation in crude oil or hydrocarbon-contaminated environments has not been directly linked with the activity of the hydrocarbon-degrading microorganisms (Eckford *et al*, 2002; Piehler *et al*., 1999; Toccalino *et al*., 1993).

Heterotrophic nitrogen fixation during growth on polar substrates (carbon compounds other than hydrocarbons) is well known, and it has been demonstrated that an addition of plant structural (alginate, xylan) or storage polysaccharides (glycogen, laminarin) to marine sediments strongly stimulates nitrogen fixation (Tibbles *et al*., 1994). However, it is possible that the high oxygen requirement for hydrocarbon biodegradation or the formation of some free oxygen radicals as by-products by mono- or dioxygenases may be incompatible with nitrogenase activity.

Table 4. Examples of known hydrocarbon-degrading bacteria able to fix atmospheric nitrogen

Strain	Substrates used for nitrogen fixation	Reference
Azomonas agilis ATCC 7494 *Azospirillum brazilense* Sp7 *A. lipoferum* Sp 59b *Azotobacter chroococcum* ATCC 9043 *A. vinelandii* strain UW *Beijerinckia mobilis* ATCC 35011	Naphthalene	Chen *et al*., 1993
Agrobacterium sp. strain 3A *Alcaligenes* sp. strain 5A	Gasoline Benzene Toluene Xylene	Prantera *et al*., 2002

High rates of nitrogen fixation have been measured in cyanobacterial mats from temperate and tropical environments (Herbert, 1999). In such communities, formation of microoxic niches and excretion of fermentation products or polysaccharides by cyanobacteria may stimulate nitrogen fixation by aerobic heterotrophs.

In contrast to aerobic bacteria, the capability for nitrogen fixation in active anaerobic bacteria is never impeded by oxygen, and they often posses nitrogenases and may contribute to the bound nitrogen in oil-contaminated environments. For instance, the strictly anaerobic bacteria such as sulfate reducers are known to have the ability to fix atmospheric nitrogen (Widdel, 1987; Postgate and Kent, 1985; Widdel and Bak, 1992). Also, it has been demonstrated that sulfate-reducing bacteria are responsible for the bulk nitrogen fixation associated with the roots or rhizomes of seagrass meadows (*Spartina alterniflora, Zostera noltii* and *Z. marina*) (Welsh *et al*., 1996; Gandy and Yoch, 1988; Nielsen *et al*., 2001).

Sulfate-reducing bacteria are important constituents of microbial mats and many strains have been isolated that can degrade hydrocarbons (Aeckersbers *et al*., 1991; Rabus *et al*., 1993; Rueter *et al*., 1994; Harms *et al*., 1999, as examples).

So far however, the ability of cyanobacteria or sulfate-reducing bacteria to fix nitrogen in oil-contaminated environments has not been studied, and thus still presents an attractive hypothesis for experimental and environmental studies.

A.3. Objectives of the present work

Contamination of coastal marine environments with crude oil is likely to trigger the development of dense microbial communities on and in the oiled sediments; members of these communities may directly or indirectly participate in the oil biodegradation process.

(a) To gain a functional understanding of the development and activities of oil-associated microbial groups and of the long-term fate of compact oil layers, a controllable procedure for surface contamination of marine sediment with crude oil was developed in the present work. The development of microbial communities on and in the oil layers was described by observation of color changes and microsensor measurements. Pristine sediments maintained under the same conditions were used as controls.

(b) To date, there are a vast number of studies dealing with the biodegradation of crude oil or individual hydrocarbons. Significant knowledge has been gained about biodegradation of different crude oil fractions in natural environments, for example in investigations of oil biodegradation upon accidental or experimental spills, and in laboratory experiments. Biodegradation pathways of individual hydrocarbons, the enzymes involved, the genes and genetic regulation of catabolic operons for hydrocarbon oxidation have been described in much detail. However, there is little information about the extent of oil-carbon mineralization under conditions without mineral limitation for bacterial growth. The extent of aerobic oil mineralization was therefore quantified by measurement of the CO_2 produced during oil biodegradation in closed oxic systems, with supplementation of inorganic nutrients and optimum distribution of the petroleum to avoid uncontrollable limitations.

(c) The availability of fixed nitrogen strongly limits the extent of oil biodegradation. It is possible that cyanobacteria growing on oil-contaminated sediments may indirectly enhance biodegradation of crude oil by providing fixed nitrogen to the heterotrophic oil-degrading bacteria. Physiological and molecular methods were used in this work to investigate nitrogen fixation by microbial communities growing on oil-contaminated marine sediments in microcosm experiments. Nitrogen fixation was investigated using the acetylene-reduction assay and quantification of total nitrogen. Molecular methods based on the amplification and reverse-transcription of the nitrogenase *nifH* genes or their transcripts, respectively, were applied to reveal the identity and activity of the nitrogen-fixing bacteria.

B. Results and discussion – an overview

For a functional understanding of the biotic and abiotic factors that influence biodegradation of crude oil on marine sediments, experimental approaches under controlled but *in situ* or near-*in situ* conditions are needed (Oh *et al*., 2001; Prince *et al*., 2002; Shi *et al*., 1999; Röling *et al*., 2002). A first important goal of this study was the establishment of microbial guilds that develop under near *in situ* conditions upon surface contamination of marine sediments with crude oil and contribute to its degradation. Once such microcosms had been established, it was observed that oil layers were still present after prolonged incubation, suggesting incomplete degradation. The next objective was therefore to quantify biodegradation of petroleum distributed on inert solid mineral matrices in separate experiments. The experiments with the added oil layer also revealed that fertilization with inorganic nutrients greatly stimulated biodegradation of oil, thus indicating severe nitrogen limitation of the oil-degrading community. A third objective was, therefore, to investigate possible fixation of atmospheric nitrogen in the microbial communities growing on oil-contaminated sediments in the experimental microcosms.

B.1. Establishment of stratified microbial communities on oil-contaminated marine sediments in experimental microcosms

As a starting material for the establishment of microbial guilds on oil-contaminated sediments oxic, clay-like sediment from a mud flat (Wadden Sea) of the North Sea was used. If incubated anoxically, this sediment did not turn dark, indicating that sulfate reduction and hence the load of readily utilizable organic compounds are not significant. The content of organic carbon of this sediment was relatively low (8 mg per g dry sediment) and therefore interference by other heterotrophic degradation processes was not significant in comparison to oil degradation.

The wet but not flooded sediment was placed in glass aquaria and covered by a layer of similar, dried sediment. Crude oil was added (0.2 ml per cm^2, Fig. 7.A) and allowed to loose the volatile fraction by evaporation, a process that very often occurs *in situ*, following accidental oil spills. Occasionally, dried sediment was powdered over the oil layer, to secure the adherence to the mineral support. Oil that has lost the volatile fraction by evaporation will hereafter be referred to as weathered oil. Following evaporation, the oil-sediment layers became increasingly viscous, such that water addition did not cause buoyancy of the oil. To provide a potentially high diversity of oil-degrading bacteria, the sediment was inoculated

with a mixture of samples collected from geographically different hydrocarbon-contaminated sites. The aquaria were placed inside a greenhouse and maintained under light/dark conditions.

Within four weeks of incubation, the oiled sediment regained its initial color, suggesting aerobic oil degradation. Also, growth of cyanobacteria over the oil layers was observed (Fig. 6.B, C). Occasionally, patches of sulfur-oxidizing bacteria developed (Fig. 6.C, highlighted). However, below the surface (~ 1 mm), the oil layer was still present, even after incubation for more than 1 year. Below the oil layer, the sediment appeared black, suggesting that the added oil promoted anoxia and sulfate reduction. This was indeed confirmed by microsensor measurements, showing complete oxygen consumption at 1 mm depth and elevated concentrations of sulfide below this zone (Fig. 7). The sediment in the oil-free aquarium (control) was gradually covered by a loose layer of cyanobacteria, and maintained its initial color at all depths.

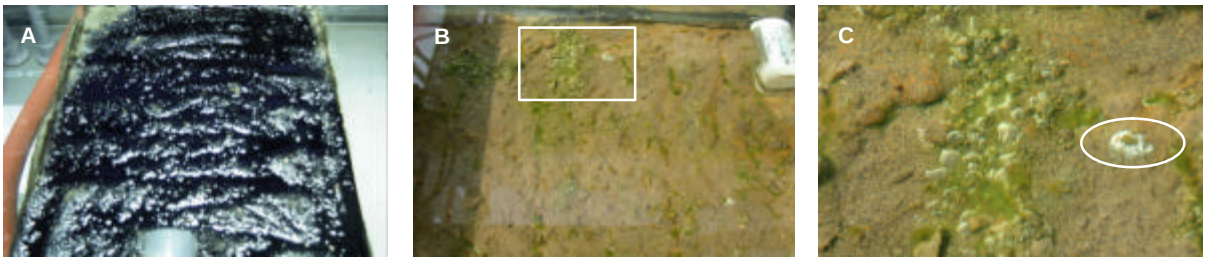


Fig. 6. Visible differences between the sediment directly upon contamination with oil (A), and after four weeks of incubation (B). A section (framed in B) with a patch of whitish bloom of sulfide-oxidizing bacteria (circled) is shown at higher magnification (C); also, oxygen bubbles formed in a flock of filamentous phototrophs (Cyanobacteria, Algae) are visible. Figure adapted from Musat *et al.*, 2004 (see Part II, 1).

During the first two months, there was no apparent stimulation of oxygenic phototrophic organisms by the oil in comparison to the pristine sediment. Patchy layers of filamentous cyanobacteria developed in both, the oil-contaminated and the pristine aquaria. However, microscopic examination of the developing patches of phototrophic organisms revealed that non-heterocystous cyanobacteria (*Oscillatoria*-like) tended to dominate the contaminated sediment whereas there was more heterogeneity on the pristine sediment; the most notable among the latter were heterocystous cyanobacteria (*Anabaena*-like) and diatoms. Upon prolonged incubation, however, the oil layer became gradually felt-like by increasing development of cyanobacteria and possibly other filamentous organisms on its upper surface. It is presently unclear whether the oil-associated phototrophic organisms present a higher biomass than those in the pristine controls, and which factors cause association of the

filaments with the oil surface. On the one hand, there may be a purely physical explanation for the later increased development of cyanobacterial layers, viz. the aged oil may present a favorable substratum for attachment and growth of cyanobacteria. On the other hand, biodegradation of oil may enhance the supply of CO_2 for photosynthesis. So far, experiments with axenic strains of cyanobacteria in the presence of hydrocarbons do not indicate any degradative ability (Musat and Widdel, unpublished), in agreement with other observations (Abed and Köster, 2005). Moreover, according to recent reports, the growth of cyanobacterial layers on oil-contaminated sediments in the Arabian Gulf is only opportunistic (Barth, 2003).

Oxygen and sulfide profiles were measured in the light and in the dark for the oil-contaminated and pristine sediments (Fig. 7). The surface layers of the oil-contaminated sediment were photosynthetically active, as indicated by the high levels of oxygen produced. However, the oxygen concentration decreased in a steep gradient to depletion at 1.0 mm depth (Fig. 7). One explanation for this observation is that crude oil represents a highly concentrated source of reducing equivalents, and biodegradation of oil requires a large amount of oxygen per amount of substrate. Consequently, oxygen is rapidly scavenged by aerobic oil-degrading bacteria, leading to anoxic conditions below the oil layer. Once installed, such conditions are likely to persist, because diffusion of oxygen from the overlying water is severely limited by oil. In the pristine sediment, oxygen diffusion is not hampered, reaching depths more than 2.0 mm.

The pronounced sulfidogenesis, indicated by the sulfide profiles measured in light and in dark, can be explained by the direct utilization of oil compounds by sulfate-reducing bacteria (Rabus *et al.*, 1996; Rueter *et al.*, 1994). Alternatively, sulfate-reducing bacteria may use oxygenated products from the incomplete oxidation of hydrocarbons by aerobic oil-degrading bacteria (Belyaev *et al.*, 1982; Nazina *et al.*, 1985), organic metabolites excreted by cyanobacteria, or dead microbial biomass. Photosynthesis ceased during incubation in dark, leading to the expansion of the anoxic area in the upper layers. In the pristine sediments, no free sulfide could be measured in the light or in the dark (Fig. 7).

In conclusion, the microcosms established in this study offer a valuable experimental tool for investigating, under defined conditions, phenomena observed at oil-contaminated sites. For instance, the significance of light for the oil-degrading community or of oxygen for the formation of substrates for sulfate-reducing bacteria may be studied by the use of parallel incubations under dark versus light or anoxic versus oxic conditions, respectively.

Furthermore, the influence of nutrient addition (e.g. ammonia, nitrate, phosphate) on oil degrading and associated microbial communities may be studied in such systems.

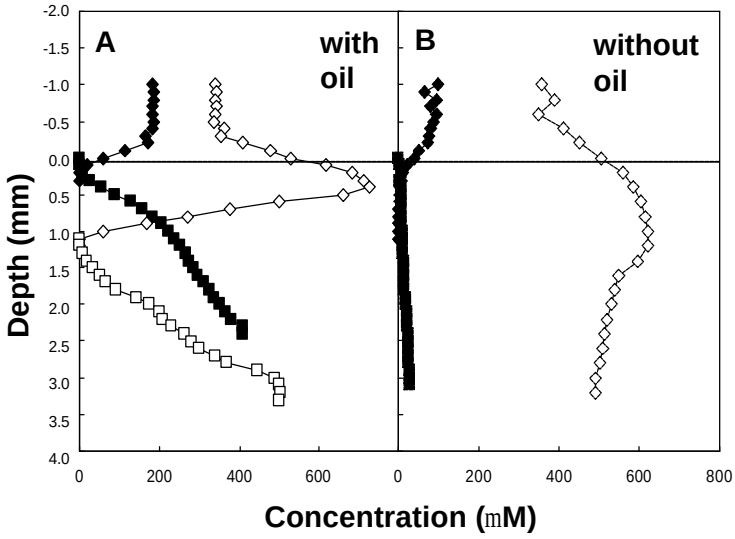


Fig. 7. Microprofiles of oxygen in light (◇) and dark (◆), and sulfide in light (□) and dark (■) in the oil-contaminated (A) and pristine (B) sediments after 2 months of incubation. No free sulfide was measured in the pristine sediment in light. From Musat *et al*., 2004 (see Part II, 1); and Abed and Musat, unpublished.

However, the observation that oil layers were still present even after incubation for more than one year showed that biodegradation was rather incomplete, even though the oil layer was rather thin. This observation led to the central question as to which extent petroleum can be mineralized biologically under optimum conditions, viz. with sufficient dispersion and biominerals.

B.2. Quantification of petroleum biodegradation on marine sediments in experimental microcosms

B.2.1. Quantification of oil degradation via CO_2 analysis

Since crude oil is a very concentrated form of reducing equivalents, the requirement of oxygen per amount of substrate is high. Conventional experiments involve high amounts of oil, which for a quantification of CO_2 in a closed system would require voluminous gas phases. If, on the other hand, very small droplets are added that do not deplete the O_2 reservoir, they are poorly accessible by bacteria. Consequently, our experiments were designed in order to obtain significant O_2 consumption and CO_2 production, but also such that O_2 was still present after growth to avoid O_2 limitation. First, the suitable amount of crude oil to be added to a closed system with a defined volume of air was calculated assuming the approximate bulk formula for crude oil $<CH_2>$, yielding the following equation for aerobic biodegradation:

$$CH_2 + 1.5\ O_2 \quad ? \quad CO_2 + H_2O \qquad (2).$$

Accordingly, 1.0 g (71.4 mmol) of crude oil requires 3.4 g oxygen (106.25 mmol O_2), corresponding to 2.6 l pure O_2 or 13 l of air under normal conditions. Consequently, a closed system with a head-space of 1 l contains the amount of oxygen theoretically needed for the complete oxidation of 77 mg of crude oil. Therefore, the amount of oil added in our biodegradation experiments in 1-l bottles never exceeded 77 mg, unless otherwise specified. Because this is a relatively small amount of oil, we attempted to obtain optimum distribution to avoid limitations in substrate accessibility. Considering that (a) a certain fraction of this oil will be lost by evaporation before the degradation experiment, that (b) crude oil is not completely oxidized to CO_2 and H_2O, and that (c) a significant fraction is incorporated into biomass, the amount of oxygen under such experimental conditions should be even present in excess. Nevertheless, during all biodegradation experiments the oxygen concentration was continuously monitored to verify that it did not become the limiting factor.

To calculate the extent of oil degradation, the experimentally quantified CO_2 was compared with the theoretical amount of CO_2 that would be formed by complete oxidation of the added oil. The theoretical amount of CO_2 is equimolar with the total carbon content of crude oil as determined by combustion analyses (Table 5). Only oil that has lost the volatile fraction via evaporation was used. Oil depleted in the volatile fraction was prepared by dissolving defined amounts of petroleum (between 5 and 8 mg) in dichloromethane (DCM),

distribution on sand, and by allowing the volatile fraction to evaporate. The determined mass of the volatile fraction amounted to up to 34% of the lightest crude oil (Table 5). When weight changes were no longer recorded (usually after 5 to 6 days), the carbon content of the weathered oil was determined by combustion analysis (Table 5).

Table 5. The extent of evaporation of crude oils and the carbon content of oils depleted in the volatile fraction.

Crude oil	Amount lost by evaporation at 25 °C (% wt/wt)	Carbon in the weathered oil	
		(% by mass)	(mmol [g oil]$^{-1}$)
Gulf of Mexico	18 ± 1.4	90.0	75
Casablanca	27 ± 2.9	93.5	78
North Sea	24 ± 0.7	73.5	61
Sinstorf	34 ± 1.0	94.6	79

The same procedure was used to prepare weathered oil for the biodegradation experiments. Optimal conditions for the quantitative oil degradation experiments were determined by investigating the influence of the addition of inorganic nutrients, and oil distribution on different solid mineral substrata on the rate of microbial CO_2 production.

B.2.2. Establishment of optimal conditions for quantitative study of oil degradation

It has been shown that fertilization with inorganic nutrients (ammonia, phosphate and iron(III)) greatly stimulated biodegradation of petroleum in laboratory experiments and field applications (Atlas and Bartha, 1972; Dibble and Bartha, 1976; Olivieri *et al*., 1976; Pritchard and Costa, 1991; Prince and Bragg, 1997). Nevertheless, an exact quantification of the extent of mineralization of crude oil spread on marine sediments, under conditions of fertilization with inorganic nutrients, has not been attempted so far. The effect of supplementation with inorganic nutrients on crude oil biodegradation was investigated first using Gulf of Mexico crude oil distributed on coarse-grain sand. As expexted, the rate of oil mineralization was significantly higher if inorganic nutrients were supplemented. However, the extent of mineralization under such conditions was still less than one would expect in view of the apparent decrease of oil that is often obvious in degradation experiments. Only 13% of the initial oil in the experiments supplemented with inorganic nutrients, and 8% in the experiments without addition of inorganic nutrients were mineralized within 360 days of incubation (Fig. 8).

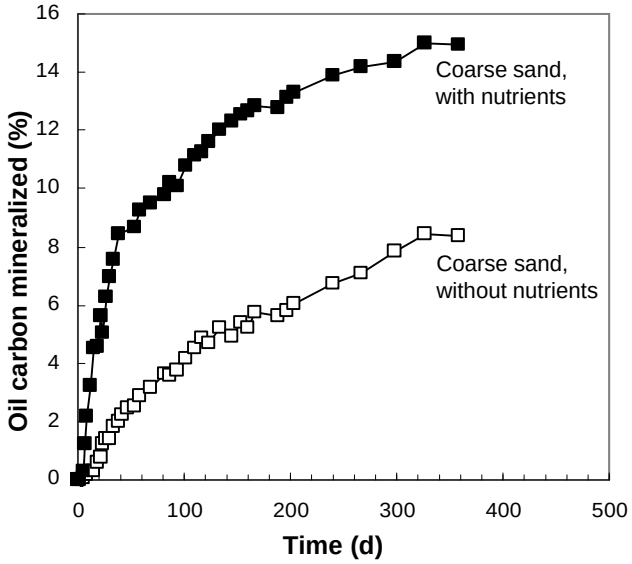


Fig. 8. Mineralization of weathered Gulf of Mexico crude oil (75 mg) finely distributed on coarse-grain sand (ca. 50 g) in experimental microcosms (1-l bottles), with and without supplementation of inorganic nutrients (NH_4Cl, KH_2PO_4, Fe(III)).

Furthermore, the influence of the solid mineral matrices on biodegradability of crude oil was investigated. Mineralization of Gulf of Mexico crude oil was investigated upon distribution on (a) clay-like sediment, (b) limestone gravel (0.5 to 1.0 cm), (c) fine-grain sand (<0.1 to 0.1 mm) and (d) coarse-grain sand (0.3 to 0.7 mm). All experiments, including the oil-free controls were supplemented with inorganic nutrients. The use of clay-like minerals and gravel yielded high levels of CO_2 release also in the oil-free controls (data not shown). This may be due to the release of CO_2 from carbonate minerals or to the degradation of organic compounds present in these materials. Because low background CO_2 production is required for quantitative determinations of oil biodegradation, these materials were not included in further quantitative studies. The use of fine- and coarse-grain sand always yielded low CO_2 release in the control experiments, indicating that sand was a suitable carrier material. However, the results with fine-grain sand in paralel incubations were inconsistent due to buoyancy of the oil-sand particles. Thus, the coarse-grain sand, as it has been used already in the initial experiments, was the carrier material of choice for all other experiments.

Knowing the importance of fertilization with inorganic nutrients and the optimum solid substratum, the effect of the extent of distribution on biodegradation of crude oil was further studied. To obtain different extents of distribution, four methods of oil addition were used: Gulf of Mexico crude oil dissolved in DCM was (a) finely distributed on coarse-grain sand, or (b) added on the glass surface in bottles that received no sand; alternatively, oil that was

not dissolved in organic solvents was (c) added directly over water or (d) as a compact droplet on sand. The highest mineralization rates were obtained with oil finely distributed on sand (Fig. 9). Significantly lower mineralization rates were obtained with oil added over glass, due to inhomogeneous distribution and adherence of oil to the glass. Addition of oil over water yielded degradation rates comparable with those obtained with oil finely distributed on sand; however, the results with parallel incubations were poorly reproducible, due to the irregular adherence of oil to the glass. The lowest extent of mineralization was obtained when oil was added as a droplet to the sand. Although inorganic nutrients were supplemented as in the other experiments, the extent of biodegradation was limited by the poor distribution that ultimately affects substrate availability (Fig. 9).

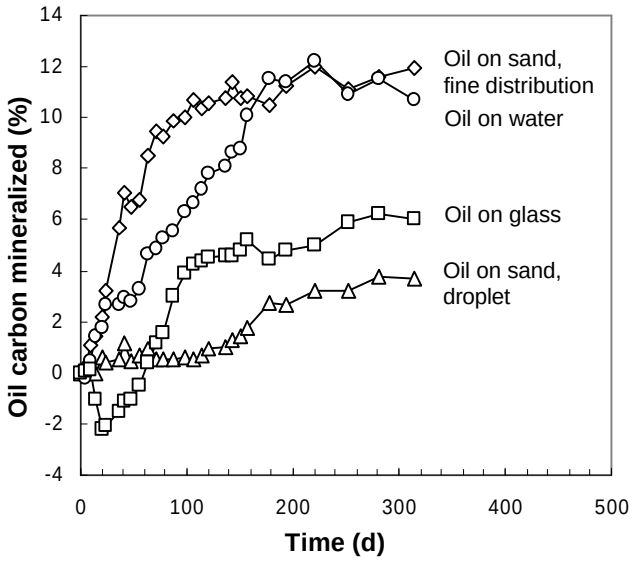


Fig. 9. The influence of distribution on the extent of petroleum mineralization. Crude oil was differently distributed on coarse-grain sand, and all experiments were supplemented with inorganic nutrients.

B.2.3. Quantitative mineralization experiments with different types of crude oil

Considering fertilization with inorganic nutrients and fine distribution of crude oil on coarse-grain sand as optimum conditions, biodegradation of different types of crude oil depleted of the volatile fraction was investigated. For comparison with respect to degradability, naphthalene as a poorly soluble yet defined hydrocarbon and glucose as a well-degradable defined soluble substrate were included in parallel experiments. Four different crude oil types, Gulf of Mexico, Casablanca, North Sea and Sinstorf, were used. Mineralization of all crude oils was very low in comparison to mineralization of naphthalene and glucose (Fig. 10). Thus, the extent of oil mineralization within 240 days of incubation ranged from 15%

(Gulf of Mexico) to 20% (Sinstorf) of the initial oil-carbon, while 65 and 77% of the added naphthalene and glucose, respectively, were mineralized.

Previous studies on the extent of mineralization of crude oil in seawater showed that as much as 42% of petroleum has been mineralized within 32 days (Atlas and Bartha, 1972a, b). However, the authors used a paraffinic crude oil that contained high amounts of *n*-alkanes (paraffins), compounds known to be relatively easily biodegradable according to field studies and laboratory investigations (Leahy and Colwell, 1990; Bragg *et al*., 1992; Prince 1993). In the present study, however, the situation upon oil spills in nature was simulated, viz. the oil was allowed to loose the light fraction through evaporation and to adhere to solid mineral support. With respect to bioavailability, viz. distribution of the oil, the results are expected to allow the maximum extent of oil biodegradation.

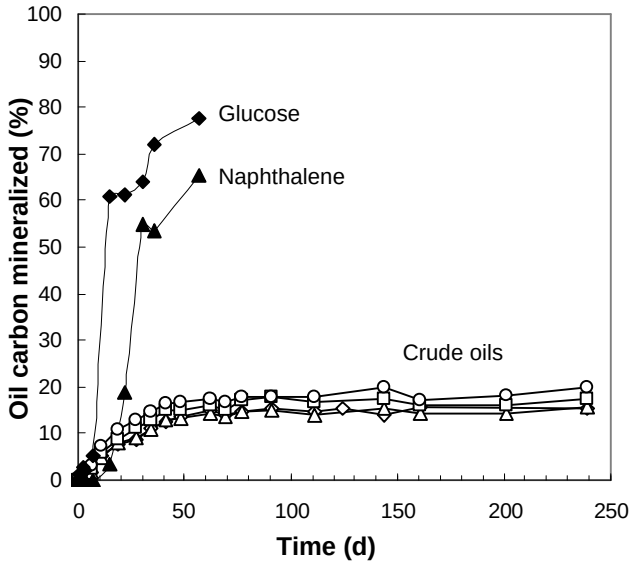


Fig. 10. Mineralization of Gulf of Mexico, Casablanca, North Sea and Sinstorf crude oils under optimum conditions. Crude oils (75 mg each) were dissolved in DCM and finely distributed on coarse-grain sand (ca. 50 g) in 1-l bottles. Mineralization of the defined compounds, naphthalene (80 mg) and glucose (280 mg) is shown for comparison.

Biodegradation is certainly accompanied by assimilation of oil-carbon into biomass. In growth studies with bacteria or yeasts on defined hydrocarbons it has been shown that the assimilated proportion is as high as 50% of the supplied carbon (Morgan and Watkinson, 1994; Linton and Stephenson, 1978; Shennan, 1984). If we consider similar carbon assimilation in the present study, the total extent of oil-carbon removal due to biodegradation (assimilation and mineralization) will range from 23 to 30% of the initially, non-weathered oil (Fig. 11). However, such estimates were obtained with pure cultures in bioreactors optimized for biomass production. In an enriched complex microbial community, grazing,

cell death and further biodegradation of the assimilated carbon is expected to decrease the actually present biomass significantly. When glucose and naphthalene were used as substrates in the degradation experiment, more than 50% of the original carbon was recovered as CO_2. Consequently, it would be more reasonable to consider the present living and dead biomass to be around 35% of the biodegraded carbon. In such conditions, the biomass (assimilation) presented in Fig. 11 would be overestimates.

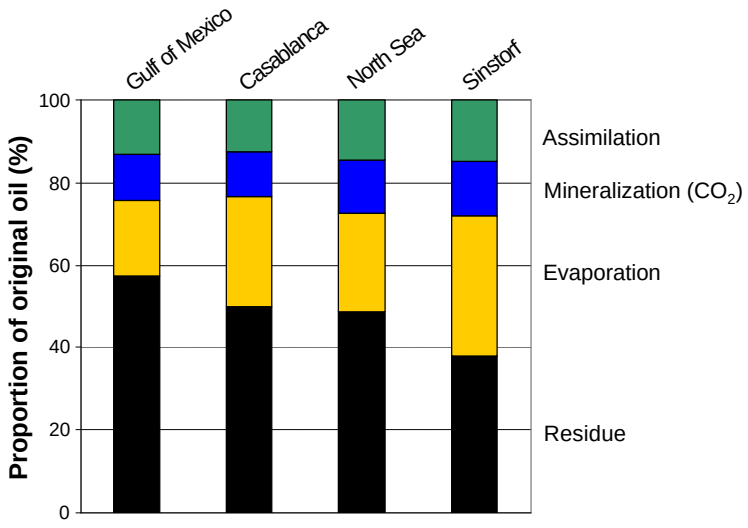


Fig. 11. The calculated extent of petroleum removal by evaporation and biodegradation (mineralization and assimilation) under optimum distribution conditions and with supplementation of inorganic nutrients.

Chemical analyses of the residual fraction clearly showed extensive biodegradation of the branched and normal alkanes. With inorganic nutrient supplementation, *n*-alkanes with up to 39 carbon atoms were completely biodegraded (Fig. 10). The branched alkanes pristane (Pri) and phytane (Phy) were also degraded, but to a lesser extent. Without supplementation with inorganic nutrients biodegradation of saturated hydrocarbons was significant, but less extensively.

The molecular ratios Pri/*n*C17 and Phy/*n*C18 are widely used to estimate the extent of biodegradation. Increasing values of these ratios are considered to indicate biodegradation of oil, based on the assumption that branched alkanes are less susceptible to biodegradation than *n*-alkanes. The molecular ratios Pri/*n*C17 and Phy/*n*C18 determined for the biodegraded samples were clearly higher than those of the weathered oil, indicating loss of *n*-alkanes due to biodegradation (Table 6). Most importantly, there was no significant difference of these ratios between the weathered and the original oil (Table 6).

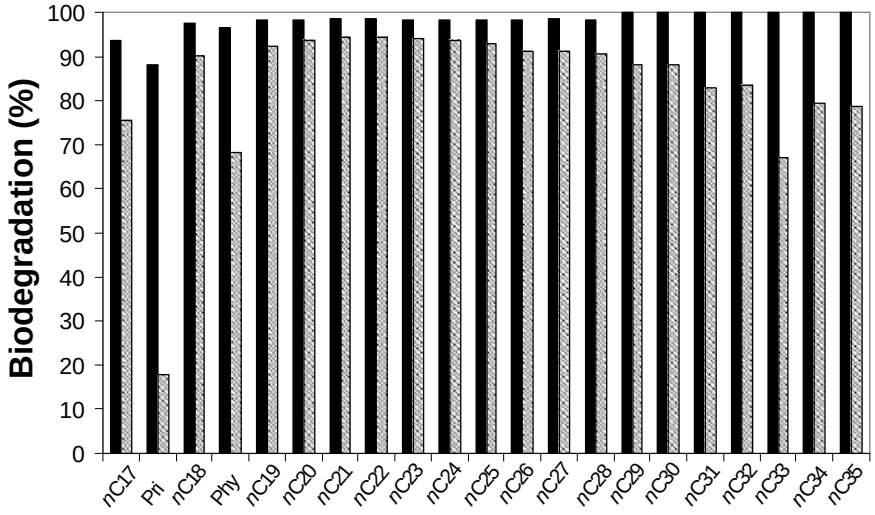


Fig. 12. Biodegradation of normal and branched alkanes of weathered Gulf of Mexico crude oil finely distributed on coarse-grain sand. Solid columns: biodegradation with supplementation of inorganic nutrients; hatched columns: biodegradation without inorganic nutrients.

There is no information yet concerning the nature of the persistent fraction. It has been assumed that, for example, the structurally complex polar fraction of crude oil is very resistant to degradation by microorganisms (Atlas, 1981; Atlas and Bartha, 1992). Also, polycyclic aromatic hydrocarbons (PAH) with more than 3 rings may be only incompletely oxidized, leading to oxygen consumption but no CO_2 production. Such partly oxidized PAH may covalently bind to macromolecules, for example to humic or fulvic acids, or may form dimers or trimers via covalent ether or ester bonds, thus significantly reducing their availability for microbial degradation (Richnow *et al*., 1993, 1997; Kästner *et al*., 1999). Although humic or fulvic acids are commonly found in soils, such compounds may be introduced into the marine environment through river inputs, or similar compounds may be formed for instance by decomposition of algal biomass.

Table 6. Molecular ratios of branched- versus *n*-alkanes for original, weathered and biodegraded Gulf of Mexico crude oil.

Sample	Pri/*n*C17	Phy/*n*C18
Gulf of Mexico crude oil	0.31	0.47
Gulf of Mexico weathered oil	0.32	0.49
Degradation with inorganic nutrients	0.62	0.72
Degradation without inorganic nutrients	1.08	1.58

Although biodegradation obviously accounts for only a small fraction, probably this particular fraction is the most problematic with respect to environmental effects. Following biodegradation of this fraction, the remaining residue most likely consists of large compounds, e.g. asphaltenes or partly oxidized PAH, with considerably reduced toxicity. Moreover, such large compounds are solid and are as inert as the macromolecules encountered in the composition of soils.

Quantification of crude oil mineralization revealed that biodegradation was strongly stimulated by addition of inorganic nutrients. Since the ability to fix atmospheric N_2 as a source of inorganic nitrogen is widespread among prokaryotes and may contribute significantly to the nitrogen budget of certain marine environments (see p. 17-20), the next major objective was to investigate the capacity to fix atmospheric N_2 of microbial communities growing on oil-contaminated and pristine marine sediments.

B.3. Nitrogen fixation in microbial communities growing on oil-contaminated and pristine marine sediments

The microcosms established in the aquaria with and without oil represented a suitable source of samples to analyze the presence, phylogenetic affiliation and expression of *nifH* genes. For direct measurement of nitrogen fixation activity by the acetylene reduction (AR) assay, the aquaria were less suitable, because of a certain patchiness and the relatively high amounts of samples needed for various parallel incubations. For AR assays, microcosms were established *de novo* in several small bottles but with the same starting material as used in the aquaria.

B.3.1. Molecular characterization of the diazotrophic bacteria

Nitrogenase (*nifH*) genes in the enriched microbial communities were analyzed using universal PCR primers for the dinitrogense reductase gene (Zehr and McReynolds, 1989). Via a gene library, 43 sequences were retrieved from the oil-contaminated sediment and 41 from the pristine sediment. The deduced protein sequences were aligned to the SwissProt NifH database using the ARB software package (Ludwig *et al*., 2004). Phylogenetic trees were reconstructed using the neighbor joining method in ARB and the phylogenetic position of representative NifH sequences is shown in Fig. 13 and 14.

A high diversity of NifH sequences was obtained from the microbial community developed on oil-contaminated sediments. The NifH sequences obtained from the upper section were most similar with NifH from Cyanobacteria (*Anabaena* sp, *Lyngbya* sp., and *Phormidium* sp.), Gammaproteobacteria (*Vibrio* sp., *Pseudomonas* sp., *Marichromatium* sp. and *Methylobacter* sp.), Betaproteobacteria (*Azoarcus* sp.) and Deltaproteobacteria (*Desulfovibrio* sp. and *Geobacter* sp.) (Fig. 13, 14). All sequences recovered from the lower section fall within Cluster III, and were closely related to NifH from Deltaproteobacteria (*Desulfovibrio* sp, *Desulfobacter* sp., *Geobacter* sp.) and green-sulfur bacteria (*Chlorobium* sp.) (Fig. 14). The *nifH* genes retrieved reflect a selection of functional groups due to oil contamination and stratification.

The NifH sequences obtained from the upper section of the pristine sediment fall within Cluster I. Most of them (15 out of 26) were closely related (91 to 97% similarity) to the second nitrogenase gene from the heterocystous cyanobacterium *Anabaena variabilis* ATCC 29413 (Thiel *et al*., 1995). Other sequences were closely related with NifH from non-heterocystous cyanobacteria (*Phormidium* sp., *Lyngbya lagerheimii* and *Cyanothece* sp.) and Gammaproteobacteria (*Pseudomonas* sp.) (Fig. 13). The sequences obtained from the lower

section fall within Cluster III and were similar to NifH from *Desulfovibrio* sp. and *Geobacter sulfurreducens* (Fig. 14).

In addition, the expression of *nifH* was investigated under both light and dark conditions using reverse transcription and PCR (RT-PCR). Expression analysis showed that sequences related with the second nitrogenase (*nif2*) from *Anabaena variabilis* ATCC 29413 were expressed in the light in both, the oil-contaminated and pristine microcosms. The second nitrogenase of *A. variabilis* is expressed only under anoxic conditions in both vegetative cells and heterocysts (Thiel *et al*., 1995). These findings suggest that genes similar with *nif2* could be expressed in heterocysts or vegetative cells in micro-oxic or anoxic microniches within the laminated, mat-like structures. Alternatively, since the distribution of *nif2*-like genes among heterocystous cyanobacteria is not known, the found sequences could be also expressed during the day under oxic conditions.

Sequences related to NifH from non-heterocystous cyanobacteria such as *Lyngbya* and *Phormidium* were expressed in the dark in both microcosms (Fig. 13). These sequences were similar with NifH previously described for well-developed *Microcoleus* and *Lybgya* mats (Omoregie *et al*., 2004a). It has been shown that nitrogen fixation in these mats was maximal at night (Omoregie *et al*., 2004b). This suggests that in addition to N_2 fixation during daytime, the oil-contaminated and pristine microcosms may also fix nitrogen at night.

Sequences related to NifH from Deltaproteobacteria were amplified from both microcosms. Expression analysis showed that these sequences were expressed only in the oil-contaminated sediment, irrespective of the illumination conditions (Fig. 14). Sulfate-reducing bacteria are known to have the ability to fix atmospheric nitrogen (Postgate and Kent, 1985; Widdel, 1987; Widdel and Bak, 1992). Also, nitrogen fixation by sulfate-reducing bacteria has been demonstrated in marine environments such as the rhizosphere of seagrass meadows (Gandy and Yoch, 1988; Herbert, 1999; Nielsen *et al*., 2001) or pristine, natural microbial mats (Steppe and Paerl, 2002). In such environments, sulfate-reducing bacteria benefit from the supply of organic carbon by the plant roots. Similarly, cyanobacteria, known to excrete glycollate or fermentation products such as acetate and lactate, could support growth and nitrogen fixation of the sulfate-reducing bacteria (Stal, 1995). However, the contribution of sulfate-reducing bacteria to the nitrogen balance in our experiments is probably marginal, since sediments incubated permanently under anoxic conditions revealed very low acetylene reduction activity (data not shown).

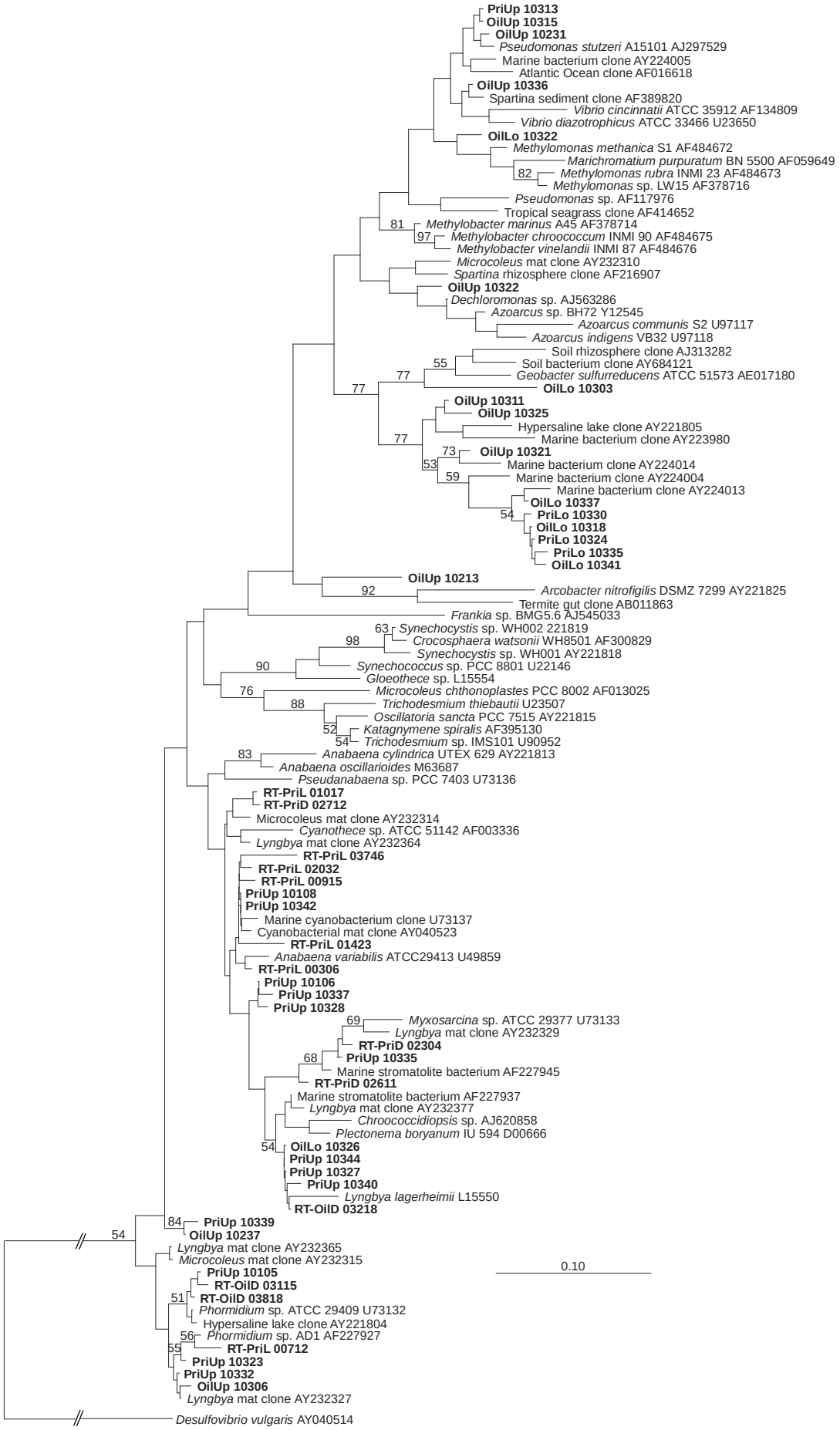

PriUp 10313
OilUp 10315
OilUp 10231
Pseudomonas stutzeri A15101 AJ297529
Marine bacterium clone AY224005
Atlantic Ocean clone AF016618
OilUp 10336
Spartina sediment clone AF389820
Vibrio cincinnatii ATCC 35912 AF134809
Vibrio diazotrophicus ATCC 33466 U23650
OilLo 10322
Methylomonas methanica S1 AF484672
Marichromatium purpuratum BN 5500 AF059649
82
Methylomonas rubra INMI 23 AF484673
Methylomonas sp. LW15 AF378716
Pseudomonas sp. AF117976
Tropical seagrass clone AF414652
81
Methylobacter marinus A45 AF378714
97
Methylobacter chroococcum INMI 90 AF484675
Methylobacter vinelandii INMI 87 AF484676
Microcoleus mat clone AY232310
Spartina rhizosphere clone AF216907
OilUp 10322
Dechloromonas sp. AJ563286
Azoarcus sp. BH72 Y12545
Azoarcus communis S2 U97117
Azoarcus indigens VB32 U97118
Soil rhizosphere clone AJ313282
55
Soil bacterium clone AY684121
77
Geobacter sulfurreducens ATCC 51573 AE017180
77
OilLo 10303
OilUp 10311
OilUp 10325
Hypersaline lake clone AY221805
77
Marine bacterium clone AY223980
73
OilUp 10321
53
Marine bacterium clone AY224014
59
Marine bacterium clone AY224004
Marine bacterium clone AY224013
OilLo 10337
54
PriLo 10330
OilLo 10318
PriLo 10324
PriLo 10335
OilLo 10341
OilUp 10213
92
Arcobacter nitrofigilis DSMZ 7299 AY221825
Termite gut clone AB011863
Frankia sp. BMG5.6 AJ545033
63
Synechocystis sp. WH002 221819
98
Crocosphaera watsonii WH8501 AF300829
Synechocystis sp. WH001 AY221818
90
Synechococcus sp. PCC 8801 U22146
Gloeothece sp. L15554
76
Microcoleus chthonoplastes PCC 8002 AF013025
88
Trichodesmium thiebautii U23507
Oscillatoria sancta PCC 7515 AY221815
52
Katagnymene spiralis AF395130
54
Trichodesmium sp. IMS101 U90952
83
Anabaena cylindrica UTEX 629 AY221813
Anabaena oscillarioides M63687
Pseudanabaena sp. PCC 7403 U73136
RT-PriL 01017
RT-PriD 02712
Microcoleus mat clone AY232314
Cyanothece sp. ATCC 51142 AF003336
Lyngbya mat clone AY232364
RT-PriL 03746
RT-PriL 02032
RT-PriL 00915
PriUp 10108
PriUp 10342
Marine cyanobacterium clone U73137
Cyanobacterial mat clone AY040523
RT-PriL 01423
Anabaena variabilis ATCC29413 U49859
RT-PriL 00306
PriUp 10106
PriUp 10337
PriUp 10328
69
Myxosarcina sp. ATCC 29377 U73133
Lyngbya mat clone AY232329
RT-PriD 02304
68
PriUp 10335
Marine stromatolite bacterium AF227945
RT-PriD 02611
Marine stromatolite bacterium AF227937
Lyngbya mat clone AY232377
Chroococcidiopsis sp. AJ620858
Plectonema boryanum IU 594 D00666
54
OilLo 10326
PriUp 10344
PriUp 10327
PriUp 10340
Lyngbya lagerheimii L15550
RT-OilD 03218
84
PriUp 10339
54
OilUp 10237
Lyngbya mat clone AY232365
Microcoleus mat clone AY232315
PriUp 10105
RT-OilD 03115
51
RT-OilD 03818
Phormidium sp. ATCC 29409 U73132
Hypersaline lake clone AY221804
56
Phormidium sp. AD1 AF227927
55
RT-PriL 00712
PriUp 10323
PriUp 10332
OilUp 10306
Lyngbya mat clone AY232327
Desulfovibrio vulgaris AY040514
0.10

Fig. 13. Phylogenetic tree of Cluster I NifH showing the position of representative sequences obtained in this study (bold). Pri: pristine microcosm; Oil: oil-contaminated microcosm; Up: upper section; Lo: lower section; L: light; D: dark; RT: reverse-transcription.

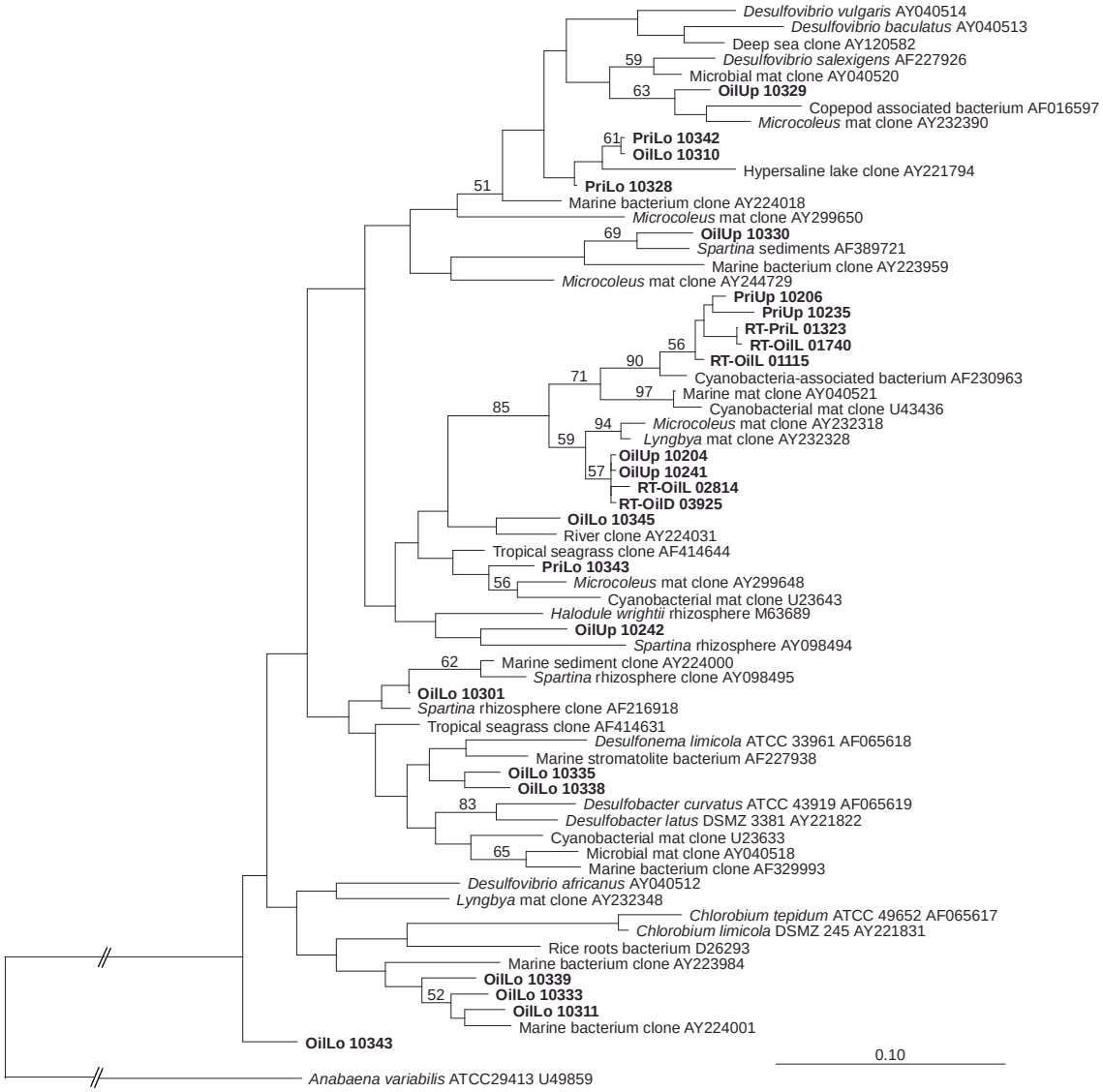


Fig. 14. Phylogenetic tree of Cluster III NifH showing the position of representative sequences obtained in this study (bold). Pri: pristine microcosm; Oil: oil-contaminated microcosm; Up: upper section; Lo: lower section; L: light; D: dark; RT: reverse-transcription.

Sequences related to NifH from Betaproteobacteria, Gammaproteobacteria and green-sulfur bacteria that were obtained by direct amplification were not retrieved by RT-PCR, suggesting that the corresponding organisms were not actively involved in N_2 fixation.

B.3.2. Nitrogenase activity in microbial communities on oil-contaminated and pristine sediments

The nitrogen fixation activity was measured for microbial functional groups developed on oil-contaminated and pristine sediments in experimental microcosm by the acetylene-reduction (AR) assay (Stewart *et al*., 1967). Activity measurements with samples from the previously established oil-contaminated and pristine microcosms (Musat *et al*., 2004) were inconsistent due to patchiness of the system. Moreover, whenever the laminated structure of the mat-like community was disturbed during sampling, the AR rates were significantly reduced (data not shown). For these reasons, microbial communities were *de novo* established in bottles supplied with sediment or with sediment contaminated with crude oil (Gulf of Mexico). In order to distinguish between nitrogen fixation by phototrophic or heterotrophic microorganisms, parallel bottles were established that were either incubated under natural light-dark conditions or permanently in the dark. This resulted in the development of mat-like structures, dominated by filamentous phototrophic microorganisms in the light, and of exclusively heterotrophic microbial communities in the experiments incubated in the dark. As positive control for heterotrophic nitrogen fixation, parallel bottles supplied with alginate instead of crude oil were incubated in the dark.

The nitrogenase activity of the microcosms incubated under light/dark conditions was determined both in the light and dark. Both, the oil-contaminated and the pristine sediment exhibited highest AR in the light (Fig. 15) with the maximum rate within 32 days of incubation (Fig. 15, 16).

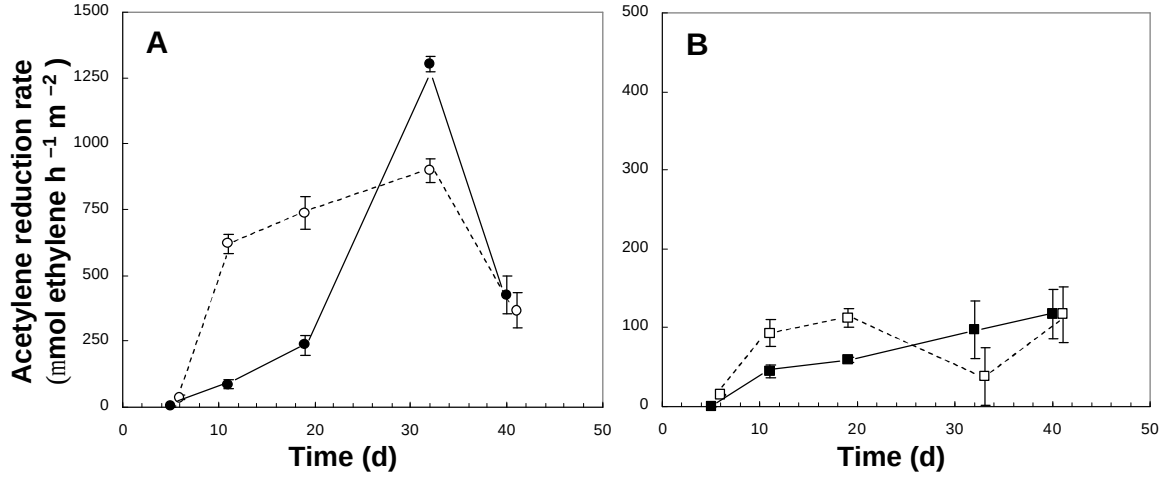


Fig. 15. Acetylene reduction activity of the microbial communities developed on oil-contaminated and pristine sediments under light/dark conditions. **A.** The activity in the light for microbial communities developed on oil-contaminated (●) and pristine sediments (○). **B.** The AR activity in the dark for the microbial communities on oil-contaminated (■), and pristine (□) sediments. Each point represents the average of measurements of triplicate bottles which were sacrificed for analysis (dicontinuos monitoring).

During the initial 20 days of incubation, the AR rates of the oil-contaminated sediment were consistently lower than those of the pristine sediment, suggesting that the development of diazotrophic organisms was initially inhibited by oil (Fig. 15).

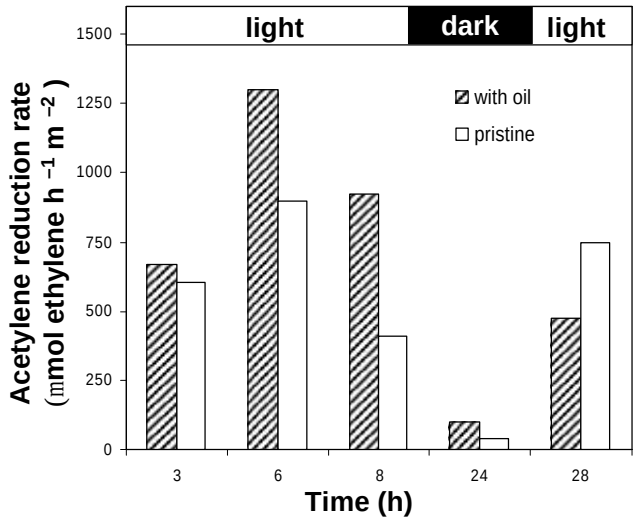


Fig. 16. Acetylene reduction activity of the microbial communities developed on oil-contaminated and pristine sediments incubated under light/dark conditions. The activity was measured after 32 days of incubation. The sediments were incubated in the light for 8 h, in the dark for 16 h and shifted back in light for 4 h.

The AR activity of the oil-contaminated and pristine sediments incubated permanently in the dark, i.e. pure heterotrophic nitrogen fixation, was much lower (Fig. 17). For the oil-contaminated sediment the AR activity was almost undetectable within 30 days of incubation; low activity was measured after 40 days of incubation (Fig. 17). The AR activity of pristine sediments incubated permanently in the dark was undetectable. However, when such sediments were provided with alginates, significant AR rates were determined after relatively short incubation time (Fig. 17). The activity decreased upon longer incubations, probably due to depletion of the substrate.

Quantification of total bound nitrogen suggested a small increase as a result of N_2 fixation for the oil-contaminated and pristine microcosms incubated under light/dark conditions. The increase, calculated for sample dry mass, was about 8 mmol N mg^{-1} for the oil-contaminated sediment and about 15 mmol N mg^{-1} for the pristine sediment. However, an exact quantification was difficult to obtain, due to the heterogeneity of the clay-like sediment which caused significant scattering in triplicate determinations (data not shown).

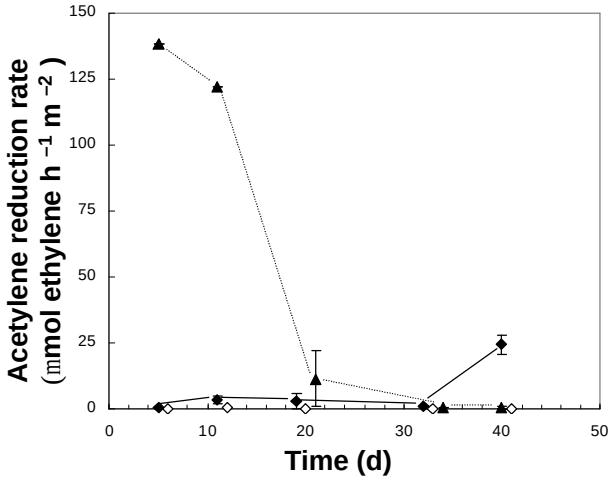


Fig. 17. Acetylene reduction activity in the microbial communities developed on oil-contaminated (◆) and pristine (◇) sediments during permanent incubation in the dark. Higher rates of acetylene reduction were obtained if alginates were added to the pristine sediments (▲) (discontinuous monitoring as in Fig. 15).

In conclusion, the present study indicates that cyanobacteria, most notably heterocystous cyanobacteria, were responsible for the bulk nitrogen fixation in the oil-contaminated and pristine microcosms. The ability to fix N_2 is not common among hydrocarbon-degrading bacteria, as indicated by the low number of studies reporting heterotrophic N_2 fixation with hydrocarbons as growth substrate (Chen *et al.*, 1993; Prantera *et al.*, 2002). Moreover, biodegradation of crude oil is strongly limited by the availability of bound nitrogen (see p. 15). The nitrogen fixed by cyanobacteria may be transferred, for example upon death and decay of the cyanobacterial filaments, to the oil-degrading bacteria and thus stimulate biodegradation of crude oil.

References

Abed, R. M. M., and J. Köster. 2005. The direct role of the aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds. International Biodet Biodeg **55:**29-37.

Aeckersberg, F., F. Bak, and F. Widdel. 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch Microbiol **156:**5-14.

Aitken, C. M., D. M. Jones, and S. R. Larter. 2004. Anaerobic hydrocarbon biodegradation in deep subsurface oil reservoirs. Nature **431:**291-294.

Al-Hasan, R. H., N. A. Sorkhoh, D. Al Bader, and S. S. Radwan. 1994. Utilization of hydrocarbons by cyanobacteria from microbial mats on oily coasts of the Gulf. Appl Microbiol Biotechnol **41:**615-619.

Al-Hasan, R. H., D. A. Al-Bader, and N. A. Sorkoh. 1998. Evidence for *n*-alkane consumption and oxidation by filamentous cyanobacteria from oil-contaminated coasts of the Arabian Gulf. Mar Biol **130:**521-527.

Al-Hasan, R. H., M. Khanafer, M. Eliyas, and S. S. Radwan. 2001. Hydrocarbon accumulation by picocyanobacteria from the Arabian Gulf. J Appl Microbiol **91:**533-540.

Arzayus, K. M., R. M. Dickhut, and E. A. Canuel. 2001. Fate of atmospherically deposited polycyclic aromatic hydrocarbons (PAHs) in Chesapeake Bay. Environmental Science & Technology **35:**2178-2183.

Assinder, S. J., and P. A. Williams. 1990. The TOL plasmids: determinants of the catabolism of toluene and the xylenes, p. 1-69, Adv Microbial Physiol, vol. 31. Academic Press.

Atlas, R. M., and R. Bartha. 1972. Degradation and mineralization of petroleum by two bacteria isolates from coastal waters. Biotechnol Bioeng **14:**297-308.

Atlas, R. M., and R. Bartha. 1972. Degradation and mineralization of petroleum in sea water: limitation by nitrogen and phosphorus. Biotechnol Bioeng **14:**309-318.

Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev **45:**180-209.

Atlas, R. M., and R. Bartha. 1992. Hydrocarbon biodegradation and oil spill bioremediation. Adv Microb Ecol **12:**287-338.

Atlas, R. M. 1995. Bioremediation of petroleum pollutants. International Biodeterioration and Biodegradation**:**317-327.

Atlas, R. M. 1995. Petroleum biodegradation and oil spill bioremediation. Marine Pollution Bulletin **31:**178-182.

Austin, B., J. J. Calomiris, J. D. Walker, and R. R. Colwell. 1977. Numerical taxonomy and ecology of petroleum-degrading bacteria. Appl Environ Microbiol **34:**60-68.

Barth, J. J. 2003. The influence of cyanobcateria on oil polluted intertidal soils at the Saudi Arabian Gulf shores. Marine Pollution Bulletin **46:**1245-1252.

Bateman, J. N., B. Speer, L. Feduik, and R. A. Hartline. 1986. Naphthalene association and uptake in *Pseudomonas putida*. J Bacteriol **166:**155-161.

Bazylinski, D. A., C. O. Wirsen, and H. W. Jannasch. 1989. Microbial utilization of naturally occuring hydrocarbons at the Guaymas Basin hydrothermal vent site. Appl Environ Microbiol **55:**2832-.

Belyaev, S. S., K. S. Laurinavichus, S. N. Obratsova, S. N. Gorlatov, and M. V. Ivanov. 1982. Microbiological processes in the critical zone of injection wells in oil-fields. Microbiology (Transl. from Mikrobiologiya, Moscow) **51:**793-797.

Bergman, B., J. R. Gallon, A. N. Rai, and L. J. Stal. 1997. N_2 fixation by non-heterocystous cyanobacteria. FEMS Microbiol Rev **19:**139-185.

Bossert, I., and R. Bartha. 1984. The fate of petroleum in soil ecosystems, p. 435-475. *In* R. M. Atlas (ed.), Petroleum Microbiology. Macmillan Publishing Company, New York.

Bouchez-Naitali, M., H. Rakatozafy, R. Marchal, J.-Y. Leveau, and J. P. Vandecasteele. 1999. Diversity of bacterial strain degrading hexadecane in relation to the mode of substrate uptake. J Appl Microbiol **86:**421-428.

Braddock, J. F., J. E. Lindstrom, and E. J. Brown. 1995. Distribution of hydrocarbon-degrading microorganisms in sediments from Prince William Sound, Alaska, following the Exxon Waldez oil spill. Marine Pollution Bulletin **30:**125-132.

Bragg, J. R., R. C. Prince, J. B. Wilkinson, and R. M. Atlas. 1992. Bioremediation for shoreline cleanup following the 1989 Alaskan oil spill. Exxon Co.

Bragg, J. R., R. C. Prince, E. J. Harner, and R. M. Atlas. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. Nature **368:**413-418.

Bregnard, T. P. A., A. Haener, P. Hoehener, and J. Zeyer. 1997. Anaerobic degradation of pristane in nitrate-reducing microcosms and enrichment cultures. Appl Environ Microbiol **63**.

Capone, D. G. 2001. Marine nitrogen fixation: what's the fuss? Current Opinion in Microbiology **4:**341-348.

Cerniglia, C. E., C. Van Baalen, and D. T. Gibson. 1980. Oxidation of biphenyl by the cyanobacterium, *Oscillatoria* sp., strain JCM. Arch Microbiol **125:**203-207.

Cerniglia, C. E., C. Van Baalen, and D. T. Gibson. 1980. Metabolism of naphthalene by the cyanobacterium *Oscillatoria* sp., strain JCM. J Gen Microbiol **116:**485-494.

Cerniglia, C. E., D. T. Gibson, and C. Van Baalen. 1980. Oxidation of naphthalene by cyanobacteria and microalgae. J Gen Microbiol **116:**495-500.

Cerniglia, C. E., J. P. Freeman, J. R. Althaus, and C. Van Baalen. 1983. Metabolism and toxicity of 1- and 2-methylnaphthalene and their derivatives in cyanobacteria. Arch Microbiol **136:**177-183.

Cerniglia, C. E. 1984. Microbial metabolism of polycylclic aromatic hydrocarbons. Advances in Applied Microbiology **16:**31-71.

Cerniglia, C. E. 1984. Microbial transformation of aromatic hydrocarbons, p. 99-129. *In* R. M. Atlas (ed.), Petroleum Microbiology. Macmillan Publishing Co., New York.

Chen, Y. P., G. Lopez-de-Victoria, and C. R. Lovell. 1993. Utilization of aromatic compounds as carbon and energy sources during growth and N_2-fixation by free-living nitrogen fixing bacteria. Arch Microbiol **159:**207-212.

Chen, Y. B., B. Dominic, M. T. Mellon, and J. P. Zehr. 1998. Circadian rhythm of nitrogenase geen expression in the diazotrophic filamentous nonheterocystous cyanobacterium Trichodesmium sp. Strain IMS 101. J Bacteriol **180:**3598-3605.

Chen, J., G. Henderson, C. C. Grimm, S. W. Lloyd, and R. A. Laine. 1998. Termites fumigate their nests with naphthalene. Nature **392:**558-559.

Cohen, Y., and M. Gurevitz. 1992. The cyanobacteria - ecology, physiology, and molecular genetics, p. 2079-. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications., Second ed. Springer-Verlag.

Cohen, Y. 2002. Bioremediation of oil by marine microbial mats. Int Microbiol **5:**189-193.

Cooney, J. J. 1984. The fate of petroleum pollutants in freshwater ecosystems, p. 399-435. *In* R. M. Atlas (ed.), Petroleum Microbiolgy. Macmillan Publishing Company, New York.

Dibble, J. T., and R. Bartha. 1976. Effect of iron on the biodegradation of petroleum in sea water. Appl Environ Microbiol **31:**544-546.

Dyksterhouse, S. E., J. P. Gray, R. P. Herwig, J. C. Lara, and J. T. Staley. 1995. *Cycloclasticus pugetii* gen. nov., sp. nov., an aromatic hydrocarbon-degrading bacterium from marine sediments. Int J Syst Bacteriol **45:**116-123.

Eady, R. R. 1996. Structure-function relationships of alternative nitrogenases. Chem Rev **96:**3013-3030.

Eckford, R., F. D. Cook, D. Saul, J. Aislabie, and J. Foght. 2002. Free-living heterotrophic nitrogen-fixing bacteria isolated from fuel-contaminated antarctic soils. Appl Environ Microbiol **68:**5181-5185.

Ehrenreich, P., A. Behrends, J. Harder, and F. Widdel. 2000. Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Arch Microbiol **173:**58-64.

Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young. 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl Environ Microbiol **57:**1139-1145.

Evans, P. J., W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young. 1992. Metabolites formed during anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl Environ Microbiol **58:**496-501.

Fischer-Romero, C., B. J. Tindall, and F. Jüttner. 1996. *Tolumonas auensis* gen. nov., sp. nov., a toluene-producing bacterium from anoxic sediments of a freshwater lake. Int J Syst Bacteriol **46:**183-188.

Floodgate, G. D. 1984. The fate of petroleum in marine ecosystems, p. 355-399. *In* R. M. Atlas (ed.), Petroleum Microbiolgy. Macmillan Publishing Company, New York.

Frontera-Suau, R., F. D. Bost, T. J. McDonald, and P. J. Morris. 2002. Aerobic biodegradation of hopanes and other biomarkers by crude oil-degrading enrichment cultures. Environ Sci Technol **36:**4585-4592.

Galushko, A., D. Minz, B. Schink, and F. Widdel. 1999. Anaerobic degradation of naphthalene by a pure culture of a novel type of marine sulphate-reducing bacterium. Environ Microbiol **1:**415-420.

Gandy, E. L., and D. C. Yoch. 1988. Relationship between nitrogen-fixing sulfate reducers and fermenters in salt marsh sediments and roots of *Spartina alterniflora*. Appl Environ Microbiol **54:**2031-2036.

Garrett, R. M., I. J. Pickering, C. E. Haith, and R. C. Prince. 1998. Photooxidation of crude oils. Environ Sci Technol **32:**3719-3723.

Gauthier, M. J., B. Lafay, R. Christen, L. Fernandez, M. Acquaviva, P. Bonin, and J. C. Bertrand. 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new

extremely halotolerant, hydrocarbon-degrading marine bacterium. Int J Syst Bacteriol **42:**568-576.

Geiselbrecht, A. D., R. P. Herwig, J. W. Deming, and J. T. Staley. 1996. Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon-degrading marine bacteria from Puget Sound sediment. Appl Environ Microbiol **62:**3344-3349.

Gibson, D. T. 1984. Microbial degradation of organic compounds. Marcel Dekker, New York.

Gibson, D. T., and R. E. Parales. 2000. Aromatic hydrocarbon dioxygenases in environmental biotechnology. Current Opinion in Biotechnology **11:**236-243.

Golyshin, P. N., T. N. Chernikova, W. R. Abraham, H. Lunsdorf, K. N. Timmis, and M. M. Yakimov. 2002. *Oleiphilaceae* fam. nov., to include *Oleiphilus messinensis* gen. nov., sp. nov., a novel marine bacterium that obligatory utilizes hydrocarbons. Int J Syst Evol Microbiol **52:**901-911.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser **41:**95-98.

Hara, A., K. Syutsubo, and S. Harayama. 2003. *Alcanivorax* which prevails in oil-contaminated seawater exhibits broad substrate specificity for alkane degradation. Environ Microbiol **5:**746-753.

Harayama, S., H. Kishira, Y. Kasai, and K. Shutsubo. 1999. Petroleum biodegradation in marine environments. J Mol Microbiol Biotechnol **1:**63-70.

Harayama, S., Y. Kasai, and A. Hara. 2004. Microbial communities in oil-contaminated seawater. Current Oppinion in Biotechnolgy **15:**205-214.

Harms, G., K. Zengler, R. Rabus, F. Aeckersberg, D. Minz, R. Rossello-Mora, and F. Widdel. 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl Environ Microbiol **65:**999-1004.

Hedlund, B. P., A. D. Geiselbrecht, T. J. Bair, and J. T. Staley. 1999. Polycyclic aromatic hydrocarbon degradation by a new marine bacterium, *Neptunomonas naphthovorans*, gen. nov., sp. nov. Appl Environ Microbiol **65:**251-259.

Hedlund, B. P., A. D. Geiselbrecht, and J. T. Staley. 2001. *Marinobacter* strain NCE312 has a *Pseudomonas*-like naphthalene dioxygenase. FEMS Microbiol Lett **201:**47-51.

Heider, J., A. M. Spormann, H. R. Beller, and F. Widdel. 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiology Reviews **22:**459-473.

Heitkamp, M. A., J. P. Freeman, D. W. Miller, and C. E. Cerniglia. 1988. Pyrene degradation by a *Mycobacterium* sp.: identification of oxidation and ring fission products. Appl Environ Microbiol **54:**2256-2265.

Herbert, R. A. 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews **23:**563-590.

Hoffman, L. 1996. Recolonization of the intertidal flats by microbial mats after the Gulf War oil spill. *In* F. Krupp, A. H. Abuzinada, and I. A. Nader (ed.), A marine wildlife sanctuary for the Arabian Gulf. NCWCD, Riyadh.

Hommel, R. K. 1990. Formation and physiological role of biosurfactants produced by hydrocarbon-utilizing microorganisms. Biodegradation **1**.

Hurek, T., L. L. Handley, B. Reinhold-Hurek, and Y. Piche. 2002. *Azoarcus* grass endophytes contribute fixed nitrogen to the plant in an unculturable state. Mol Plant-Microbe Interacts **15:**233-242.

Hurley, M. D., O. Sokolov, T. J. Wallington, H. Takekawa, M. Karasawa, B. Klotz, I. Barnes, and K. H. Becker. 2001. Organic aerosol formation during the atmospheric degradation of toluene. Environmental Science & Technology **35:**1358-1366.

Iwabuchi, N., M. Sunairi, M. Urai, C. Itoh, H. Anzai, M. Nakajima, and S. Harayama. 2002. Extracellular polysaccharides of *Rhodococcus rhodochrous* S-2 stimulate the degradation of aromatic components in crude oil by indigenous marine bacteria. Appl Environ Microbiol **68:**2337-2343.

Juck, D., T. Charles, L. G. Whyte, and C. W. Greer. 2000. Polyphasic microbial community analysis of petroleum hydrocarbon-contaminated soils from two northern Canadian communities. FEMS Microbiology Ecology **33:**241-249.

Juhasz, A. L., and R. Naidu. 2000. Bioremediation of high molecular weight polycyclic aromatic hydrocarbons: a review of the microbial degradation of benzo(a)pyrene. International Biodeterioration & Biodegradation **45:**57-88.

Kasai, Y., H. Kishira, and S. Harayama. 2002. Bacteria belonging to the genus *Cycloclasticus* play a primary role in the degradation of aromatic hydrocarbons released in a marine environment. Appl Environ Microbiol **68:**5625-5633.

Kästner, M., S. Streibich, M. Beyrer, H. H. Richnow, and W. Fritsche. 1999. Formation of bound residues during microbial degradation of [^{14}C]anthracene in soil. Appl Environ Microbiol **65:**1834-1842.

Kelly, I., J. P. Freeman, F. E. Evans, and C. E. Cerniglia. 1991. Identification of a carboxylic acid metabolite from the catabolism of fluoranthene by *Mycobacterium* sp. Appl Environ Microbiol **57:**636-641.

Kim, K., Y. P. Zhang, and G. P. Roberts. 1999. Correlation of activity regulation and substrate recognition of the ADP-ribosyltransferase that regulates nitrogenase activity in Rhodospirillum rubrum. J Bacteriol **181:**1698-1702.

Klug, M. J., and A. J. Markowetz. 1967. Thermophilic bacteria isolated on *n*-tetradecane. Nature **215:**1082-1083.

Kniemeyer, O., T. Fischer, H. Wilkes, F. O. Glockner, and F. Widdel. 2003. Anaerobic degradation of ethylbenzene by a new type of marine sulfate-reducing bacterium. Appl Environ Microbiol **69:**760-768.

Leahy, J. G., and R. R. Colwell. 1990. Microbial degradation of hydrocarbons in the environment. Microbiol Rev **54:**305-315.

Lee, K., and F. X. Merlin. 1999. Bioremediation of oil on shoreline environments: development of techniques and guidelines. Pure Appl Chem **71:**161-171.

Lindstrom, J. E., R. C. Prince, J. C. Clark, M. J. Grossman, T. R. Yeager, J. F. Braddock, and E. J. Brown. 1991. Microbial populations and hydrocarbon biodegradation potentials in fertilized shoreline sediments affected by the T/V Exxon Valdez oil spill. Appl Environ Microbiol **57:**2514-2522.

Linton, J. D., and R. J. Stephenson. 1978. A preliminary study on growth yields in relation to the carbon and energy content of various organic growth substrates. FEMS Microbiol Lett **3:**95-98.

Lovell, C. R., Y. M. Piceno, J. M. Quattro, and C. E. Bagwell. 2000. Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, *Spartina alterniflora*. Appl Environ Microbiol **66:**3814-3822.

Lovley, D. R., M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Siegel. 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature **339:**297-300.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, Arno Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüssmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. 2004. ARB: a software environment for sequence data. Nucl Acid Res **32:**1363-1371.

Margesin, R., and F. Schinner. 1997. Efficiency of indigenous and inoculated cold-adapted soil microorganisms for biodegradation of diesel oil in alpine soils. Appl Environ Microbiol **63:**2660-2664.

Mateles, R. I., J. N. Baruah, and S. R. Tannebaum. 1967. Growth of a thermophilic bacterium on hydrocarbons: a new source of single cell protein. Science **157:**1322-1323.

McGowan, L., R. Herbert, and G. Muyzer. 2004. A comparative study of hydrocarbon degradation by *Marinobacter* sp., *Rhodococcus* sp. and *Corynebacterium* sp. isolated from different mat systems. Ophelia **58:**271-283.

Meckenstock, R. U., E. Annweiler, W. Michaelis, H. Richnow, and B. Schink. 2000. Anaerobic naphthalene degradation by a sulfate-reducing enrichment culture. Appl Environ Microbiol **66:**2743-2747.

Meckenstock, R. U., M. Safinowski, and C. Griebler. 2004. Anaerobic degradation of polycyclic aromatic hydrocarbons. FEMS Microbiol Ecol **49:**27-36.

Miller, R. M., and R. Bartha. 1989. Evidence from liposome encapsulation from transport-limited microbial metabolism of solid alkanes. Appl Environ Microbiol **55:**269-274.

Morgan, P., and R. J. Watkinson. 1994. Biodegradation of components of petroleum, p. 1-31. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publisher.

Musat, F., A. Wieland, and F. Widdel. 2004. Marine sediment with surface contamination by oil in microcosms for microbiological studies. Ophelia **58:**217-222.

Narro, M. L., C. E. Cerniglia, C. Van Baalen, and D. T. Gibson. 1992. Metabolism of phenanthrene by the marine cyanobacterium *Agmenellum quadruplicatum* PR-6. Appl Environ Microbiol **58:**1351-1359.

Nazina, T. N., E. P. Rozanova, and S. I. Kuznetsov. 1985. Microbial oil transformation processes accompanied by methane and hydrogen sulfide formation. Geomicrobiol J **4:**103-130.

Nielsen, L. B., K. Finster, D. T. Welsh, A. Donelly, R. A. Herbert, R. de Wit, and B. A. Lomstein. 2001. Sulphate reduction and nitrogen fixation rates associated with roots, rhizomes and sediments from *Zostera noltii* and *Spartina maritima* meadows. Environmental Microbiology **3:**63-71.

Oh, Y. S., D. S. Sim, and S. J. Kim. 2001. Effects of nutrients on crude oil biodegradation in the upper intertidal zone. Marine Pollution Bulletin **42:**1367-1372.

Okhuma, M., S. Noda, and T. Kudo. 1999. Phylogenetic diversity of nitrogen fixation genes in the symbiotic microbial community in the gut of diverse termites. Appl Environ Microbiol **65:**4926-4934.

Olivieri, R., P. Bacchin, A. Robertiello, N. Oddo, L. Degen, and A. Tonolo. 1976. Microbial degradation of oil spills enhanced by a slow-release fertilizer. Appl Environ Microbiol **31:**629-634.

Omoregie, E. O., L. L. Crumbliss, B. M. Bebout, and J. P. Zehr. 2004. Comparison of diazotroph community structure in *Lyngbya* sp. and *Microcoleus chthonoplastes* dominated microbial mats from Guerrero Negro, Baja, Mexico. FEMS Microbiology Ecology **47:**305-318.

Owens, E. H., G. S. Mauseth, C. A. Martin, A. Lamarche, and J. Brown. 2002. Tar ball frequency data and analytical results from a long-term beach monitoring program. Marine Pollution Bulletin **44:**770-780.

Piehler, M. F., J. G. Swistak, J. L. Pinckney, and H. W. Paerl. 1999. Stimulation of diesel fuel biodegradation by indigenous nitrogen fixing bacterial consortia. Microbial Ecology **38:**69-78.

Postgate, J. R., and H. M. Kent. 1985. Diazotrophy within *Desulfovibrio*. J Gen Microbiol **131:**2119-2122.

Prantera, M. T., A. Drozdowicz, S. G. Leite, and A. S. Rosado. 2002. Degradation of gasoline hydrocarbons by two N_2-fixing soil bacteria. Biotechnology Letters **24:**85-89.

Prince, R. C. 1993. Petroleum spill bioremediation in marine environment. Critical Reviews in Microbiology **19:**217-242.

Prince, R. C., D. L. Elmendorf, J. R. Lute, C. S. Hsu, C. E. Halth, J. D. Senius, G. J. Dechert, G. S. Douglas, and E. L. Butler. 1994. 17? (H),21? (H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ Sci Technol **28:**142-145.

Prince, R. C., and J. R. Bragg. 1997. Shoreline bioremediation following the Exxon Valdez oil spill in Alaska. Bioremed J **1:**97-104.

Prince, R. C., R. E. Bare, R. M. Garrett, M. J. Grossman, C. E. Haith, L. G. Keim, K. Lee, G. J. Holtom, P. Lambert, G. A. Sergy, E. H. Owens, and C. C. Guenette. 1999. Presented at the Proceeding from the fifth international in situ and on site bioremediation symposium., San Diego, California.

Prince, R. C. 2002. Petroleum and other hydrocarbons, biodegradation of, p. 2402-2416. *In* G. Bitton (ed.), Encyclopedia of environmental microbiology. John Wiley, New York.

Prince, R. C., R. M. Garrett, R. E. Bare, M. J. Grossman, T. Townsend, J. M. Suflita, K. Lee, E. H. Owens, G. A. Sergy, J. F. Braddock, J. E. Lindstrom, and R. R. Lessard. 2003. The roles of photooxidation and biodegradation in long-term weathering of crude and heavy fuel oils. Spill Science and Technology Bulletin **8:**145-156.

Prince, R. C., R. E. Bare, R. M. Garrett, M. J. Grossman, C. E. Haith, L. G. Keim, K. Lee, G. J. Holtom, P. Lambert, G. A. Sergy, E. H. Owens, and C. C. Guenette. 2003. Bioremediation of stranded oil on an Arctic shoreline. Spill Science &Technology Bulletin **8:**303-312.

Pritchard, P. H., and C. F. Costa. 1991. EPA's Alaska oil spill bioremediation project. Environmental Science & Technology **25:**372-379.

Pritchard, P. H., J. G. Mueller, J. C. Rogers, F. V. Kremer, and J. A. Glaser. 1992. Oil spill bioremediation: experiences, lessons and results from the Exxon Valdez oil spill in Alaska. Biodegradation **3:**315-335.

Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdel. 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl Environ Microbiol **59:**1444-1451.

Rabus, R., and F. Widdel. 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol **163:**96-103.

Rabus, R., M. Fukui, H. Wilkes, and F. Widdel. 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridizytion of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl Environ Microbiol **62:**3605-3613.

Rabus, R., H. Wilkes, A. Behrends, A. Armstroff, T. Fischer, A. J. Pierik, and F. Widdel. 2001. Anaerobic initial reactions of *n*-alkanes: evidence for involvement of an organic radical in the metabolism of n-hexane in a denitrifying bacterium. J Bacteriol **183:**1707-1715.

Rabus, R., M. Kube, A. Beck, F. Widdel, and R. Reinhardt. 2002. Genes involved in the anaerobic degradation of ethylbenzene in a denitrifying bacterium, strain EbN1. Arch Microbiol **178:**506-516.

Rees, D. C., and J. B. Howard. 2000. Nitrogenase: standing at the crossroads. Current Oppinion in Chemical Biology **4:**559-566.

Richnow, H. H., R. Seifert, J. Hefter, M. Kaestner, B. Mahro, and W. Michaelis. 1993. Metabolites of xenobiotica and mineral oil constituents linked to macromolecular organic matter in polluted environments. Adv Org Geochem **22:**671-681.

Richnow, H. H., R. Seifert, J. Hefter, M. Link, W. Francke, G. Schaefer, and W. Michaelis. 1997. Organic pollutants associated with macromolecular soil organic matter: mode of binding. Org Geochem **26:**745-758.

Rippka, R., J. Deruelles, J. B. Waterbury, M. Herdman, and R. Y. Stanier. 1979. Generic assignments, strain histories and properties of pure cultures of cyanobacteria. J Gen Microbiol **111:**1-61.

Rippka, R. 1988. Isolation and purification of cyanobacteria., p. 3-27. *In* L. Packer and A. N. Glazer (ed.), Methods in enzymology, vol. 167. Academic Press, INC.

Rockne, K. J., J. C. Chee-Sanford, R. A. Sanford, B. P. Hedlund, J. T. Staley, and S. E. Strand. 2000. Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. Appl Environ Microbiol **66:**1595-1601.

Roubal, G., and R. M. Atlas. 1978. Distribution of hydrocarbon-utilizing microorganisms and hydrocarbon biodegradation potentials in Alaskan continental shelf areas. Appl Environ Microbiol **35:**897-905.

Röling, W. F. M., M. G. Milner, D. M. Jones, K. Lee, F. Daniel, R. J. P. Swannell, and I. M. Head. 2002. Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. Applied and Environmental Microbiology **68:**5537-5548.

Rueter, P., R. Rabus, H. Wilkes, F. Aeckersberg, F. A. Rainey, H. W. Jannasch, and F. Widdel. 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature **372:**455-458.

Sekelsy, A. M., and G. S. Shreve. 1999. Kinetic model of biosurfactant-enhanced hexadecane biodegradation by *Pseudomonas aeruginosa*. Biotechnol Bioeng **63:**401-409.

Shennan, J. L. 1984. Hydrocarbons as substrates in industrial fermentation, p. 643-685. *In* R. M. Atlas (ed.), Petroleum Microbiology. Macmillan Publishing Company, New York.

Shiaris, M. P., and J. J. Cooney. 1983. Replica plating method for estimating phenanthrene-utilizing and phenanthrene-cometabolizing microorganisms. Appl Environ Microbiol **45:**706-710.

Shields, M. S., M. O. Montgomery, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard. 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl Environ Microbiol **55:**1624-1629.

Sikkema, J., J. A. M. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews **59:**201-222.

Smith, M. R., M. Ewing, and C. Ratledge. 1991. The interaction of variuos aromatic substrates degraded by *Pseudomonas* sp. NCIB 10643: synergistic inhibition of growth by two compounds that serve as growth substrates. Appl Microbiol Biotechnol **34:**536-538.

So, C. M., and L. Y. Young. 1999. Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl Environ Microbiol **65:**2969-2976.

Sorkhoh, N., R. Al-Hasan, S. Radwan, and T. Hoepner. 1992. Self-cleaning of the Gulf. Nature **359:**109.

Stal, L. J. 1995. Physiological ecology of cyanobacteria in microbial mats and other communities. New Phytol **131:**1-32.

Stal, L. J. 2000. Cyanobacterial mats and stromatolites, p. 61-120. *In* B. A. Whitton and M. Potts (ed.), The ecology of cyanobacteria. Kluwer Academic Publishers.

Steppe, T. F., J. B. Olson, H. W. Paerl, R. W. Litaker, and J. Belnap. 1996. Consortial N_2 fixation - a strategy for meeting nitrogen requirements of marine and terrestrial cyanobacterial mats. FEMS Microbiol Ecol **21:**149-156.

Steppe, T. F., J. L. Pinckney, J. Dyble, and H. W. Paerl. 2001. Diazotrophy in modern marine Bahamian stromatolites. Microbial Ecology **41:**36-44.

Steppe, T. F., and H. W. Paerl. 2002. Potential N_2 fixation by sulfate-reducing bacteria in a marine intertidal microbial mat. Aquatic Microbial Ecology **28:**1-12.

Stewart, W. D. P., G. P. Fitzgerald, and R. H. Burris. 1967. In situ studies of N_2 fixation using the acetylene reduction technique. Proc Natl Acad Sci USA **58:**2071-2078.

Swannell, R. P. J., B. C. Croft, A. L. Grant, and K. Lee. 1995. Evaluation of bioremediation agents in beach microcosm. Spill Science and Technology Bulletin **2:**151-159.

Swannell, R. P. J., K. Lee, and M. McDonagh. 1996. Field evaluations of marine oil spill bioremediation. Microbiological Reviews **60:**342-365.

Syutsubo, K., H. Kishira, and S. Harayama. 2001. Development of specific oligonucleotide probes for the identification and in situ detection of hydrocarbon-degrading *Alcanivorax* strains. Environ Microbiol **3:**371-379.

Thiel, T., L. E. M., J. C. Erker, and A. Ernst. 1995. A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium. Proc Natl Acad Sci USA **92:**9358-9362.

Tibbles, B. J., M. I. Lucas, V. E. Coyne, and T. S. Newton. 1994. Nitrogenase activity in marine sediments from a temperate salt marsh lagoon: modulation by complex polysaccharides, ammonium and oxygen. J Exp Mar Biol **184:**1-20.

Tissot, B. P., and D. H. Welte. 1984. Petroleum formation and occurence. Springer-Verlag, Berlin, Germany.

Tkalich, P., and E. S. Chan. 2002. Vertical mixing of oil droplets by breaking waves. Marine Pollution Bulletin **44:**1219-1229.

Toccalino, P. L., R. L. Johnson, and D. R. Boone. 1993. Nitrogen limitation and nitrogen fixation during alkane biodegradation in a sandy soil. Appl Environ Microbiol **59:**2977-2983.

Ueda, T., Y. Suga, N. Yahiro, and T. Matsuguchi. 1995. Remarkable N_2 fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of the *nifH* gene sequences. J Bacteriol **177:**1414-1417.

Van Beilen, J. B., M. G. Wubbolts, and B. Witholt. 1994. Genetics of alkane oxidation by *Pseudomonas oleovorans*. Biodegradation **5:**161-174.

Van Hamme, J. D., and O. P. Ward. 2001. physical and metabolic interactions of *Pseudomonas* sp. strain JA5-B45 and *Rhodococcus* sp. strain F9-D79 during growth on crude oil and the affect of a chemical surfactant on them. Appl Environ Microbiol **67:**4874-4879.

Van Hamme, J. D., A. Singh, and O. P. Ward. 2003. Recent advances in petroleum microbiology. Microbiology and Molecular Biology Reviews **67:**503-549.

Walker, J. D., and R. R. Colwell. 1976. Enumeration of petroleum-degrading microorganisms. Appl Environ Microbiol **31:**198-207.

Walker, J. D., and R. R. Colwell. 1976. Measuring the potential activity of hydrocarbon-degrading bacteria. Appl Environ Microbiol **31:**189-197.

Welsh, D. T., S. Bourgues, R. de Wit, and R. A. Herbert. 1996. Seasonal variations in nitrogen fixation (acetylene reduction) and sulphate reduction rates in the rhizosphere of Zostera noltii: nitrogen fixation by sulfate reducing bacteria. Mar Biol **125:**619-628.

Widdel, F. 1987. New types of acetate-oxidizing, sulfate-reducing *Desulfobacter* species, *D. hydrogenophilus* sp. nov., *D. latus* sp. nov., and *D. curvatus* sp. nov. Arch Microbiol **148:**286-291.

Widdel, F., and F. Bak. 1992. Gram-negative mesophilic sulfate-reducing bacteria., p. 3352-3378. *In* H. G. Balows, M. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes. Springer Verlag, New York.

Widdel, F., and R. Rabus. 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Current Opinion in Biotechnology **12:**259-276.

Wilkes, H., S. Kuhner, C. Bolm, T. Fischer, A. Classen, F. Widdel, and R. Rabus. 2003. Formation of *n*-alkane- and cycloalkane-derived organic acids during anaerobic growth of a denitrifying bacterium with crude oil. Org Geochem **34:**1313-1323.

Wilson, R. D., P. H. Monaghan, A. Osanik, L. C. Price, and M. A. Rogers. 1974. Natural marine oil seepage. Science **184:**857-865.

Yakimov, M. M., P. N. Golyshin, S. Lang, E. R. Moore, W. R. Abraham, H. Lunsdorf, and K. N. Timmis. 1998. *Alcanivorax burkumensis* gen nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. Int J Syst Bacteriol **48:**339-348.

Yakimov, M. M., L. Giuliano, G. Gentile, E. Crisafi, T. N. Chernikova, W. R. Abraham, H. Lunsdorf, K. N. Timmis, and P. N. Golyshin. 2003. *Oleispira antarctica* gen. nov., sp. nov., a novel hydrocarbonoclastic marine bacterium isolated from Antarctic coastal sea water. Int J Syst Evol Microbiol **53:**779-785.

Yakimov, M. M., G. Gentile, V. Bruni, S. Cappello, G. D'Auria, P. N. Golyshin, and L. Giuliano. 2004. Crude oil-induced shift of coastal bacterial communities of rod bay (Terra Nova Bay, Ross Sea, Antarctica) and characterization of cultured cold-adapted hydrocarbonoclastic bacteria. FEMS Microbiology Ecology **49:**419-432.

Yoo, S. J., H. C. Angove, V. Papaefthymiou, B. K. Burgess, and E. Münk. 2000. Mössbauer study of the MoFe protein of nitrogenase from *Azotobacter vinelandii* using selective 57Fe enrichment of the M-centers. J Am Chem Soc **122:**4926-4936.

Zehr, J. P., and L. A. McReynolds. 1989. Use of degenerate oligonucleotides for the amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium* spp. Appl Environ Microbiol **61:**2522-2526.

Zehr, J. P., M. T. Mellon, and S. Zani. 1998. New nitrogen-fixing microorganisms detected in oligotrophic oceans by amplification of nitrogenase (*nifH*) genes. Appl Environ Microbiol **64:**3444-3450.

Zehr, J. P., J. B. Waterbury, P. J. Turner, J. P. Montoya, E. Omoregie, G. F. Steward, A. Hansen, and D. M. Karl. 2001. Unicellular cyanobacteria fix N_2 in the subtropical North Pacific Ocean. Nature **412:**635-638.

Zehr, J. P., B. D. Jenkins, S. M. Short, and G. F. Steward. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental Microbiology **5:**539-554.

Zengler, K., H. H. Richnow, R. Rossello-Mora, W. Michaelis, and F. Widdel. 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature **401:**266-269.

Zengler, K., J. Heider, R. Rossello-Mora, and F. Widdel. 1999. Phototrophic utilization of toluene under anoxic conditions by a new strain of *Blastochloris sulfoviridis*. Arch Microbiol **172:**204-212.

Zhang, Y., and R. M. Miller. 1992. Enhanced octadecane dispersion and biodegradation by a *Pseudomonas* rhamnolipid surfactant (biosurfactant). Appl Environ Microbiol **58:**3276-3282.

Zhang, Y., and R. M. Miller. 1994. Effect of a *Pseudomonas* rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Appl Environ Microbiol **60:**2101-2106.

Zhang, X., and L. Y. Young. 1997. Carboxylation as an initial reaction in the anaerobic metabolism of naphthalene and phenanthrene by sulfidogenic consortia. Appl Environ Microbiol **63:**4759-4764.

Part II. Manuscripts

Content and explanation of contribution

1. **Marine sediment with surface contamination by oil in microcosms for microbiological studies**
   Florin Musat, Andrea Wieland, and Friedrich Widdel

   Concepts of this study were developed in cooperation with F. Widdel. Establishment of experimental microcosms was done by F. Musat. A. Wieland contributed with chemical microprofile measurements.

2. **Quantification of petroleum biodegradation on marine sediments in experimental microcosms**
   Florin Musat, Heinz Wilkes, and Friedrich Widdel

   Concepts of this study were developed in cooperation with F. Widdel. Establishment of optimal conditions for biodegradation and quantification experiments were carried out by F. Musat. Chemical analyses of crude oil were performed by H. Wilkes.

3. **Nitrogen fixation in microbial communities of oil-contaminated marine sediments**
   Florin Musat, Jens Harder, and Friedrich Widdel

   Concepts of this study were developed in cooperation with F. Widdel. Molecular investigations and measurements of nitrogenase activity were performed by F. Musat. J. Harder contributed to phylogenetic analyses of NifH.

1

Marine sediment with surface contamination by oil in microcosms for microbiological studies

Florin Musat[1], Andrea Wieland[2] and Friedrich Widdel[1]*

Ophelia 58 (2004): 217–222

[1] Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany

[2] Marine Biological Laboratory, Strandpromenaden 5, DK-3000 Helsingør, Denmark

*** Corresponding author**

Abstract

Contamination of tidal sediment with oil and the development of microbial populations associated with oil was simulated in aquaria in the laboratory. Even distribution of a thin viscous mixture of oil and natural sediment over pristine sediment was a controllable method to achieve development of bioactive horizons (mat-like stratification) attributable to distinct microbial activities (phototrophic, aerobic, anaerobic). Microsensor measurements and observations of color changes showed that the addition of oil significantly promoted subsurface oxygen consumption and microbial sulfate reduction. There was so far no clear indication that cyanobacterial growth was stimulated by the added oil. Sediment microcosms as established in the present study are promising systems for the future study of the long-term fate of oil on and in marine sediments and of the significance of bacterial guilds and environmental parameters (light, oxygen, fertilization, etc.) for biodegradation of oil.

Key words: Petroleum, oil, pollution, marine sediments, aerobic degradation, sulfate reduction, gradients

Introduction

Oil pollution is most serious in aqueous environments such as the sea (Bragg *et al*. 1994; Harayama *et al*. 1999; Swanell *et al*. 1996), lakes or deep aquifers (Beller 2000; Gieg and Suflita 2002; Richnow *et al*. 2003). Whereas oil in normal soils that are not water-saturated can spread three-dimensionally and undergo aerobic degradation in a porous system, water forces oil to spread horizontally, i.e. only two-dimensionally, and hence to remain relatively compact. Vertical spreading into aquatic sediments is only possible to limited extent if interstitial space becomes temporarily accessible with sinking water level, for instance in tidal zones, or if sediment surfaces are restructured by hydromechanical forces. Since oil is a hydrophobic, concentrated and highly reduced form of organic carbon, microbial degradation of the compact layers in aquatic habitats is often strongly limited by oxygen and nutrient salts such as nitrogen compounds, phosphate, iron (Bragg *et al*. 1994; Oh *et al*. 2001) and the relatively small surface area for microbial colonization relative to the oil volume. Evaporation of the volatile hydrocarbons increases the viscosity of the remaining oil, such that rather coherent, long-lasting oil layers incorporating clay or sand are formed on sediment surfaces and significantly resist hydromechanical emulsification. Emulsification, however, is an important prerequisite for microbial degradation. Nevertheless, even such compact oil layers are likely to foster the development of complex microbial communities that may slowly oxidize organic fractions of the oil.

For a functional understanding of the abiotic and biotic factors that determine the persistence or microbial degradation of oil in sediments, experimental approaches under controlled but in *situ or* near-*in situ* conditions are needed (for instance, Oh *et al*. 2001; Prince *et al*. 2002; Shi *et al*. 1999). Here we describe a controllable procedure for explicit surface contamination of marine sediment with crude oil in a laboratory system and its maintenance. This system allows the experimental study of the development and activities of stratified (mat-like) oil-associated microbial guilds (functional groups) as well as the long-term fate of compact oil layers.

Materials and Methods

Clay-like marine sediment for the long-term establishment of artificially contaminated microcosms was collected from the tidal area (Wadden Sea of North Sea) in Horumersiel near Wilhelmshaven (Germany). Only the upper olive-gray sediment was used. Stocks were

stored under a shallow layer (few millimeters) of seawater in sealed plastic boxes in the dark to avoid evaporation and pre-growth of photosynthetic organisms.

To prepare the carrier for crude oil, a quantity of the sediment was centrifuged, dried and homogenized in a mortar and pestle. Then, the dry sediment was soaked with crude oil (Maya oil; see other articles of this issue) so as to obtain a liquid slurry. This slurry was spread in a glass dish and incubated under a fume hood for four days to allow evaporation of the most volatile hydrocarbons. The viscosity of the slurry increased but still allowed spreading on the sediment surface (see below).

Saline sediment samples for the inoculation of the artificially oil-contaminated sediment were obtained from a small harbor near l'Ampolla (Spain), a saltwater-covered deposit of petroleum residues from the Etang de Berre (France), and a small harbor in Horumersiel (see above). Both, the oxic (upper olive-gray) and anoxic (lower black, below approx. 1 cm) part were collected from these environments and mixed directly before inoculation.

Oil-contaminated sediments were established inside a greenhouse with artificial light in addition to daylight. A thick layer (4 cm) of the pristine sediment was placed in aquaria (38×23×14 cm, inner L×W×H). The sediment was fully seawater-saturated but not flooded. Then, the inoculum from contaminated sites was evenly distributed over the surface and by means of spatula pushed ("punctured") into the sediment. The total inoculum size was one twentieth of the pristine sediment volume. This procedure allowed a rather even distribution but still maintained small anoxic microniches that would have disappeared upon complete homogenization. The pristine (bulk) sediment was then covered with the oil-sediment mixture (corresponding to ~0.2 ml oil per cm^2). After one day, seawater (8 cm) was gently added without loosening the oil layer. The aquaria were connected to a seawater circulation system with a cooling unit. The aquaria were exposed to daylight and in addition to mercury lamps (daylight-like spectrum, 200 W; Osram) at a distance of 80 cm operated on a 12 h on – 12 h off regime. The temperature was kept between 22 and 32 °C. Temperature fluctuations were mainly due to daily or seasonal changes of sunlight.

Total carbon was analyzed via combustion (~1,800 °C) and gas chromatography of formed CO_2 using a Nitrogen Analyzer NA (Fisons Instruments). Inorganic carbon was analyzed by gas chromatographic head space analysis of acidified (0.1 M H_2SO_4, final conc.) sediment samples in butyl rubber-sealed vials. Organic carbon was calculated as the difference between the measured values.

Microprofiles of oxygen in the mats were measured by means of microsensors as described (Kühl & Jørgensen, 1992; Wieland & Kühl, 2000).

Results and Discussion

The primary goal of the present work was the establishment of microbial guilds that develop under *in situ*-like conditions upon surface contamination of tidal sediments with oil and contribute to its degradation. To minimize interference by other heterotrophic degradation processes, we used sediment without significant organic load before the application of oil. Even though the effect of oil pollution on organic-rich sediments such as those populated by invertebrates or oxygenic phototrophic mats is also of significant environmental interest, the complexity of the processes in such systems renders a causal understanding of the observed microbiological parameters difficult. As a starting material, our experiments employed clay-like oxic (olive-gray) sediment from a mud flat (Wadden Sea) of the North Sea. If incubated anoxically, this sediment does not turn dark indicating that sulfate reduction and hence the load of organic compounds is not significant. Carbon analyses indeed revealed only 12.5 mg total C and 8 mg organic C per g dry sediment. Black (sulfidic) spots were only observed around dead biomass particles (e.g. remnants of snails); these were not included in the experiments.

The sediment was placed in glass aquaria (Fig. 1) inside a greenhouse. To provide a potentially great diversity of oil-degrading microorganisms, the sediment was homogeneously inoculated with a defined volume of a mixture of sediment samples from geographically different sites with hydrocarbon contamination.

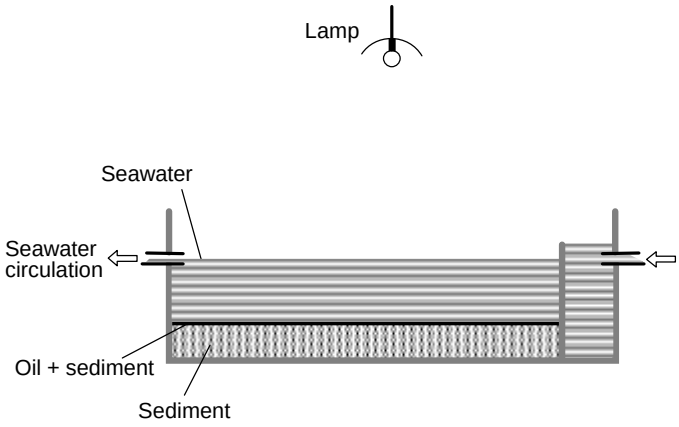


Fig. 1. Simplified scheme of experimental microcosm employed in this study showing oil-contaminated sediment and seawater circulation (integrated cooling unit not shown).

Controllable contamination with oil for microbiological studies was achieved by the addition of a layer of another sediment portion that had been dried, soaked with oil and allowed to loose the most volatile hydrocarbon fractions (Fig. 1, 2A), very similar as it usually occurs *in situ*. If sediment contaminated in this way was flooded with seawater, the rather viscous, coherent oil-rich layer remained associated with the sediment surface. An alternative approach we tested was to add the oil directly to the wet (but not flooded) sediment in the aquaria; however, most of the oil in this experiment was released from the sediment upon flooding such that experimental conditions were not controllable.

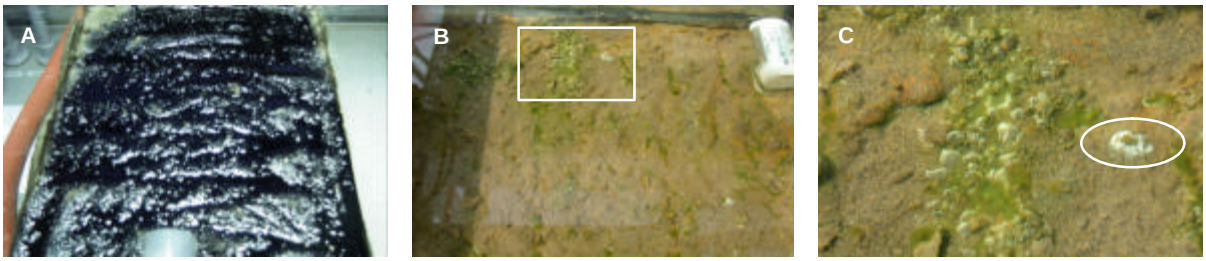


Fig. 2. Visible differences between the sediment directly upon contamination with oil (A), and after four weeks of incubation (B). A section (framed in B) with a small patch of whitish bloom of sulfide-oxidizing bacteria (circled) is shown at higher magnification (C); also, oxygen bubbles formed in a flock of filamentous phototrophs (Cyanobacteria, Algae) are visible.

Within four weeks, the oil-containing, initially dark-brown sediment surface had completely regained its original color indicating aerobic oil degradation (Fig. 2B). However, the regained sediment color did not extend deeper than ~1 mm. Below this depth, the dark oil layer was still present, even after incubation for one year. At sites where the oil layer was occasionally not coherent, patches with whitish blooms developed that were indicative of sulfide-oxidizing bacteria (Fig. 2B, C). Furthermore, if small areas of the oil layer were removed, an underlying black sediment became visible. The sediment in oil-free control aquaria remained olive-grey at all depths. These observations suggest that the added oil stimulated anoxia and sulfate reduction. Indeed, microsensor measurements revealed oxygen depletion even in the light, viz. during photosynthetic oxygen production in the contaminated aquaria (Fig. 3). Also free sulfide was only measured in sediment covered by oil (Abed and Musat, unpublished data).

The pronounced depletion and sulfidogenesis due to oil addition are explained by oxygen scavenge by aerobic oil-degrading bacteria, the supply of substrates from the oil to sulfate-reducing bacteria (Fig. 4), and by the oil layer severely limiting oxygen diffusion. Sulfate-reducing bacteria may utilize a certain number of hydrocarbons directly from the oil (Rabus *et al*. 1996; Rueter *et al*. 1994; Widdel and Rabus 2001), or they may also utilize

oxygenated products from an incomplete oxidation of hydrocarbons by aerobic bacteria (Belyaev *et al*. 1982; Nazina *et al*. 1985); oxygen limitation in the microoxic part may favor such incomplete oxidation. Furthermore, dead microbial biomass may contribute to electron donors for sulfate reduction.

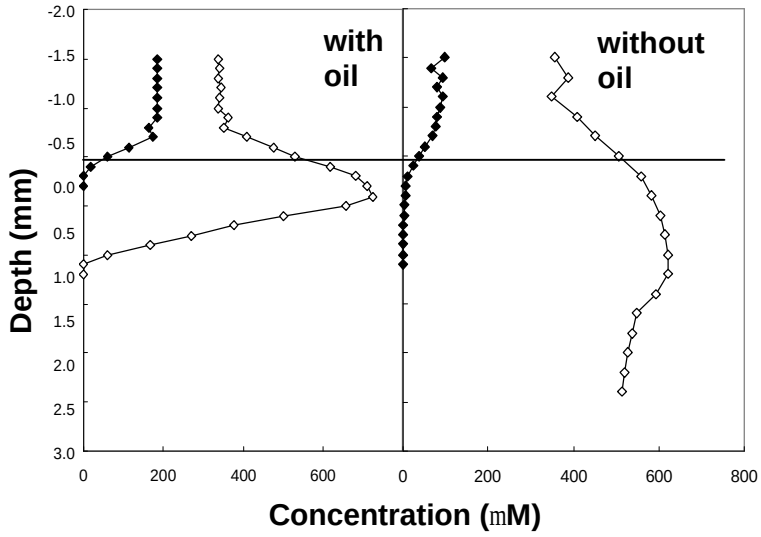


Fig. 3. Microprofiles of oxygen in light (◇) and dark (◆), incubations of oil contaminated (left) and pristine (right) sediment after 2 months of incubation (see also Fig. 1).

During the first two months, there was no apparent stimulation of oxygenic phototrophic organisms by the oil in comparison to the pristine control aquarium. Patchy layers of greenish filamentous organisms developed in both, the contaminated and the pristine aquaria. However, microscopical examination of developing patches of phototrophic organisms revealed that *Oscillatoria*-like cyanobacteria tended to dominate on the contaminated sediment whereas there was more heterogeneity among the phototrophs on the pristine sediment; the most notable among the latter were diatoms and *Anabaena*-like cyanobacteria. Upon prolonged incubation, the oil layer became gradually felt-like by increasing development of cyanobacteria and possibly other filamentous organisms on its upper surface. It is presently unclear whether the oil-associated organisms present a higher phototrophic biomass than that in the pristine controls and which factors cause association of the filaments with the oil surface. One the one hand, there may be a purely physical explanation, viz. the aged oil may present a favorable substratum for attachment and growth of cyanobacteria. On the other hand, oil in the course of biodegradation may enhance the supply of CO_2 for photosynthesis. So far, experiments with axenic cyanobacteria in the presence of hydrocarbons do not indicate that they have any degradative capacity (Musat & Widdel, unpublished), in agreement with other observations (Abed & Köster 2004). Also

from the perspective of the function of cyanobacteria as typical photoautotrophic organisms, a direct participation in the biodegradation of oil hydrocarbons is unlikely.

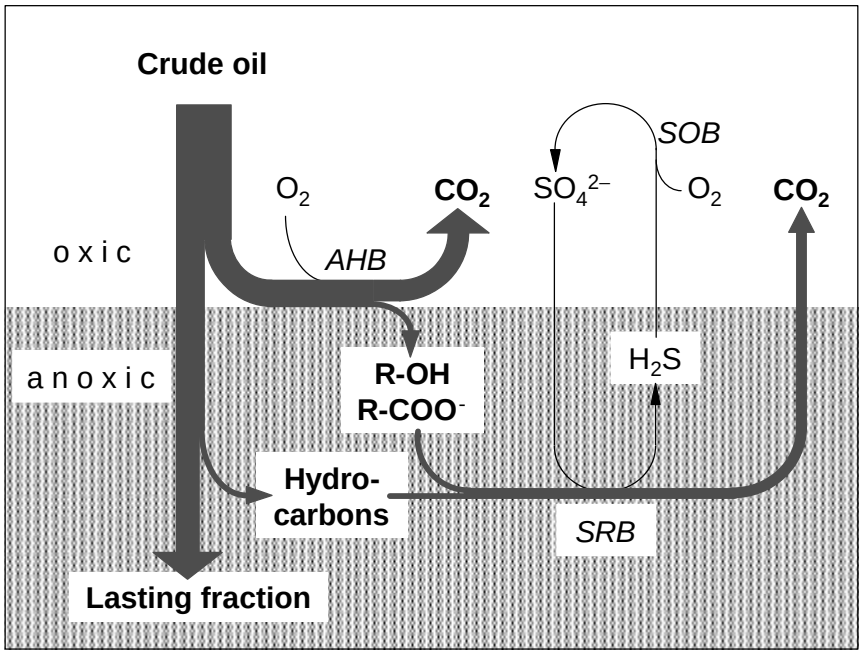


Fig. 4. Scheme of proposed heterotrophic processes on and in the oil-contaminated marine sediment. Aerobic heterotrophic bacteria (*AHB*) oxidize a substantial part of the oil to CO_2, but may release oxygenated organic products. The latter and also a number of hydrocarbons from the oil are utilized by sulfate-reducing bacteria (*SRB*). Produced hydrogen sulfide is aerobically reoxidized by sulfide-oxidizing bacteria (*SOB*). Cyanobacteria that may benefit from produced CO_2 are not included.

In conclusion, the experimental microcosms described in this study offer considerable potential for investigating, under defined conditions, phenomena observed at oil-contaminated marine sites. For instance, the significance of light for the oil-degrading community or of oxygen for the formation of substrates for sulfate-reducing bacteria may be studied by the use of parallel incubations under dark or anoxic conditions, respectively. Furthermore, the influence of nutrient additions (e.g., ammonia, nitrate, phosphate) fertilization on oil-degrading and associated microbial communities may be studied in such systems.

Acknowledgments

We thank Jens Harder for help with chemical analyses and Dirk de Beer for support of microsensor work. This work was supported by the European project MATBIOPOL (EVK3-CT1999-00010).

References

Abed, R. M. M. and J. Köster. 2004. The direct role of aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds. Inter. Biodet. Biodegr. (in press).

Beller, H.R. 2000. Metabolic indicators for detecting *in situ* anaerobic alkylbenzene degradation. Biodegradation **11:** 125-139.

Belyaev, S. S., K. S. Laurinavichus, S. N. Obratsova, S. N Gorlatov, and M. V. Ivanov. 1982. Microbiological processes in the critical zone of injection wells in oilfields. – Microbiology (Transl. from *Mikrobiologiya*, Moscow) **51:** 793-797.

Bragg, J. R., R. C. Prince, E. J. Harner, and R. M Atlas. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. Nature **368:** 413-418.

Gieg, L. M. and J. M. Suflita. 2002. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. Environ. Sci. Technol. **36:** 3755-3762.

Harayama, S., H. Kishira, Y. Kasai, and K. Shutsubo. 1999. Petroleum biodegradation in marine environments. J. Mol. Microbiol. Biotechnol. **1:** 63-70.

Kühl, M. and B. B. Jørgensen B. B. 1992 Microsensor measurement of sulfate reduction and sulfite oxidation in compact microbial communities of aerobic biofilms. Appl. Environ. Microbiol. **58:** 1164-1174.

Nazina, T. N., E. P. Rozanova, and S. I. Kuznetsov. 1985. Microbial oil transformation processes accompanied by methane and hydrogen sulfide formation. Geomicrobiol. J. **4:** 103-130.

Oh, Y.-S., D.-S. Sim, and S.-J. Kim. 2001. Effects of nutrients on crude oil biodegradation in the upper intertidal zone. Marine Pollut. Bull. **42:** 1367-1372.

Prince, R.C., E.H. Owens, and G.A. Sergy. 2002. Weathering of an Arctic oil spill over 20 years: The BIOS experiment revisited. Marine Pollut. Bull. **44:** 1236-1242.

Rabus, R., M. Fukui, H. Wilkes, and F. Widdel. 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. Appl. Environ. Microbiol. **62:** 3605-3613.

Richnow, H. H., E. Annweiler, W. Michaelis, and R. U. Meckenstock. 2003. Microbial in situ degradation of aromatic hydrocabons in contaminated aquifer monitored by carbon isotope fractionation. J. Contam. Hydrol. **65:** 101-120.

Rueter, P., R. Rabus, H. Wilkes, F. Aeckersberg, F. A. Rainey, H. W. Jannasch, and F. Widdel. 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature **372:** 455-458.

Shi, Y., M. D. Zwolinski, M. E. Schreiber, J. M. Bahr, G. W. Sewell, and W. J. Hickey. 1999**.** Molecular analysis of microbial community structures in pristine and contaminated aquifers: field and laboratory microcosm experiments. Appl. Environ. Microbiol. **65:** 2143-2150.

Swannell R. P. J., K. Lee, and M. McDonagh. 1996. Field evaluations of marine oil spill bioremediation. Microbiol. Rev. **60:** 342-365.

Widdel, F. and R. Rabus 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr. Opinion Biotechnol. **12:** 259-276.

Wieland, A. and M. Kühl, M. 2000 Short-term temperature effects on oxygen and sulfide cycling in a hypersaline cyanobacterial mat (Solar Lake, Egypt). Mar. Ecol. Prog. Ser. **196:** 87-102.

2

Quantification of petroleum biodegradation on marine sediments in experimental microcosms

Florin Musat[1], Heinz Wilkes[2] and Friedrich Widdel[1]*

Manuscript in preparation

[1] Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany

[2] GeoForschungZentrum Potsdam, Aufgabenbereich 4.3: Organische Geochemie, Telegrafenberg, D-14473 Potsdam, Germany

*** Corresponding author**

Abstract

Biodegradation of petroleum on marine sediments was investigated in microcosms by measuring mineralization of the oil-carbon to CO_2. To simulate conditions after oil pollution in natural open systems, the oil was allowed to loose the volatile fraction by evaporation before the degradation experiments. The mass of the volatile fraction ranged from 18 to 34% wt/wt of the original crude oils used in the present study. The highest extent of biodegradation was obtained upon fertilization with inorganic nutrients (ammonia, phosphate, iron[III]) and fine distribution of petroleum on solid inert matrices. But even under such optimum conditions, mineralization of the different weathered crude oils was only 15 to 20% within 240 days of incubation. Assuming an assimilation of 50% of the degraded organic carbon in oil into biomass (based on published reports), between 42 and 62% of the original (non-weathered) oil was removed by the combined processes of evaporation, mineralization and assimilation. Accordingly, between 38 and 58% are likely to persist for a long, indefinite period of time. Gas-chromatographic analysis of the residual fraction showed that normal and branched alkanes were almost completely degraded. To our knowledge, this is the first accurate quantification of the extent of mineralization of petroleum on marine sediments.

Introduction

Crude oil is a major pollutant of the marine environment with significant amounts of petroleum hydrocarbons being released into the oceans from accidental spills. The spilled hydrocarbons are subject to a variety of physical, chemical and biological transformations (Atlas, 1981; Prince, 1993) such as evaporation of compounds with less than 15 carbon atoms (Bragg *et al*., 1994; Prince, 2002) or photooxidation of the aromatic oil fraction (Garrett *et al*., 1998; Prince *et al*., 2003). Nevertheless, the major mechanism of oil removal from the environment is biodegradation which, therefore, has received most attention in the study and treatment of oil pollution over the past decades (Atlas, 1981; Leahy and Colwell, 1990; Atlas and Bartha, 1992; Prince, 1993; Atlas, 1995a, b; Van Hamme *et al*, 2003).

The activity of the hydrocarbon-degrading bacteria is strongly affected by environmental factors, most notably by the availability of oxygen and inorganic nutrients (nitrogen, phosphorus, and iron) and accessibility of petroleum hydrocarbons for the hydrocarbon-degrading bacteria (Leahy and Colwell, 1990; Prince, 1993). Although a large number of petroleum hydrocarbons have been proven to be degraded under anoxic conditions (Heider *et al*., 1999; Widdel and Rabus, 2001), it is actually considered that aerobic biodegradation is quantitatively most significant for oil bioremediation in the environment (Widdel and Rabus, 2001; Prince, 2002). The nitrogen and phosphorus content in crude oils is generally very low (Tissot and Welte, 1984), and the activity of the hydrocarbon-degrading bacteria is strongly limited by the availability of these elements. Hydrocarbon degradation was strongly stimulated in both laboratory experiments (Atlas and Bartha, 1972a; Oh *et al*, 2001) and field applications (Prince and Bragg, 1997; Atlas, 1995a; Bragg *et al*, 1994; Leahy and Colwell, 1990) by fertilization with combined nitrogen and phosphorus. But also iron has been recognized as a strongly growth-limiting element in the marine environment, and supplementation with iron has been proven to increase the rate of crude oil degradation (Dibble and Bartha, 1976). However, it is generally assumed that even under conditions without inorganic nutrient limitation crude oils are not completely degraded, such that a certain fraction persists indefinitely in the environment (Atlas, 1995a).

Because petroleum is a complex mixture of hundreds of hydrocarbons and other organic compounds, quantitative estimation of bulk petroleum biodegradation has proven to be difficult. Biodegradation of petroleum has been commonly quantified by gas chromatography (GC) analyses of the GC-resolvable compounds, and comparison of the changes in the chemical composition of the biodegraded sample relative to the original oil or to conserved internal markers, mainly pristane, phytane and hopanes (Atlas, 1981; Atlas and

Bartha, 1992; Bragg *et al*., 1994; Prince *et al*., 1994; Swannell *et al*., 1996). The extent of biodegradation of the GC-resolvable compounds determined by this approach may be higher than 90%, but estimates vary considerably depending on the nature of the conserved markers used, the nature of crude oil, availability of inorganic nutrients, environmental conditions and time of sampling (Prince, 1993; Swannell *et al*., 1996). On the other hand, not all compounds in crude oil can be resolved by GC, and biodegradation of the unresolved fraction cannot be estimated. Also, high-molecular mass alkanes and polycyclic aromatic hydrocarbons (PAH) with 30 and more atoms of carbon cannot be analyzed by this method since they lack sufficient volatility (Atlas and Bartha, 1992). Moreover, markers considered resistant to biodegradation may actually be biodegraded, as demonstrated for pristane and phytane (Dibble and Bartha, 1976), and more recently for hopanes (Frontera-Suau *et al*, 2002). In such conditions, the estimates based on internal markers may represent underestimates of the extent of biodegradation.

A completely different approach based on measurement of total carbon has been attempted, and it indicated degradation to CO_2 of 42% of a paraffinic, i.e. *n*-alkane-rich petroleum (Atlas and Bartha, 1972a, b). But studies on biodegradation of petroleum under laboratory or *in situ* conditions have shown that in particular normal alkanes are very susceptible to biodegradation and therefore quite often completely degraded (Haines and Alexander, 1974; Atlas and Bartha, 1992). It is thus likely that mineralization of aliphatic-rich petroleum is not comparable with mineralization of more complex crude oils, with significant content of various aromatics or polar compounds. Moreover, contamination of the marine environment with crude oil is most serious in coastal areas, where biodegradation is further impaired by the adherence of oil to sediments (Weissenfels *et al*., 1992; Weise *et al*., 1999; Lee *et al*., 2003).

To our knowledge, quantification of the biodegradation of crude oil distributed on marine sediments by measurements of the total oil-carbon has not been attempted so far. In a previous study on biodegradation of oil on marine sediments in experimental microcosms, we observed that biodegradation of oil was rather incomplete even after prolonged incubation time (Musat *et al*., 2004). The present study was undertaken to determine the biodegradability of oil spread on sediment surface, under conditions that favor evaporation of the volatile fraction. The extent of biodegradation was determined with and without fertilization with inorganic nutrients. At the same time we studied the influence of the degree of oil distribution and the role of different mineral matrices on oil biodegradability. Measurement of total oil-carbon mineralization suggested that even under the experimentally

achieved optimum conditions (fine distribution of oil and fertilization with inorganic nutrients) only a relatively small fraction of the weathered oil was degraded to CO_2 within 240 days of incubation. GC analysis of the residual oil fraction showed that the normal and branched alkanes were completely biodegraded.

Materials and methods

Origin of mineral support materials, petroleum and inoculum

Clay-like sediment was collected from the tidal area (Wadden Sea of North Sea) in Horumersiel near Wilhelmshaven (Germany). This material was chosen because of its relatively low content of fresh (i.e. readily utilisable) organic substrates. Limestone gravel (0.5 – 1.0 cm diameter, irregular shape) was collected near Bremen, Germany. Fine-grain (particles of <0.1 to 0.1 mm diameter) and coarse-grain (particles of 0.3 to 0.7 mm diameter) silicate sands were commercially available from Baltus (Bremen, Germany). Before use, gravel and sand were washed twice with 100 mM HCl (each 15 min), rinsed thoroughly with distilled water and dried in an oven at 50 ^{o}C. The four crude oil types used in this study were from the Gulf of Mexico, Casablanca, North Sea and Sinstorf (near Hamburg).

Preparation of weathered oil and determination of the carbon content

Weathered oil for the degradation experiments was prepared by dissolving defined amounts (usually 75 mg) of petroleum in dichloromethane (DCM), distribution on sand in 1-l bottles (Ochs GmbH, Bovenden-Lenglern, Germany); the bottles were placed in a fume hood at 24 – 25 ^{o}C to allow evaporation of the light oil fraction. The mass of the volatile fraction was determined by distribution of small amounts of DCM-dissolved crude oil (between 5 and 8 mg) on sand in tin cups and determination of the weigh loss with a high-precision balance (Sartorius AG, Göttingen, Germany). The low boiling point of DCM ensured rapid evaporation of the solvent. Thereafter, up to 34% of the lightest crude oil evaporated within 140 h (Fig. 1). Oil that had lost the light fraction via evaporation is referred to as weathered oil. When weight changes were no longer recorded (usually after 5 to 6 days of evaporation) (Table 1), the weathered oil in the tin cups was analysed for its carbon content by combustion using a Nitrogen Analyzer NA 1500 (Fisons Instruments GmbH, Mainz-Kastel, Germany). The weathered oil distributed on sand in bottles was used as a starting material for degradation experiments.

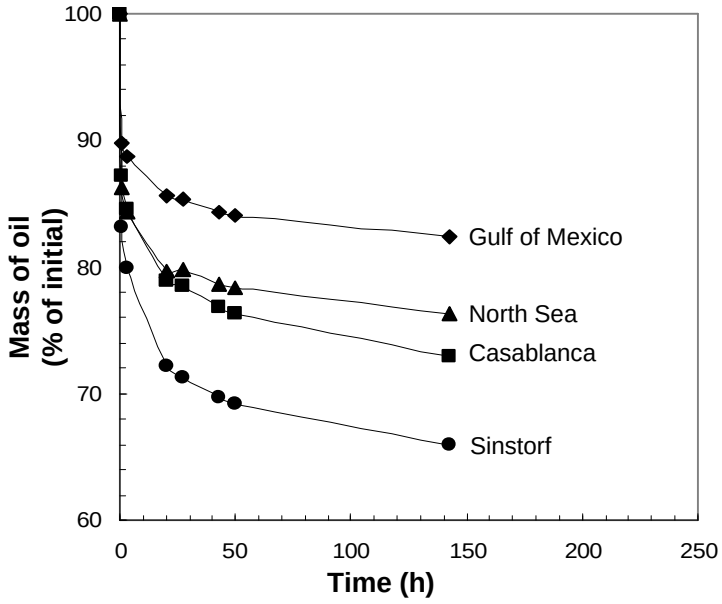


Fig. 1. Determination of the mass of crude oils remaining during loss of the volatile fractions. Between 5 and 8 mg of crude oil were dissolved in dichloromethane, distributed on sand and placed in a fume hood at 25 °C. Weight changes due to evaporation of the light fraction were monitored for up to 6 days. Weight changes due to evaporation of the solvent are not shown.

Table 1. The carbon content of crude oils depleted in the volatile fraction as determined by combustion analysis

Crude oil	(% by mass)	(mmol [g oil]$^{-1}$)
Gulf of Mexico	90.0	75
Casablanca	93.5	78
North Sea	73.5	61
Sinstorf	94.6	79

Biodegradation experiments

All degradation experiments were performed in duplicate in Duran bottles with a total volume of 1165 ml which were provided with crude oil on different matrices: (a) 2.0 ml from a solution of 37.5 mg oil per ml DCM were spread over the entire surface of 50 g of coarse-grain sand; (b) 2.0 ml of the DCM-dissolved oil was spread over the glass surface in bottles that received no sand; (c) 80 mg of undiluted crude oil was added as a droplet to sand; (d) and 80 mg of undiluted crude oil was added on top of 45 ml sea-water with a bottom layer of sand (volume of sand and water, 60 ml; head-space, 1100 ml). Other mineral matrices used and the amounts added per bottle were (e) fine-grain sand (50 g), (f) clay-like sediment (5 mm thick layer), and (g) gravel (12 to 15 pieces). All experiments were placed in a fume hood for 5 days to allow evaporation of the organic solvent (if present) and the

light oil fraction. Evaporation of the sea-water was compensated by addition of distilled water if needed. After evaporation, also the water-free bottles (viz. those with crude oil added over sand, clay-like sediment and gravel) were provided with 45 ml of natural sea water. The source of inoculum was a mixture of samples from oil-contaminated marine sediments described elsewhere (Musat *et al*., 2004). Cores of 0.5 cm diameter and 1.0 cm lenght were collected from these sediments and mixed with natural sea water (North Sea, Germany, salinity of 3.5 %). All bottles except for two sterile controls were inoculated with 2 ml from the oil-free sediment suspension. The seawater was amended with NH_4Cl (200 mg l^{-1}), NaH_2PO_4 (32 mg l^{-1}) and $FeSO_4$ (2.1 mg l^{-1}). Following water and inoculum addition, all bottles were immediately closed with thick, black rubber stoppers (Ochs GmbH, Bovenden-Lenglern, Germany) hold in place with perforated screw caps, and incubated in the dark at 24 – 25 ^{o}C.

Chemical analyses

Samples for CO_2 analysis were withdrawn through the stopper via syringes. Measurements were done with a Shimadzu 8A gas chromatograph (Shimadzu Corp, Kyoto, Japan) equipped with a thermal conductivity detector (TCD) and a 2 m Porapak Q column (Sigma Aldrich GmbH, Taufkirchen, Germany). Injector and detector were maintained at 110 ^{o}C, and the oven was operated isothermally at 40 ^{o}C. Nitrogen was used as carrier gas.

For analysis of oil hydrocarbons in the experiments with coarse sand as carrier matrix, samples were extracted with *n*-hexane using ultrasonification. After evaporation to dryness the extract yields were determined gravimetrically. Based on the extract mass, a specific amount of 5α-androstane was added as internal standard. The samples were fractioned into aliphatic hydrocarbons, aromatic hydrocarbons and polar compounds by MPLC as previously described (Radke *et al*, 1980). The alkane fractions were analysed by gas chromatography using a 6890 Gas Chromatograph (Agilent Technologies) equipped with a PTV injection system, a FID, and an Ultra 1 fused silica capillary column (length 50 m, inner diameter 0.2 mm, film thickness 0.33 μm). Helium was used as carrier gas. The oven temperature was programmed from 40 ^{o}C (initial hold time 2 min) to 300 ^{o}C (final hold time 65 min) at a rate of 5 ^{o}C min^{-1}. Individual compounds were quantified relative to the known amounts of the internal standard.

Results

Approach for quantitative study of bulk oil degradation on solid substrata

Since crude oil is a very concentrated form of reducing equivalents, the requirement of oxygen per amount of substrate is high. Conventional experiments involve high amounts of oil, which in a closed system for quantification of CO_2 would require voluminous gas phases. If, on the other hand, very small droplets are added that do not deplete the O_2 reservoir, they are poorly accessible by bacteria. Consequently, our experiments were designed in order to obtain significant O_2 consumption and CO_2 production, but also such that O_2 was still present after growth to avoid O_2 limitation. First, the amount of crude oil to be added to a closed system with a defined volume of air was calculated assuming the approximate general formula $<CH_2>$ yielding the following equation for aerobic biodegradation:

$$CH_2 + 1.5\ O_2 \ ?\ \ CO_2 + H_2O \qquad (1).$$

Accordingly, 1.0 g (71.4 mmol) of crude oil requires 3.4 g oxygen (106.25 mmol O_2), corresponding to 13 l of air under normal conditions. Consequently, a closed system with a head-space of 1 l contains the amount of oxygen theoretically needed for the complete oxidation of 77 mg of crude oil. Therefore, the amount of oil added in our biodegradation experiments never exceeded 77 mg, unless otherwise specified. Because this is a relatively small amount of oil, we attempted to obtain optimum distribution to avoid limitations in substrate accessibility. Considering that a certain fraction of this oil will be lost by evaporation, that crude oil is not completely degraded to CO_2 and H_2O, and that a significant fraction is incorporated into biomass, the amount of oxygen under such experimental conditions should be even present in excess. Nevertheless, during all biodegradation experiments the oxygen concentration was continuously monitored to verify that it did not become the limiting factor.

To calculate the extent of oil degradation, the experimentally quantified CO_2 was compared with the theoretical amount of CO_2 that would be formed by complete oxidation of the added oil. The theoretical amount of CO_2 was considered to be equimolar with the total carbon content of crude oil as determined by combustion analyses (Table 1).

The influence of inorganic nutrient addition on biodegradation of weathered oil

Although the influence of inorganic nutrients (nitrogen, phosphorus and iron(III)) on biodegradation of crude oil has been extensively studied (Atlas and Bartha, 1972b; Dibble

and Bartha, 1976; Olivieri *et al*., 1976; Pritchard and Costa, 1991; Prince and Bragg, 1997), an exact quantification of the extent of mineralization of crude oil spread on marine sediments under conditions of fertilization with inorganic nutrients has not been attempted so far. We investigated the effect of supplementation with inorganic nutrients on crude oil biodegradation using Gulf of Mexico crude oil distributed on coarse-grain sand. As expected, the rate of oil mineralization was significantly higher if inorganic nutrients were supplemented (Fig 2). Within the initial 40 days of incubation, the biodegradation rates in the experiments with addition of inorganic nutrients were 4 to 5 times higher than in the experiments without such additions.

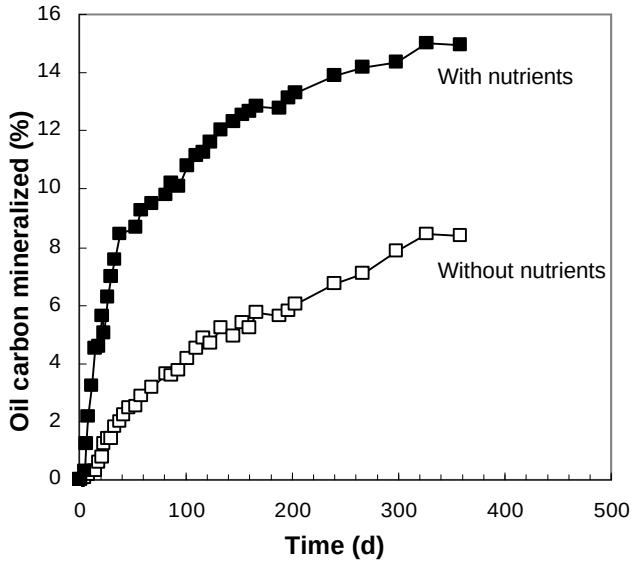


Fig. 2. Mineralization of weathered Gulf of Mexico crude oil (75 mg) finely distributed on coarse-grain sand (ca. 50 g) in experimental microcosms (1-l bottles), with and without supplementation of inorganic nutrients.

The decrease of the biodegradation rates between 40 and 360 days of incubation (Fig. 2) may be explained by the depletion of readily biodegradable compounds. However, the extent of mineralization within 360 days of incubation was only 13% of the initial oil in the experiments with added inorganic nutrients, and only 8% in the experiments without inorganic nutrients (Fig. 2). Our results, which confirm the importance of fertilization with inorganic nutrients, show that even under such favourable conditions, only a small fraction of the oil-carbon is mineralized within relatively long incubation times. To gain further insights into mineralization of petroleum spread on marine sediments, the influences of the solid mineral substrata and of the degree of petroleum distribution on the extent of degradation were investigated.

Biodegradation of crude oil under different conditions of physical distribution

To determine the influence of different mineral matrices, biodegradation of Gulf of Mexico crude oil distributed over clay-like sediment, fine- and coarse-grain sand, and gravel was investigated. Since addition of inorganic nutrients significantly stimulated biodegradation of oil, all experiments, including the oil-free controls were supplemented with ammonia, phosphorus and iron. The use of gravel and clay-like material yielded high levels of CO_2 production in the controls (data not shown). This was most probably due to the release of CO_2 from carbonate minerals (clay-like sediment) or to degradation of substrates existing in both these materials. Because low background CO_2 production is required for accurate quantitative estimate of petroleum degradation, gravel and clay-like materials were not considered for further quantitative studies. If fine- and coarse-grain sand were used as mineral support, CO_2 production in the control experiments was low and allowed for quantitative estimation of petroleum biodegradation. However, the results obtained with fine-grain sand were inconsistent due to buoyancy of oil-sand particles. The coarse-grain sand, used also in the initial experiments, proved to be the most suitable material for quantitative oil mineralization studies. For comparison, degradation of oil was also studied if oil was adhering to the glass bottle, as a compact droplet on sand, and on the water surface.

The highest mineralization rates were obtained with oil finely distributed on sand (Fig. 3). Addition of oil over water yielded comparable extent of mineralization within 320 days of incubation. However, the results with oil added over water in parallel incubations were poorly reproducible, due to the inconsistent adherence of oil to the glass. Oil added over glass also resulted in inconsistent distribution and adherence to the glass. Consequently, the extent of mineralization was low (Fig. 3) and poorly reproducible. The lowest mineralization extent was obtained with the oil added as a droplet to the sand. Although inorganic nutrients were supplemented as in all other experiments, the extent of biodegradation was limited by the poor distribution that ultimately affects substrate availability (Fig. 3).

These results are in agreement with previous investigations on the fate of petroleum spilled in marine environment showing that biodegradation is significantly influenced by the availability of oil-compounds for the oil-degrading microorganisms (Floodgate, 1984; Leahy and Clowell, 1990; Atlas and Bartha, 1992). For example, formation of tarballs or asphalt pavements as a result of interaction of crude oil with sediments greatly reduced the extent of biodegradation (Atlas, 1981; Atlas and Bartha, 1992) and it has been shown that such structures persist for a very long time in the environment (Owens *et al*., 2002).

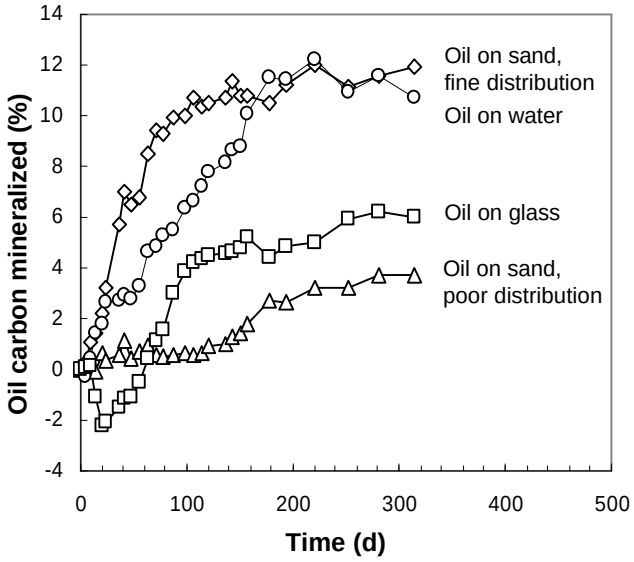


Fig. 3. The influence of distribution on the extent of petroleum mineralization. Crude oil (75 to 80 mg) was differently distributed on coarse-grain sand (ca. 50 g), and all experiments were supplemented with inorganic nutrients.

However, even with fine distribution of petroleum and fertilization with inorganic nutrients, the extent of mineralization of weathered crude oil within 320 days was relatively low (Fig. 3). In order to investigate whether this is a principle characteristic of biodegradability of crude oils, mineralization of other types of crude oil depleted of the volatile fraction was quantified.

Quantitative mineralization experiment wit different types of crude oil

Under the established favourable conditions of fertilization with inorganic nutrients and fine distribution of crude oil on coarse-grain sand, biodegradation of different types of crude oil depleted of the volatile fraction was investigated. For comparison, analogous degradation experiments were carried out with naphthalne and glucose as easily degradable defined compounds. Four different crude oil types, from Gulf of Mexico, Casablanca, North Sea and Sinstorf, were used. Mineralization of all crude oils was very low in comparison to mineralization of naphthalene and glucose (Fig. 4). Thus, the extent of oil mineralization within 240 days of incubation ranged from 15% (Gulf of Mexico) to 20% (Sinstorf) of the initial oil-carbon, while 65 and 77% of the added naphthalene and glucose, respectively, were mineralized (Fig. 4).

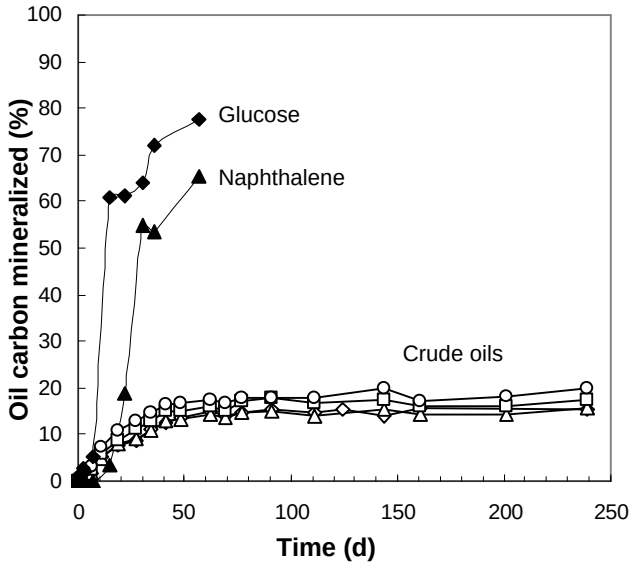


Fig. 4. Mineralization of Gulf of Mexico, Casablanca, North Sea and Sinstorf crude oils under optimum conditions. Crude oils (75 mg each) were dissolved in dichloromethane and finely distributed on coarse-grain sand (ca. 50 g) in 1-l bottles. Mineralization of the defined compounds, naphthalene (80 mg) and glucose (280 mg) is shown for comparison.

Chemical analysis of the residual fraction of Gulf of Mexico crude oil collected after 174 days of incubation clearly showed extensive biodegradation of the normal and branched alkanes (Fig. 5). With inorganic nutrient supplementation, *n*-alkanes with up to 39 carbon atoms were completely biodegraded (Fig. 5). The branched alkanes pristane and phytane were also degraded, but to a lesser extent. Without supplementation with inorganic nutrients, biodegradation of saturated hydrocarbons was also significant, but less extensive.

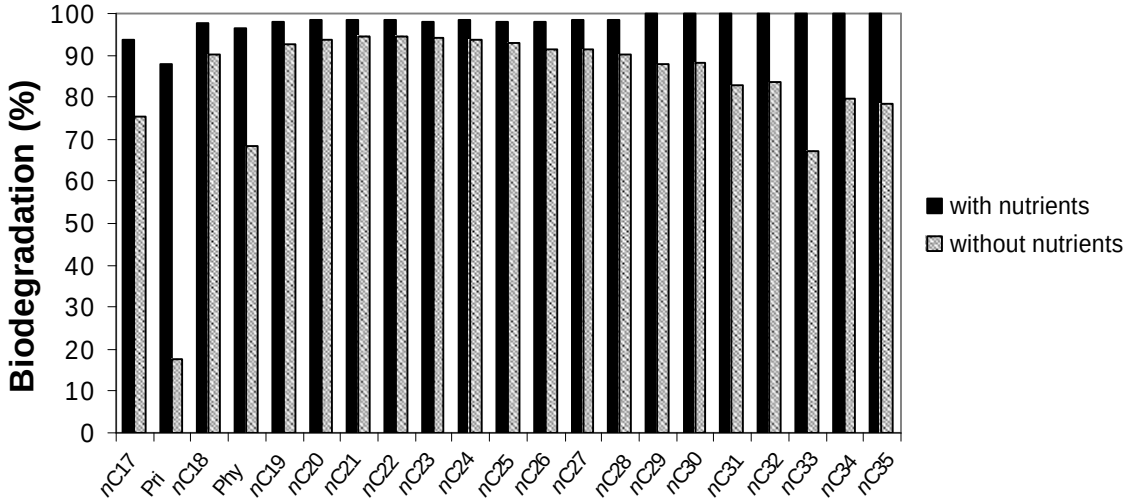


Fig. 5. Biodegradation of normal and branched alkanes of weathered Gulf of Mexico crude oil finely distributed on coarse-grain sand, with and without addition of inorganic nutrients.

The molecular ratios Pri/*n*C17 and Phy/*n*C18 determined for the biodegraded samples were clearly higher than those of the weathered oil, indicating loss of *n*-alkanes due to biodegradation (Table 2). Most importantly, there was no significant difference of these ratios between the weathered and the original oil (Table 2).

Table 2. Molecular ratios of branched- versus *n*-alkanes for original, weathered and biodegraded Gulf of Mexico crude oil.

Sample	Pri/*n*C17	Phy/*n*C18
Gulf of Mexico crude oil	0.31	0.47
Gulf of Mexico weathered oil	0.32	0.49
Degradation with inorganic nutrients	0.62	0.72
Degradation without inorganic nutrients	1.08	1.58

Discussion

Investigation of the extent of oil biodegradation revealed that even under optimum distribution conditions and fertilization with inorganic nutrients, only a relatively small fraction of the total oil-carbon was mineralized to CO_2. Thus, between 15 and 20% of the initial weathered oil were mineralized within 1 year of incubation. Previous studies on the extent of mineralization of crude oil in seawater showed that as much as 42% of a paraffinic petroleum has been mineralized within 32 days (Atlas and Bartha, 1972a, b). Paraffinic crude oils contain high amounts of *n*-alkanes (paraffins), compounds known to be relatively easily biodegradable as reported from field studies and laboratory investigations (Leahy and Colwell, 1990; Bragg *et al*., 1992; Prince 1993). In our study, we focused on pre-evaporated oil that had lost a significant amount of the *n*-alkane fraction through evaporation. Moreover, we investigated biodegradation of crude oil on solid mineral support, a situation that very often occurs in nature following an oil spill. In view of the high degree of oil distribution on sediments, which we achieved with the help of organic solvents and which is unlikely to occur in the environment, we consider that our results represent the maximum extent of oil biodegradation if optimum distribution is achieved.

Biodegradation is certainly accompanied by assimilation of oil-carbon into biomass. In growth studies with bacteria or yeasts on defined hydrocarbons it has been shown that the assimilated proportion is as high as 50% of the supplied carbon (Linton and Stephenson,

1978; Shennan, 1984; Morgan and Watkinson, 1994). Assuming similar carbon assimilation, between 42 and 62 % of the original (non-weathered) oil was removed by the combined processes of evaporation, mineralization and assimilation within 240 days of incubation (Fig. 6). However, such biomass estimates were obtained with pure cultures in bioreactors optimized for biomass production. In an enriched complex microbial community, grazing, cell death and further biodegradation of the assimilated carbon is expected to decrease the biomass and increase the CO_2 yields. If glucose and naphthalene were used as substrates in the degradation experiment, 77 and 65%, respectively, of the original carbon was recovered as CO_2. Consequently, it would be more reasonable to consider the biomass yields amounting to around 35% of the biodegraded carbon, as suggested by mineralization of naphthalene in the present study. In such conditions, the biomass yields presented in Fig. 6 would be overestimates.

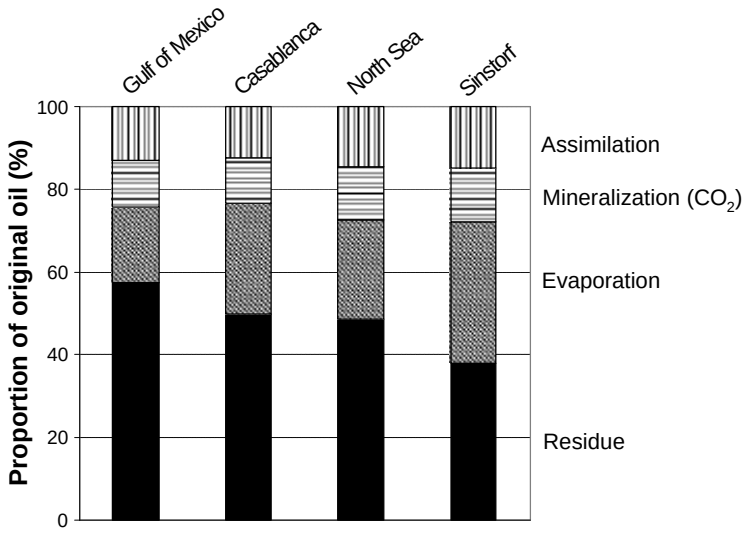


Fig. 6. The calculated extent of petroleum removal by evaporation and biodegradation (mineralization and assimilation) under optimum distribution conditions and with supplementation of inorganic nutrients.

The residual fraction, ranging from 38 to 58%, is likely to persist for an indefinite period of time in the environment. We have no information yet concerning the nature of this fraction. It is considered, for example that the polar fraction of crude oil is very resistant to degradation by microorganisms (Atlas, 1981; Atlas and Bartha, 1992). Polycyclic aromatic hydrocarbons with more than 3 rings may be only incompletely oxidized, leading to oxygen consumption but no CO_2 production. Such partly oxidized PAH may bind covalently to macromolecules, for example to humic or fulvic acids, or may form dimers or trimers via ether or ester covalent bonds, thus significantly reducing their availability for microbial

degradation (Richnow *et al*., 1993, 1997; Kästner *et al*., 1999). Although humic or fulvic acids are commonly found in soils, such compounds may be introduced into the marine environment through river inputs, or similar compounds may be formed for instance by decomposition of algal biomass.

Although biodegradation accounts for only a small fraction, probably this particular fraction is the most problematic with respect to environmental effects. Following biodegradation of this fraction, the remaining residue most likely consists of large compounds, e.g. asphaltenes or partly oxidized PAH, with considerably reduced toxicity. Moreover, such large compounds are solid and are as inert as the macromolecules encountered in the composition of soils.

Acknowledgments

We thank Jens Harder and Cristina Probian for help with chemical analyses. This work was supported by the European project MATBIOPOL (EVK3-CT1999-00010).

References

Atlas, R. M., and R. Bartha. 1972. Degradation and mineralization of petroleum by two bacteria isolates from coastal waters. Biotechnol and Bioeng **14:**297-308.

Atlas, R. M., and R. Bartha. 1972. Degradation and mineralization of petroleum in sea water: limitation by nitrogen and phosphorus. Biotechnol and Bioeng **14:**309-318.

Atlas, R. M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiol Rev **45:**180-209.

Atlas, R. M., and R. Bartha. 1992. Hydrocarbon biodegradation and oil spill bioremediation. Adv Microb Ecol **12:**287-338.

Atlas, R. M. 1995. Petroleum biodegradation and oil spill bioremediation. Mar Poll Bull **31:**178-182.

Atlas, R. M. 1995. Bioremediation of petroleum pollutants. Int Biodet Biodeg**:**317-327.

Bragg, J. R., R. C. Prince, J. B. Wilkinson, and R. M. Atlas. 1992. Bioremediation for shoreline cleanup following the 1989 Alaskan oil spill. Exxon Co.

Bragg, J. R., R. C. Prince, E. J. Harner, and R. M. Atlas. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. Nature **368:**413-418.

Dibble, J. T., and R. Bartha. 1976. Effect of iron on the biodegradation of petroleum in sea water. Appl Environ Microbiol **31:**544-546.

Floodgate, G. D. 1984. The fate of petroleum in marine ecosystems, p. 355-399. *In* R. M. Atlas (ed.), Petroleum Microbiolgy. Macmillan Publishing Company, New York.

Frontera-Suau, R., F. D. Bost, T. J. McDonald, and P. J. Morris. 2002. Aerobic biodegradation of hopanes and other biomarkers by crude oil-degrading enrichment cultures. Environ Sci Technol **36:**4585-4592.

Garrett, R. M., I. J. Pickering, C. E. Haith, and R. C. Prince. 1998. Photooxidation of crude oils. Environ Sci Technol **32:**3719-3723.

Haines, J. R., and M. Alexander. 1974. Microbial degradation of high-molecular weight alkanes. Appl Microbiol **28:**1084-1085.

Heider, J., A. M. Spormann, H. R. Beller, and F. Widdel. 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiol Rev **22:**459-473.

Kästner, M., S. Streibich, M. Beyrer, H. H. Richnow, and W. Fritsche. 1999. Formation of bound residues during microbial degradation of [14C]anthracene in soil. Appl Environ Microbiol **65:**1834-1842.

Leahy, J. G., and R. R. Colwell. 1990. Microbial degradation of hydrocarbons in the environment. Microbiol Rev **54:**305-315.

Lee, K., P. Stoffyn-Egli, G. H. Tremblay, E. H. Owens, G. A. Sergy, C. C. Guenette, and R. C. Prince. 2003. Oil-mineral aggregate formation on oiled beaches: natural attenuation and sediment relocation. Spill Sci & Technol Bull **8:**285-296.

Linton, J. D., and R. J. Stephenson. 1978. A preliminary study on growth yields in relation to the carbon and energy content of various organic growth substrates. FEMS Microbiol Lett **3:**95-98.

Morgan, P., and R. J. Watkinson. 1994. Biodegradation of components of petroleum, p. 1-31. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publisher.

Musat, F., A. Wieland, and F. Widdel. 2004. Marine sediment with surface contamination by oil in microcosms for microbiological studies. Ophelia **58:**217-222.

Oh, Y. S., D. S. Sim, and S. J. Kim. 2001. Effects of nutrients on crude oil biodegradation in the upper intertidal zone. Mar Poll Bull **42:**1367-1372.

Olivieri, R., P. Bacchin, A. Robertiello, N. Oddo, L. Degen, and A. Tonolo. 1976. Microbial degradation of oil spills enhanced by a slow-release fertilizer. Appl Environ Microbiol **31:**629-634.

Owens, E. H., G. S. Mauseth, C. A. Martin, A. Lamarche, and J. Brown. 2002. Tar ball frequency data and analytical results from a long-term beach monitoring program. Mar Poll Bull **44:**770-780.

Prince, R. C. 1993. Petroleum spill bioremediation in marine environment. Crit Rev Microbiol **19:**217-242.

Prince, R. C., D. L. Elmendorf, J. R. Lute, C. S. Hsu, C. E. Halth, J. D. Senius, G. J. Dechert, G. S. Douglas, and E. L. Butler. 1994. 17α(H),21β(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ Sci Technol **28:**142-145.

Prince, R. C., and J. R. Bragg. 1997. Shoreline bioremediation following the Exxon Valdez oil spill in Alaska. Bioremed J **1:**97-104.

Prince, R. C. 2002. Petroleum and other hydrocarbons, biodegradation of, p. 2402-2416. *In* G. Bitton (ed.), Encyclopedia of Environmental Microbiology. John Wiley, New York.

Prince, R. C., R. M. Garrett, R. E. Bare, M. J. Grossman, T. Townsend, J. M. Suflita, K. Lee, E. H. Owens, G. A. Sergy, J. F. Braddock, J. E. Lindstrom, and R. R. Lessard. 2003. The roles of photooxidation and biodegradation in long-term weathering of crude and heavy fuel oils. Spill Sci & Technol Bull **8:**145-156.

Pritchard, P. H., and C. F. Costa. 1991. EPA's Alaska oil spill bioremediation project. Environ Sci & Technol **25:**372-379.

Radke, M., H. Willsch, and D. H. Welte. 1980. Preparative hydrocarbon group determination by automated medium pressure liquid chromatography. Analytical Chemistry **52:**406-411.

Richnow, H. H., R. Seifert, J. Hefter, M. Kaestner, B. Mahro, and W. Michaelis. 1993. Metabolites of xenobiotica and mineral oil constituents linked to macromolecular organic matter in polluted environments. Adv Org Geochem **22:**671-681.

Richnow, H. H., R. Seifert, J. Hefter, M. Link, W. Francke, G. Schaefer, and W. Michaelis. 1997. Organic pollutants associated with macromolecular soil organic matter: mode of binding. Org Geochem **26:**745-758.

Shennan, J. L. 1984. Hydrocarbons as substrates in industrial fermentation, p. 643-685. *In* R. M. Atlas (ed.), Petroleum Microbiology. Macmillan Publishing Company, New York.

Swannell, R. P. J., K. Lee, and M. McDonagh. 1996. Field evaluations of marine oil spill bioremediation. Microbiol Rev **60:**342-365.

Tissot, B. P., and D. H. Welte. 1984. Petroleum formation and occurence. Springer-Verlag, Berlin, Germany.

Van Hamme, J. D., A. Singh, and O. P. Ward. 2003. Recent advances in petroleum microbiology. Microbiol Mol Biol Rev **67:**503-549.

Weise, A. M., C. Nalwajko, and K. Lee. 1999. Oil-mineral fine interactions facilitate oil biodegradation in seawater. Environ Technol **20:**811-824.

Weisenfels, W. D., H.-J. Klewer, and J. Langhoff. 1992. Adsorption of polycyclic aromatic hydrocarbons by soil particles: influence on biodegradability and biotoxicity. Appl Microbiol Biotechnol **36:**689.

Widdel, F., and R. Rabus. 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol **12:**259-276.

3

Nitrogen fixation in microbial communities of oil-contaminated marine sediments

Florin Musat, Jens Harder and Friedrich Widdel*

Manuscript in preparation

[1] Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany

*** Corresponding author**

Abstract

The potential of different phylogenetic and physiological microbial groups growing on oil-contaminated and pristine marine sediments in experimental microcosms to fix atmospheric nitrogen was studied. Molecular investigation based on amplification and sequencing of the dinitrogenase reductase structural gene (*nifH*) revealed a high NifH diversity in the oil-contaminated microcosm. Sequences related to heterocystous and non-heterocystous cyanobacteria, green-sulfur bacteria, Betaproteobacteria, Gammaproteobacteria and Deltaproteobacteria were obtained by direct amplification. A less diverse diazotrophic community, dominated by cyanobacteria, was observed in the pristine microcosm. Expression analyses showed that *nifH* genes similar to those of heterocystous and non-heterocystous cyanobacteria were expressed in the light and dark, respectively, in both microcosms. In addition, *nifH* genes similar to those of Deltaproteobacteria were expressed in the oil-contaminated sediments. Nitrogenase activity was measured by the acetylene reduction (AR) assay with oil-contaminated and pristine sediments incubated under light/dark conditions or permanently in the dark. For both oil-contaminated and pristine microcosms developed under light/dark conditions, highest AR activity was observed in the light, suggesting that heterocystous cyanobacteria were the dominant diazotrophs. Nitrogenase activity in the experiments incubated permanently in the dark was very low. The results demonstrated that cyanobacteria were responsible for the bulk nitrogen fixation in the experimental oil-contaminated and pristine marine sediments.

Introduction

Crude oil is a major pollutant of the marine environment and biodegradation of oil or oil constituents as the principal mechanism of remediation have been extensively studied (Prince, 1993; Atlas, 1995; van Hamme *et al*., 2003). Since the main constituents of crude oil are hydrocarbons, microorganisms in environments contaminated with petroleum must deal with a high load of organic carbon in comparison to essential inorganic nutrients, in particular nitrogen, phosphorus, and iron. It has indeed been shown that biodegradation of crude oil is often significantly stimulated by fertilization with combined nitrogen and phosphorus compounds (Leahy and Colwell, 1990; Bragg *et al*., 1994; Atlas, 1995; Prince and Bragg, 1997). On the other hand, the high load of organic carbon and low availability of inorganic nitrogen may, in principle, favor the selection of microorganisms with the capacity to fix atmospheric nitrogen as observed, for example, in the gut of marine invertebrates (Proctor, 1997), natural, pristine microbial mats (Zehr *et al*., 1995; Steppe and Paerl, 2002; Omoregie *et al*., 2004a), sediment and salt marsh environments (Bagwell *et al*., 1997; Lovell *et al*., 2000, 2001).

Following massive contamination with crude oil of coastal areas in the Arabian Gulf, growth of mat-like layers of cyanobacteria on the oil-contaminated sediments has been observed (Sorkhoh *et al*., 1992; Hoffman, 1996; Höpner *et al*., 1996). This raised the question as to their possible role at such highly contaminated sites. While the direct role of cyanobacteria in biodegradation of hydrocarbons is unlikely in the view of new results (Barth, 2003; Abed and Köster, 2005), they may indirectly contribute to the biodegradation of petroleum, for instance by providing fixed nitrogen for the oil-degrading heterotrophs. All major groups of cyanobacteria, including unicellular and non-heterocystous species, have representatives able to fix nitrogen (Bergman *et al*., 1997; Berman-Frank *et al*., 2003). Moreover, cyanobacteria are well known for their ability to fix N_2 in a wide variety of marine environments, including benthic microbial communities such as cyanobacterial mats (Bebout *et al*., 1993; Omoregie *et al*., 2004b). But also associated heterotrophs may contribute fixed nitrogen, since the ability to fix N_2 is widespread among members of the major prokaryotic phylogenetic groups (Zehr *et al*., 2003). Heterotrophic nitrogen-fixation during growth on naturally common polar substrates, for example glucose or polysaccharides, is well known (Herbert, 1975; Tibbles *et al*., 1994). Although crude oil is a rich source of organic carbon, only a relatively small number of studies have shown heterotrophic nitrogen fixation in oil- or hydrocarbon-contaminated environments (Toccalino *et al*., 1993; Eckford *et al*, 2002) and stimulation of hydrocarbon degradation by nitrogen-

fixing bacteria (Piehler *et al.*, 1999). Nevertheless, the heterotrophic nitrogen-fixation in these environments has not been directly linked with the activity of the hydrocarbon-degrading bacteria. Only a limited number of oil-degrading bacteria have been reported to fix N_2 using hydrocarbons as growth substrates (Chen *et al.*, 1993; Prantera *et al.*, 2002).

In the present study, we investigated the capacity for nitrogen fixation in microbial communities growing on oil-contaminated and pristine marine sediments in experimental microcosms. Experimental oil-contaminated and pristine microcosms were established as described previously (Musat *et al.*, 2004). The microorganisms involved in N_2 fixation were identified by amplification and sequencing of nitrogenase *nifH* genes. Nitrogenase activity was examined by expression analysis of the *nifH* gene phylotypes using reverse-transcription-PCR and by measurement of acetylene reduction rates. The results obtained suggest that nitrogen fixation occured mainly in the light and was most notably due to heterocystous cyanobacteria in both the oil-contaminated and pristine microcosms.

Materials and methods

Establishment of microcosms

Establishment of microbial communities on oil-contaminated and pristine sediments in experimental microcosms has been described previously (Musat *et al*, 2004). For nitrogenase (acetylene reduction) assays, microbial guilds were newly and separately established in miniaturized sediment systems in 50 ml serum bottles (Ochs GmbH, Bovenden-Lenglern, Germany). The bottles were supplied with a 5 mm layer of clay-like sediment (Horumersiel, North Sea, Germany). Addition of crude oil (Gulf of Mexico, 0.07 ml cm^{-2}) and natural seawater (7 ml per bottle, salinity 3.5 %) was done as described previously (Musat *et al.*, 2004). Each experiment was inoculated with 1 ml of the oil-free suspension from an oil-contaminated microcosm (Musat *et al.*, 2004). Bottles were incubated under light/dark conditions as described (Musat *et al.*, 2004); in addition, duplicate bottles were incubated permanently in the dark. Experiments with alginate (50 mg $bottle^{-1}$) were incubated in the dark and used as positive control for heterotrophic nitrogen fixation.

Nucleic acid extraction

Cores of 10 mm diameter and 10 mm thickness were collected from the oil-contaminated and pristine microcosms using sectioned syringe barrels. The cores were divided into an

upper layer of 0 to 2 mm and a lower layer of 3 to 10 mm. About 0.5 g of the upper layer and 1.5 g of the lower layer were used for DNA extraction. Total DNA was extracted by freeze-thaw, proteinase K and SDS treatments, chloroform extraction, and isopropanol precipitation (Zhou *et al*., 1996). Total RNA was extracted from cores (3.5 g sediment) collected in the light or dark as previously described (Stahl *et al*., 1988). The quality and purity or RNA was checked using an Agilent 2100 Bioanalyzer (Agilent Technologies, Ambion). Residual DNA was removed by incubation with DNaseI (Fermentas GmbH, St. Leon-Rot, Germany).

Amplification and reverse transcription

Amplification of partial *nifH* fragments was done with the universal primers NIFH1 (5'-TGYGAYCCNAARGCNGA-3', positions 639 to 655 of *A. vinelandii* M11579) and NIFH2 (5'-ADNGCCATCATYTCNCC-3', positions 984 to 1000 of *A. vinelandii* M11579) (Y = C or T; N = A, G, C or T; D = A, G or T; R = A or G) (Zehr *et al*., 1989). The PCR reactions contained 1× PCR buffer, 1 mg BSA ml^{-1}, 100 pmol of each primer, 0.2 mM total dNTP, 1.5 mM $MgCl_2$, 2.5 U RedTaq DNA polymerase (Sigma-Aldrich GmbH, Taufkirchen, Germany), and 20 ng of total DNA. The final volume was adjusted to 50 µl with PCR water. The cycling conditions were as follows: 95 ^oC for 5 min (initial denaturation), followed by 30 cycles of 94 ^oC for 1 min (denaturation), 57 ^oC for 1 min (annealing), and 72 ^oC for 1 min (elongation), and 72 ^oC for 5 min (final elongation).

The reverse transcription reactions contained 1 µg total RNA, 20 pmol of the reverse primer (NIFH2), 5 × reverse transcription buffer, 1 mM total dNTP and 200 U of RevertAid M-MuLV Reverse Transcriptase (Fermentas GmbH, St. Leon-Rot, Germany). The reactions were incubated at 42 ^oC for 90 min. Two µl from the reverse transcription reaction were used as template for PCR amplification with the NIFH1 and NIFH2 primers in the conditions described above. Control reactions without reverse transcriptase were performed.

Construction of *nifH* gene libraries

PCR and RT-PCR products were extracted from agarose gels and purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). The purified products were ligated into the pGEM-T Easy vector (Promega, Madison, Wis.) using the standard Promega protocol. The recombinant vectors were transformed into chemically competent *E. coli* cells. The transformation reactions were plated on LB agar plates with ampicillin (100 µg ml^{-1}), 0.5 mM IPTG and 80 µg X-gal ml^{-1}. The clone libraries were screened by PCR with the

M13F and M13R primers. Positive clones were transferred to 2 ml tubes containing 1 ml LB broth supplemented with ampicillin (100 μg ml^{-1}) and incubated with shaking (180 rpm) at 37°C for 18 h. Plasmids were purified using a QIAprep Spin Miniprep Kit (Qiagen) and sequenced in one direction with the M13F or M13R primer, using the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, USA).

Sequence analysis

Sequences were routinely edited and aligned with the BioEdit Sequence Alignment Editor (Hall, 1999). The amino acid sequences were predicted in BioEdit. For the purpose of phylogenetic reconstruction, the entire NifH database was downloaded from SwissProt and aligned using the ARB software package (Ludwig *et al*., 2004). All NifH sequences obtained in this study were added and aligned to the database. The alignment was corrected manually. Phylogenetic trees were reconstructed using the neighbor joining method in ARB with the application of Kimura corrections.

Acetylene reduction assay

Nitrogenase activity was measured by the acetylene reduction assay (Stewart *et al*., 1967). At defined time intervals (5, 11, 19, 32 and 40 days), triplicate bottles were sealed with rubber stoppers and aluminium crimps and 10% of the headspace volume was replaced by acetylene. Acetylene reduction (AR) was monitored over 24 hours, unless otherwise specified. Ethylene concentrations were quantified by injecting 0.1 ml from the air headspace of the sealed incubated bottles into a Shimadzu GC14A (Shimadzu Corp., Japan) gas chromatograph equipped with a 50 m HP-PLOT U column (Sigma Aldrich GmbH, Taufkirchen, Germany) and an FID detector. Hydrogen was used as carrier gas. The GC was operated as follows: injector 130 °C, detector 150 °C, and oven 55 °C. Each activity time point represents the median of measurements of triplicate microcosms.

Determination of total nitrogen

Bottles from different time points for AR assay were analyzed for the total nitrogen content. Homogenized, triplicate sub-samples were freeze-dried and analyzed by combustion with a Nitrogen Analyzer NA1500 (Fisons Instruments GmbH, Mainz-Kastel, Germany) with sulfanilamide as a calibration standard.

Results

Establishment of microbial communities on oil-contaminated and pristine marine sediments (Musat *et al*., 2004) and qualitative observation of growth stimulation by addition of ammonia suggested that these microcosms were limited by the availability of bound nitrogen. Therefore, the capability of these communities to fix atmospheric nitrogen was investigated by amplification and expression analyses of nitrogenase *nifH* genes, and measurements of nitrogenase activity.

Detection of *nifH* genes and analysis of group affiliation

Nitrogenase *nifH* genes were amplified from both the oil-contaminated (43 sequences) and pristine (41 sequences) microcosms. In general, nitrogenase genes cluster into four basic phylogenetic groups designed Clusters I–IV (Chien and Zinder, 1996; Zehr *et al*., 2003). Cluster I and III contain conventional (Mo-containing) nitrogenases, Cluster II contains "second alternative" (containing Fe instead of Mo or V) nitrogenases, and Cluster IV contains nitrogenase homologues (Zehr *et al*., 2003).

All *nifH* sequences obtained in this study fall within Cluster I and Cluster III (Fig. 1, 2). A high diversity of NifH sequences was obtained from the microbial community developed on oil-contaminated sediments. The NifH sequences obtained from the upper section were most similar with NifH from Cyanobacteria (*Anabaena* sp, *Lyngbya* sp., and *Phormidium* sp.), Gammaproteobacteria (*Vibrio* sp., *Pseudomonas* sp., *Marichromatium* sp. and *Methylobacter* sp.), Betaproteobacteria (*Azoarcus* sp.) and Deltaproteobacteria (*Desulfovibrio* sp. and *Geobacter* sp.) (Fig. 1, 2). All sequences recovered from the lower section fall within Cluster III, and were closely related to NifH from Deltaproteobacteria (*Desulfovibrio* sp, *Desulfobacter* sp., *Geobacter* sp.) and green-sulfur bacteria (*Chlorobium* sp.) (Fig. 2).

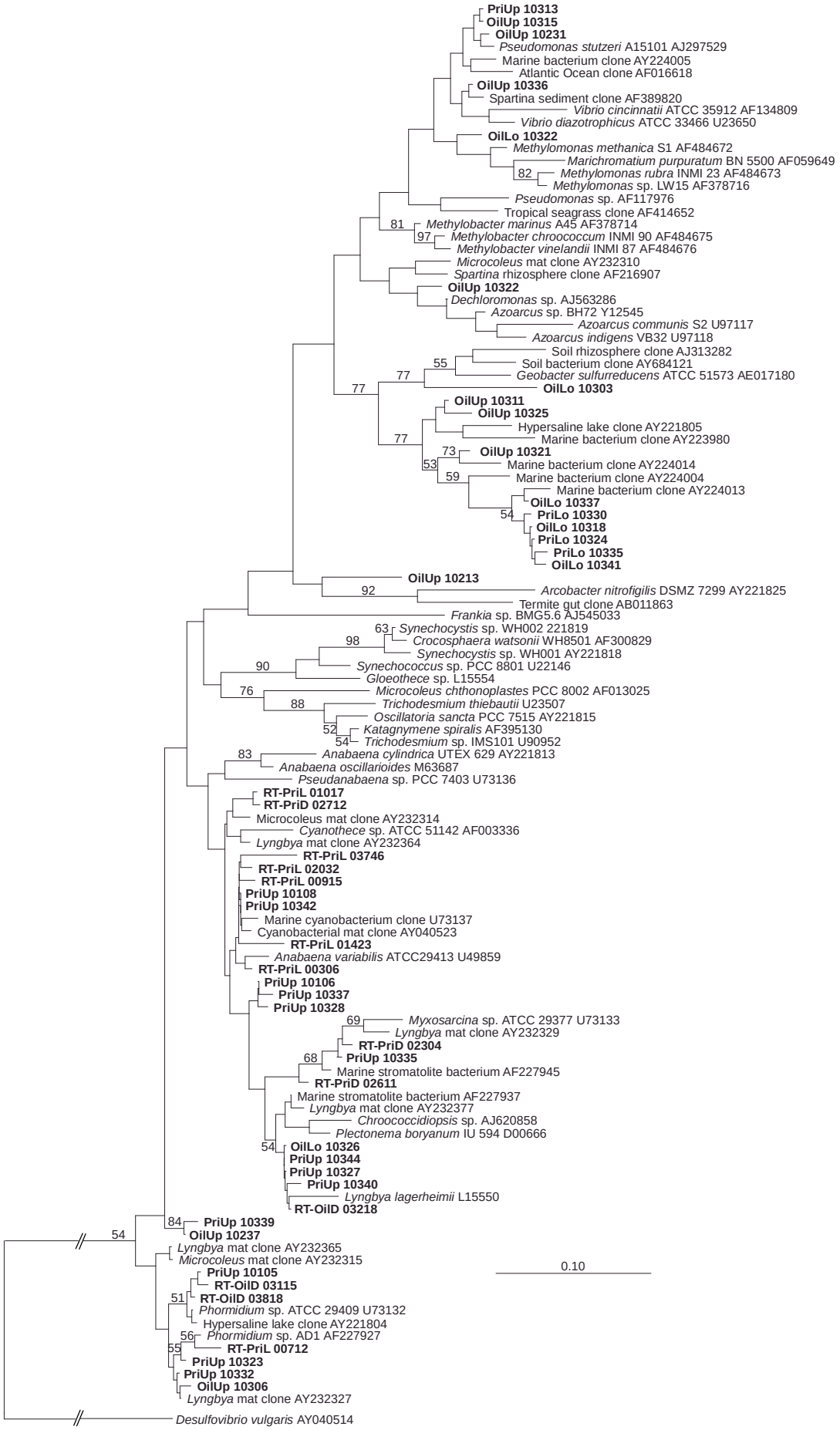
The NifH sequences obtained from the upper section of the pristine sediment fall within Cluster I. Most of them (15 out of 26) were closely related (91 to 97% similarity) to the second nitrogenase gene from the heterocystous cyanobacterium *Anabaena variabilis* ATCC 29413 (Thiel *et al*., 1995). Other sequences were closely related with NifH from non-heterocystous cyanobacteria (*Phormidium* sp., *Lyngbya lagerheimii* and *Cyanothece* sp.) and Gammaproteobacteria (*Pseudomonas* sp.) (Fig. 1). The sequences obtained from the lower section fall within Cluster III and were similar to NifH from *Desulfovibrio* sp. and *Geobacter sulfurreducens* (Fig. 2).

No attempts were made to amplify *nifH* from the microcosms incubated permanently in the dark because nitrogenase activity was not detectable (see below).

Expression of *nifH* phylotypes

Because sequences closely related to NifH from heterocystous and non-heterocystous cyanobacteria were detected via gene libraries, expression of the *nifH* genes was investigated both in the light and dark. Investigation of *nifH* gene expression in the microbial community growing on oil-contaminated sediments revealed that genes for NifH related to the second nitrogenase of the heterocystous cyanobacterium *Anabaena variabilis* ATCC 29413 were expressed during day time, while sequences from relatives of non-heterocystous cyanobacteria (*Lyngbya* sp. and *Phormidium* sp.) were expressed in the dark (Fig. 1). Gene sequences for NifH related to that of *Desulfovibrio* sp. were expressed independently of the light conditions (Fig. 2). No transcripts corresponding to NifH from green-sulfur bacteria, Betaproteobacteria, Gammaproteobacteria, or to some of the Deltaproteobacteria (*Desulfobacter* sp.and *Geobacter* sp.) were recovered by reverse-transcription, even though the genes could be retrieved directly.

All reverse-transcription sequences from the pristine sediment were closely related to NifH of filamentous heterocystous and non-heterocystous cyanobacteria. NifH corresponding to the transcripts retrieved for the light incubation were most similar with the second nitrogenase of *Anabaena variabilis* ATCC 20413 while the NifH retrieved in the dark were closely related to sequences from the non-heterocystous cyanobacteria *Lyngbya lagerheimii*. No transcripts corresponding to the sequences closely related to Deltaproteobacteria were obtained, suggesting that these organisms may not actively fix N_2 in the pristine sediments (Fig. 1, 2).


PriUp 10313
OilUp 10315
OilUp 10231
Pseudomonas stutzeri A15101 AJ297529
Marine bacterium clone AY224005
Atlantic Ocean clone AF016618
OilUp 10336
Spartina sediment clone AF389820
Vibrio cincinnatii ATCC 35912 AF134809
Vibrio diazotrophicus ATCC 33466 U23650
OilLo 10322
Methylomonas methanica S1 AF484672
Marichromatium purpuratum BN 5500 AF059649
82
Methylomonas rubra INMI 23 AF484673
Methylomonas sp. LW15 AF378716
Pseudomonas sp. AF117976
Tropical seagrass clone AF414652
81
Methylobacter marinus A45 AF378714
97
Methylobacter chroococcum INMI 90 AF484675
Methylobacter vinelandii INMI 87 AF484676
Microcoleus mat clone AY232310
Spartina rhizosphere clone AF216907
OilUp 10322
Dechloromonas sp. AJ563286
Azoarcus sp. BH72 Y12545
Azoarcus communis S2 U97117
Azoarcus indigens VB32 U97118
Soil rhizosphere clone AJ313282
55
Soil bacterium clone AY684121
77
Geobacter sulfurreducens ATCC 51573 AE017180
77
OilLo 10303
OilUp 10311
OilUp 10325
Hypersaline lake clone AY221805
77
Marine bacterium clone AY223980
73
OilUp 10321
53
Marine bacterium clone AY224014
59
Marine bacterium clone AY224004
Marine bacterium clone AY224013
OilLo 10337
54
PriLo 10330
OilLo 10318
PriLo 10324
PriLo 10335
OilLo 10341
OilUp 10213
92
Arcobacter nitrofigilis DSMZ 7299 AY221825
Termite gut clone AB011863
Frankia sp. BMG5.6 AJ545033
63
Synechocystis sp. WH002 221819
98
Crocosphaera watsonii WH8501 AF300829
Synechocystis sp. WH001 AY221818
90
Synechococcus sp. PCC 8801 U22146
Gloeothece sp. L15554
76
Microcoleus chthonoplastes PCC 8002 AF013025
88
Trichodesmium thiebautii U23507
Oscillatoria sancta PCC 7515 AY221815
52
Katagnymene spiralis AF395130
54
Trichodesmium sp. IMS101 U90952
83
Anabaena cylindrica UTEX 629 AY221813
Anabaena oscillarioides M63687
Pseudanabaena sp. PCC 7403 U73136
RT-PriL 01017
RT-PriD 02712
Microcoleus mat clone AY232314
Cyanothece sp. ATCC 51142 AF003336
Lyngbya mat clone AY232364
RT-PriL 03746
RT-PriL 02032
RT-PriL 00915
PriUp 10108
PriUp 10342
Marine cyanobacterium clone U73137
Cyanobacterial mat clone AY040523
RT-PriL 01423
Anabaena variabilis ATCC29413 U49859
RT-PriL 00306
PriUp 10106
PriUp 10337
PriUp 10328
69
Myxosarcina sp. ATCC 29377 U73133
Lyngbya mat clone AY232329
RT-PriD 02304
68
PriUp 10335
Marine stromatolite bacterium AF227945
RT-PriD 02611
Marine stromatolite bacterium AF227937
Lyngbya mat clone AY232377
Chroococcidiopsis sp. AJ620858
Plectonema boryanum IU 594 D00666
54
OilLo 10326
PriUp 10344
PriUp 10327
PriUp 10340
Lyngbya lagerheimii L15550
RT-OilD 03218
84
PriUp 10339
54
OilUp 10237
Lyngbya mat clone AY232365
Microcoleus mat clone AY232315
0.10
PriUp 10105
RT-OilD 03115
51
RT-OilD 03818
Phormidium sp. ATCC 29409 U73132
Hypersaline lake clone AY221804
56
Phormidium sp. AD1 AF227927
55
RT-PriL 00712
PriUp 10323
PriUp 10332
OilUp 10306
Lyngbya mat clone AY232327
Desulfovibrio vulgaris AY040514

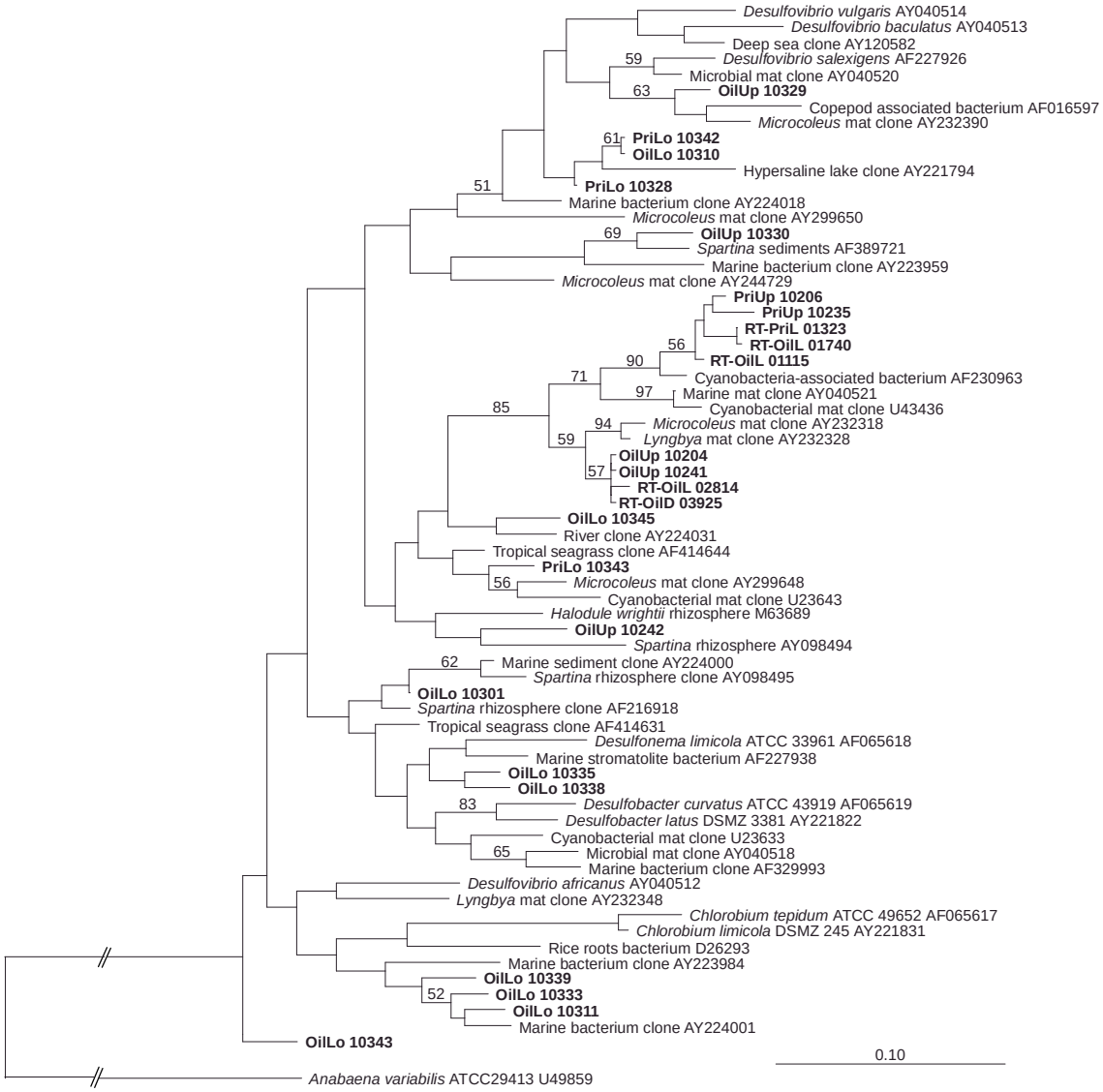


Fig. 2. Phylogenetic tree of Cluster III NifH showing the position of representative sequences obtained in this study (bold). Pri: pristine microcosm; Oil: oil-contaminated microcosm; Up: upper section; Lo: lower section; L: light; D: dark; RT: reverse-transcription.

Measurement of nitrogen fixation in oil-contaminated and pristine sediment microcosms

Nitrogenase (acetylene reduction, AR) activity measurements with samples from the previously established oil-contaminated and pristine microcosms (Musat *et al*., 2004) were inconsistent due to patchiness of the system. Moreover, whenever the laminated structure of the mat-like community was disturbed during sampling, the AR rates were significantly reduced (data not shown). For these reasons, measurements of nitrogenase activity were

Fig. 1 (page before). Phylogenetic tree of Cluster I NifH showing the position of representative sequences obtained in this study (bold). Pri: pristine microcosm; Oil: oil-contaminated microcosm; Up: upper section; Lo: lower section; L: light; D: dark; RT: reverse-transcription.

performed with newly established miniaturized oil-contaminated and pristine microcosms in bottles.

The nitrogenase activity of the microcosms grown under light/dark conditions was determined by incubation with acetylene both in the light and dark. Both, the oil-contaminated and the pristine sediment exhibited highest AR in light (Fig. 3, 4) with a maximum rate after 32 days of development (Fig. 3). During the initial 20 days of incubation, the AR rates of the oil-contaminated sediment were consistently lower than those of the pristine sediment, suggesting that the development of diazotrophic organisms was initially inhibited by oil (Fig. 3).

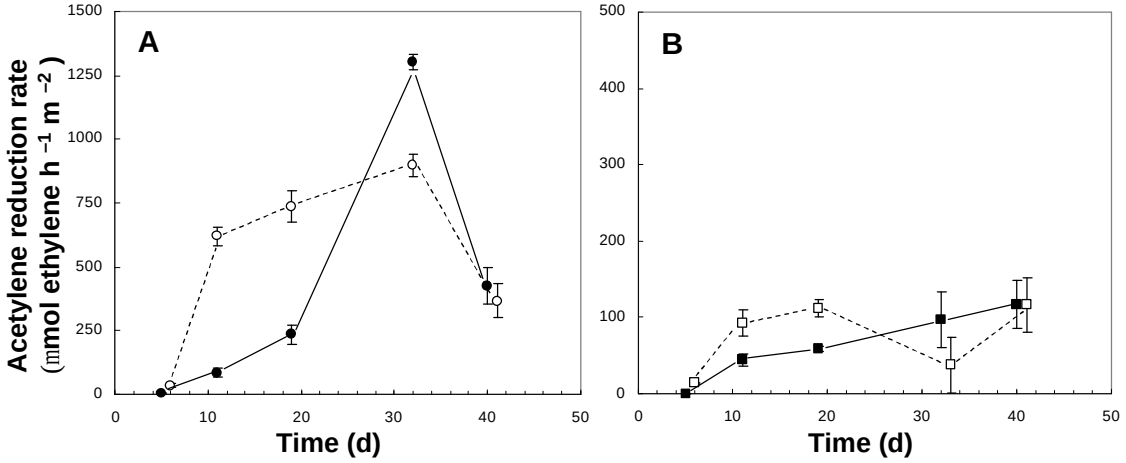


Fig. 3. Acetylene reduction activity of the microbial communities developed on oil-contaminated and pristine sediments under light/dark conditions. **A.** The activity in light for microbial communities developed on oil-contaminated (●) and pristine sediments (○). **B.** The AR activity in the dark for the microbial communities on oil-contaminated (■), and pristine (□) sediments. Each point represents the average of measurements of triplicate bottles which were sacrificed for analysis.

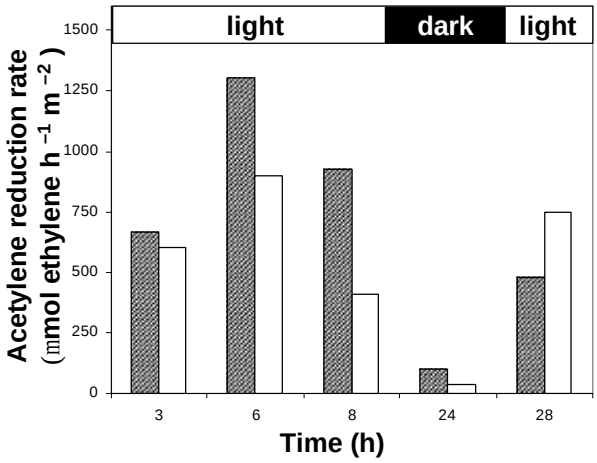


Fig. 4. Acetylene reduction activity of the microbial communities developed on oil-contaminated (hatched columns) and pristine (open columns) sediments incubated under light/dark conditions. The activity measured after 32 days of development is shown. The sediments were incubated in the light for 8 h, in the dark for 16 h and shifted back in light for 4 h.

The AR activity of the oil-contaminated and pristine sediment communities grown permanently in the dark was much lower. For the oil-contaminated sediment community the AR activity was almost undetectable within 30 days of incubation; low activity was measured after 40 days of incubation (Fig. 5). The AR activity of pristine sediment communities developed permanently in the dark was undetectable. However, when such sediments were provided with alginates, significant AR rates were determined after relatively short incubation time (Fig. 5). The activity decreased upon longer incubations, probably due to depletion of the substrate.

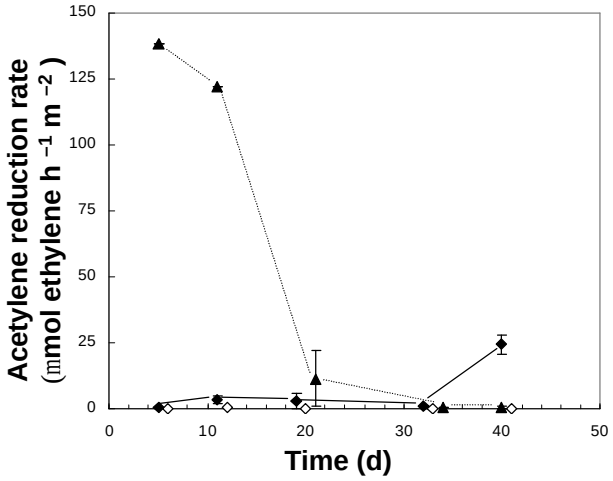


Fig. 5. Acetylene reduction activity in the microbial communities developed on oil-contaminated (◆) and pristine (◇) sediments incubated in the dark. Higher rates of acetylene reduction were obtained if alginates were added to the pristine sediments (▲).

Quantification of total bound nitrogen suggested a small increase as a result of nitrogen fixation for the oil-contaminated and pristine microcosms incubated under light/dark conditions. The increase, calculated for sample dry mass, was of about 8 mmol N mg^{-1} for the oil-contaminated sediment and about 15 mmol N mg^{-1} for the pristine sediment. However, an exact quantification was difficult to obtain, due to heterogeneity of the clay-like sediment causing significant scattering in triplicate determinations (data not shown).

Discussion

In a previous study we reported the establishment of experimental microcosms to study biodegradation of crude oil on marine sediments (Musat *et al*., 2004). Subsequent qualitative observations indicated that development of microbial communities in such experimental systems was limited by the availability of bound nitrogen (data not shown). Therefore, the ability of the microbial communities growing on oil-contaminated and pristine sediments to fix N_2 as a growth-limiting element was investigated.

A high diversity of NifH sequences was retrieved by direct amplification from the oil-contaminated sediment, reflecting a selection of various phylogenetic or physiological groups due to oil contamination and stratification (causing gradients e.g. of light or O_2). Sequences related to NifH from both heterocystous and non-heterocystous cyanobacteria were amplified from the upper layer, suggesting that these sediments in principle harbour the potential for N_2- fixation in the light and dark. Expression analysis showed that sequences related to the second nitrogenase (*nif2*) from *Anabaena variabilis* ATCC 29413 were expressed in the light. The second nitrogenase of *A. variabilis* is expressed only under anoxic conditions in both vegetative cells and heterocysts (Thiel *et al*., 1995). Our findings suggest that genes similar to *nif2* could be expressed in heterocysts or vegetative cells in micro-oxic or anoxic microniches within the laminated, mat-like structures. Alternatively, since the distribution and expression of *nif2*-like genes among heterocystous cyanobacteria is not known and thus not necessarily restricted to *A. variabilis*, the sequences we found could be also expressed in other cyanobacteria even during the day under oxic conditions.

Sequences related to NifH from non-heterocystous cyanobacteria such as *Lyngbya* and *Phormidium* were also amplified from the upper section of the oil-contaminated sediment (Fig. 1). These sequences were expressed in the dark (Fig. 1) and were similar to NifH previously described for well-developed *Microcoleus* and *Lybgya* mats (Omoregie *et al*., 2004a). It has been shown that nitrogen fixation in these mats was maximal at night (Omoregie *et al*., 2004b). This suggests that in addition to N_2 fixation in the light the oil-contaminated microcosms may also fix nitrogen at night. Sequences related to NifH from Betaproteobacteria, Gammaproteobacteria and green-sulfur bacteria were also amplified from the upper section of the oil-contaminated sediment. However, these sequences were not retrieved by RT-PCR, suggesting that the corresponding organisms were not actively involved in N_2 fixation.

Sequences related to NifH from Deltaproteobacteria were amplified from both the upper and lower sections of the oil-contaminated sediment. Expression analysis showed that these sequences were expressed irrespective of the illumination conditions (Fig. 2). Sulfate-reducing bacteria are known to have the ability to fix atmospheric nitrogen (Postgate and Kent, 1985; Widdel, 1987; Widdel and Bak, 1992). Also, nitrogen fixation by sulfate-reducing bacteria has been demonstrated in marine environments such as the rhizosphere of seagrass meadows (Gandy and Yoch, 1988; Herbert, 1999; Nielsen *et al*., 2001) or microbial mats (Steppe and Paerl, 2002). In such environments sulfate-reducing bacteria benefit from the supply of organic carbon by the plant roots. Similarly, cyanobacteria, known to excrete glycolate or fermentation products such as acetate and lactate, could support growth and nitrogen fixation of the sulfate-reducing bacteria (Stal, 1995). However, the contribution of sulfate-reducing bacteria to the nitrogen balance in our experiments is probably marginal, since sediments incubated permanently under anoxic conditions revealed very low AR activity (data not shown).

The diversity of NifH obtained by direct amplification from the pristine sediment was low (Fig. 1 and 2). Sequences obtained from the upper section were dominated by NifH related to those of cyanobacteria (Fig. 1). Only sequences related to NifH from Deltaproteobacteria were amplified from the lower section (Fig. 2). Expression analysis showed that only sequences related to NifH from cyanobacteria were expressed in the pristine microcosms. Thus, sequences related with NifH of heterocystous cyanobacteria were expressed in the light, and sequences related to NifH from non-heterocystous cyanobacteria were expressed in the dark (Fig. 1 and 2).

The results of expression analysis were confirmed by activity measurements using the AR assay. Nitrogenase activity measured for the oil-contaminated and pristine microcosms incubated under light/dark conditions showed a distinct diel pattern, with the highest AR rates during incubation in light (Fig 3, 4). This pattern is typical for heterocystous cyanobacteria, such as *Anabaena*, known to confine nitrogen fixation to heterocysts and thus able to fix nitrogen during the day (Bebout *et al.*, 1993; Bergman *et al*., 1997, Berman-Frank *et al*., 2003). However, nitrogenase activity was also measured in the dark which according to extensive physiological investigations (Fay, 1992; Berman-Frank, 2003) cannot be due to *Anabaena*. Although some non-heterocystous cyanobacteria have been reported to be able to fix nitrogen during the day (Stal and Krumbein, 1981; Janson *et al*., 1994), it is more typical for these organisms to show such activity at night, viz. to protect their nitrogenase by

temporally separating nitrogen fixation and photosynthesis (Bergman *et al*., 1997; Berman-Frank *et al*., 2003).

The nitrogenase activity of the pristine sediment was much higher than the activity of the oil-contaminated sediment within the first 20 days of incubation, probably due to inhibition of cyanobacterial growth by the crude oil (Fig. 3). Nevertheless, the toxic oil fraction may be removed by biodegradation and evaporation of further oil components, thus explaining the higher activity measured for the oil-contaminated sediments after 30 days of incubation (Fig. 3).

Nitrogen fixation by microcosms incubated permanently in the dark, i.e. pure heterotrophic nitrogen fixation, with crude oil as a growth substrate was very low (Fig. 2). In other studies it has been shown that heterotrophic bacteria from oil-contaminated environments can fix N_2 (Eckford *et al*, 2002; Toccalino *et al*., 1993; Piehler *et al*., 1999), but only few strains could use hydrocarbons as substrates for nitrogen fixation (Chen *et al*., 1993; Prantera *et al*., 2002). Although nitrogenase gene sequences closely related to NifH from *Pseudomonas* and *Vibrio*, genera known to contain oil-degrading species, were amplified from the oil-contaminated sediment (Fig. 1), these sequences were not recovered by reverse-transcription, suggesting that they are not actively expressed. If alginates were added as substrate instead of crude oil, high nitrogenase activity was measured already within 5 days of incubation (Fig. 2). This clearly indicates the growth of heterotrophic microorganisms able to fix nitrogen by using natural polar compounds, but not hydrocarbons as growth substrates, in accordance with previous reports (Laguerre *et al*., 1987; Tibbles *et al*., 1994; Herbert, 1999).

In conclusion, the present study indicates that cyanobacteria, most notably heterocystous cyanobacteria, were responsible for the bulk nitrogen fixation of the oil-contaminated and pristine microcosms. The fixed nitrogen may be transferred, for example by death and decay of the cyanobacterial filaments, to the oil-degrading bacteria and thus it may stimulate biodegradation of crude oil. However, studies with defined mixed cultures are required to demonstrate that biodegradation of crude oil is indeed stimulated by fixed nitrogen provided by diazotrophic cyanobacteria.

Acknowledgments

Cristina Probian is acknowledged for help with chemical analyses. Olav Grundman is acknowledged for help with molecular investigations. This work was supported by the European project MATBIOPOL (EVK3-CT1999-00010).

References

Abed, R. M. M., and J. Köster. 2004. The direct role of the aerobic heterotrophic bacteria associated with cyanobacteria in the degradation of oil compounds. International Biodeterioration and Biodegradation **55:**29-37.

Atlas, R. M. 1995. Petroleum biodegradation and oil spill bioremediation. Marine Pollution Bulletin **31:**178-182.

Bagwell, C. E., Y. M. Piceno, A. Ashburne-Lucas, and C. R. Lovell. 1998. Physiological diversity of the rhizosphere diazotroph assemblages of selected salt marsh grasses. Appl Environ Microbiol **64:**4276-4282.

Barth, J. J. 2003. The influence of cyanobcateria on oil polluted intertidal soils at the Saudi Arabian Gulf shores. Marine Pollution Bulletin **46:**1245-1252.

Bebout, B. M., M. W. Fitzpatrick, and H. W. Paerl. 1993. Identification of the sources of energy for nitrogen fixation and physiological characterization of nitrogen-fixing members of a marine microbial mat community. Appl Environ Microbiol **59:**1495-1503.

Bergman, B., J. R. Gallon, A. N. Rai, and L. J. Stal. 1997. N_2 fixation by non-heterocystous cyanobacteria. FEMS Microbiol Rev **19:**139-185.

Berman-Frank, I., P. Lundgren, and P. Falkowski. 2003. Nitrogen fixation and photosynthetic oxygen evolution in cyanobacteria. Research in Microbiology **154:**157-164.

Bragg, J. R., R. C. Prince, E. J. Harner, and R. M. Atlas. 1994. Effectiveness of bioremediation for the Exxon Valdez oil spill. Nature **368:**413-418.

Chen, Y. P., G. Lopez-de-Victoria, and C. R. Lovell. 1993. Utilization of aromatic compounds as carbon and energy sources during growth and N_2-fixation by free-living nitrogen fixing bacteria. Arch Microbiol **159:**207-212.

Chien, Y.-T., and S. H. Zinder. 1996. Cloning, functional organization, trancript studies, and phylogenetic analysis of the complete nitrogenase structural genes (*nifHDK2*) and associated genes in the archaeon *Methanosarcina barkeri* 227. J Bacteriol **178:**143-148.

Eckford, R., F. D. Cook, D. Saul, J. Aislabie, and J. Foght. 2002. Free-living heterotrophic nitrogen-fixing bacteria isolated from fuel-contaminated antarctic soils. Appl Environ Microbiol **68:**5181-5185.

Fay, P. 1992. Oxygen relations of nitrogen fixation in cyanobacteria. Microbiological Reviews **56:**340-373.

Gandy, E. L., and D. C. Yoch. 1988. Relationship between nitrogen-fixing sulfate reducers and fermenters in salt marsh sediments and roots of *Spartina alterniflora*. Appl Environ Microbiol **54:**2031-2036.

Hall, T. A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser **41:**95-98.

Herbert, R. A. 1975. Heterotrophic nitrogen fixation in shallow estuarine sediments. J Exp Mar Biol Ecol **18:**215-225.

Herbert, R. A. 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiology Reviews **23:**563-590.

Hoffman, L. 1996. Recolonization of the intertidal flats by microbial mats after the Gulf War oil spill. *In* F. Krupp, A. H. Abuzinada, and I. A. Nader (ed.), A marine wildlife sanctuary for the Arabian Gulf. NCWCD, Riyadh.

Höpner, T., M. Yousef, L. Berthe-Corti, H. Felzmann, H. Struck, and A. Al-Thukair. 1996. Cyanobacterial mats on oil-polluted sediments - start of a promising self-remediation process? *In* F. Krupp, A. H. Abuzinada, and I. A. Nader (ed.), A marine wildlife sanctuary for the Arabian Gulf. Environmental research and conservation following the 1991 Gulf war oil spill, Riyadh, Frankfurt a.M.

Janson, S., E. J. Carpenter, and B. Bergman. 1994. Compartmentalization of nitrogenase in a nonheterocystous cyanobacterium: *Trichodesmium contortum*. FEMS Microbiol Lett **118:**9-14.

Laguerre, G., B. Bossand, and R. Bardin. 1987. Free-living dinitrogen-fixing bacteria isolated from petroleum refinery oily sludge. Appl Environ Microbiol **53:**1674-1678.

Leahy, J. G., and R. R. Colwell. 1990. Microbial degradation of hydrocarbons in the environment. Microbiol Rev **54:**305-315.

Lovell, C. R., M. J. Friez, J. W. Longshore, and C. E. Bagwell. 2001. Recovery and phylogenetic analysis of nifH sequences from diazotrophic bacteria associated with dead aboveground biomass of Spartina alterniflora. Appl Environ Microbiol **67:**5308-5314.

Lovell, C. R., Y. M. Piceno, J. M. Quattro, and C. E. Bagwell. 2000. Molecular analysis of diazotroph diversity in the rhizosphere of the smooth cordgrass, *Spartina alterniflora*. Appl Environ Microbiol **66:**3814-3822.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, Arno Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüssmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. 2004. ARB: a software environment for sequence data. Nucl Acid Res **32:**1363-1371.

Musat, F., A. Wieland, and F. Widdel. 2004. Marine sediment with surface contamination by oil in microcosms for microbiological studies. Ophelia **58:**217-222.

Nielsen, L. B., K. Finster, D. T. Welsh, A. Donelly, R. A. Herbert, R. de Wit, and B. A. Lomstein. 2001. Sulphate reduction and nitrogen fixation rates associated with roots, rhizomes and sediments from *Zostera noltii* and *Spartina maritima* meadows. Environmental Microbiology **3:**63-71.

Omoregie, E. O., L. L. Crumbliss, B. M. Bebout, and J. P. Zehr. 2004. Comparison of diazotroph community structure in *Lyngbya* sp. and *Microcoleus chthonoplastes* dominated microbial mats from Guerrero Negro, Baja, Mexico. FEMS Microbiology Ecology **47:**305-318.

Omoregie, E. O., L. L. Crumbliss, B. M. Bebout, and J. P. Zehr. 2004. Determination of nitrogen-fixing phylotypes in *Lyngbia* sp. and *Microcoleus chthonoplastes* cyanobacterial mats from Guerrero Negro, Baja California, Mexico. Appl Environ Microbiol **70:**2119-2128.

Piehler, M. F., J. G. Swistak, J. L. Pinckney, and H. W. Paerl. 1999. Stimulation of diesel fuel biodegradation by indigenous nitrogen fixing bacterial consortia. Microbial Ecology **38:**69-78.

Postgate, J. R., and H. M. Kent. 1985. Diazotrophy within *Desulfovibrio*. J Gen Microbiol **131:**2119-2122.

Prantera, M. T., A. Drozdowicz, S. G. Leite, and A. S. Rosado. 2002. Degradation of gasoline hydrocarbons by two N_2-fixing soil bacteria. Biotechnology Letters **24:**85-89.

Prince, R. C. 1993. Petroleum spill bioremediation in marine environment. Critical Reviews in Microbiology **19:**217-242.

Prince, R. C., and J. R. Bragg. 1997. Shoreline bioremediation following the Exxon Valdez oil spill in Alaska. Bioremed J **1:**97-104.

Proctor, L. M. 1997. Nitrogen-fixing, photosynthetic, anaerobic bacteria associated with pelagic copepods. Aquatic Microbial Ecology **12:**105-113.

Sorkhoh, N., R. Al-Hasan, S. Radwan, and T. Hoepner. 1992. Self-cleaning of the Gulf. Nature **359:**109.

Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. Appl Environ Microbiol **54:**1079-1084.

Stal, L. J. 1995. Physiological ecology of cyanobacteria in microbial mats and other communities. New Phytol **131:**1-32.

Stal, L. J., and W. E. Krumbein. 1981. Aerobic nitrogen fixation in pure cultures of a benthic marine *Oscillatoria* (cyanobacteria). FEMS Microbiol Lett **11:**295-298.

Steppe, T. F., and H. W. Paerl. 2002. Potential N_2 fixation by sulfate-reducing bacteria in a marine intertidal microbial mat. Aquatic Microbial Ecology **28:**1-12.

Stewart, W. D. P., G. P. Fitzgerald, and R. H. Burris. 1967. In situ studies of N_2 fixation using the acetylene reduction technique. Proc Natl Acad Sci USA **58:**2071-2078.

Thiel, T., L. E. M., J. C. Erker, and A. Ernst. 1995. A second nitrogenase in vegetative cells of a heterocyst-forming cyanobacterium. Proc Natl Acad Sci USA **92:**9358-9362.

Tibbles, B. J., M. I. Lucas, V. E. Coyne, and T. S. Newton. 1994. Nitrogenase activity in marine sediments from a temperate salt marsh lagoon: modulation by complex polysaccharides, ammonium and oxygen. J Exp Mar Biol Ecol **184:**1-20.

Toccalino, P. L., R. L. Johnson, and D. R. Boone. 1993. Nitrogen limitation and nitrogen fixation during alkane biodegradation in a sandy soil. Appl Environ Microbiol **59:**2977-2983.

Van Hamme, J. D., A. Singh, and O. P. Ward. 2003. Recent advances in petroleum microbiology. Microbiology and Molecular Biology Reviews **67:**503-549.

Widdel, F. 1987. New types of acetate-oxidizing, sulfate-reducing *Desulfobacter* species, *D. hydrogenophilus* sp. nov., *D. latus* sp. nov., and *D. curvatus* sp. nov. Arch Microbiol **148:**286-291.

Widdel, F., and F. Bak. 1992. Gram-negative mesophilic sulfate-reducing bacteria., p. 3352-3378. *In* H. G. Balows, M. Trüper, M. Dworkin, W. Harder, and K. H. Schleifer (ed.), The prokaryotes. Springer Verlag, New York.

Zehr, J. P., M. Mellon, S. Braun, W. Litaker, T. Steppe, and H. W. Paerl. 1995. Diversity of heterotrophic nitrogen fixation genes in a marine cyanobacterial mat. Appl Environ Microbiol **61:**2527-2532.

Zehr, J. P., B. D. Jenkins, S. M. Short, and G. F. Steward. 2003. Nitrogenase gene diversity and microbial community structure: a cross-system comparison. Environmental Microbiology **5:**539-554.

Zehr, J. P., and L. A. McReynolds. 1989. Use of degenerate oligonucleotides for the amplification of the nifH gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl Environ Microbiol **55:**2522-2526.

Zhou, J., M. A. Bruns, and J. M. Tiedje. 1996. DNA recovery from soils of diverse composition. Appl Environ Microbiol **62:**316-322.

Further contribution

Anaerobic biodegradation of cyclohexane by a nitrate-reducing enrichment culture with scavenge of nitrite by anaerobic ammonium oxidization

Florin Musat[1], Astrid Behrends[1], DagmarWoebken[1], Heinz Wilkes[2] and Friedrich Widdel[1]*

Manuscript in preparation

[1] Max Planck Institute for Marine Microbiology, Celsiusstr. 1, 28359 Bremen, Germany

[2] GeoForschungZentrum Potsdam, Aufgabenbereich 4.3: Organische Geochemie, Telegrafenberg, D-14473 Potsdam, Germany

*** Corresponding author**

Abstract

An anaerobic freshwater enrichment culture was established with cyclohexane as the only organic substrate and with nitrate as electron acceptor. Quantitative growth experiments to reveal the electron and nitrogen balance showed consumption of cyclohexane and nitrate, but also of ammonia. Dissapearance of ammonium ions was much more than could be accounted for by nitrogen assimilation into biomass, indicating the occurrence of anaerobic ammonia oxidation (Anammox) with nitrite from cyclohexane-dependent nitrate reduction. Occurrence of Anammox was further substantiated by stimulation of nitrite depletion upon *de novo* addition of ammonia. Analysis of 16S rRNA genes and subsequent *in situ* hybridization revealed that members of the *Geobacteriaceae* accounted for ca. 75% and relatives of the anaerobic ammonium oxidizer, '*Candidatus Brocadia anammonoxidans*' (related to Planctomycetes), for ca. 18% of the enriched population. According to these results, *Geobacteriaceae* may oxidize cyclohexane and reduce nitrate mainly to nitrite (and partly to ammonia) that is scavenged by anaerobic ammonia oxidizers. Gas chromatography-mass spectrometry (including an authentic standard) revealed cyclohexylsuccinate among organic metabolites, in accordance with an activation of cyclohexane analogous to that of *n*-alkanes, i.e. by carbon-carbon addition to fumarate.

Introduction

Cycloaliphatic hydrocarbons may represent a large proportion of petroleum and derived refined products such as fuels (Tissot and Welte, 1984; Morgan and Watkinson, 1994). This and the widespread use of cyclic alkanes as solvents or raw materials for organic syntheses may lead to the release of significant quantities of such compounds into the environment (Perry, 1984; Tissot and Welte, 1984). Cyclic alkanes are more toxic than open-chain alkanes (even though less toxic than aromatic hydrocarbons) and therefore as pollutants cause concern (Sikkema *et al*., 1994; Sikkema *et al*., 1995). Unlike saturated non-cyclic hydrocarbons which are known to be easily biodegradable, unsubstituted cycloalkanes are particularly resistant to biodegradation and relatively few studies have been successful in growing enrichments or isolating pure cultures able to degrade such compounds (Solano-Serena *et al*., 2000a, b; Cheng *et al*., 2002). Most studies on biodegradation of cycloalkanes have focused on biodegradation of cyclohexane as representative cycloalkane (Stirling and Watkinson, 1977; Anderson *et al*., 1980; Trower *et al*., 1985; Solano-Serena *et al*., 2000b; Rouviere and Chen, 2003). Aerobically, cyclohexane is typically activated by cyclohexane monooxygenase, yielding cyclohexanol, which is further metabolized through cyclohexanone and caprolactone to adipic acid (Perry, 1984; Cheng *et al*., 2002).

During the past decade, anaerobic biodegradation of several saturated hydrocarbons has been demonstrated (Aeckersberg *et al*., 1991, 1998; Rueter *et al*., 1994; So and Young, 1999; Zengler *et al*., 1999; Ehrenreich *et al*., 2000; Spormann and Widdel, 2000; Widdel and Rabus, 2001). Normal and branched akanes for example have been shown to serve as growth substrates for bacteria using nitrate (Bregnard *et al*., 1997; Ehrenreich *et al*., 2000) or sulfate (Aeckersberg *et al*., 1991; Rueter *et al*., 1994; Aeckersberg *et al*., 1998) as electron acceptors, as well as for methanogenic mixed cultures (Zengler *et al*., 1999; Anderson and Lovley, 2000). More recently, anaerobic biodegradation of cyclohexane under sulfate-reducing and methanogenic conditions has been demonstrated in sediment samples from a gas condensate-contaminated aquifer ammended with complex mixtures of hydrocarbons (e.g. gasoline) (Townsend *et al*., 2004).

To our knowledge, a culture capable of cyclohexane biodegradation under nitrate-reducing conditions has not been reported so far. In the present study, we describe and analyse a nitrate-reducing enrichment culture growing with cyclohexane as sole substrate. Unlike other, described enrichment and pure cultures obtained with *n*-alkanes and nitrate, the present one neither contained Beta- nor Gammaproteobacteria, but was rather dominated by *Geobacteriaceae* of the Deltaproteobacteria. Furthermore, the new enrichment culture

performed an anaerobic oxidation of ammonia as a simultaneous yet separate process, probably due to a group of *Planctomycetes.*

Materials and methods

Source of organisms, media and cultivation techniques

Anoxic sediment from a fresh-water lake (Bad Zwischenahner Meer, Germany) was used as inoculum for enrichment cultures. A volume of 5 ml of homogenized sediment was used to inoculate 50 ml of defined bicarbonate/CO_2-buffered fresh-water medium (Widdel and Bak, 1992; Rabus and Widdel, 1995) in a flat 100 ml bottle. The medium was overlaid with 3 ml of 2,2,4,4,6,8,8-heptamethylnonane (HMN) as inert carrier phase containing 25 µl cyclohexane and incubated anoxically under a N_2-CO_2 mixture (90/10, v/v) at 28 ^oC. The bottles were incubated nearly horizontally with orifices below the medium surface so as to yield a large surface, but to avoid contact between the hydrocarbon phase and the stopper (Rabus and Widdel, 1995). Sediment-free subcultures were incubated with constant horizontal shaking (50 – 70 rpm). Transfers to fresh medium were done with 10% (v/v) inoculum.

Chemical analyses

Measurement of nitrate and nitrite by HPLC was done as described elsewhere (Rabus and Widdel, 1995). Ammonium was measured colorimetrically using the method of indophenol formation (Boltz and Taras, 1978).

Cyclohexane concentrations in the carrier (HMN) were measured by headspace analysis (samples withdrawn at 28 ^oC). A volume of 0.1 ml gas phase was removed via a N_2-flushed gas-tight syringe and injected into a Shimadzu GC14B gas chromatograph (Shimadzu Corp., Japan), equipped with an FID and a 50 m HP-PLOT U column (Sigma Aldrich GmbH, Taufkirchen, Germany). Nitrogen was used as a carrier gas. The oven was operated isothermally at 140 ^oC, detector 280 ^oC and injector 150 ^oC.

Metabolites were extracted from acidified 400-ml cultures as described previously (Wilkes *et al*., 2000; Rabus *et al*., 2001). Prior to extraction, the HMN phase was removed via a separatory funnel. Methylated culture extracts were analyzed by gas-chromatography-mass spectrometry (GC-MS) with a type 5890 gas chromatograph (Hewlett Packard, Waldbronn, Germany) connected to a type 95SQ mass spectrometer (Finnigan

MAT/ThermoFinnigan, Egelsbach, Germany) as described (Wilkes *et al*., 2000; Rabus *et al*., 2001). Standard cyclohexylsuccinic acid was purchased from Sigma-Aldrich (Sigma-Aldrich, Deisenhofen, Germany).

Clone library construction, sequencing and phylogenetic analysis

Nearly the complete 16S rRNA gene was amplified using bacterial specific primers 8f (Hicks *et al*., 1992) and 1492r (Kane *et al*., 1993). The PCR reactions contained 50 pmol (1 µl) of each primer, 1 mM (10 µl) total dNTPs, 1 × PCR buffer (5 µl), 0.3 mg BSA ml^{-1} (5 µl), and 2 U (2 µl) of RedTaq polymerase (Sigma-Aldrich). The amplification was done with a Thermocycler Mastercycler (Eppendorf, Hamburg, Germany) as follows: an initial denaturation step at 95 ^{o}C for 10 min, followed by 30 cycles of 1 min denaturation at 95 ^{o}C, 1 min annealing at 50 ^{o}C, and 3 min elongation at 72 ^{o}C, with a final extension step of 10 min at 72 ^{o}C.

The PCR products were purified with the QIAquick Purification Kit (Qiagen, Hilden, Germany) and cloned into the pCR4 vector (Invitrogen, Gröningen, Netherlands) according to the manufacturer's recommendation. The recombinant vectors were transformed into chemically competent *E. coli* cells using a previously described method (Ravenschlag *et al*., 1999). The 16S rRNA gene libraries were screened by PCR. Positive clones were partially sequenced with the primers M13F or M13R. The clones were grouped based on similarity of partial sequences, and representatives were chosen for full sequencing. Plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen) and sequenced using vector primers and universal primers for bacterial rRNA genes, the ABI Prism BigDye Terminator v 3.0 cycle sequencing kit and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems Inc., Foster City, USA). The ribosomal rRNA gene of the apparent Anammox bacteria was amplified using specific primers (Schmid *et al*., 2000), purified and sequenced on both strands without cloning. Sequence data were analyzed with the ARB software package (Ludwig *et al*., 2004).

Fluorescence in situ hybridization

For preparation of fluorescence *in situ* hybridization (FISH) 1.0 ml aliquots of the enrichment culture were withdrawn with N_2-flushed syringes and fixed for 2 – 3 h with 3% (final concentration) formaldehyde at 4 ^{o}C, washed twice with 1× phosphate-buffered saline (PBS: 10 mM sodium phosphate [pH 7.2], 130 mM NaCl) and stored in 1× PBS-ethanol (1:1) at –20 °C. For FISH, aliquots were filtered onto 0.2-µm pore GTTP polycarbonate

filters (Millipore, Eschborn, Germany). The cells were hybridized, additionally stained with 4´,6´-diamidino-2-phenylindole (DAPI) and microscopically counted as described previously (Snaidr *et al*., 1997; Pernthaler *et al*., 2002; Woebken *et al*., in prep.). FISH with catalyzed reporter deposition (CARD-FISH) with the probe AMX820 specific for the Anammox organisms (Schmid *et al*., 2000) was performed as desribed (Pernthaler *et al*., 2002). Values were corrected for the signals counted with the probe NON338. The oligonucleotide probes used in this study, GEO825 (30% FA, Lowe *et al*., 2000), EUB338 (Amann *et al*., 1990), AMX368 (Schmid *et al*., 2000) and AMX820 were purchased from Interactiva (Ulm, Germany).

Results

Enrichment and growth experiment

Enrichment of cyclohexane-degrading nitrate-reducing bacteria was attempted from freshwater sediments that were incubated in mineral medium overlaid with an inert organic carrier (heptamethylnonane) containing 0.8% cyclohexane. Whenever depleted, nitrate was re-supplied in portions of 5 mM. When after approximately 9 weeks reduction of nitrate was significantly faster than in the cyclohexane-free controls, fresh mineral medium again supplied with cyclohexane and nitrate was inoculated with 10% (v/v) of the grown culture. After several transfers to new media, a sediment-free enrichment culture mainly of rod-shaped cells was obtained. Newly inoculated cultures consumed 5 mM of nitrate within approximately 3 weeks. Upon growth on cyclohexane, the color of the settled biomass was pink to red. Visible spectra of whole cells presented an absorption maximum at 408 nm, indicative of a cytochrome.

Attempts to isolate the anaerobic cyclohexane-degrading bacteria in pure culture were not succesfull so far. For this reason, further physiological investigations were carried out with the enrichment culture. Since different physiological types of dissimilatory nitrate reducers may be present, all possible reduction products of nitrate were investigated. Surprisingly, we found that ammonium was completely consumed simultaneously with reduction of nitrate (Fig. 1A). Moreover, as long as ammonium was present in the culture medium, nitrite occurred only transiently at low concentration (Fig. 1A). After depletion of ammonium, nitrate was reduced to nitrite at significant concentrations, which accumulated in

the medium. New supplementation with ammonium resulted in rapid consumption of nitrite, accompanied by cyclohexane consumption and growth (Fig 1A, B).

Results of a quantitative growth experiment of the nitrate-reducing enrichment culture with cyclohexane are summarized in Table 1. N_2O was not detectable, which leaves N_2 as the only end product of nitrogen catabolism. The final electron balance showed a surplus of the electrons from the consumed electron donors (cyclohexane and ammonium) in comparison to the consumed electron acceptor (the ratio of electrons dissimilated: electrons consumed was 1.34). This can be explained by the assimilation of organic carbon from cyclohexane and as well as assimilation of ammonium. Measurements of cyclohexane and nitrate showed consumption of cyclohexane with nitrate as electron acceptor (Table 1). No consumption of cyclohexane or ammonium was observed in cultures without nitrate. Also, ammonium was not consumed in controls with nitrate but lacking cyclohexane.

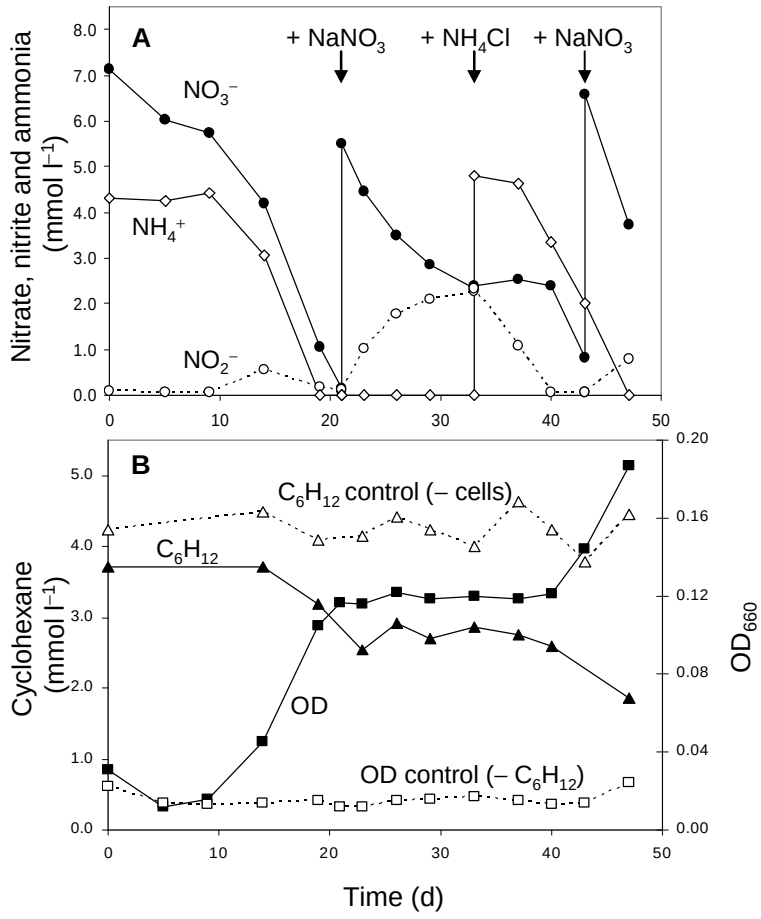


Fig. 1. Anaerobic consumption of cyclohexane and ammonium in a nitrate-reducing enrichment culture growing with cyclohexane as the sole organic substrate. **A.** Time courses of the concentration of nitrate (●), ammonium (◇) and nitrite (○). **B.** Time courses of the concentration of cyclohexane in the enrichment culture (▲) and a sterile control (△); development of cell density with cyclohexane (■) and without cyclohexane (□). Experiments were carried out in anoxic bottles with a culture volume of 175 ml and 10 ml heptamethylnonane as inert carrier for cyclohexane.

Table 1. Quantification of the anaerobic consumption of cyclohexane, ammonium and nitrate in the enrichment culture monitored in Fig. 1.

Electron source or sink (mmol)	Culture with C_6H_{12}	Culture without C_6H_{12}	Culture without NO_3^-
C_6H_{12} added	0.65	0	0.74
C_6H_{12} consumed	0.33	0	0
Electrons from C_6H_{12}	11.88	0	0
NH_4^+ added	1.59	0.88	0.88
NH_4^+ consumed	1.59	0	0
Electrons from NH_4^+	4.77	0	0
Electrons from C_6H_{12} + NH_4^+ (sum)	16.65	0	0
NO_3^- added	3.22	1.07	0
NO_3^- consumed	2.57	0.27	0
NO_2^- formed	0.14	0	0
Electrons for NO_3^- reduction	12.43	1.35	0

[a] The complete oxidation of 1 mol cyclohexane to CO_2 yields 36 mol electrons. Oxidation of 1 mol ammonium to N_2 yields 3 mol electrons.
[b] Electrons consumed = 5 × (nitrate added – nitrate remaining) – 3 × (nitrite remaining).

Molecular characterization of the enrichment culture

A 16S rRNA gene library constructed with specific bacterial primers was dominated by sequences related to members of the family *Geobacteriaceae*, with highest sequence similarity to those of *Geobacter humireducens* and *G. metallireducens* (93%). FISH with a *Geobacteriaceae*-specific probe showed that these organisms were dominant in the enrichment culture, accounting for as much as 75% of the total cell number determined by counting of the DAPI-stained cells (Fig. 2A, B).

The 16S rRNA genes amplified with primers specific for the Anammox bacteria were similar with those of *Candidatus Brocadia anammoxidans* (94% sequence similarity). Hybridization with specific probes revealed that the Anammox bacteria represented up to 18% of the total cell number (Fig. 2C, D).

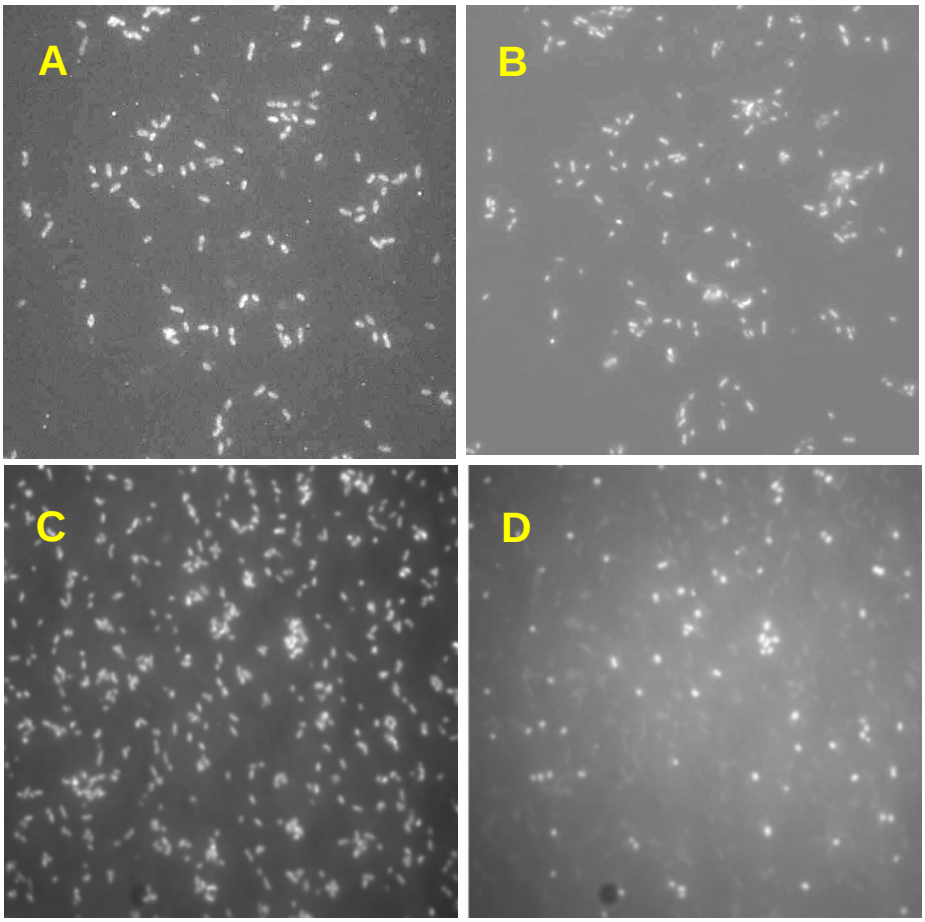


Fig. 2. Fluorescence in situ hybridization of the anaerobic enrichment culture on cyclohexane and nitrate with the GEO825 probe (**A**) showed dominance of *Geobacteraceae*-related organisms, accounting for up to 75% of the DAPI-stained cells (**B**). Hybridization with AMX368 (**C**) indicated that Anammox-related organisms represented up to 18% of the DAPI-stained cells (**D**).

Identification of cyclohexane metabolites

Gas chromatography–mass spectrometry analysis of extracts from the culture grown with cyclohexane revealed a complex mixture of organic acids that were not detectable in controls lacking nitrate or cyclohexane. A metabolite with the same retention time as the standard cyclohexylsuccinate dimethyl ester was identified in the ion chromatogram (Fig. 3). The fragmentation pattern yielded fragment ions at *m/z* 114, 146, 155 and 197, in agreement with the fragmentation pattern of standard cyclohexylsuccinate dimethyl ester (Fig. 4 A). Furthermore, the total ion chromatogram also revealed the presence of two fatty acids which based on the mass spectra were tentatively identified as 9-cyclohexylnonanoic acid and 11-cyclohexylundecanoic acid (Fig. 4B, C).

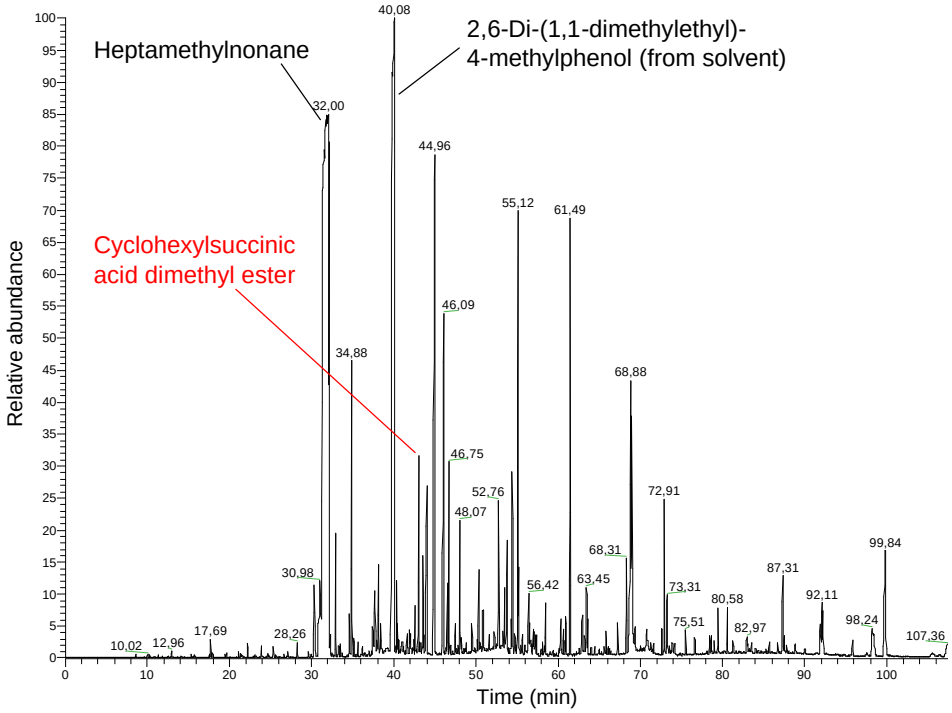


Fig. 3. Total ion chromatogram of the extract of the enrichment culture grown with cyclohexane indicating cyclohexylsuccinate as a metabolite of cyclohexane degradation.

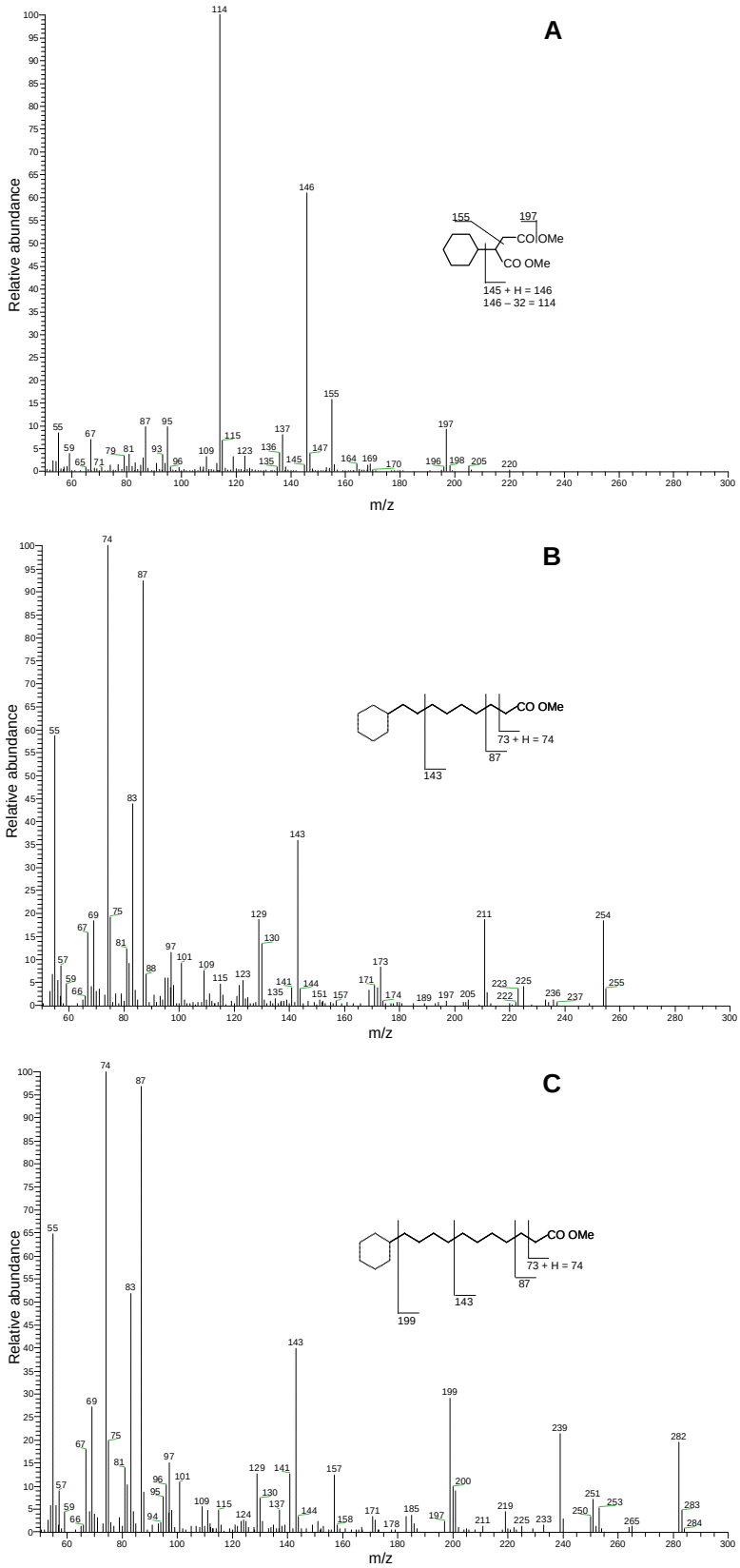


Fig. 4. Mass spectra of cyclohexylsuccinate (**A**), 9-cycloheyxlnonanoate (**B**) and 11-cyclohexylundecanoate (**C**) (di)methyl esters.

Discussion

During the past decade, anaerobic degradation of normal or branched alkanes by enriched or pure cultures has been clearly demonstrated (Aeckersberg *et al*., 1991, 1998; Rueter *et al*., 1994; Bregnard *et al*., 1997; So and Young, 1999; Zengler *et al*., 1999; Ehrenreich *et al*., 2000; Spormann and Widdel, 2000; Widdel and Rabus, 2001). However, anaerobic biodegradation of unsubstituted cycloalkanes, which are particularly resistant to biodegradation even under oxic conditions, has been so far demonstrated only with aquifer sediments (Townsend *et al*., 2004). The present study is the first yielding an enrichment culture capable of anaerobic biodegradation of cyclohexane under nitrate-reducing conditions.

Furthermore, the development of members of the Deltaproteobacteria and microorganisms performing anaerobic ammonia oxidation in sediment-derived cultures with hydrocarbons and nitrate has not been described before. Freshwater enrichment cultures with these substrates so far yielded Beta- or Gammaproteobacteria (Anders *et al*., 1995; Ehrenreich *et al*., 2000; Rabus and Widdel, 1995; Harms *et al*., 1999; Song *et al*., 1999; Rockne *et al*., 2000). A functional interpretation of the microbial composition of the enrichment culture as determined by FISH (Fig. 2) would be that the *Geobacteriaceae*-related cells (ca. 75% of total cell number) were responsible for the bulk of cyclohexane and nitrate consumption, and the Planctomycetales-related cells for anaerobic ammonium consumption. Members of the genus *Geobacter* are mainly Fe(III) or Mn(IV) reducers and some species have been shown to anaerobically oxidize aromatic compounds, including phenol and toluene (Lovley *et al*., 1989). Some *Geobacter* species (e.g. *G. metallireducens*) have been shown to use also nitrate as terminal electron acceptor (Lovley and Phillips, 1988). However, similar with nitrate utilization as electron acceptor in the related *Desulfuromonas* (Widdel and Pfennig, 1992), reduction of nitrate by *Geobacteriaceae* yields ammonia (Lovley and Phillips, 1988). Measurements of possible reduction products of nitrate in growth experiments revealed that, surprisingly, ammonia was depleted during growth of the enrichment with cyclohexane as sole substrate. Given the high amount of ammonium consumed (9.1 mmol l^{-1}, Fig. 1A), depletion cannot be explained by assimilation. Based on previous determinations of the correlation between biomass yields and OD in anaerobic bacteria (Rabus and Widdel, 1996), the increase in OD determined here (Fig. 1B) may correspond to approximately 50 mg cell dry mass l^{-1}. According to the Redfield biomass ratio (Redfield *et al*., 1963) this means approximately 0.5 mmol N l^{-1},

which is very low in comparison with the consumed ammonium and indicates that depletion was rather due to a dissimilatory process, the only conceivable one being anaerobic ammonium oxidation described as Anammox and being linked to the presence of members of the *Planctomycetales* (eq. 2, Mulder *et al*., 1995; Jetten *et al*., 1999). The assumption is also substantiated by the observation that nitrite was only present at low concentration as long as ammonium was present in the culture medium, but accumulated after depletion of ammonium. Also, new addition of ammonium resulted in rapid reduction of the accumulated nitrite, simultaneously with consumption of ammonium. The presence of Anammox organisms accounting for up to 18% of the total cell number and closely related to *Candidatus Brocadia anammoxidans* (Schmid *et al*., 2000), was also demonstrated on the molecular level by sequencing of the 16S rRNA gene and FISH with specific probes. Therefore, it appears that the dominant *Geobacteriaceae* responsible for degradation of cyclohexane reduce nitrate mainly to nitrite (eq. 1); the relatively slow continuous release of the latter by the slowly growing cyclohexane degraders may have favoured the enrichment of Anammox organisms. More rapid and more complete reduction of nitrite to ammonia would deprive the culture of sufficient nitrite for the anammox process. In turn, anaerobic oxidation of ammonium favoured degradation of cyclohexane by preventing accumulation of nitrite, a potentially harmful compound, in the culture medium.

$$C_6H_{12} + 18NO_3^- \quad ? \quad 18NO_2^- + 6HCO_3^- + 6H^+ \qquad (1)$$

$$NH_3 + NO_2^- + H^+ \quad ? \quad N_2 + 2H_2O \qquad (2; \text{Jetten } \textit{et al}., 1999)$$

If ammonium was no longer present in the medium, the ratio of nitrate reduced / nitrite formed was 1.4 (Fig. 1A) indicating that approximately 29% of the formed nitrite was further reduced by the cyclohexane-degrading bacteria; these may be a *Geobacter* species, or low numbers of other, denitrifying bacteria, that may also scavenge polar products of cyclohexane. However, any further reduction of nitrite was obviously slow, thus allowing for the enrichment of anaerobic ammonium oxidizers which require equimolar amounts of nitrite and ammonium (eq. 2).

The intense pink-red color observed in settled pellet of the enrichment culture may be due to a high content of cytochromes, which has been documented for members of the genera *Geobacter* and the related *Desulfuromonas* (Pfennig and Biebl, 1976; Bache *et al*., 1983), but also for the Anammox organisms (Jetten *et al*., 1999).

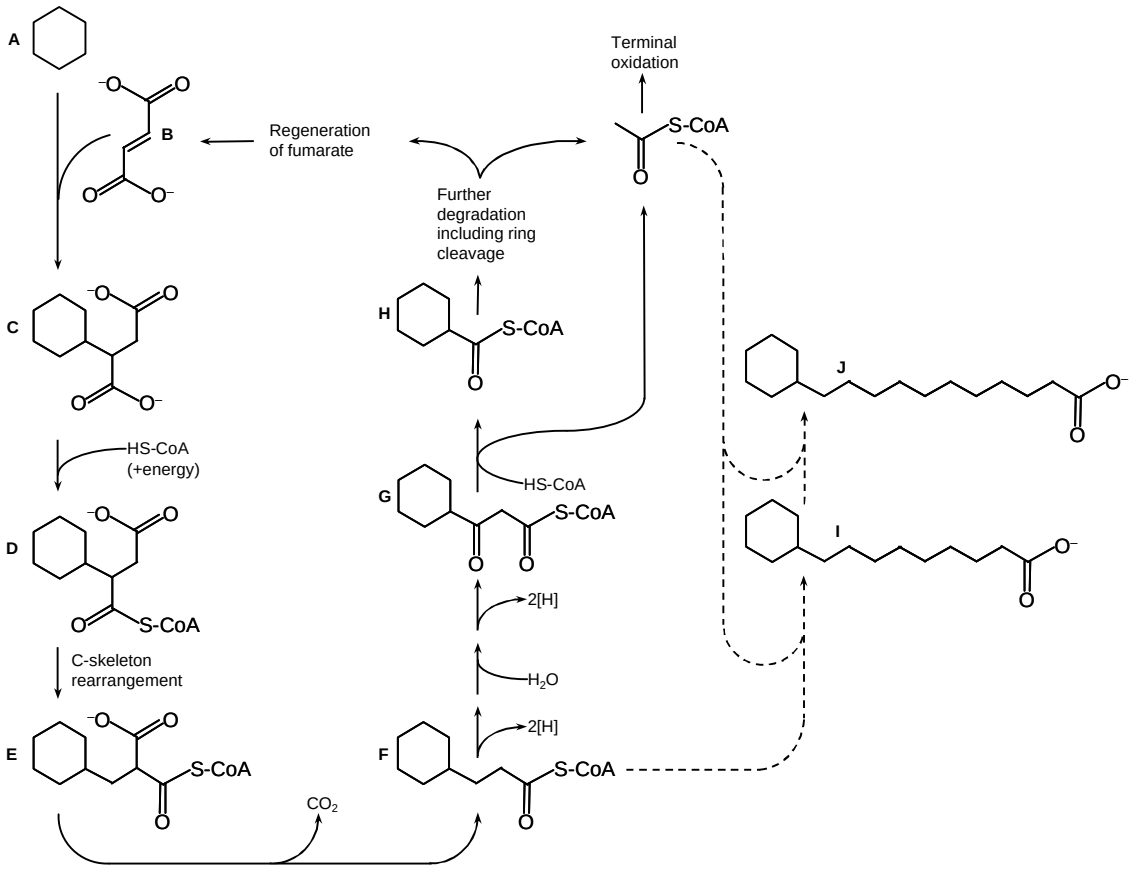


Fig. 5. Proposed pathway for the anaerobic degradation of cyclohexane. Cyclohexane (A) is activated by addition to fumarate (B), yielding cyclohexylsuccinate (C) which may be then activated with coenzyme A to cyclohexylsuccinyl-CoA (D). The latter may undergo rearrangement of the C-skeleton to (2-methylcyclohexyl)malonyl-CoA (E), which via decarboxylation may yield cyclohexylpropionyl-CoA (F). Further degradation may proceed via 3-oxo-3-cyclohexyl-propionyl-CoA (G), cyclohexyl-carboxyl-CoA (H), and ring cleavage yielding fumarate and acetyl-CoA for the general metabolism. The fatty acids 9-cyclohexylnonanoate (I) and 11-cyclohexylundecanoate (J) may be synthesized from cyclohexylpropionyl-CoA (F) via addition of C_2-units addition in the fatty acids synthesis cycle.

Based on the metabolites identified, we propose that cyclohexane is anaerobically activated like *n*-alkanes (Kropp *et al.*, 2000; Rabus *et al.*, 2001) by addition to fumarate yielding cyclohexylsuccinate, and further metabolized analogous to the pathway of activated *n*-alkanes (Fig. 5, Wilkes *et al.*, 2002, 2003). The thioester of cyclohexylsuccinate, cyclohexylsuccinyl-CoA, may via rearrangement of the C-skeleton and decarboxylation yield cyclohexylpropionyl-CoA (Fig. 5). Further degradation of cyclohexylpropionate may yield cyclohexylcarboxyl-CoA which undergoes ring cleavage yielding fumarate (regeneration of fumarate required for activation) and acetyl-CoA units for the general metabolism. Identification of 9-cyclohexylnonanoate and 11-cyclohexylundecanoate suggests that cyclohexylpropionyl-CoA may enter the lipid fatty acid synthesis cycle, and upon 3 or 4 cycles of C_2-unit addition yield the mentioned fatty acids.

Acknowledgments

Simon Kühner is acknowledged for help with extraction of metabolites. This work was supported by the Max Planck Society.

References

Aeckersberg, F., F. Bak, and F. Widdel. 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Arch Microbiol **156:**5-14.

Aeckersberg, F., F. A. Rainey, and F. Widdel. 1998. Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long chain alkanes under anoxic conditions. Arch Microbiol **170:**361-369.

Amann, R. I., L. Krumholz, and D. A. Stahl. 1990. Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. Journal of Bacteriology **172:**762-770.

Anders, H. J., A. Kaetzke, P. Kämpfer, W. Ludwig, and G. Fuchs. 1995. Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov., respectively, members of the β 3ubclass of the Proteobacteria. Int J Syst Bacteriol **45:**327-333.

Anderson, M. S., R. A. Hall, and M. Griffin. 1980. Microbial metabolism of alicyclic hydrocarbons: cyclohexane catabolism by a pure strain of *Pseudomonas* sp. J Gen Microbiol **120:**89-94.

Anderson, R. T., and D. R. Lovley. 2000. Hexadecane decay by methanogenesis. Nature **404:**722-723.

Bache, R., P. M. H. Kroneck, M. Merkle, and H. Beinert. 1983. A survey of EPR-detectable components in sulfur-reducing bacteria. Biochim Biophys Acta **722:**417–426.

Boltz, D. F., and M. J. Taras. 1978. Nitrogen, p. 197-251. *In* D. F. Boltz and J. A. Howell (ed.), Colorimetric determination of nonmetals, second ed. Wiley, New York.

Bregnard, T. P. A., A. Haener, P. Hoehener, and J. Zeyer. 1997. Anaerobic degradation of pristane in nitrate-reducing microcosms and enrichment cultures. Appl Environ Microbiol **63**.

Cheng, Q., S. M. Thomas, and P. E. Rouviere. 2002. Biological conversion of cyclic alkanes and cyclic alcohols into dicarboxylic acids: biochemical and molecular basis. Appl Microbiol Biotechnol **58:**704-711.

Ehrenreich, P., A. Behrends, J. Harder, and F. Widdel. 2000. Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Arch Microbiol **173:**58-64.

Harms, G., K. Zengler, R. Rabus, F. Aeckersberg, D. Minz, R. Rossello-Mora, and F. Widdel. 1999. Anaerobic oxidation of *o*-xylene, *m*-xylene, and homologous alkylbenzenes by new types of sulfate-reducing bacteria. Appl Environ Microbiol **65:**999-1004.

Hicks, R. E., R. I. Amann, and D. A. Stahl. 1992. Dual staining of natural bacterioplancton with 4',6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl Environ Microbiol **58:**2158-2163.

Jetten, S. M. S., M. Strous, K. T. van de Pas-Schoonen, J. Schalk, G. J. M. van Dongen, A. A. van de Graaf, S. Logemann, G. Muyzer, M. C. M. van Loosdrecht, and J. G. Kuenen. 1999. The anaerobic oxidation of ammonium. FEMS Microbiology Reviews **22:**421-437.

Kane, M. D., L. K. Poulsen, and D. A. Stahl. 1993. Monitoring the enrichment and isolation of sulfate-reducing bacteria by using oligonucleotide hybridization probes designed from environmentally derived 16S rRNA sequences. Appl. Env. Microbiol. **59:**682-686.

Kropp, K. G., I. A. Davidova, and J. M. Suflita. 2000. Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. Appl Environ Microbiol **66:**5393-5398.

Lovley, D. R., and E. J. P. Phillips. 1988. Novel mode of microbial energy metabolism: organic carbon oxidation coupled to dissimilatory reduction of iron or manganese. Appl Environ Microbiol **54:**1472-1480.

Lovley, D. R., M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Siegel. 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature **339:**297-300.

Lowe, K. L., T. J. Dichristina, A. N. Roychoudhury, and P. Van Cappellen. 2000. Microbiological and geochemical characterization of microbial Fe(III) reduction in salt marsh sediments. Geomicrobiol J **17:**163-178.

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, Arno Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W.

Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüssmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. 2004. ARB: a software environment for sequence data. Nucl Acid Res **32:**1363-1371.

Morgan, P., and R. J. Watkinson. 1994. Biodegradation of components of petroleum, p. 1-31. *In* C. Ratledge (ed.), Biochemistry of microbial degradation. Kluwer Academic Publisher.

Mulder, A., A. A. van de Graaf, L. A. Robertson, and J. G. Kuenen. 1995. Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. FEMS Microbiol Ecol **16:**177-183.

Pernthaler, A., J. Pernthaler, and R. Amann. 2002. Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. Appl. Environ. Microbiol. **68:**3094-3101.

Perry, J. J. 1984. Microbial metabolism of cyclic alkanes, p. 61-99. *In* R. M. Atlas (ed.), Petroleum Microbiology. Macmillan Publishing Co., New York.

Pfennig, N., and H. Biebl. 1976. *Desulfuromonas acetoxidans* gen. nov. and sp. nov., a new anaerobic, sulfur-reducing, acetate-oxidizing bacterium. Arch Microbiol **110:**3–12.

Rabus, R., and F. Widdel. 1995. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Arch Microbiol **163:**96-103.

Rabus, R., and F. Widdel. 1996. Utilization of alkylbenzenes during anaerobic growht of pure cultures of denitrifying bacteria on crude oil. Appl Environ Microbiol **62:**1238-1241.

Rabus, R., H. Wilkes, A. Behrends, A. Armstroff, T. Fischer, A. J. Pierik, and F. Widdel. 2001. Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. J Bacteriol **183:**1707-1715.

Ravenschlag, K., K. Sahm, J. Pernthaler, and R. Amann. 1999. High bacterial diversity in permanently cold marine sediments. Applied and Environmental Microbiology **65:**3982-3989.

Redfield, A., B. Ketchum, and F. Richards. 1963. The influence of organisms on the composition of sea-water, p. 26-77. *In* M. Hill (ed.), The sea. Interscience, New York.

Rockne, K. J., J. C. Chee-Sanford, R. A. Sanford, B. P. Hedlund, J. T. Staley, and S. E. Strand. 2000. Anaerobic naphthalene degradation by microbial pure cultures under nitrate-reducing conditions. Appl Environ Microbiol **66:**1595-1601.

Rouviere, P. E., and M. W. Chen. 2003. Isolation of *Brachymonas petroleovorans* CHX, a novel cyclohexane-degrading β-proteobacterium. FEMS Microbiol Lett **227:**101-106.

Rueter, P., R. Rabus, H. Wilkes, F. Aeckersberg, F. A. Rainey, H. W. Jannasch, and F. Widdel. 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature **372:**455-458.

Schmid, M., U. Twachtmann, M. Klein, M. Strous, S. Juretschko, M. Jetten, J. W. Metzger, K. H. Schleifer, and M. Wagner. 2000. Molecular evidence for genus level diversity of bacteria capable of catalyzing anaerobic ammonium oxidation. Systematic & Applied Microbiology **23:**93-106.

Sikkema, J., J. A. M. de Bont, and B. Poolman. 1994. Interactions of cyclic hydrocarbons with biological membranes. J Biol Chem **269:**8022-8028.

Sikkema, J., J. A. M. de Bont, and B. Poolman. 1995. Mechanisms of membrane toxicity of hydrocarbons. Microbiological Reviews **59:**201-222.

Snaidr, J., R. Amann, I. Huber, W. Ludwig, and K. H. Schleifer. 1997. Phylogenetic analysis and *in situ* identification of bacteria in activated sludge. Appl. Environ. Microbiol. **63:**2884-2896.

So, C. M., and L. Y. Young. 1999. Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl Environ Microbiol **65:**2969-2976.

Solano-Serena, F., R. Marchal, J. M. Lebeault, and J. P. Vandecasteele. 2000. Selection of microbial populations degrading recalcitrant hydrocarbons of gasoline by monitoring of culture-headspace composition. Letters in Applied Microbiology **30:**19-22.

Solano-Serena, F., R. Marchal, S. Casaregola, C. Vasnier, J. M. Lebeault, and J. P. Vandecasteele. 2000. A *Mycobacterium* strain with extended capacities for degradation of gasoline hydrocarbons. Appl Environ Microbiol **66:**2392-2399.

Song, B., M. M. Häggblom, J. Zhou, J. M. Tiedje, and N. J. Palleroni. 1999. Taxonomic characterization of denitrifying bacteria that degrade aromatic compounds and description of *Azoarcus toluvorans* sp. nov. and *Azoarcus toluclasticus* sp. nov. Int J Syst Bacteriol **49:**1129-1140.

Spormann, A. M., and F. Widdel. 2000. Metabolism of alkylbenzenes, alkanes, and other hydrocarbons in anaerobic bacteria. Biodegradation **11:**85-105.

Stirling, L. A., and R. J. Watkinson. 1977. Microbial metabolism of alicyclic hydrocarbons: isolation and properties of a cyclohexane-degrading bacterium. J Gen Microbiol **99:**119-125.

Tissot, B. P., and D. H. Welte. 1984. Petroleum formation and occurence. Springer-Verlag, Berlin, Germany.

Townsend, G. T., R. C. Prince, and J. M. Suflita. 2004. Anaerobic biodegradation of alicyclic constituents of gasoline and natural gas condensate by bacteria from an anoxic aquifer. FEMS Microbiol Ecol **49:**129-135.

Trower, M. K., R. M. Buckland, R. Higgins, and M. Griffin. 1985. Isolation and characterization of cyclohexane-metabolizing *Xanthobacter* sp. Appl Environ Microbiol **49:**1282-1289.

Widdel, F., and F. Bak. 1992. Gram-negative mesophilic sulfate-reducing bacteria. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The Prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications., 2nd ed, vol. 4. Springer-Verlag, Berlin, Heidelberg, New York.

Widdel, F., and N. Pfennig. 1992. The genus *Desulfuromonas* and other gram-negative sulfur-reducing eubacteria, p. 3379-3389. *In* A. Balows, H. G. Trüper, M. Dworkin, W. Harder, and K.-H. Schleifer (ed.), The Prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications., 2nd ed, vol. 4. Springer-Verlag, Berlin, Heidelberg, New York.

Widdel, F., and R. Rabus. 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Current Opinion in Biotechnology **12:**259-276.

Wilkes, H., C. Boreham, G. Harms, K. Zengler, and R. Rabus. 2000. Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. Org Geochem **31:**101-115.

Wilkes, H., R. Rabus, T. Fischer, A. Armstroff, A. Behrends, and F. Widdel. 2002. Anaerobic degradation of *n*-hexane in a denitrifying bacterium: further degradation on the initial intermediate (1-methylpentyl)succinate via C-skeleton rearrangement. Arch Microbiol **177:**235-243.

Wilkes, H., S. Kuhner, C. Bolm, T. Fischer, A. Classen, F. Widdel, and R. Rabus. 2003. Formation of *n*-alkane- and cycloalkane-derived organic acids during anaerobic growth of a denitrifying bacterium with crude oil. Org Geochem **34:**1313-1323.

Zengler, K., H. H. Richnow, R. Rossello-Mora, W. Michaelis, and F. Widdel. 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature **401:**266-269.

Acknowledgments

Thanks first to my academic supervisor, Prof. Dr. Friedrich Widdel (MPI Bremen), for giving me the opportunity to work at the institute, for his valuable guidance and for motivating and inspiring discussions.

Special thanks to my second supervisor, Prof. Dr. Heribert Cypionka (ICBM, Oldenburg), for his willingness to read my thesis.

Thanks to Andrea Wieland (University of Hamburg), Raeid Abed and Dirk de Beer for their support with the microsensor work. I especially thank Heinz Wilkes (GeoForschungZentrum Potsdam) for his valuable help with chemical analyses of crude oil samples and metabolites. Thanks to Jens Harder for countless suggestions and help with phylogenetic analyses; Ramona Apple for teaching me the anaerobic techniques, significant help in the lab and the nice working atmosphere; Cristina Probian for the gas chromatographs and HPLC's; Olav Grundmann, for support with molecular methods; Martin Krüger for interesting discussions and proof-reading the introductory part of my thesis; Enoma Omoregie for sharing tips and tricks about *nifH* genes phylogeny; Simon Kühner for help with extraction of metabolites; my (actual and former) office colleagues, Dörte Gade, Sabrina Schübbe, Judith Amann, Insa Werner and Claus Lang, for such a nice and friendly atmosphere; and all my colleagues from the Department of Microbiology for the nice environment, after-lunch cookies and Octoberfest tours.

Special thanks to Ileana Stoica and Tatiana Vassu (University of Bucharest) who guided me at the beginning of my scientific career.

Finally, I wish to thank my wife and best friend, Niculina, for invaluable, constant support and encouragement through good and bad times. Thanks to my parents and my family who were always besides me.