Molecular diversity studies of bacterial communities of oil polluted microbial mats from the Etang de Berre (France)

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Abstract

The biodiversity of microbial mats inhabiting the oil-contaminated lagoon *Etang de Berre* was determined by molecular approaches. The fingerprint of denaturing gradient gel electrophoresis (DGGE) and automatic ribosomal intergenic spacer analysis (ARISA) of mats exposed to different pollution levels showed specific microbial communities for each site but similar diversity richness. Species composition of the mats were compared by constructing 16S rRNA libraries. Amplified rDNA restriction analysis (ARDRA) of clone libraries confirmed their similar level of diversity richness. Phylogenetic analysis of the 16S rRNA sequences showed that the classes γ and α of Proteobacteria were abundantly present in both sites whereas phylotypes related to the δ-Proteobacteria and to the uncultured WS3 group were mainly found in the site with the highest pollution. Identification of the species involved in oil degradation by combining culture-based approaches and DGGE, showed that enrichment cultures were constituted by members of the Rhodobacterales and species related to *Rhodococcus*, *Sphingomonas*, *Xanthomonas* and *Microbacterium*, all of them known for their ability to degrade hydrocarbons. Our findings suggest that oil pollution has not affected the biodiversity richness of the mats. However, the populations involved in hydrocarbon degradation represent a minor fraction of the mat communities in the *Etang de Berre*.

Introduction

The structure of microbial communities has become an increasingly important issue for scientists, especially in ecosystems polluted by human activities. Characterizing microbial diversity and identifying microorganisms playing a key role in the degradation of pollutants could be useful in defining new strategies for bioremediation.

Marine coastal ecosystems are important aquatic environments which, due to their location, are frequently affected by industrial run-off, shipping activities and oil transport accidents. In coastal lagoons and marine intertidal flats, the development of microbial mats is frequently observed. Microbial mats are stratified microbial communities dominated by photoautotrophic cyanobacteria (Van den Ende & Van Gemerden, 1994; Van Gemerden, 1994). While cyanobacteria are the key photosynthetic supporters of the microbial mats, purple sulphur bacteria, purple nonsulphur bacteria, as well as heterotrophic aerobic and anaerobic bacteria (Van Gemerden, 1994), are also present. These different phylogenetic and physiological groups form laminated ecosystems in virtue of their varied response to oxygen and sulphide gradients in the mat (Wieland *et al.*, 2003; Fourçans *et al.*, 2004).

Microbial mats are able to degrade a variety of organic compounds including oil. Colonization of oil-polluted

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regions by microbial mats was observed after the Gulf War, suggesting that these communities have a central role in oil biodegradation (Hoffman, 1996). In recent studies, special care has been taken to analyse the biodiversity of phototropic bacterial and sulphate-reducing populations present in the mats (Minz *et al.*, 1999; Nübel *et al.*, 2000; Wieland *et al.*, 2003; Martinez-Alonso *et al.*, 2005). In contrast, very few studies have examined the impact of oil pollution on the community structure of the mats or on the presence of oil-degrading microorganisms in the mats. Yet, our studies on hypersaline microbial mats have demonstrated the impact of oil contamination on the microbial populations inhabiting microbial mats (Bordenave *et al.*, 2004a, b). From these results it appears that further studies are needed to understand the role of microorganisms in hydrocarbon degradation in complex mat communities.

The ability of aerobic organisms to degrade polycyclic aromatic hydrocarbons (PAHs) is well known and has been observed in studies involving a wide variety of soil and sediments (Atlas & Cerniglia, 1995). However, only recently have a few studies been focused on the identification of aerobic heterotrophic bacteria present in the mats and their role in oil biodegradation (Abed *et al*, 2002; Jonkers & Abed, 2003; Abed & Köster, 2005). In this study, we investigated the bacterial mat communities inhabiting oil-contaminated sites using different molecular techniques: denaturing gradient gel electrophoresis (DGGE), automatic ribosomal intergenic spacer analysis (ARISA) and amplified ribosomal DNA restriction analysis (ARDRA). We report on the phylogenetic identification of the dominant bacterial groups present in the mats and in aerobic communities degrading PAHs obtained by molecular and culture-dependent methods.

Materials and methods

Environmental samples

The Etang de Berre is a coastal lagoon bordering the Mediterranean Sea in southern France (43° 29′05″N; 5°11′17″E). The lagoon is surrounded by a zone of intensive industrial activity and has been receiving oil refinery waste for the last 30 years. The selected sites B1, B2 and B3 were chosen according to their relative distance from waste input. Samples were taken as follow: B1, outside the retention barrier (50 m from pollution input); B2, near to the retention barrier (25 m); and B3, immediately adjacent to pollution input. Mat samples were collected in September 2001. Three core samples of 15 cm (i.d) were taken by hand with a glass cylinder from the top 2 cm of the thin microbial mats (10 mm thickness) developed above the sediment. Samples were conserved in glass containers at 4 °C during transportation to the laboratory (6 h). From each sample, three subcores were taken at equidistant point with sterile plastic tubes (27 mm i.d, 10 mm depth). Samples were used immediately for enrichment cultures. For hydrocarbon analysis and DNA extraction, samples were frozen at − 20 °C until further preparation.

Chemical analysis

Hydrocarbon concentrations in mats were determined by gas chromatography equipped with a mass spectrometer detector (GC/MS). For each mat, triplicates (100 mg) of composite samples from the three subcores were extracted by dissolving in methylene chloride and sonicating 10 min (Bordenave *et al.*, 2004b). Then, samples were purified by low-pressure liquid chromatography on an open silica-alumina column (saturates and aromatics were eluted with a mixture of pentane/methylene chloride 80/20). Solvent was then evaporated to 2 mL and the samples were analysed by a Hewlett-Packard model 6890N GC equipped with a Hewlett-Packard model 5973 mass selective detector (Hewlett-Packard, Palo Alto, CA). The MS was operated in the selected ion monitoring mode. The operating conditions of the GC/MS instrument have been described (Mazeas & Budzinski, 2002).

PAH enrichment cultures

Aerobic enrichment cultures with Berre mats were obtained using hydrocarbons as the sole carbon source in a mineral medium (MM). The MM contained (per litre of distilled water): KH_2PO_4, 0.25 g; NH_4Cl, 0.50; $CaCl_2 \cdot 2H_2O$, 0.05 g; NaCl, 20 g; $MgSO_4 \cdot 7H_2O$, 0.8 g; Na_2SO_4, 2 g; trace element solution, 1 mL; vitamin solution, 1 mL (Pfennig & Trüper, 1992). The trace element solution (modified from Overmann *et al.*, 1992) contained (per litre of distilled water): H_3BO_3, 300 mg; $FeSO_4 \cdot 7H_2O$, 1.1 g; $CoCl_2 \cdot 6H_2O$, 190 mg; $MnCl_2 \cdot 2H_2O$, 50 mg; $ZnCl_2$, 42 mg; $NiCl_2 \cdot 6H_2O$, 24 mg; $Na_2MoO_4 \cdot 2H_2O$, 18 mg; $CuCl_2 \cdot 2H_2O$, 2 mg. The pH was adjusted to 7.0–7.2 and the medium was sterilized (121 °C, 20 min) prior to the addition of the vitamin solution (filter sterilized) and organic substrates. Selected hydrocarbons, containing an increasing number of carbon atoms – naphthalene, phenanthrene, fluorene, fluoranthene- and a representative PAH containing sulphur – benzothiophene – were added at a final concentration of 100 mg L^{-1}. All hydrocarbons were supplied by Aldrich. They were prepared as acetone stock solutions (30–50 g L^{-1}) and small volumes (20–30 μL) were added to sterilized flasks (160 °C, 120 min). The acetone was allowed to evaporate until dryness, then 10 mL of MM were added. Inoculum was prepared by mixing 2 g of mats B1, B2 and B3 (fresh weight) in 10 mL of medium. Cells were released from the mat particles by shaking the mats vigorously then sediment residues were removed by centrifugation (2000 ***g***, 5 min). Cultures were

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inoculated with the supernatant (1 mL). All cultures were carried out in sealed 80 mL penicillin flasks and incubated at 30 °C with shaking (200 r.p.m.) in the dark. An abiotic control, prepared and inoculated as above but acidified to pH 2 (HCl 10%), was included as a reference for turbidity. After 1 month of incubation, 2 mL of cultures showing obvious growth as compared with the respective abiotic control were used to inoculate a fresh medium containing the same hydrocarbon as the sole carbon source. After four transfers, total DNA was extracted from enrichment cultures for microbial diversity analysis.

DNA Extraction

Direct DNA extractions from environmental samples were performed in triplicate using 250 mg (fresh weight) of each mat subsample and the bead beater UltraClean™ Soil DNA isolation kit (MoBio Laboratories, Carlsbad) following the manufacturer's instructions. Isolation of genomic DNA from the hydrocarbon-degrading cultures was performed by filtering (0.2 µm, Whatman) 10 mL of culture under sterile conditions. The filters were cut into small pieces, suspended in the TES buffer (20 mM Tris, 50 mM EDTA, 150 mM NaCl, pH 7.9) with the addition of SDS (20%) and boiled for 5 min. DNA was purified by phenol:chloroform:i-soamyl-alcohol (25 : 24 : 1) extraction and ethanol (70%) precipitation. Purified DNA was suspended in 50 µL TE. This procedure results in the extraction of high molecular mass DNA showing only a low degree of fragmentation.

PCR amplification and molecular analysis

DGGE

Partial 16S rRNA genes were PCR-amplified using the universal primers 341F-GC and 907RM (Schäffer & Muyzer, 2001). Ten nanograms of the total nucleic acid extracted were used for PCR amplification. The final 50 µL reaction mixture contained 12.5 µL PCR hot start *Taq* Master mix (Quiagen, Courtaboeuf, France), 0.5 µL of each primer (20 µM) and 2 µL $MgCL_2$ (2.5 mM). PCR was performed in a PTC-100 thermocycler (MJ Research) using the following protocol: 15 min at 94 °C for DNA-polymerase activation; 30 cycles at 94 °C for 60 s, 58 °C for 60 s, and 72 °C for 5 min, followed by 10 min at 72 °C and incubation at 4 °C until further processing. DGGE was performed with the D GENE System (Bio-Rad Inc., Hercules, CA) following the manufacturer's instructions. 1 mm thick 6% acrylamide/bisacrilamide gels with a 20–70% urea-formamide gradient were used. Gels were run in a 1 × -TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA; pH 8.3) for 16 h at 100 V at a constant temperature of 60 °C. Following electrophoresis, gels were stained with an ethidium bromide solution (0.5 µg mL^{-1}), visualized on a UV transilluminator table and photographed. Photographs were scanned in an Arcus II scanner (AGFA) and similarity coefficients then calculated with the ImageMaster 1D software (Amersham Biosciences, NJ). For further analysis, only bands clearly distinguishable from the background were considered. For the diversity analysis of enrichment cultures, individual DGGE bands were excised from gels and eluted in 15 µL MilliQ water for 24 h at 4 °C. One microlitre was used for reamplification (same protocol as above) and analysed by DGGE alongside the original sample. Pure reamplified bands were separated from nonincorporated primers using a GFX PCR DNA purification kit (Amersham Biosciences, NJ) and sequenced by BaseClear Labservices (Leiden, the Netherlands).

ARISA

Intergenic spacer regions (IGS) between 16S and 23S rRNA genes were amplified with primers 1390F-Tet (universal, small subunit) and 23S-B-21-R (bacteria-specific, large subunit) (Borneman & Triplett, 1997; Garcia-Martinez *et al.*, 1999). 1390F-Tet was 5′-fluorescently labelled with TET (Tetrachloro-Fluorescein). The PCR reaction mix was similar to that above but without additional $MgCl_2$. After activation at 94 °C for 15 min, 35 cycles of PCR were run at 94 °C for 60 s, 56 °C for 90 s, 72 °C for 60 s followed by 72 °C for 10 min. The concentration of PCR-amplified products was estimated spectrophotometricly at 260 nm in a Unikon 931 (Kontron, Germany). Between 1 and 3 µL of PCR product were added with deionized formamide and 1 µL sized internal standard TAMRA 2500 (Applied Biosystems) to make up a total volume of 20 µL. Mixtures were denatured at 95 °C for 5 min followed by 5 min on ice. Different IGS were separated by capillary electrophoresis using a POP-4 polymer on ABI PRISM 310 genetic analyser (Applied Biosystems Instruments, Foster City, CA). In the electrophoregram, the peak size was estimated by comparison to fragments from the internal size standard using the GeneScan 3.1 software programme (Applied Biosystems). With the primers used in this study, *c.* 189 bp of both the 16S and 23S RNA encoding genes were amplified in addition to the spacer region (shorter fragments are artefacts). Only IGS fragments longer than this value were considered for further analysis.

ARDRA

ARDRA was used to characterize 16S rRNA gene diversity within clone libraries obtained from the least (B1) and most (B3) polluted sites. PCR amplifications of total DNA extracted from mat samples were performed using the universal primer pair R1nF and U2R (Weider *et al.*, 1996) that amplify a fragment of *c.* 1060 bp of the 16S rRNA gene. For clone library construction, PCR products were ligated

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into a pCR2.1-Topo vector following the manufacturer's instructions (Invitrogen). Ligation mixtures were transformed into chemically competent TOP10 cells (TOPO TA cloning kit, Invitrogen). From each library, 80 positive clones were subjected to reamplification for ARDRA analysis using TOP1 and TOP2 primers that surrounded the cloning site (Precigou *et al.*, 2001). The reamplified PCR products were digested with *Taq*I and *Rsa*I (New England Biolabs, Beverly, UK) to generate restriction fragment profiles. Restriction analysis was performed in 15 μL total volume containing 10 μL of PCR product, 5 U of enzyme and 1.5 μL of appropriate buffer. Incubations were done overnight at 65 and 37 °C, respectively, for *Taq*I and *Rsa*I. Restriction patterns of PCR products were separated by electrophoresis on 2% Methaphor gel (FMC BioProducts, ME) in 1% TAE and compared by running a molecular marker 100 bp DNA Ladder (New England Biolabs, Beverly, UK). For each different restriction pattern, one representative PCR product was purified and sequenced by ESGS-QBiogene (Evry, France). Rarefaction and coverage analysis were applied to the clone libraries. Rarefaction curves were produced using the analytical algorithm of Hulbert (1971) and 95% confidence intervals were estimated as described by Heck *et al.* (1975) using the software Analytic Rarefaction [version 1.3, S.M Holland (www.uga.edu/~strata/software)]. Libraries coverage (C) were calculated by the equation $C = 1 - (n/N) \times 100$, where n is the number of unique clones and N is the total number of clones analysed (Good, 1953). The frequency distribution of the observed ARDRA types in clone libraries was determined and used to calculate the Shannon–Weaver index $\{H = -\Sigma[n_i \log (n_i)]$, where n_i is the relative contribution of clone type i to the whole library} (Shannon & Weaver, 1949). Cloned 16S rRNA gene sequences were first compared with their closest relatives available in databases, using BLAST from the National Center for Biotechnology Information. Then all sequences and their closest relatives were fitted into an alignment of about 12 000 full and partial 16S rRNA gene sequences using the automated tools of the ARB software package (http://www.arb-home.de) (Ludwig *et al.*, 2004). Phylogenetic trees containing only total 16S rRNA gene sequences were constructed using different methods: parsimony, maximum likelihood and neighbour-joining with the Jukes and Cantor correction (Jukes & Cantor, 1969; Saitou & Nei, 1987). All trees gave similar sequence identification. Finally, partial 16S rRNA gene sequences were incorporated into the final tree according to the maximum parsimony criteria without allowing changes to the existing tree topology, using a special tool of the ARB software. The 16S rRNA partial gene sequences obtained in this study have been deposited in the sequence database at the European Molecular Biology Laboratory (EMBL) under accession numbers AM229449–AM229534.

Results

Hydrocarbon composition in mats

The composition of hydrocarbons extracted from microbial mats and resolved by GC/MS is shown in Fig. 1. The aliphatic and aromatic hydrocarbons detected, grouped in function of their carbon atoms content, were: (C10) naphthalene; (C12) dibenzothiophene; (C13) tridecane, methyl-dibenzothiophene; (C14) tetradecane, phenanthrene, ethyl-dibenzothiophene, dimethyl-dibenzothiophene; (C15) pentadecane, trimethyl-dibenzothiophene, methyl-phenanthrene, ethyl-phenanthrene; (C16) hexadecane, dimethyl-phenanthrene, pyrene, fluoranthene; (C17) heptadecane, trimethyl-phenanthrene; (C18) octadecane, pristane, chrysene, benzo[*a*]anthracene; (C19) nonadecane; (C20) eicosane, benzo[*a*]pyrene, benzo[*b,k,j*]-fluoranthene, perylene, benzoperylene; and (C22) docosane, indeno[1,2,3-*cd*]pyrene, benzo[*ghi*]perylene. Hydrocarbon concentrations in mats vary in function of the distance to the oil pollution input; the site B3 showing the highest hydrocarbon concentration and site B1 the lowest. The major compounds detected were hydrocarbons containing 15–17 carbon atoms. All the compounds detected were found in the different studied sites reflecting the chronic pollution of the Berre lagoon.

Comparison of microbial mat community fingerprinting

DGGE

Analysis of microbial mat communities by DGGE exhibited high diversity demonstrated by the presence of many distinguishable bands and a high number of unresolved bands in the separation profiles (Fig. 2a). Nevertheless, each mat had a

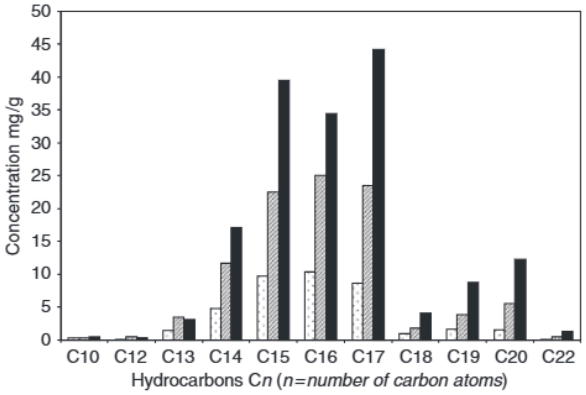

Fig. 1. Hydrocarbon concentration in Berre microbial mats at the points sampled: B1 (dotted column), outside of the retention barrier; B2 (hatched column), near to the retention barrier; B3 (black column), immediately adjacent to the oil pollution input.

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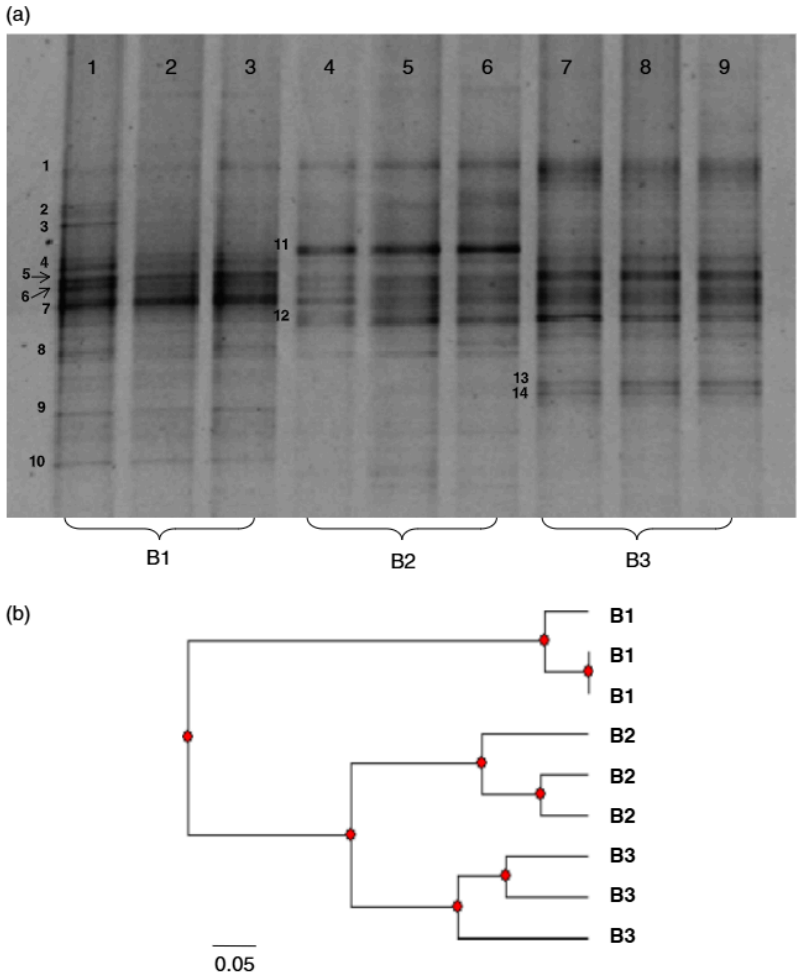

Fig. 2. DGGE community analysis of contaminated mats. (a) Stained gel of PCR-amplified 16S rRNA encoding genes from three subsamples of Berre microbial mats exposed to increasing oil pollution levels. Subsamples from mats exposed to low (B1 site, lanes 1–3) medium (B2 site, lanes 4–6) and high (B3 site, lanes 7–9) oil pollution levels. (b) Similarity analysis between the mat samples; the dendogram is based on the Jaccard's coefficient.

specific bacterial community fingerprint. The total number of clearly discernible bands was as follow: site B1 (low pollution level), 10 bands; site B2 (medium pollution level), eight bands; site B3 (high pollution level), nine bands. Triplicate extracts from the same mat showed almost identical DGGE profiles, thus demonstrating the reproducibility of the method. Profile comparison by the construction of similarity dendrograms (Fig. 2b) revealed a higher degree of similarity between replicates than between samples from mats exposed to different pollution levels. However, the comparison made it possible to group the major DGGE bands into three distinct ensembles. The first group was constituted by bands found in all sites (bands 1, 4, 6 and 7; Fig. 2a), whatever the contamination level. The second group was formed by bands 2, 5, 8 and 12 (Fig. 2a) that were detected in mats from different locations. The third group included bands specific to each site: bands 3, 9 and 10 were found in mats exposed to a low pollution level; band 11 in mats of medium pollution level; and bands 13 and 14 in highly polluted mats.

ARISA

The lengths of amplified spacer regions between 16S and 23S rRNA genes were resolved by capillary electrophoresis. Fingerprinting (electrophoregrams) of bacterial communities results in a series of peaks corresponding to different IGS sizes in which fluorescence intensity is correlated to their specific abundance. A very high degree of reproducibility of ARISA profiles was observed by running three different PCR products from each site (data not shown). Representative profiles, presented in Fig. 3, show IGS regions with lengths ranging from 290 to 750 bp. These values are typical for IGS lengths found in the GenBank database that range between 143 and 1529 bp (Fisher &

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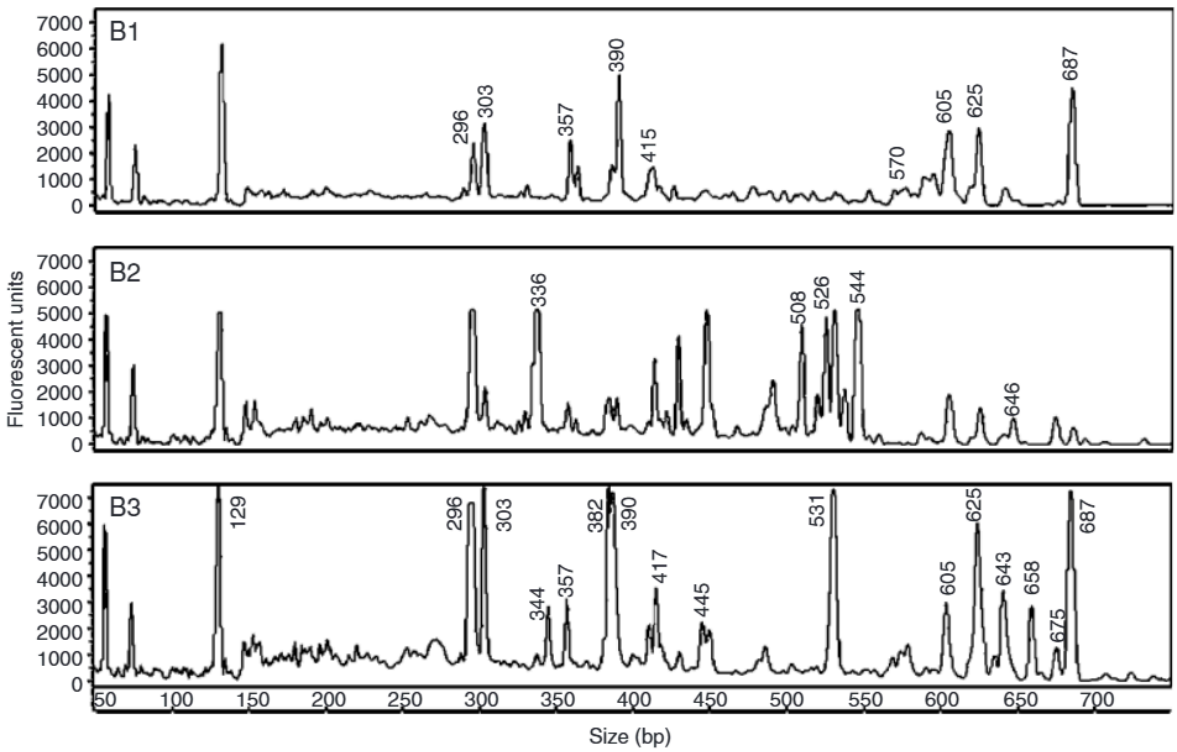

Fig. 3. ARISA profiles of the bacterial communities from Berre microbial mats exposed to (B1) low, (B2) medium and (B3) high oil pollution levels. Each panel presents a representative electropherogram that was performed on three subsamples for each site.

Triplett, 1999). The diversity of these communities was determined by the number of peaks observed on each site, with a threshold of 500 fluorescence units in peak height from the baseline. Using this criterion, the number of peaks per site is as follows: B1, 21; B2, 27; and B3, 26. This reflects high IGS diversity on all sites. When comparing the number of the major peaks (defined as peaks representing at least 5% of the total profile height) to the number of major bands on the DGGE profiles, the same order of magnitude is evident, with seven, eight and seven dominant IGS fragments on sites B1, B2 and B3, respectively. Bacterial diversity for the three sites was similar, whether all the fragments detected (threshold of 500 fluorescence units in peak height) or only the main fragments were considered. However, mat communities from sites receiving different levels of pollution showed specific ARISA profiles which differed from site to site. IGS fingerprint analysis showed that most of the main peaks were shared by all sites (peaks 296, 303, 390, 605, 625 and 687 bp) but changed in their relative fluorescence intensity.

Community analysis

In order to identify the populations involved in oil degradation, we had first hoped to determine the phylogeny of bands excised from DGGE gels. However, the presence of a high number of thin bands prevented any separation of a single band for use in cloning and made the analysis more difficult. Similarly, the separation of the amplified IGS by Methaphor-TAE gel electrophoresis was unsuccessful.

As an alternative approach, we then constructed clone libraries by amplifying 16S rRNA genes. The sites offering the greatest contrast, those with the lowest (B1) and highest (B3) pollution, were chosen for this analysis. 80 cloned 16S rRNA genes from each site were analysed by restriction endonuclease digestion. Using *Taq*I and *Rsa*I, a total of 65 ARDRA profiles were found in sites B1 and B3. For each site, 38 different restriction profiles were detected. Rarefaction analysis applied to ARDRA profiles from both libraries showed very similar curves patterns (Fig. 4) without clear saturation. These curves indicate that more diversity could be revealed by increasing the number of clones analysed; however, both mats communities have been sampled with similar intensity relative to their overall richness. The calculated clone libraries coverage were of 72.5 and 68.75 for B1 and B3 libraries, respectively, indicating that about three quarters of the total diversity was covered for both mats. The analysis of the relative clone abundance distribution (data not shown) revealed that a large proportion of them (62%) were observed only once in the libraries studied and that sites B1 and B3 shared only 11 restriction profiles (17%). These results

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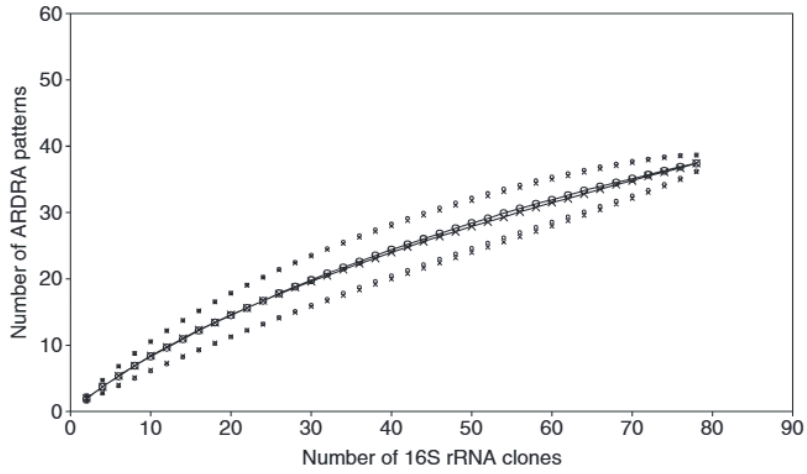

Fig. 4. Rarefaction curves for the different ARDRA patterns of 16S rRNA gene clones libraries of Berre mats B1 and B3, exposed to low (×) and high (○) oil pollution levels, respectively. The small symbols represent the respective limits of 95% confidence intervals for each site.

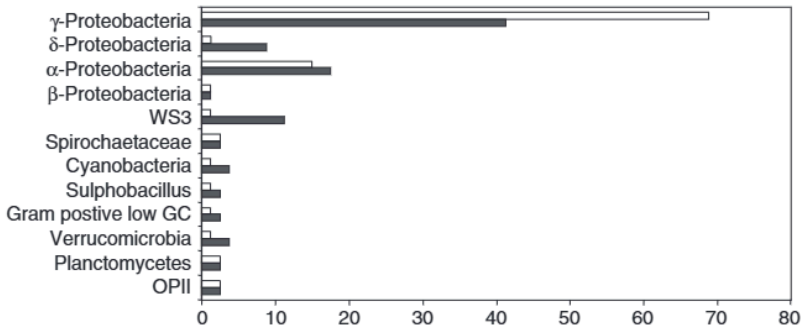

Fig. 5. Percentage distribution of sequences related to different phylogenetic groups found in the clone libraries from Berre microbial mats exposed to low (□) and high (■) oil pollution levels.

confirm the high microbial diversity and the distinct community composition of the mats studied.

To affiliate each restriction profile to a specific phylogenetic group, one representative clone of each ARDRA profile was sequenced. The distribution of the 65 clones sequenced across 16S rRNA gene phylogenetic groups is shown in Fig. 5. The major taxon represented was the class of Proteobacteria. Most of the sequenced clones were related to the *Gammaproteobacteria* with a relative abundance of 69% and 41% on sites B1 and B3, respectively. Similar proportions of *Alphaproteobacteria* relatives were found in both sites, representing 15% on B1 and 18% on B3. The groups of *Deltaproteobacteria* and WS3 were found mainly on site B3 (9% and 11%, respectively), rarely on site B1 (1% for both groups). Other groups were detected at a lower frequency ($< 4\%$). The dominant *Gammaproteobacteria* phylotypes detected in mats (Fig. 6) were closely related to the genera *Oceanospirillum* and *Idiomarina* which represented, respectively, 21% and 4% on site B1 and 5% and 21% on site B3. Clone sequences related to *Marinobacter*, *Thioalkalivibrio* and *Halomonas* were also abundantly present on site B1 (10%, 11% and 11%, respectively) but in a lower proportion on site B3 (5%, 4% and 1%, respectively). Sequences related to unclassified sulphur-oxidizing *Gammaproteobacteria* were present in both sites in similar proportions. In the α-subclass of the Proteobacteria, the most abundant clones in both polluted sites were affiliated to the *Rhodobactereaceae* group. Sequences related to *Hyphomonadaceae* and *Sphingomonadales* were found mainly on sites B3 (6%) and B1 (5%), respectively. Clones related to *Deltaproteobacteria*, found on site B3, clustered with *Bdellovibrionales* (4%), *Desulfobacterales* (3%) and *Desulfuromonadales* (3%). Only a minor proportion of sequences related to noncultured *Deltaproteobacteria* were detected on the B1 site. 1% and 9% of sequences that clustered with noncultured bacteria, in the division WS3, were observed on sites B1 and B3, respectively. To estimate the community diversity of each ecosystem we calculated the Shannon–Weaver index for the clone libraries studied. The sites with the lowest and highest pollution display a Shannon–Weaver index of 0.97 and 1.07, respectively; these values reflect similar community diversity in both sites.

Bacterial populations in PAH enrichment cultures

Microbial mat populations involved in hydrocarbon degradation were further studied by combining a culture-

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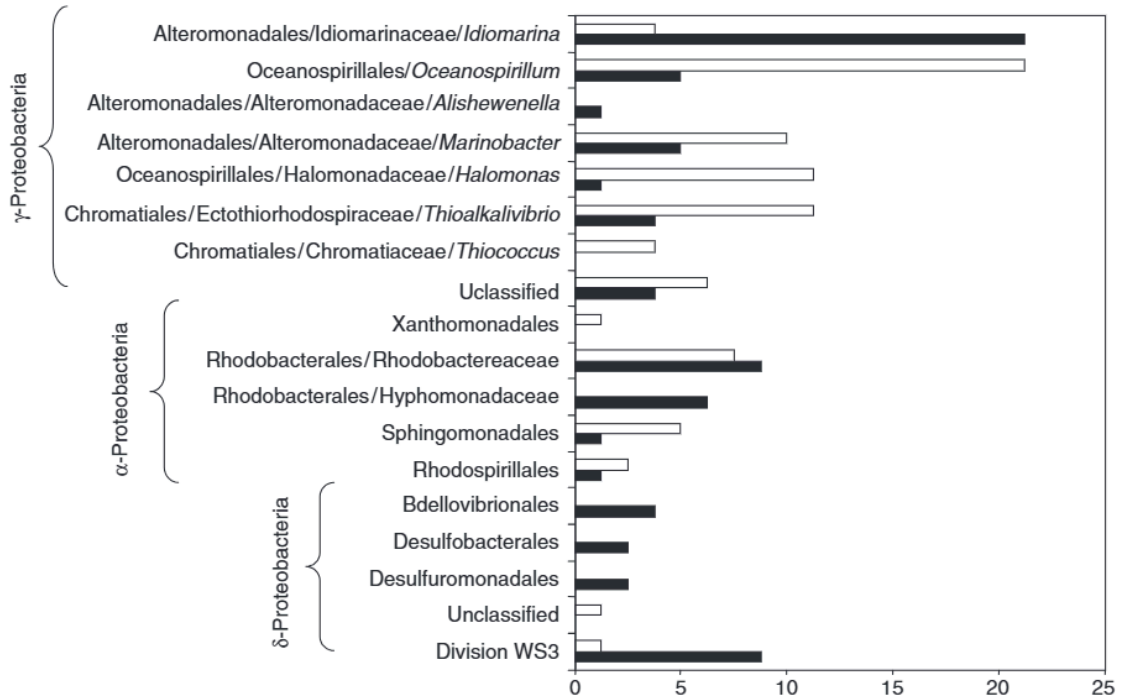

Fig. 6. Percentage distribution of the major phylotypes found in Berre microbial mats exposed to low (□) and high (■) oil pollution levels.

dependent method and DGGE. DGGE profiles (Fig. 7) show a total of 20 clearly distinct bands in the PAH enrichment cultures. Diversity richness, as estimated from the number of DGGE bands, was low, with only three to five clear bands observed for each substrate. Bands were excised from gel, reamplified and sequenced, except bands 1, 6 and 12 for which no sequences could be obtained. Most of the bacteria growing in the enrichment cultures (10 bands) belonged to the *Alphaproteobacteria* (Fig. 8). The other bands were related to different phylogenetic groups (% of similarity is indicated in brackets). Bands 5 and 10 were affiliated with *Rhodococcus* (99%) and *Microbacterium* (99%), respectively; both belonging to the *Actinobacteria* group. Band 15 was related to the *Xanthomonas* genera (97%) from the *Gammaproteobacteria* group. Marked differences in DGGE profiles were found, depending on the culture substrate used. Analysis of DGGE fingerprints and the phylogenetic affiliation of bands revealed that some microbial groups grew on several PAHs while others were specific to a single substrate. Comigrating bands 7, 14 and 17, observed in FH, NH and PHE cultures, respectively, clustered together and were closely related to genera *Sphingomonas* (99%). Bands 9 and 19, detected in FH and PHE cultures, respectively, formed a cluster with *Paracoccus* (99%). Band 10, related to *Microbacterium*, was sequenced from the FH profile; comigrating bands were detected, with lower intensity, on BT, NH and PHE-DGGE profiles. A similar pattern was observed for band 15, related to *Xanthomonas* (97%), which was sequenced from the NH profile but a comigrating band was also found in the PHE enrichment culture.

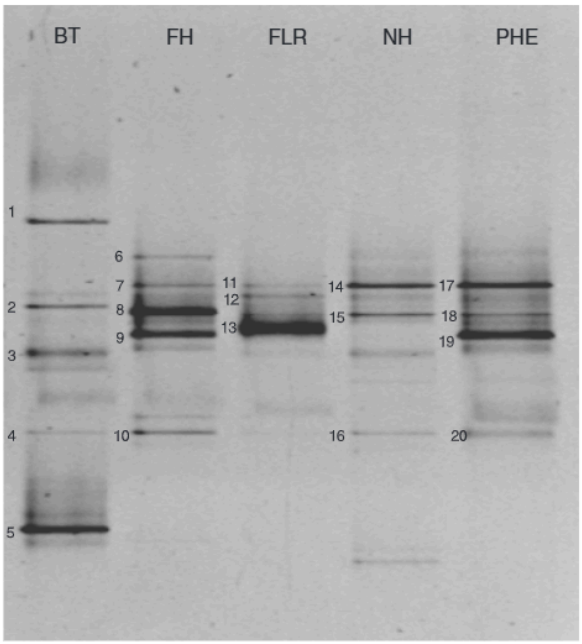

Fig. 7. DGGE analysis of mat enrichment cultures containing different PAHs as the sole carbon source: benzothiophene (BT), fluoranthene (FH), fluorene (FLR), naphthalene (NH) and phenanthrene (PHE).

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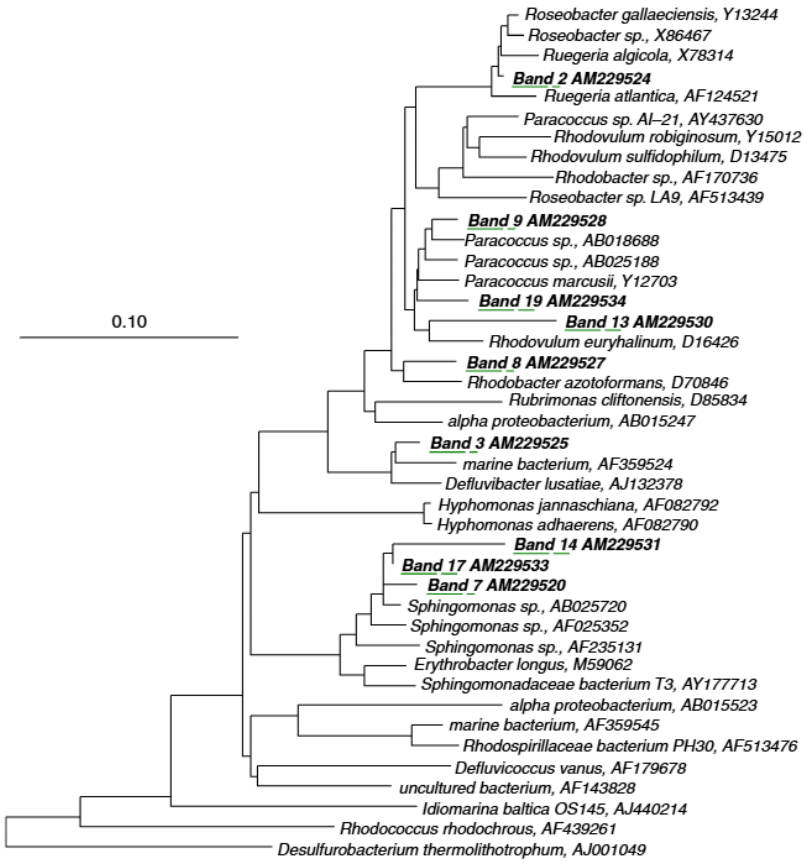

Fig. 8. Phylogenetic tree showing the affiliation of the 16S rRNA encoding gene sequences retrieved from DGGE bands from microbial mat cultures with reference sequences obtained from ARB (accession numbers are indicated). DGGE bands have the prefix 'Band' and the same number as indicated in Fig. 6. The sequence of *Methanobacterium bryantii* AF028688 was used as outgroup to root the tree. The scale represents 10% estimated sequence divergence.

Discussion

Since the introduction of molecular techniques in the analysis of biodiversity, a wide variety of methods have proved very useful for comparing microbial ecosystems. In this study, DGGE, ARISA and ARDRA methods have shown that Berre microbial mats exposed to increasing levels of oil pollution display similar bacterial diversity but a different community composition.

16S rRNA encoding gene PCR-DGGE profiles (Fig. 2) showed between eight and 10 major bands and a high number of unresolved bands. With this technique, each band is assumed to correspond to a single bacterial species inhabiting the microbial communities. In IGS-PCR-ARISA fingerprints (Fig. 3) a higher number of major peaks were observed, with a high level of very small peaks, suggesting a higher diversity richness in ARISA than in DGGE profiles. This apparent difference with DGGE data could be attributed to the ARISA method's higher sensitivity, due to the laser detection of fluorescently-labelled DNA. Nevertheless, the number of major peaks observed in ARISA elecrophoregrams was around seven to eight, which is quite similar to the number of bands detected by the DGGE method. In fact, both methods revealed similar diversity richness in the microbial communities exposed to different pollution levels. Both fingerprint methods showed that some of the 16S rRNA encoding gene bands or IGS peaks were shared by the three mats studied while others were found only in a single mat. However, the relative abundance of the shared phylotypes was different in each site. These observations suggest a difference in bacterial community structure in mats inhabiting the same environment (Berre lagoon) but exposed to different oil contamination levels. A very similar picture was derived from ARDRA analysis of the 16S rRNA gene clone libraries. Sequencing of clones and phylogenetic analysis resulted in the identification of 21 and 23 phylotypes in the sites of lowest and highest oil pollution, respectively. These phylotypes were distributed differently on each site (Figs 5 and 6). These results allowed us to conclude that, independently of the method used to estimate the species

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biodiversity of mats exposed to increasing oil pollution levels, no changes were observed in the diversity richness in Etang de Berre mats. Nevertheless, the community structure was impacted by oil. These observations are consistent with previous results reported by Kasai *et al.* (2001) and Röling *et al.* (2002) when they analysed the bacterial community make-up of oil-polluted sand in field and laboratory experiments. Their studies revealed that oil pollution had impacted the community structure but not the diversity richness.

As an alternative method for characterizing communities developing in oil-polluted environments, we combined a culture-dependent method and DGGE. The diversity richness, as determined by the number of DGGE bands obtained from PAH enrichment cultures (Fig. 7), was substantially lower compared to that observed on mat samples (Figs 2, 3 and 5), showing a decrease in biodiversity during enrichment. Many authors have concluded that organisms living in natural environments are not adapted to growth under defined laboratory conditions. This behaviour could be attributed to the lack of growth promoters, the level of organic carbon sources, or the general ignorance of the growth requirements for most environmental bacteria [Kaeberlein *et al.* (2002) and references cited therein].

In order to identify the microbial mat populations involved in oil degradation, we performed phylogenetic analysis of the clone libraries from microbial mats and of the excised bands from PAH-enrichment-DGGE. The study of the clone libraries of the mats exposed to the lowest and highest levels of pollution showed that the dominant group of bacteria in both mats was represented by members of the γ subclass of the Proteobacteria (Fig. 5). The presence of *Gammaproteobacteria* has been reported in studies carried out on beaches contaminated after the Nakhodka spill accident in the Sea of Japan (Kasai *et al.*, 2001), on beach sediment microcosms contaminated with oil (Röling *et al.*, 2002) and on microbial mats exposed to high pollution levels (Abed *et al.*, 2002). Moreover, different organisms related to *Marinobacter*, *Halomonas* or *Alcanivorax*, all belonging to the *Gammaproteobacteria* group and known as a hydrocarbon-degrading bacteria, are frequently found in marine environments contaminated with oil (Yakimov *et al.*, 1998; Kasai *et al.*, 2001; Gerdes *et al.*, 2005). These observations are consistent with our results. However, in other studies a decline of *Gammaproteobacteria* has been observed. Macnaughton *et al.* (1999), in experiments conducted with sand artificially contaminated with oil, reported a disappearance of *Gammaproteobacteria* and a dominance of *Alphaproteobacteria*. Nevertheless, the authors remarked that the *Gammaproteobacteria* group was found at week 8 after the contamination by oil and the disappearance of the subclass of *Gammaproteobacteria* occurred also in the control which was not exposed to oil pollution. A similar result was observed by comparing the microbial communities growing just beyond the periphery of marine hydrocarbon seep sediment with those found at the edge (LaMontagne *et al.*, 2004). A decline of *Gammaproteobacteria* was found at the edge which was exposed to higher, and probably toxic, concentrations of hydrocarbons. The main genera in the γ subclass of the Proteobacteria detected in the Etang de Berre mats were affiliated to *Idiomarina*, *Oceanospirillum*, *Halomonas*, *Marinobacter* and *Thioalkalivibrio* (Fig 6). These genera have frequently been found in marine environments and in microbial mats (Kasai *et al.*, 2001; Jonkers & Abed, 2003; Gerdes *et al.*, 2005). Members of *Idiomarina*, that were dominant in the highly polluted site (21%) compared to the least polluted site (4%), have been detected in oil bioremediation experiments (Röling *et al.*, 2004) but their direct role in hydrocarbon degradation is still to be determined. Surprisingly, representatives of aerobic heterotrophic bacteria able to degrade hydrocarbons, such as *Marinobacter* and *Halomonas* (Gauthier *et al.*, 1992; Melcher *et al.*, 2002), were scarcely found on the highly polluted site.

The second most abundant group in the Etang de Berre microbial mats was the *Alphaproteobacteria*. In this subclass, a group of sequences related to *Rhodobacterales* (Fig. 6) was found more abundantly in the mats exposed to the high pollution level (15%) compared to mats at the least polluted level (8%). This phylogenetic group, and especially the species related to *Roseobacter*, has been detected frequently in seawater hydrocarbon degradation experiments, especially during the early stage of degradation (Chang *et al.*, 2000; Brakstad & Lodeng, 2005). In our study, however, the sequences related to *Roseobacter (Rodobactereaceae*) represent only a minor fraction of the mat communities (8% and 9%). Possibly, the dominance of the γ or other subclass of Proteobacteria in the different studies could be explained by the availability of inorganic nutrients. For example, Röling *et al.* (2002) reported a dominance of *Alphaproteobacteria* when they supplemented the oil-polluted sand microcosms with nitrogen but not in the microcosms receiving only oil. In our experiment, nitrogen availability in the mats was not determined. The dominance of *Gammaproteobacteria* observed in this study may result from the complexity of microbial mat communities and their chronic exposition to oil compared to the one-off pollution of sand populations cited above. Moreover, the presence of high levels of carbon from oil pollution may limit the development of hydrocarbon-degrading communities because of a limited supply of inorganic nutrients (nitrogen, phosphor) or lower oxygen transfer (Atlas & Cerniglia, 1995).

This last hypothesis seems more probable thanks to the identification of microorganisms mainly found in the highly polluted site. Such microorganisms were related to organisms from anaerobic environments: sulphur-reducers from

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the *Deltaproteobacteria* group, methanogenic and anoxic organisms from the WS3 division (Godon *et al.*, 1997; Derakshani *et al.*, 2001). Possibly the presence of high oil levels affected the oxygen availability which resulted in an increased growth of anaerobic organisms at the most polluted site.

Using this cloning and sequencing approach, the community structures of different mats exposed to low and high pollution levels were determined. However, it was not possible to establish a functional relationship between the phylogenetic groups detected and the oil pollution degradation activity. Such a relationship was made clear by combining enrichment cultures and DGGE analysis which together made possible the identification of microorganisms able to grow on PAHs under fixed conditions. The diversity of the PAH-enrichment cultures revealed a higher number of sequences related to hydrocarbon-degrading species. All PAH enrichment cultures presented at least one DGGE band related to *Roseobacter* and others *Rhodobacterales*, phylotypes that have been associated to PAH and crude oil degradation in marine environments (Melcher *et al.*, 2002; Buchan *et al.*, 2005). Moreover, independently of the PAH used as the carbon source, phylogenetic characterization of main DGGE bands indicated that enrichment cultures were constituted by organisms related to *Sphingomonas* (FH, FLR, NH and PHE), *Microbacterium* (BT, FH, NH and PHE), *Rhodococcus* (BT) or *Xanthomonas* (NH, PHE), all genera containing members able to degrade hydrocarbons (Bastiaens *et al.*, 2000; Sharma & Pant, 2000; Widada *et al.*, 2002; Schippers *et al.*, 2005). Some of these genera were also found in the field samples but they represent a minor fraction in the natural microbial mat communities. Nevertheless, this last approach gives functional information on the microorganisms useful in implementing *in situ* bioremediation strategies.

In both culture-independent and culture-based methods, there remain numerous potential limitations. Fingerprint methods are subject to different bias which have been reviewed elsewhere (Fisher & Triplett, 1999; Muyzer, 1999). Culture-dependent methods, by modifying the environmental conditions, permit the growth of organisms adapted to selected culture conditions but, generally, these organisms are not abundant in the field. However, the combination of both a broader spectrum of culture-dependent and culture-independent methods, could be very useful in improving the study of complex ecosystems and in identifying organisms involved in PAH degradation as well as in others specific functions.

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