

Bacterial diversity of a cyanobacterial mat degrading petroleum compounds at elevated salinities and temperatures

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Abstract

Cyanobacterial mats of the Arabian Gulf coast of Saudi Arabia experience extreme conditions of temperature and salinity. Because they are exposed to continuous oil pollution, they form ideal models for biodegradation under extreme conditions. We investigated the bacterial diversity of these mats using denaturing gradient gel electrophoresis and 16S rRNA cloning, and tested their potential to degrade petroleum compounds at various salinities (fresh water to 16%) and temperatures (5 to 50 °C). Cloning revealed that c. 15% of the obtained sequences were related to unknown, possibly novel bacteria. Bacteria belonging to *Beta*-, *Gamma*- and *Deltaproteobacteria*, *Cytophaga*–*Flavobacterium*–*Bacteroides* group and *Spirochetes*, were detected. The biodegradation of petroleum compounds at different salinities by mat microorganisms showed that pristine and *n*-octadecane were optimally degraded at salinities between 5 and 12% (weight per volume NaCl) whereas the optimum degradation of phenanthrene and dibenzothiophene was at 3.5% salinity. The latter compounds were also degradable at 8% salinity. The same compounds were degraded at temperatures between 15 and 40 °C but not at 5 and 50 °C. The optimum temperature of degradation was 28–40 °C for both aliphatics and aromatics. We conclude that the studied microbial mats from Saudi Arabia are rich in novel halotolerant and thermotolerant microorganisms with the potential to degrade petroleum compounds at elevated salinities and temperatures.

Introduction

Cyanobacterial mats develop well under extreme conditions, where the abundance and activity of grazing organisms is limited (Javor & Castenholz, 1984; Cohen, 1989; Farmer, 1992). The Arabian Gulf is a harsh environment, situated in a semi-arid region with high evaporation rates (200 cm year⁻¹) (Khan & Al-Ajmi, 1998). The seawater salinity may reach up to 16% during warm seasons, when the temperature often exceeds 50 °C. The coastal flats of the Gulf are covered by a variety of microbial mat systems (Al-Thukair & Al-Hinai, 1993) that are subjected to significant seasonal changes in temperature and salinity as well as to daily cycles of dehydration and rewetting by tides. Owing to the tidal regime, these mats experience highly variable environmental conditions that change quickly from moderate (3–4% salinity, 25 °C) to extreme (16% salinity, 50 °C) on a daily basis. These mats are of additional interest because of their frequent exposure to oil pollution from nearby terminals. Indeed, some of these mats became

dominant only after the Gulf War in 1991, when more than 10.8 million barrels of crude oil was released into the Arabian Gulf (Sorkhoh *et al.*, 1992; Fayad & Overton, 1995). In spite of the unique environmental settings of the mats, the diversity of their microbial communities has been poorly investigated. Earlier reports were based mainly on microscopy and cultivation techniques (Sorkhoh *et al.*, 1993, 1995; Hoffmann, 1996; Höpner *et al.*, 1996), where diversity is often underestimated.

Salinity and temperature are important key environmental parameters that influence the degradation process of petroleum compounds. These parameters influence the structure and physiology of existing microbial communities and change the physical and chemical properties of the pollutants (e.g. solubility and viscosity). The diversity and metabolic potential of degrading bacteria are considered to decrease as the environmental conditions become more extreme (Foght & McFarlane, 1999; Margesin & Schinner, 2001). Previous studies showed that rates of hydrocarbon biodegradation decreased with increasing salinity, and

biodegradation could not be detected above 15% salinity (Ward & Brock, 1978; Rhykerd *et al.*, 1995). Nevertheless, bacterial strains capable of performing pollutant degradation at high salt concentrations were isolated (Oren *et al.*, 1992). The increase in temperature was shown to enhance biodegradation (Ward & Brock, 1976; Margesin & Schinner, 2001), although high temperatures are known to reduce the diversity of microorganisms. Saudi Arabia's contaminated mats experience these extreme parameters simultaneously, and thus the adaptation of bacterial communities and the chances for bioremediation are difficult to predict from present knowledge. Therefore, additional information on the capability of microbial communities within the mats to degrade oil components at variable salinities and temperatures is required.

The aim of this study was to explore the bacterial diversity of cyanobacterial mats along the coasts of Saudi Arabia using culture-independent approaches such as denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene cloning. The potential of the mat communities to degrade aliphatic (pristane and *n*-octadecane) and aromatic (phenanthrene and dibenzothiophene) compounds at different salinities and temperatures was also examined. The compounds were selected to represent major groups of oil constituents (branched alkane, straight-chain alkane, aromatic hydrocarbons and organosulphur compounds, respectively).

Materials and methods

Mat samples and environmental settings

Microbial mats were collected in November 2002 from Dawhat Al-Daffi in Saudi Arabia, north of Jubail. The site was selected because of its accessibility and location close to

oil terminals. The mats developed directly after the 1991 oil spill and they are currently growing on top of sandy sediments that still contain oil residues. These mats are frequently exposed to occasional oil-spill incidents from nearby oil terminals. The air temperature at the sampling site is on average 17 °C in winter, and may reach 50 °C in a hot summer. At the time of sampling, the air temperature was 30 °C and the salinity of the water was 5%. During high tide the entire site is flooded, whereas during low tide the seawater retreats and the area is exposed to air. High rates of evaporation during the low tide lead to salt precipitation and frequent desiccation of the mats (Fig. 1, left panel). The upper intertidal zone is usually drier than the lower intertidal zone. The continuous flushing of seawater by tides results in the formation of channels throughout the entire mat system (Fig. 1, right panel). These channels retain seawater during low tides, forming small lagoons that are surrounded by dry, slightly elevated sediments. Microbial mats from these channels were collected either frozen in liquid N₂ for molecular analysis, or kept viable for biodegradation experiments.

Molecular analysis

Mat cores from low, middle and high intertidal zones (*c.* 300–500 mg each) were subjected to nucleic acid extraction, PCR and DGGE, as previously described (Abed *et al.*, 2002b). PCR amplification of 16S rRNA genes was carried out using two sets of oligonucleotide primers. CYA359F (with a 40 nucleotide GC clamp at the 5' end) and CYA781R were used as cyanobacteria-specific primers (Nübel *et al.*, 1997), and GM5F with GC clamp in combination with the reverse primer 907R for the domain bacteria (Muyzer *et al.*, 1995). Thermocycling was performed using a Mastercycler gradient cyler (Eppendorf, Hamburg, Germany). A hot-



Fig. 1. The studied microbial mats from Saudi Arabia, showing a whitish top layer of salt resulting from high evaporation (left panel) and the channelling system within these mats (right panel).

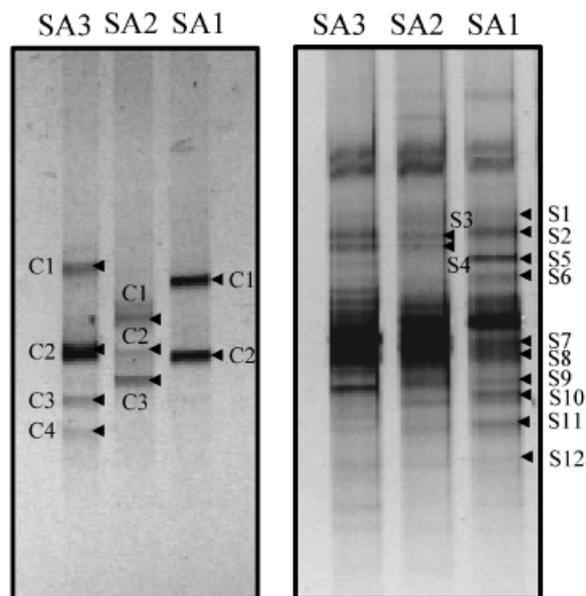


Fig. 2. Denaturing gradient gel electrophoresis fingerprints of PCR-amplified 16S rRNA gene fragments obtained from microbial mats present at low (SA1), middle (SA2) and high (SA3) intertidal, using cyanobacteria-specific (left panel) and universal bacterial (right panel) primers. The indicated bands were excised, reamplified and sequenced.

start program was performed for the cyanobacteria-specific primers as described by Nübel *et al.* (1997). A hot-start touch-down program was used for the bacterial universal primers (GM5 and 907R) in order to minimize nonspecific amplification (Muyzer *et al.*, 1995). DGGE was carried out using a Bio-Rad D-Code system and was run at 60 °C and at a constant voltage of 200 V for 3.5 h. DGGE bands were excised manually, the DNA left to diffuse out in buffer overnight, and PCR reamplified. The amplification products were sequenced in both directions.

Phylogenetic analysis

Cyanobacterial phylogenetic trees were constructed based on long 16S rRNA gene sequences (more than 1300 bp) by applying a variety of methods integrated in the ARB software (Ludwig *et al.*, 1998) such as maximum likelihood, maximum parsimony and neighbour joining (a maximum likelihood tree is presented in Figs 3 and 4). Partial sequences were not included in the calculation of trees. The 16S rRNA gene sequences of *Escherichia coli* and *Bacillus subtilis* were included in the calculations as outgroup sequences. Trees calculated using different methods were essentially equivalent, and maximum likelihood trees are presented here. The cyanobacterial forward and reverse complementary sequences obtained from DGGE bands were aligned against each other in order to obtain consensus sequences. These sequences were then aligned to the se-

quences in the ARB database using the alignment tool of the ARB software package and were inserted into the pre-established tree using the parsimony ARB tool and maintaining the overall tree topology without changes. Bacterial partial sequences were also inserted within a pre-established stable tree containing all bacterial sequences in the ARB database without allowing changes of the overall tree topology. The final tree was minimized for simplicity in presentation.

Construction of a clone library

To expand the results from DGGE analysis, a clone library from the low intertidal mat was constructed. PCR was performed on the DNA extracted from this mat using the GM3 and GM4 primers (Muyzer *et al.*, 1995). PCR products were purified using the QIAquick PCR purification kit (Diagen, Düsseldorf, Germany) and were cloned by applying a pGEM-T plasmid vector system and competent *E. coli* JM 109 cells (Promega, Heidelberg, Germany). The clones obtained were screened for the presence of inserts. Plasmids were prepared from positive clones using the Wizard Plus Minipreps Kit (Promega). The plasmid inserts were sequenced with an ABI PRISM 3100 genetic analyser (Applied Biosystems, Foster City, CA).

Preparation of organo-clay complexes

n-Octadecane, pristane, phenanthrene, and dibenzothio-*phene* were selected as model compounds for petroleum constituents. Hydrophobic clay was used as a carrier substance for the petroleum model compounds (Grötzschel *et al.*, 2002). To a 2% aqueous suspension of montmorillonite KSF (Aldrich, Steinheim, Germany), 10 mM trimethylammonium (BTMA) chloride was added slowly to obtain 0.8 mmol BTMA g⁻¹ clay [modified after (El-Nahhal *et al.*, 2000)]. The mixture was stirred for 24 h, washed three times to remove excess BTMA chloride, and then freeze-dried. To adsorb the model compounds, the hydrophobic clay (BTMA-montmorillonite) was suspended in *n*-hexane. The mixture of the model compounds (in total 20 mg per 100 mg of hydrophobic clay dissolved in *n*-hexane) was slowly added under continuous stirring. The slurry was dried in a vacuum rotary evaporator, which yielded a homogeneous powder of organo-clay complex loaded with 16.67 wt% of petroleum model compounds (hereafter designated as OCC). The adsorbed amount of model compounds was re-extracted with dichloromethane (DCM) and analysed by gas chromatography.

Biodegradation experiments

Two sets of 250 mL Erlenmeyer flasks were prepared: one to test degradation under different salinities and the other to

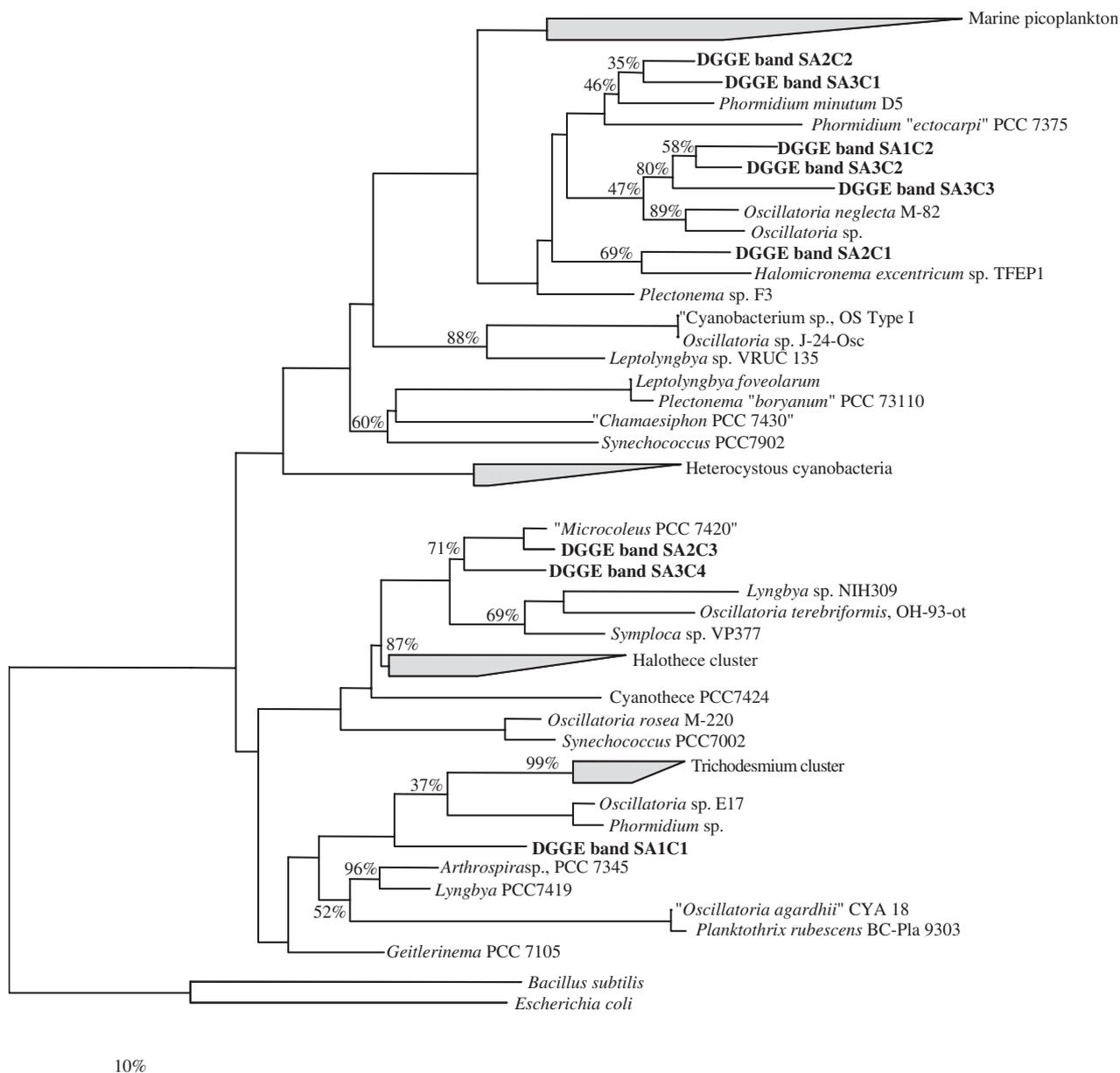


Fig. 3. Phylogenetic tree (maximum likelihood) for cyanobacteria based on publicly available, almost complete 16S rRNA genes from members of the cyanobacterial line. *Escherichia coli* and *Bacillus subtilis* were used as outgroups. The partial 16S rRNA gene sequences from our excised denaturing gradient gel electrophoresis bands were placed phylogenetically using parsimony criteria without changing the topology of the pre-established tree. The bar indicates 10% sequence divergence. Bootstrap values from 1000 trees are included and indicated as % at relevant nodes.

test degradation under different temperatures. Hypersaline seawater media of 5%, 8%, 12% and 16% weight per volume (w/v) final total salinity were prepared by adding appropriate amounts of NaCl to natural seawater. Nitrogen (3.75 mM NH_4Cl) and phosphorus (1.1 mM KH_2PO_4) sources were added, and the media were then autoclaved. All flasks received 100 mL of medium, 1 g of mat material, and 100 mg OCC. The following controls were used: (i) medium with OCC without mat material (abiotic control);

and (ii) medium with OCC and autoclaved mat core. The latter control was used to correct for the adsorption of OCC to mat material. Degradation of the compounds was tested at salinities of 3.5%, 5%, 8%, 12%, and 16% (w/v NaCl) and incubation at 28 °C. Degradation was also tested at 3.5% salinity and at temperatures of 5, 15, 28, 40 and 50 °C. All flasks were incubated on a rotary shaker at 100 rpm with a light regime of 12 h light/12 h dark. The light intensity was $100 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ (photosynthetically available

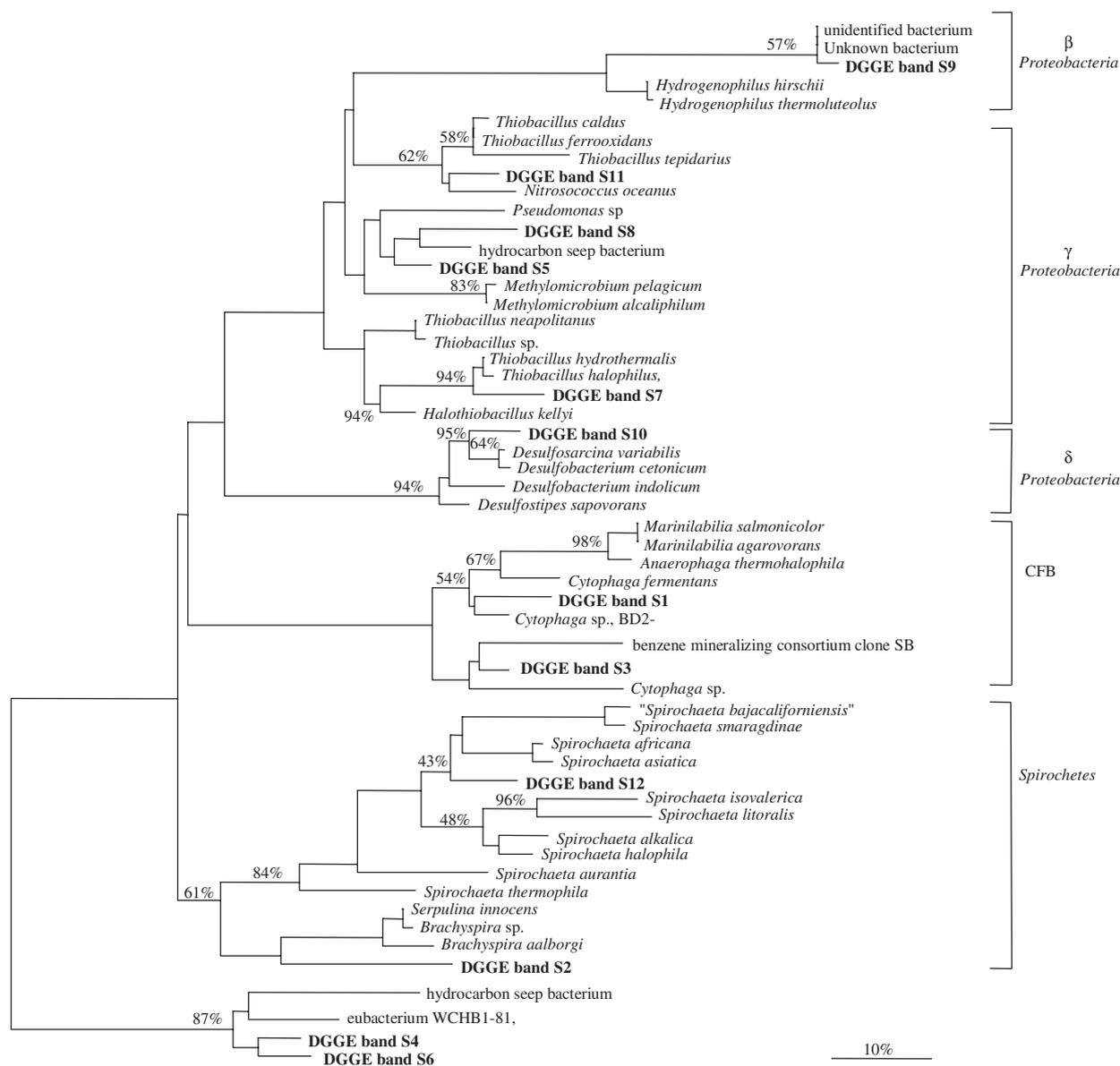


Fig. 4. Unrooted phylogenetic tree showing affiliations based on partial bacterial 16S rRNA genes and selected sequences from members of various bacterial clusters, including *Beta*, *Gamma* and *Delta* subclasses of the *Proteobacteria*, the *Cytophaga-Flavobacterium-Bacteroides* group (CFB) and *Spirochetes*. The tree was simplified for clarity by omitting all sequences between clusters. The scale bar indicates 10% estimated sequence divergence. Bootstrap values from 1000 trees are included and indicated as % at relevant nodes.

radiation). Flasks were sampled at days 2, 6, 12, 18 and 26. Samples (2 mL) were collected after vigorous shaking of the flasks in order to suspend the solid OCC and to obtain homogenous samples. These samples were stored in 20 mL glass tubes sealed with Teflon-coated screw caps at -20°C prior to extraction.

Chemical analyses

The samples were extracted with 3.5 volumes of dichloromethane (7 mL) by vertical shaking for 3 h at room

temperature. The organic phase was collected and immediately analysed with a Perkin-Elmer AutoSystem gas chromatograph (Perkin-Elmer GmbH) equipped with a CTC A200S Auto-sampler (CTC Analytics), a flame ionization detector (FID) and an Optima-5 column (50 m \times 0.32 mm; film thickness 0.25 μm). The oven was operated for 2 min at 60°C , heated at a rate of $20^{\circ}\text{C min}^{-1}$ to 150°C and at a rate of $5^{\circ}\text{C min}^{-1}$ to 310°C , and then kept at 310°C for 10 min. The injection port and FID were operated at 250 and 350°C respectively. The model hydrocarbons were quantified by integration of the FID signals and comparison with

standards. The calculated initial amount of each model compound in 2 mL sample was 66.67 µg. Data on the abundance of the model compounds are presented relative to this value.

Results

Microbial diversity within the studied mats

Denaturing gradient gel electrophoresis analysis of the cyanobacterial populations from samples collected at different distances from the tidal water (less than 50 m apart from each other) revealed differences in the community composition (Fig. 2, left panel). The DGGE profile from the lower intertidal mat displayed two major bands (SA1C1 and SA1C2) that had sequences affiliated to sequences of *Arthrospira*, *Lyngbya*, *Phormidium* and *Oscillatoria* species (Fig. 3). The middle tidal mat showed three major bands, with sequences closely related to the newly described genus *Halomicronema* (Abed *et al.*, 2002a) with 94% sequence similarity (band SA2C1), to *Phormidium minutum* D5 with 95% sequence similarity (band SA2C2), and to *Microcoleus chthonoplastes* with 98% sequence similarity (band SA2C3). From the upper intertidal mat, the sequences of DGGE bands SA3C1 and SA3C4 were phylogenetically affiliated to *Phormidium minutum* D5 (92.5% similarity) and *Microcoleus chthonoplastes* (94.5% similarity), respectively. The closest relative to band SA3C2 and band SA3C3 sequences was the sequence of *Oscillatoria neglecta* M-82.

The DGGE profile generated using the universal bacterial primers is presented in Fig. 2, right panel. Twelve bacterial sequences, excluding those of the cyanobacteria that were also amplified by the same primers, were obtained. The phylogenetic reconstruction of the sequences showed affiliation to *Beta*-, *Gamma*- and *Deltaproteobacteria*, the *Cytophaga*–*Flavobacterium*–*Bacteroides* (CFB) group, and *Spirochetes* (Fig. 4). Most DGGE fragments (DGGE bands S5, 7, 8 and 11) were derived from *Gammaproteobacteria*, and some of those sequences were related to sulphur-oxidizing bacterial species. The sequence of DGGE band S10 was closely related to the sulphate-reducing bacterium *Desulfosarcina variabilis*. DGGE bands S5 and S8 had sequences that were related to an environmental clone from a hydrocarbon seep environment, whereas the sequence of band S9 was related to an unknown species. In the CFB group, the sequences of DGGE bands S1 and S3 were related to the known *Cytophaga fermentans*.

Cloning provided a more detailed picture of the bacterial diversity within the low intertidal mat. A total of 96 clones were randomly picked and sequenced. Most clones belonged to the *Gamma*- and *Deltaproteobacteria* (20 and 18 clones, respectively) and to the CFB group (19 clones). The remaining clones were distributed among cyanobacteria

(8 clones), *Holophaga/Acidobacteria/Geothrix* (3 clones), *Verruimicrobia* (3 clones), *Chloroflexus* (4 clones), *Alphaproteobacteria* (2 clones), *Spirochetes* (3 clones) and *Planctomycetes* (5 clones). Approximately 15% of the clones obtained were not related to known microorganisms in the database. Most of the *Gammaproteobacteria* were related to sulphur-oxidizing bacteria, and 70% of them had more than 96% sequence similarity to the sequence of the chemolithotrophic bacterium *Thiobacillus hydrothermalis* (accession number M90662). Two clones belonged to the phototrophic purple sulphur bacteria; one was affiliated to *Thiobaca trueperi* and the other to a *Halochromatium* species. Only three clones of the *Gammaproteobacteria* were related to the genera *Pseudomonas* and *Alcanivorax*, which are known to include oil degraders. The *Deltaproteobacteria* included mainly sequences from sulphate-reducing bacteria belonging to the genera *Desulfococcus*, *Desulfonema*, *Desulfosarcina*, *Desulfomonile*, and *Desulfocapsa*. The cyanobacterial clones were related mainly to filamentous cyanobacteria from the genera *Phormidium* and *Microcoleus*. A group of clones was related to species of the genus *Deinococcus*, which comprises coccoid bacteria that are resistant to UV and solar radiation (Rainey *et al.*, 1997).

Degradation of petroleum compounds at different salinities

Recovery of the four compounds was, in most cases, more than 70% of the initially added amounts (Fig. 5). A slight decrease in the concentration of the four compounds was observed in the abiotic controls throughout the experiment, mainly as a result of experimental and analytical errors. Degradation of the four studied compounds was not possible in fresh-water conditions. The degradation of pristane and *n*-octadecane was observed at salinities between 3.5% and 16%. At 3.5–12% salinities and after 12 days of incubation, the concentrations of pristane and *n*-octadecane decreased to *c.* 25% and 14% of the initially added amount, respectively, and these amounts remained unchanged until the end of the experiment. Pristane was almost nondegradable at 16% salinity, whereas *n*-octadecane was considerably degraded at this salinity. Unlike the aliphatic compounds, which were degradable at different salinities, the degradation of aromatics was only observed at 3.5% salinity and to a lesser extent at 8% salinity. Phenanthrene and dibenzothiophene showed a similar pattern of degradation. At 3.5% salinity and after incubation for 12 days, both compounds were completely degraded. Whereas the degradation of phenanthrene and dibenzothiophene was not significant at 5% salinity, a further increase to 8% resulted in a measurable degradation to 25% of the initially added concentrations. Above this salinity, no significant degradation of either compound was detected.

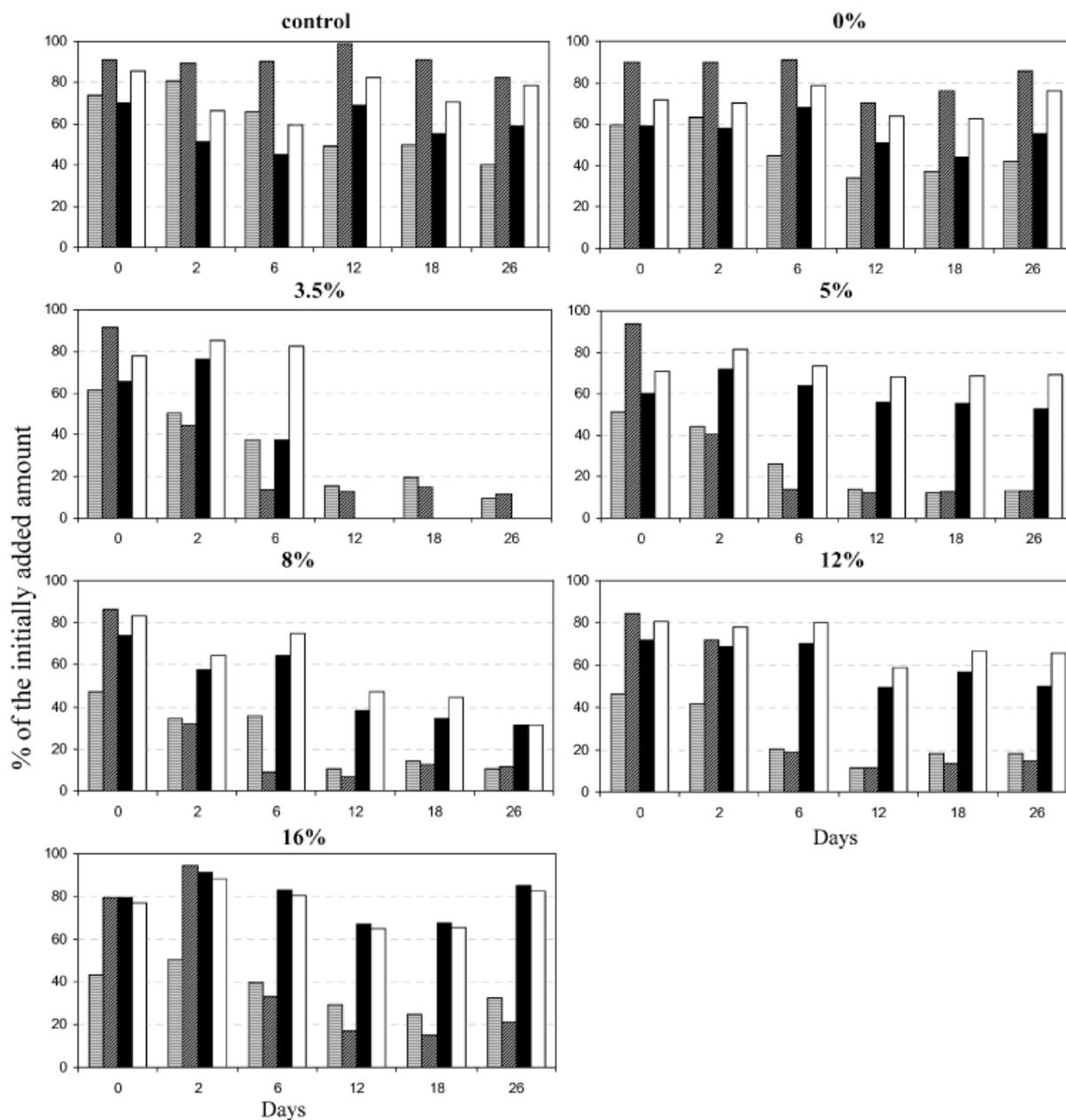


Fig. 5. Biodegradation of petroleum model compounds by microbial mats from Saudi Arabia at various salinities. The data are expressed as percentages relative to the initially added amounts (i.e. 3.33 mg of each model compound adsorbed on 100 mg of organo-clay complex particles). Dotted bars, pristane; cross-hatched bars, *n*-octadecane; black bars, phenanthrene; white bars, dibenzothiophene.

Degradation of petroleum compounds at different temperatures

The concentration of the four compounds in the controls did not change dramatically during the incubation period (Fig. 6). Pristane and *n*-octadecane showed similar degradation behaviour at different temperatures. Neither compound was degradable at 5 and 50 °C, but both exhibited a significant decrease in their amounts at temperatures be-

tween 15 and 40 °C. At 15 °C, the concentrations of *n*-octadecane and pristane decreased after incubation for 26 days to 6% and 16% of the initial amounts, respectively. After incubation for 12 days at 28 and 40 °C, *c.* 75% of pristane was degraded, whereas 88% of *n*-octadecane was degraded after just 6 days. The residues of pristane and *n*-octadecane remained undegraded even after incubation for 26 days. Phenanthrene and dibenzothiophene also exhibited a similar pattern of degradation, but one that was different

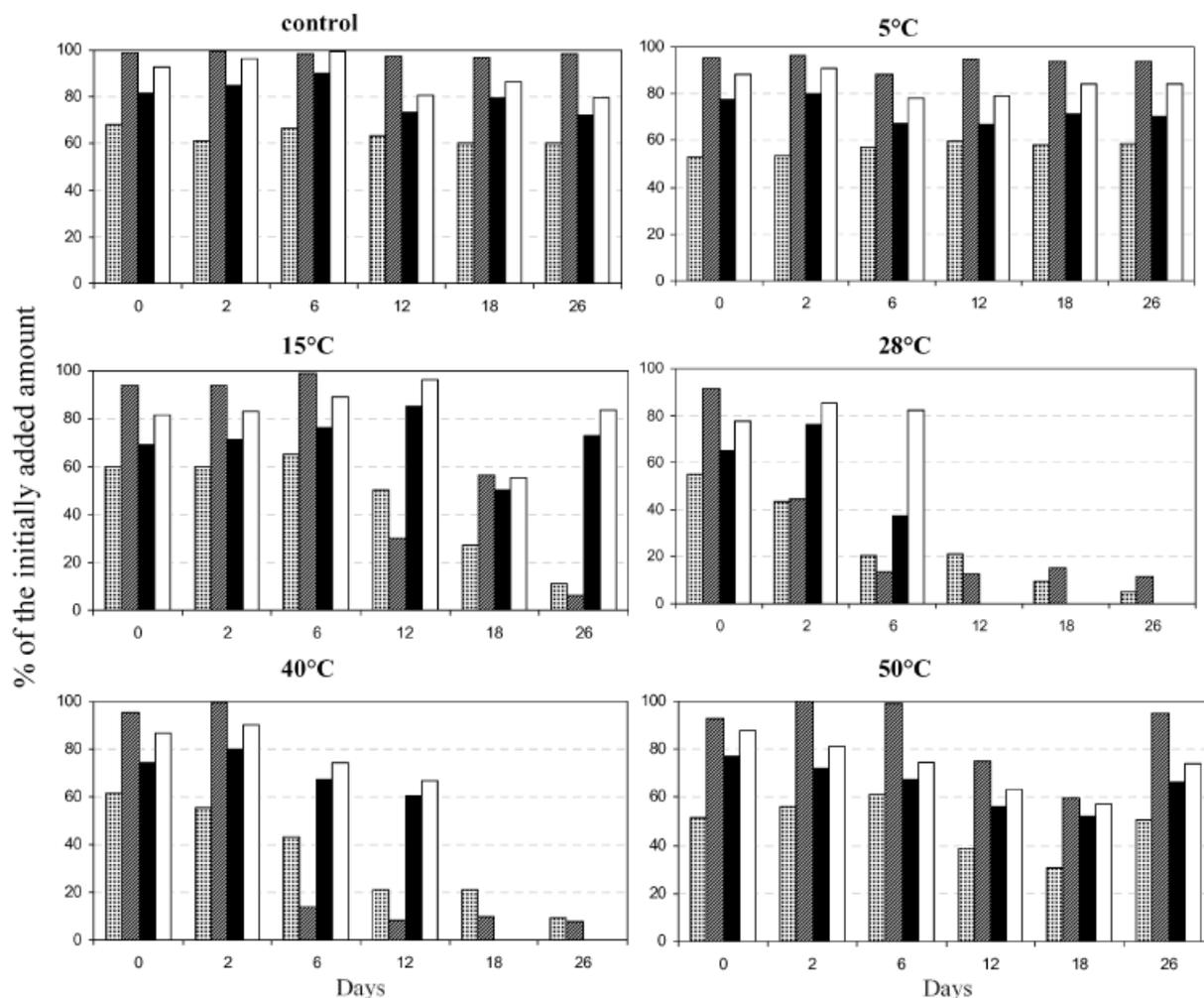


Fig. 6. Biodegradation of petroleum model compounds by microbial mats from Saudi Arabia at various temperatures. The data are expressed as percentages relative to the initially added amounts (i.e. 3.33 mg of each model compound adsorbed on 100 mg of organo-clay complex particles). Dotted bars, pristane; cross-hatched bars, n-octadecane; black bars, phenanthrene; white bars, dibenzothiophene.

from that of the aliphatics. In this case, degradation of the compounds was negligible or not at all possible at temperatures of 5, 15 and 50 °C, but in contrast they were completely degraded at 28 and 40 °C. The disappearance of the compounds from the medium was noticeable after incubation for only 12 and 18 days at 28 and 40 °C, respectively. Prior to that, a lag phase was observed during which the amounts of added compounds did not change dramatically. This lag phase was longer at 40 °C than at 28 °C. The optimum temperature for the degradation of aromatics was 28 °C.

Discussion

The gradients in environmental conditions in the studied site exert selective stresses on the microorganisms that consequently determine their diversity and distribution.

For example, the differences in the DGGE fingerprints at different tidal positions indicate an influence of the extent of wetting and desiccation on the bacterial composition of the mats. Bacteria that are more tolerant to desiccation live higher up in the intertidal zone, where they escape competition from those dominating the low intertidal zone. Studies related to the effects of environmental stress on ecosystems have concluded that microbial diversity decreases as environmental extremity increases, and that this is often accompanied by shifts in community composition (Ward *et al.*, 1998; Benlloch *et al.*, 2002; Rothrock & Garcia-Pichel, 2005). Our results are in agreement with this conclusion.

The studied microbial mats seem to be rich in novel species, because more than 15% of the obtained sequences were related to unknown bacteria. The uniqueness of these bacteria may be a result of the unusual environmental

setting. The cyanobacterium *Microcoleus chthonoplastes* found in these mats has been detected in many hypersaline environments (Prufert-Bebout & Garcia-Pichel, 1994; Garcia-Pichel *et al.*, 1996) and has been shown to grow in cultures at salinities of up to 12% (Karsten, 1996). The detection of *Deinococcus*-related sequences indicates that these mats contain bacterial populations that are resistant to UV and solar radiation (Rainey *et al.*, 1997), which is unusually high in the region. Most of the acquired sequences from *Gamma*- and *Deltaproteobacteria* were related to sulphur-oxidizing and sulphate-reducing bacteria, respectively, indicating an active sulphur cycle in these mats. The detection of phototrophic purple sulphur bacteria further supports this assumption. Oil pollution is well known to stimulate the sulphur cycle significantly (Lovely, 1997; Kleikemper *et al.*, 2002), because strains of sulphate-reducing bacteria are able to degrade petroleum compounds (Widdel & Rabus, 2001). The detection of sequences related to oil-degrading sulphate-reducing bacteria in our mat (e.g. DGGE band S10) suggests a possible role of these microorganisms in anaerobic oil degradation. Although these mats showed a strong potential in degrading hydrocarbons under aerobic conditions, few sequences related to known oil-degrading aerobic bacteria, such as *Pseudomonas* and *Alcanivorax*, were detected. Therefore, it is presumed that degradation might be performed by, in addition to these bacteria, other yet-uncultured novel bacterial populations. The identification of these novel bacteria is underway.

Effect of salinity on oil biodegradation

The studied mat was able to degrade the given compounds efficiently in hypersaline conditions, and the degradation patterns suggest its richness in halophilic oil-degrading bacteria. The inability to degrade these compounds in fresh-water conditions supports the notion that these bacteria are strictly marine. Degradation of the aromatic compounds, unlike the alkanes, was restricted to a narrow range of salinity, mainly because of their toxicity and limited solubility. Degradation of the aromatics at 3.5% and 8% and not at 5% salinity suggests the presence of two distinct aromatic-degrading bacterial populations with growth optima at these salinities. Degradation of alkanes was partial in all cases, probably as a result of the limited accessibility of the compounds by bacteria, a phenomenon that has been observed in previous experiments (Abed *et al.*, 2002b). It is probable that these compounds were trapped by strong binding in the clay mineral interlayers, decreasing bioavailability (Abed *et al.*, 2002b). In spite of these limitations, montmorillonite clay was found to serve as a good carrier system for low-soluble petroleum compounds (Abed *et al.*, 2002b; Grötzschel *et al.*, 2002).

Our results also suggest that rates of hydrocarbon degradation decrease significantly at very high salinities, in agreement with previous reports on other hypersaline environments (Ward & Brock, 1978; Rhykerd *et al.*, 1995). This decrease can only be attributed to salinity and not to other factors such as oxygen or nutrient limitations, because the slurries were continuously shaken and the medium contained N and P. Salinity influences biodegradation rates either by reducing bacterial activity (Walker & Calwell, 1975) or by limiting the solubility of hydrocarbons. This inhibitory effect of salinity was shown to be more pronounced for aromatic than for aliphatic compounds (Mille *et al.*, 1991).

Several halophilic bacteria have been shown to degrade hydrocarbons (Oren *et al.*, 1992; Margesin & Schinner, 2001). *Marinobacter hydrocarbonoclasticus* grew at salinities of up to 20% and degraded various aliphatic and aromatic hydrocarbons (Gauthier *et al.*, 1992). A *Marinobacter*-dominant halophilic culture isolated from an oil production facility in Oklahoma degraded benzene, toluene, ethylbenzene, and xylenes (BTEX) completely at 14% salinity within 1–2 weeks (Nicholson & Fathepure, 2004). Indeed, isolation of oil-degrading bacteria from our mats revealed a dominance of *Marinobacter* species that degraded various alkanes at salinities of up to 8% (data not shown).

Effect of temperature on oil biodegradation

Our experiments demonstrated a pronounced influence of temperature on the degradation of the studied compounds. The optimum temperature for degradation was 28 °C, which is close to the ambient temperature at the time of sampling. Degradation was possible at 40 °C but not at 50 °C nor below 15 °C, indicating that these mats are inhabited by moderately thermophilic oil-degrading bacteria. Other studies on mats from the same region revealed a dominance of thermophilic oil-utilizing bacteria (Sorkhoh *et al.*, 1993) with the ability to degrade crude oil at 40–45 °C (Al-Maghrabi *et al.*, 1999).

Several investigations into the effect of temperature on degradation rates in cold and hot environments were performed (Backman & Jansson, 2005; Coulon *et al.*, 2005; Leven & Schnurer, 2005; Polymenakou & Stephanou, 2005). Most studies concluded that degradation is preferably performed at higher temperatures, probably by stimulating the enzymes involved in the degradation process (Kohring *et al.*, 1989; Wu *et al.*, 1996). The metabolites of polycyclic aromatic hydrocarbon degradation under thermophilic and mesophilic conditions were shown to be different as a result of the influence of temperature on enzyme activity (Müller *et al.*, 1998; Annweiler *et al.*, 2000). Higher temperatures also reduce the viscosity of crude oil and thus increases its

diffusion through sediments, a process that renders oil components accessible to bacteria.

Extreme conditions and their implications for bioremediation

Extreme environmental conditions may represent a natural barrier to hydrocarbon degradation, rendering bioremediation in these environments problematic. Two main strategies have been developed to bioremediate high-salt environments: one is reducing the salinity (Rhykerd *et al.*, 1995); and the other is making the bacteria to function in the presence of high salt concentrations (Oren *et al.*, 1992). While the former approach involved irrigation of contaminated fields with fresh water or diluted seawater, the latter approach involved producing genetically engineered halophilic oil-degrading bacteria (Kapley *et al.*, 1999), bioaugmentation with artificial consortia, or stimulating the activity of indigenous bacteria (Al-Hadhrami *et al.*, 1996). The irrigation of polluted sediments in Kuwait facilitated hydrocarbon biodegradation (Radwan *et al.*, 1995; Al-Daher *et al.*, 1998; Balba *et al.*, 1998) but induced changes in the community composition. Inoculation of contaminated sites with genetically modified microorganisms or consortia isolated from another environment was in most cases unsuccessful (Radwan *et al.*, 1995; Vogel, 1996; Lee & Merlin, 1999). Our results show that hypersaline polluted sites can be rich in oil-degrading bacteria and that the addition of new bacteria is not needed. Therefore, attempts at the bioremediation of such sites should consider ways of stimulating existing bacteria to degrade oil components, rather than introducing new strains.

Temperature is also a key factor in determining the need and type of the most suitable bioremediation approach. Bacterial activity and biodegradation rates can change seasonally as a function of temperature. Ward & Brock (1976) demonstrated a clear correlation between seasonal changes in temperature and oxidation of hexadecane, with optimum degradation rates at 20–25 °C in the summer. Similarly, the mineralization of crude oil by soil samples from Louisiana salt marshes changed depending on the season (Jackson & Pardue, 1997). As a consequence, bioremediation might be needed in certain seasons and not in others. In hot environments, the volatile hydrocarbon fraction of oil evaporates quickly, leaving longer-chain aliphatic and aromatic components, which are more difficult to degrade and may persist in nature for a long time. In the Gulf region, this process resulted in the formation of a layer of tar that settled over large areas of the coast. This tar layer is resistant to biodegradation, and bioremediation in such cases is irrelevant. Therefore other strategies such as the mechanical collection of tar layers should be considered.

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References

- Abed RMM, Garcia-Pichel F & Hernández-Mariné M (2002a) Polyphasic characterization of benthic, moderately halophilic, moderately thermophilic cyanobacteria with very thin trichomes and the proposal of *Halomicronema excentricum* gen. nov., sp. nov. *Arch Microbiol* **177**: 361–370.
- Abed RMM, Safi NMD, Köster J, de Beer D, Rullkötter J & Garcia-Pichel F (2002b) Microbial diversity of a heavily polluted microbial mat and its community changes following degradation of petroleum compounds. *Appl Environ Microbiol* **68**: 1674–1683.
- Al-Daher R, Al-Awadhi N & El-Nawawy A (1998) Bioremediation of damaged desert environment using the windrow soil pile system in Kuwait. *Environ Int* **24**: 175–180.
- Al-Hadhrami MN, Lappin-Scott HM & Fisher PJ (1996) Effects of the addition of organic carbon sources on bacterial respiration and *n*-alkane biodegradation of Omani crude oil. *Mar Pollut Bull* **32**: 351–357.
- Al-Maghrabi IMA, Aqil AOB, Isla MR & Chaalal O (1999) Use of thermophilic bacteria for bioremediation of petroleum contaminants. *Energ Sources* **21**: 17–29.
- Al-Thukair AA & Al-Hinai K (1993) Preliminary damage assessment of algal mats sites located in the western gulf following the 1991 oil spill. *Mar Pollut Bull* **27**: 229–238.
- Anweiler E, Richnow HH, Antranikian G, Hebenbrock S, Garms C, Franke S, Francke W & Michaelis W (2000) Naphthalene degradation and incorporation of naphthalene-derived carbon into biomass by the thermophilic *Bacillus thermoleovorans*. *Appl Environ Microbiol* **66**: 518–523.
- Backman A & Jansson JK (2005) Degradation of 4-chlorophenol at low temperature and during extreme temperature fluctuations by *Arthrobacter chlorophenolicus* A6. *Microb Ecol* **48**: 246–253.
- Balba MT, Al-Daher R, Al-Awadhi N, Chino H & Tsuji H (1998) Bioremediation of oil-contaminated desert soil: the Kuwaiti experience. *Environ Int* **24**: 163–173.
- Benlloch S, López-López A, Casamayor EO, *et al.* (2002) Prokaryotic genetic diversity throughout the salinity gradient of a coastal solar saltern. *Environ Microbiol* **4**: 349–360.
- Cohen Y (1989) Photosynthesis in cyanobacterial mats and its relation to the sulfur cycle: a model for microbial sulfur interactions. *Microbial Mats Physiological Ecology of Benthic*

- Microbial Communities* (Cohen Y & Rosenberg E, eds), pp. 22–36. American Society for Microbiology, Washington.
- Coulon F, Pelletier E, Gourhant L & Delille D (2005) Effects of nutrient and temperature on degradation of petroleum hydrocarbons in contaminated sub-Antarctic soil. *Chemosphere* **58**: 1439–1448.
- El-Nahhal Y, Nir S, Serban C, Rabinovitch O & Rubin B (2000) Montmorillonite-phenyltrimethyl ammonium yields environmentally improved formulations of hydrophobic herbicides. *J Agric Food Chem* **48**: 4791–4801.
- Farmer JD (1992) Grazing and bioturbation in modern microbial mats. *The Proterozoic Biosphere A Multidisciplinary Study* (Schopf JW & Klein C, eds), pp. 247–251. Cambridge University Press, Cambridge.
- Fayad NM & Overton E (1995) A unique biodegradation pattern of the oil spilled during the 1991 Gulf War. *Mar Pollut Bull* **30**: 239–246.
- Foght JM & McFarlane DM (1999) Growth of extremophiles on petroleum. *Enigmatic microorganisms and life in extreme environments* (Seckbach J, ed), pp. 527–538. Kluwer Academic Publisher, Dordrecht, The Netherlands.
- Garcia-Pichel F, Prufert-Bebout L & Muyzer G (1996) Phenotypic and phylogenetic analyses show *Microcoleus chthonoplastes* to be a cosmopolitan cyanobacterium. *Appl Environ Microbiol* **62**: 3284–3291.
- Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P & Bertrand JC (1992) *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int J Syst Bacteriol* **42**: 568–576.
- Grötzschel S, Köster J, Abed RMM & de Beer D (2002) Degradation of petroleum model compounds immobilized on clay by a hypersaline microbial mat. *Biodegradation* **13**: 273–283.
- Hoffmann L (1996) Recolonization of the intertidal flats by microbial mats after the Gulf war oil spill. *A Marine Wildlife Sanctuary for the Arabian Gulf: Environmental Research and Conservation Following the 1991 Gulf War Oil Spill* (Krupp F, Abuzinada AH & Nader IA, eds), pp. 96–115. NCWCD, Riyad and Senckenberg Research Institute, Frankfurt.
- Höpner T, Yousef M, Berthe-Corti L, Felzmann H, Struck H & Al-Thukair A (1996) Cyanobacterial mats on oil-polluted sediments – start of a promising self-remediation process? *A Marine wildlife Sanctuary for the Arabian Gulf: Environmental Research and Conservation Following the 1991 Gulf War Oil Spill* (Krupp F, Abuzinada AH & Nader IA, eds), pp. 85–95. NCWCD, Riyad and Senckenberg Research Institute, Frankfurt.
- Jackson WA & Pardue JH (1997) Seasonal variability of crude oil respiration potential in salt and fresh marshes. *J Environ Qual* **26**: 1140–1146.
- Javor BJ & Castenholz RW (1984) Invertebrate grazers of microbial mat, Laguna Gurrero Negro, Mexico. *Microbial Mats: Stromatolites* (Cohen Y, Castenholz RW & Halvorson HO, eds), pp. 85–94. Alan R. Liss, Inc, New York.
- Kapley A, Purohit HJ, Chhatre S, Shanker R, Chakrabarti T & Khanna P (1999) Osmotolerance and hydrocarbon degradation by a genetically modified microbial consortium. *Bioresour Technol* **67**: 241–245.
- Karsten U (1996) Growth and organic osmolytes of geographically different isolates of *Microcoleus chthonoplastes* (Cyanobacteria) from benthic microbial mats: response to salinity change. *J Phycol* **32**: 501–506.
- Khan NY & Al-Ajmi D (1998) Post-war imperatives for the sustainable management of the Gulf ecosystem. *Environ Int* **24**: 239–248.
- Kleikemper J, Schroth MH, Sigler WV, Schmucki M, Bernasconi SM & Zeyer J (2002) Activity and diversity of sulfate-reducing bacteria in a petroleum hydrocarbon-contaminated aquifer. *Appl Environ Microbiol* **68**: 1516–1523.
- Kohring G-W, Rogers JE & Wiegel J (1989) Anaerobic biodegradation of 2,4-dichlorophenol in freshwater lake sediments at different temperatures. *Appl Environ Microbiol* **55**: 348–353.
- Lee K & Merlin FX (1999) Bioremediation of oil on shoreline environments: development of techniques and guidelines. *Pure Appl Chem* **71**: 161–171.
- Leven L & Schnurer A (2005) Effects of temperature on biological degradation of phenols, benzoates and phthalates under methanogenic conditions. *Int Biodeter Biodegrad* **55**: 153–160.
- Lovely DR (1997) Potential of anaerobic bioremediation of BTEX in petroleum-contaminated aquifers. *J Ind Microbiol Biotechnol* **18**: 75–81.
- Ludwig W, Strunk O, Klugbauer S, Klugbauer N, Weizenegger M, Neumaier J, Bachleitner M & Schleifer KH (1998) Bacterial phylogeny based on comparative sequence analysis. *Electrophoresis* **19**: 554–568.
- Margesin R & Schinner F (2001) Biodegradation and bioremediation of hydrocarbons in extreme environments. *Appl Microbiol Biotechnol* **56**: 650–663.
- Mille G, Almallah M, Bianchi M, Wambeke Fv & Bertrand JC (1991) Effect of salinity on petroleum biodegradation. *Fresenius J Anal Chem* **339**: 788–791.
- Müller R, Antranikian G, Maloney S & Sharp R (1998) Thermophilic degradation of environmental pollutants. *Biotechnology of Extremophiles: Advances in Biochemical Engineering/Biotechnology* (Antranikian G, ed), pp. 155–169. Springer-Verlag, Berlin, Heidelberg, NY.
- Muyzer G, Teske A, Wirsen CO & Jannasch HW (1995) Phylogenetic relationships of *Thiomicrospira* species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Arch Microbiol* **164**: 165–172.
- Nicholson CA & Fathepure BZ (2004) Biodegradation of benzene by halophilic and halotolerant bacteria under aerobic conditions. *Appl Environ Microbiol* **70**: 1222–1225.
- Nübel U, Garcia-Pichel F & Muyzer G (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl Environ Microbiol* **63**: 3327–3332.

- Oren A, Gurevich P, Azachi M & Henis Y (1992) Microbial degradation of pollutants at high salt concentrations. *Biodegradation* **3**: 387–398.
- Polymenakou PN & Stephanou EG (2005) Effect of temperature and additional carbon sources on phenol degradation by an indigenous soil *Pseudomonas*. *Biodegradation* **16**: 403–413.
- Prufert-Bebout L & Garcia-Pichel F (1994) Field and cultivated *Microcoleus chthonoplastes*: the search for clues to its prevalence in marine microbial mats. *Microbial Mats* (Stal LJ & Caumette P, eds), pp. 111–117. Springer-Verlag, Berlin, Heidelberg, NY.
- Radwan SS, Sorkhoh NA, Fardoun F & Al-Hasan RH (1995) Soil management enhancing hydrocarbon biodegradation in the polluted Kuwaiti desert. *Appl Microbiol Biotechnol* **44**: 265–270.
- Rainey FA, Nobre MF, Schumann P, Stackebrandt E & Costa MSD (1997) Phylogenetic diversity of the *Deinococci* as determined by 16S ribosomal DNA sequence comparison. *Int J Syst Bacteriol* **47**: 510–514.
- Rhykerd RL, Weaver RW & McInnes KJ (1995) Influence of salinity on bioremediation of oil in soil. *Environ Pollut* **90**: 127–130.
- Rothrock MJ & Garcia-Pichel F (2005) Microbial diversity of benthic mats along a tidal dessication gradient. *Environ Microbiol* **7**: 593–601.
- Sorkhoh N, Al-Hasan R & Radwan S (1992) Self-cleaning of the Gulf. *Nature* **359**: 109.
- Sorkhoh NA, Ibrahim AS, Ghannoum MA & Radwan SS (1993) High-temperature hydrocarbon degradation by *Bacillus stearothermophilus* from oil-polluted Kuwait desert. *Appl Microbiol Biotechnol* **39**: 123–126.
- Sorkhoh NA, Al-Hasan RH, Khanafer RH & Radwan SS (1995) Establishment of oil-degrading bacteria associated with cyanobacteria in oil-polluted soil. *J Appl Bacteriol* **78**: 194–199.
- Vogel TM (1996) Bioaugmentation as a soil bioremediation approach. *Curr Opin Biotechnol* **7**: 311–316.
- Walker JD & Calwell RR (1975) Factors affecting enumeration and isolation of actinomycetes from Chesapeake Bay and Southeastern Atlantic Ocean sediments. *Mar Biol* **30**: 193–201.
- Ward DM & Brock TD (1976) Environmental factors influencing the rate of hydrocarbon oxidation in temperate lakes. *Appl Environ Microbiol* **31**: 764–772.
- Ward DM & Brock TD (1978) Hydrocarbon biodegradation in hypersaline environments. *Appl Environ Microbiol* **35**: 353–359.
- Ward DM, Ferris MJ, Nold SC & Bateson MM (1998) A natural view of microbial biodiversity within Hot Spring cyanobacterial mat communities. *Microbiol Mol Biol Rev* **62**: 1353–1370.
- Widdel F & Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr Opin Biotechnol* **12**: 259–276.
- Wu Q, Bedard DL & Wiegel J (1996) Influence of incubation temperature on the microbial reductive dechlorination of 2,3,4,6-tetrachlorobiphenyl in two freshwater sediments. *Appl Environ Microbiol* **62**: 4174–4179.