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RESEARCH ARTICLE

Microbial community structure of a heavy fuel oil-degrading marine consortium: linking microbial dynamics with polycyclic aromatic hydrocarbon utilization

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Abstract

A marine microbial consortium obtained from a beach contaminated by the *Prestige* oil spill proved highly efficient in removing the different hydrocarbon families present in this heavy fuel oil. Seawater cultures showed a complete removal of all the linear and branched alkanes, an extensive attack on three to five-ring polycyclic aromatic hydrocarbons [PAHs; including anthracene, fluoranthene, pyrene, benzo(*a*)anthracene, chrysene, and benzo(*a*)pyrene] (30–100%), and a considerable depletion of their alkyl derivatives. Community dynamics analysis revealed that *Alcanivorax* species, known alkane degraders, predominated in the initial stages. This was followed by an increase in *Alphaproteobacteria* (i.e. *Maricaulis*, *Roseovarius*), which coincided with the depletion of low molecular PAHs. Finally, these were succeeded by *Gammaproteobacteria* (mainly *Marinobacter* and *Methylophaga*), which were involved in the degradation of the high molecular-weight PAHs. The role of these populations in the removal of the specific components was confirmed by the analysis of subcultures established using the aliphatic or the aromatic fraction of the fuel oil, or single PAHs, as carbon sources. The genus *Marinobacter* seemed to play a major role in the degradation of a variety of hydrocarbons, as several members of this group were isolated from the different enrichment cultures and grew on plates with hexadecane or single PAHs as sole carbon sources.

Introduction

Petroleum is a widespread organic pollutant in marine environments. It is estimated that each year about 0.1% of the total oil production ends up in marine systems (Albaigés, 1989). Although accidental spills account for only a small percentage of the oil released into the marine environment, large oil spills can cause extensive ecological damage to marine shorelines and have an enormous impact on local economic activities due to the associated risk to public health. A recent example is the heavy fuel oil spill from the tanker *Prestige* in 2002, which affected 1900 km of coast in the northwest of Spain. Physical and, in some cases, chemical methods are capable of rapidly removing the majority of beached oil, but they are rarely completely successful (Prince, 1997). Natural degradative processes aid in removing the remaining oil and their stimulation through nutrient and fertilizer addition has proven to enhance oil removal in a variety of coastal environments (Bragg *et al*., 1994; Venosa *et al*., 1996; Swannell *et al*., 1999).

Bacteria are considered to be the predominant agents in environmental hydrocarbon degradation (Leahy & Colwell, 1990), and hydrocarbon-degrading bacteria are ubiquitous. Marine hydrocarbon-degrading bacterial isolates belonging to more than 20 genera distributed across several groups (*Alpha*-, *Beta*- and *Gammaproteobacteria*; Gram positives; *Flexibacter–Cytophaga–Bacteroides*) have been described (Gauthier *et al*., 1992; Floodgate, 1995; Yakimov *et al*., 1998, 2007; Bruns & Berthe-Corti, 1999; Head & Swannell, 1999; Hedlund *et al*., 1999, 2001; Kodama *et al*., 2008). On the other hand, culture-independent approaches have been used to analyze changes in the structure of microbial communities from marine environments contaminated by oil spills, or from microcosms that mimic such

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environments. These 16S rRNA gene-based studies demonstrate that, after the addition of oil, diversity within the microbial community may be substantially reduced, owing to strong selection for a limited number of hydrocarbon-degrading species (Röling *et al.*, 2002; McKew *et al.*, 2007), mainly belonging to the *Gammaproteobacteria* class (Head *et al.*, 2006). Within this group, *Alcanivorax* species are regarded as the main players in the removal of aliphatic hydrocarbons from the marine environment. These species seem to have a global distribution, as *Alcanivorax*-related 16S rRNA gene sequences have been retrieved from microbial communities of different origins (Yakimov *et al.*, 2007). Little is known of the microbial populations involved in the removal of polycyclic aromatic hydrocarbons (PAHs) and their alkyl derivatives from marine environments polluted with complex oil mixtures; however, recent work suggests that members of the genus *Cycloclasticus* could play an important role (Kasai *et al.*, 2002a; McKew *et al.*, 2007).

In a previous study on the bioremediation of a beach affected by the *Prestige* oil spill we obtained a marine fuel-degrading microbial consortium (UBF) that caused an extensive degradation of the aliphatic (SF) and aromatic (AF) fractions of the fuel (Fernández-Álvarez *et al.*, 2006, 2007). Here we analyze in detail the degradative capabilities of this consortium toward different fuel components, and combine two different approaches to assign the observed actions to specific microbial populations. First, sequential changes in hydrocarbon composition during fuel biodegradation by consortium UBF are correlated to microbial community dynamics [16S rRNA gene analysis by PCR-denaturing gradient gel electrophoresis (DGGE) and clone libraries]. Secondly, our results are compared with the analysis of the microbial populations selected after subculturing the original consortium on specific fuel fractions and single PAHs.

Materials and methods

Chemicals

PAH substrates were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Solvents were obtained from J.T. Baker (Deventer, the Netherlands). 17α(H),21β(H)-hopane was purchased from Chiron AS (Trondheim, Norway), and the 16-PAH standard solution was purchased from Dr Ehrenstorfer (PAH-mix 9, Augsburg, Germany).

Media and supply of hydrocarbons

Artificial seawater (Scharlab, Barcelona, Spain) was used as the basal culture medium. Before inoculation, the sterile seawater was supplemented with 1% of the following sterile solutions: nitrogen (NH_4NO_3, 1 M), phosphorus (K_2HPO_4, 0.06 M), and metals [$MgSO_4 \cdot 7H_2O$, 20 g L^{-1}; $FeSO_4 \cdot 7H_2O$, 1.2 g L^{-1}; $MnSO_4 \cdot H_2O$, 0.3 g L^{-1}; $ZnSO_4 \cdot 7H_2O$, 0.3 g L^{-1}; $CoCl_2 \cdot 6H_2O$, 0.1 g L^{-1}; and nitrilotriacetic acid disodium salt (Sigma-Aldrich Chemie), 12.3 g L^{-1}].

The fuel oil used in this study was obtained from a water-in-oil emulsion (40% water content) of slightly weathered M-100 Russian fuel oil (no. 6 US-EPA), which was collected directly from the sea after the *Prestige* incident. The chemical composition of the fuel oil in this emulsion was 22.9% saturated hydrocarbons, 52.7% aromatic hydrocarbons, 12% resins and 12.4% asphaltenes as reported by CEDRE (http://www.cedre.fr/en/spill/prestige/prod.php). Before use, the water-in-oil emulsion was sterilized by autoclave, which caused its separation into two phases. The water phase was decanted and the fuel dissolved in methylene chloride (1 : 2, w/v). A portion of this solution was placed in empty sterile Erlenmeyer flasks, the solvent was allowed to evaporate at room temperature for 1 day, and then the sterile supplemented seawater medium was added. The final concentration of fuel oil in the culture medium was 5 g L^{-1}.

The solid medium was prepared with 15 g L^{-1} of purified agar (Pronadisa, Madrid, Spain). Plates containing hydrocarbons were prepared by adding the chosen hydrocarbon in acetone solution to the medium at 50 °C. Acetone was evaporated at room temperature for 24 h before inoculation.

Microbial consortia

The microbial consortium UBF had been obtained from an enrichment culture established in artificial seawater and *Prestige* fuel oil (5 g L^{-1}) inoculated with a composite sand sample collected from different points of the beach of Corrubedo (A Coruña, NW Spain). At the time of collection (February 2003) this beach was heavily polluted as a consequence of the *Prestige* oil spill. Cultures have been transferred twice a month for 5 years (1 : 50).

After 3 years of enrichment, different subcultures of consortium UBF were established in nutrient-supplemented artificial seawater with the aliphatic fraction (SF) or the AF of the *Prestige* fuel oil, or with single PAHs (phenanthrene, anthracene, fluoranthene, pyrene, benzo(*a*)anthracene and chrysene) as the carbon source. Each subculture was inoculated with a 1 : 50 dilution of the UBF microbial consortium culture and contained 0.2 g L^{-1} of one of the fuel fractions or PAHs. These cultures have been transferred monthly for 2 years.

Biodegradation of the fuel components by consortium UBF

The microbial consortium UBF was used to inoculate (0.5 mL) triplicate 125-mL Erlenmeyer flasks containing 25 mL of nutrient-supplemented artificial seawater and fuel oil (5 g L^{-1}). Sterile noninoculated flasks were used as controls. Cultures were incubated at 25 °C under fully aerobic conditions (rotary shaking, 200 r.p.m.). At 0, 7, 15, 30 and 60 days, the entire flask contents of triplicate cultures

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and controls was extracted five times with 10 mL of dichloromethane, acidified to pH 2 and extracted in the same manner using ethyl acetate as a solvent. Neutral and acidic extracts were dried using Na_2SO_4 and concentrated under vacuum to a final volume of 1 mL. Neutral extracts were used to obtain the saturated fraction (SF) and AF, and total petroleum hydrocarbons (TPHs) before GC-MS analysis.

Microbial community dynamics during fuel degradation

Two series of triplicate cultures of the microbial consortium UBF were set up. First, 0.5 mL of the UBF cultures were used to inoculate 250-mL Erlenmeyer flasks with 50 mL of nutrient-supplemented artificial seawater and fuel oil ($5\,g\,L^{-1}$) that were incubated as above. After 0, 1, 3, 7, 10 and 15 days of incubation, 1-mL samples from each replicate culture were centrifuged and pellets were stored at $-20\,^{\circ}C$ for further DNA extraction and PCR amplification. In parallel, 125-mL Erlenmeyer flasks containing 25 mL of medium were prepared and inoculated in the same manner to be used for the chemical analysis of the residual fuel. At the indicated sampling times, duplicates of those cultures were extracted as above and analyzed by CC-MS. Duplicate sterile noninoculated controls were also analyzed after 0 and 15 days.

Chemical analyses of residual fuel oil

TPHs present in the residual fuel oil recovered from the cultures were obtained by column chromatography of the neutral organic extracts using US EPA method 3611b. In brief, one-half of the neutral extract (0.5 mL) was concentrated to dryness under vacuum, weighed, redissolved in *n*-hexane and applied to an SPE glass column (J.T. Baker) packed with 2.5 g of alumina (Merck, Darmstad, Germany) previously activated at 120 °C. The saturated hydrocarbons were eluted with *n*-hexane, the aromatics with dichloromethane and the resins with methanol. All the fractions were concentrated to dryness under vacuum and weighed. Before GC-flame ionization detector (FID) and GC-MS analyses, the SF and AF were redissolved in dichloromethane (1 mL) and 0.1 mL of each fraction was combined to obtain the TPHs. *O*-terphenyl ($0.01\,g\,L^{-1}$) (Sigma-Aldrich Chemie) was added as an internal standard. GC-FID analyses were performed using a TRACE GC2000 (Thermo) gas chromatograph equipped with a FID. GC-MS analyses were performed in a Hewlett Packard HP5890 Series II gas chromatograph coupled to an HP5989 mass spectrometer. The GC chromatographs were equipped with DB5 (J&W Scientific, Folsom, CA) capillary columns (30×0.25 mm i.d.) with a 0.25-μm film thickness. The column temperature was held at 50 °C for 1 min and then increased to 320 °C at $5\,^{\circ}C\,min^{-1}$, this final temperature being held for 10 min. Injector, transfer line and detector temperatures were set at 290, 290 and 320 °C, respectively. The samples (1 μL) were injected in splitless mode using helium as the carrier gas, at a flow rate of $1.1\,mL\,min^{-1}$.

The extent of biodegradation of the 16 PAHs included in the US EPA list of priority pollutants and alkyl PAHs was determined from the hopane-normalized peak areas of the target analytes compared with those obtained for the same compounds in the control samples. The peak areas of the analytes were measured in the GC-MS reconstructed ion chromatograms obtained using the molecular ion for the aromatics, *m*/*z* 85 for linear and branched alkanes, *m*/*z* 230 for the internal standard *o*-terfenil, and *m*/*z* 191 for the conservative internal biomarker 17α(H),21β(H)-hopane.

DNA extraction from microbial consortia

Total DNA from the microbial consortia was extracted by a method modified from that of El Fantroussi *et al.* (1997). Pellets from 1-mL culture samples were washed with 0.4 mL of 0.1 M Na_3PO_4 buffer (pH 8), resuspended in 0.8 mL of the same buffer, and transferred to 2-mL microtubes with 0.6 g of glass beads (0.10–0.11 mm diameter) (B. Braun Biotech International, Melsungen, Germany). The mixtures were beaten (45 s) with a Hybaid RiboLyser (Thermo Electron). Then, a lysozyme solution (32 μL) ($50\,mg\,mL^{-1}$ Tris-HCl, 10 mM, pH 9) was added, followed by 30 min head-over-end shaking at 20 °C. Subsequently, 60 μL of 20% sodium dodecyl sulfate and 0.2 mL of 8 M ammonium acetate (pH 7.2) was added, followed by 10 min head-over-end shaking at 20 °C. After centrifugation (7000 ***g***), the supernatant was transferred to a new 2-mL tube and 1 volume of chloroform-isoamylalcohol (24 : 1) was added. The mixture was shaken for 60 min head-over-end at 20 °C and centrifuged at 5916 ***g*** for 15 min at 4 °C. The aqueous phase was transferred to a new tube and 0.8 volumes of isopropanol were added for overnight precipitation of the DNA at $-20\,^{\circ}C$. After centrifugation at 12 074 ***g*** for 25 min at 15 °C, the pellet was dried at room temperature for 1 h and resuspended in 250 μL of sterile milliQ water. The crude DNA extract was purified with a Wizard® DNA clean-up kit with vacuum manifold (Promega, Madison, WI) as described by the manufacturer. The purified DNA was finally recovered in 50 μL of sterile milliQ water and stored at $-20\,^{\circ}C$.

Isolation and identification of cultivable organisms

Heterotrophic bacterial strains from the different enrichment cultures were isolated after serial dilution and plating on 1 : 10 diluted Luria–Bertani (LB) agar prepared in artificial seawater. To isolate specific hydrocarbon-utilizing strains, the same dilutions were plated on artificial seawater

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agar plates prepared with purified agar (Pronadisa), containing $0.1\,g\,L^{-1}$ of hexadecane, phenanthrene or pyrene dissolved in the media, or with naphthalene crystals on the lid. The UBF microbial consortium was plated in all the culture media, whereas the subcultures SF and AF were exclusively plated on hexadecane or PAHs, respectively. Artificial seawater plates without a carbon source were used as controls. All the isolates were purified on 1 : 10 diluted LB plates and identified according to their partial 16S rRNA gene sequences.

PCR amplification of 16S rRNA gene from microbial consortia and bacterial isolates

Eubacterial 16S rRNA gene fragments from the microbial consortia were amplified by PCR using primers GC40-63f and 518r (El Fantroussi *et al.*, 1997). All the PCR amplifications were performed on an Eppendorf Mastercycler. The PCR mixture contained 1 μL of microbial consortia DNA as the template, 1.25 U *Taq* polymerase (Qiagen, Hilden, Germany), 25 pmol of each primer (Qiagen), 10 nmol of each dNTP (Invitrogen, Merelbeke, Belgium) and 1 × PCR buffer (Qiagen) in a total volume of 50 μL. After 5 min of initial denaturation at 95 °C, 40 cycles of amplification were carried out, each consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 55 °C and 1 min of primer extension at 65 °C, followed by a final primer extension of 10 min at 65 °C. These amplicons were then used either for DGGE fingerprinting analyses or to obtain clone libraries.

16S rRNA genes from bacterial isolates were directly amplified using the universal eubacterial primers 27f and 1521r (Weisburg *et al.*, 1991) and the PCR mixture previously described. After 5 min of initial denaturation at 95 °C, 30 cycles of amplification were carried out consisting of 1 min of denaturation at 95 °C, 1 min of annealing at 55 °C and 2 min of primer extension at 72 °C, followed by a final primer extension of 10 min at 72 °C. The PCR products were examined on 1.5% agarose gels, and the amplicons purified using a Qiaex II Extraction Kit (Qiagen) for further sequence analysis.

DGGE analysis

The PCR products from the microbial consortia and clone inserts were examined on 1.5% agarose gels and directly used for DGGE analysis on 6% polyacrylamide gels with denaturing gradients ranging from 40% to 60% (100% denaturant contains 7 M urea and 40% formamide). Electrophoresis was performed at a constant voltage of 100 V for 16 h in 1 × TAE buffer at 60 °C on an INGENYphorU-2 DGGE machine (INGENY International BV, Goes, the Netherlands). The gels were stained for 30 min with 1 × SYBR Gold nucleic acid gel stain (Molecular Probes Europe BV, Leiden, the Netherlands) and photographed under UV light, using a Pharmacia digital camera system (Image Master VDS, Pharmacia Biotech, Cambridge, UK) with LISCAP IMAGE CAPTURE software (version 1.0, Pharmacia Biotech).

16S rRNA gene clone libraries

Amplified 16S rRNA gene fragments from samples of the UBF microbial consortium obtained after 3 and 15 days of incubation as well as from the subcultures AF and with single PAHs, were cloned into the plasmid vector pCR® 2.1-TOPO, using a TOPO TA Cloning Kit (Invitrogen). To analyze transformants, nested PCR was performed on the clones, a first PCR with M13f and M13r primers as indicated by Invitrogen, and a second PCR with eubacterial primers GC40-63f and 518r as described above. DGGE patterns of the cloned fragments were compared with the fingerprints of the appropriate microbial consortia to identify which signals from the community fingerprint had been cloned. A selection of cloned inserts with different DGGE patterns was selected for further sequence analysis.

Sequencing and phylogenetic analysis

Sequencing was accomplished using the ABI Prism BigDye Terminator cycle-sequencing reaction kit (version 3.1) following the manufacturer's instructions. Primer M13r was used for sequencing 16S rRNA gene clone libraries, and primers 27f and 1521r were used to sequence cultured bacterial isolates. The sequences obtained were analyzed with the CHIMERA CHECK program (RDPII) (Cole *et al.*, 2003) and were compared with those in the SILVA 16S rRNA gene database using the SINA web aligner to determine the closest neighbor and the alignment with the next related sequences according to the Silva tree server (http://www.arb-silva.de/aligner) (Pruesse *et al.*, 2007).

Percent similarities to closest neighbors were then obtained by BLAST on-line searches (Altschul *et al.*, 1997). The 16S rRNA gene sequences obtained for the bacterial isolates and clones were deposited in the GenBank database with accession numbers GU565576–GU565603, and GU259658–GU259685, respectively.

Results

Biodegradation of the fuel oil TPHs by the UBF microbial consortium

The action of consortium UBF on different components of the *Prestige* fuel oil was determined in supplemented artificial seawater cultures with this fuel as sole carbon source. After 60 days of incubation the gravimetric analysis of the residual fuel recovered from cultures showed that the aliphatic fraction and AF had been reduced by 81% and

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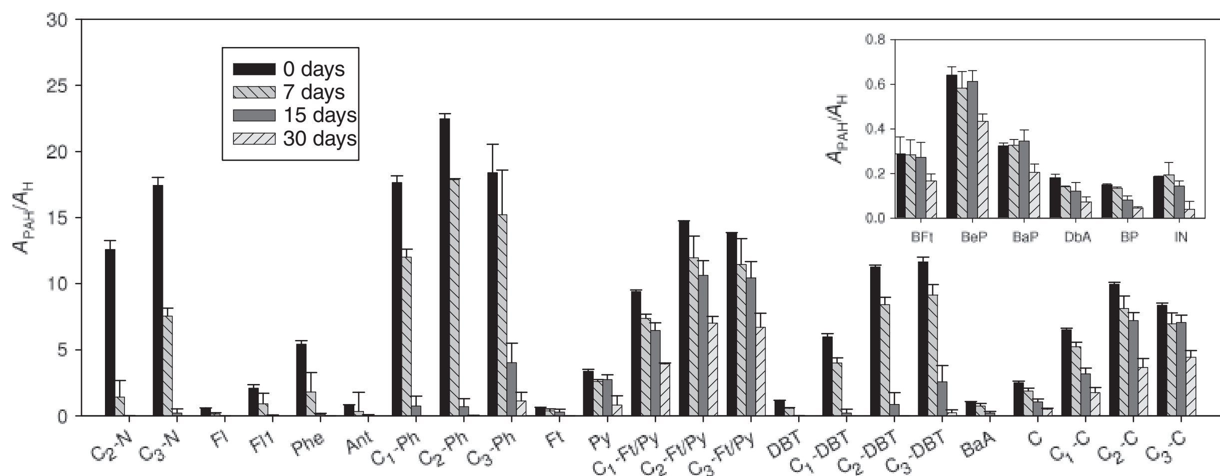

Fig. 2. Relative distribution of PAHs with respect to 17α(H),21β(H)-hopane (A_{PAH}/A_H) in abiotic controls and cultures of the microbial consortium UBF after 7, 15, 30 and 60 days of incubation. The hopane is used as a conservative internal biomarker. N, naphthalene; Fl, fluorene; P, phenanthrene; Ft, fluoranthene; Py, pyrene; DBT, dibenzothiophene; BaA, benzo(*a*)anthracene; C, chrysene; BFt, benzo(*b*+*k*)fluoranthenes; BeP, benzo(*e*)pyrene; BaP, benzo(*a*)pyrene; DbA, dibenzo(*a,h*)anthracene; BP, benzo(*g,h,i*)perilene; IN, indene-1,2,3-*c,d*-pyrene. C_1-, C_2- and C_3- indicate the number of methyl groups. Each data point represents the average of three independent replicate cultures.

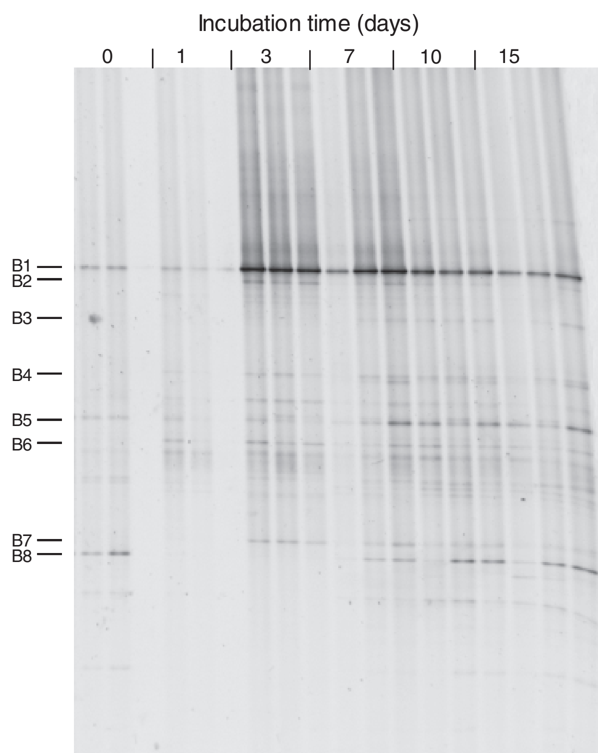

Fig. 3. DGGE profile of PCR-amplified 16S rRNA gene fragments from three independent replicate cultures of the microbial consortium UBF after 0, 1, 3, 7, 10 and 15 days of incubation.

corresponded to *Alphaproteobacteria* such as *Maricaulis* and *Roseovarius*. A minor band assigned to *Gammaproteobacteria* (B8) was particularly intense by the end of incubation (15 days).

Regarding the progress in the degradation of fuel components (Fig. 4), all the linear and the branched alkanes were depleted during the first 7 days (Fig. 4a), coinciding with maximum intensities in the *Alcanivorax* bands. As expected, the PAHs showed lower biodegradation rates. Degradation of the three-ring compounds started soon after inoculation and progressed lineally until almost complete removal at day 10, with the exception of anthracene, which persisted until the end of incubation (78% degradation). Conversely, none of the four-ring PAHs was degraded during the first week. Thereafter, fluoranthene and benzo(*a*)anthracene were degraded with similar kinetics (50% and 40% at day 15, respectively), thus suggesting that their removal could be due to the action of the same populations. Pyrene presented a slower degradation rate, reaching 18% degradation at the end of incubation.

The sequence of degradation of the different alkyl PAH families depended on their molecular weight and number of substituents, as is generally found for pure and mixed cultures and in environmental samples: C_2-N > C_3-N > C_1-P~C_1-DBT > C_2-P~C_2-DBT > C_3-P~C_3-DBT (the subscript digit after the letter C indicates the number of methyl groups) (Venosa *et al*., 1996; Kasai *et al*., 2002a; Watson *et al*., 2002; Díez *et al*., 2005). Di- and trimethylnaphthalenes were linearly removed during days 1–15 (> 95%), and the methylated phenanthrenes and dibenzothiophenes presented the highest degradation rates after day 7. Again, the identical biodegradation kinetics observed for phenanthrene and dibenzothiophene, and for their alkyl derivatives, indicates that both families are attacked by the same microbial populations.

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Table 1. Sequence analysis of clones with DGGE mobility pattern identical to bands detected in the UBF community fingerprint

Clone	Frequency (%)* 3 days	Frequency (%)* 15 days	Fragment length (bp)	Sim. (%)	Closest relative in ARB-Silva 16S rRNA gene database (accession no.)	Phylogenetic group
B1	81.6	18.8	485	100	*Alkanivorax borkumensis* SK2 (AM286690)	*Gammaproteobacteria*
B2	5.3	ND	486	99	*Alkanivorax borkumensis* MARC4D (DQ768649)	*Gammaproteobacteria*
B3	ND	6.3	440	98	Uncultured *Alphaproteobacteria* (AF143822)	*Alphaproteobacteria*
B4	ND	18.8	460	97	Uncultured *Gammaproteobacteria* (AF420370)	*Gammaproteobacteria*
B5	**5.3**	**12.5**	418	**98**	***Maricaulis* sp. MCS25 (**AJ227808**)**	***Alphaproteobacteria***
B6	**7.9**	**6.3**	428	**99**	***Roseovarius* sp. PTG4-4 (**EU603447**)**	***Alphaproteobacteria***
B7	ND	6.3	455	95	Uncultured *Alphaproteobacteria* (AY913254)	*Alphaproteobacteria*
B8	ND	9.4	464	95	Uncultured *Gammaproteobacteria* (EF648107)	*Gammaproteobacteria*

*Clone frequencies in clone libraries obtained after 3 and 15 days of incubation.
Components of the microbial communities that have been isolated are indicated in bold.

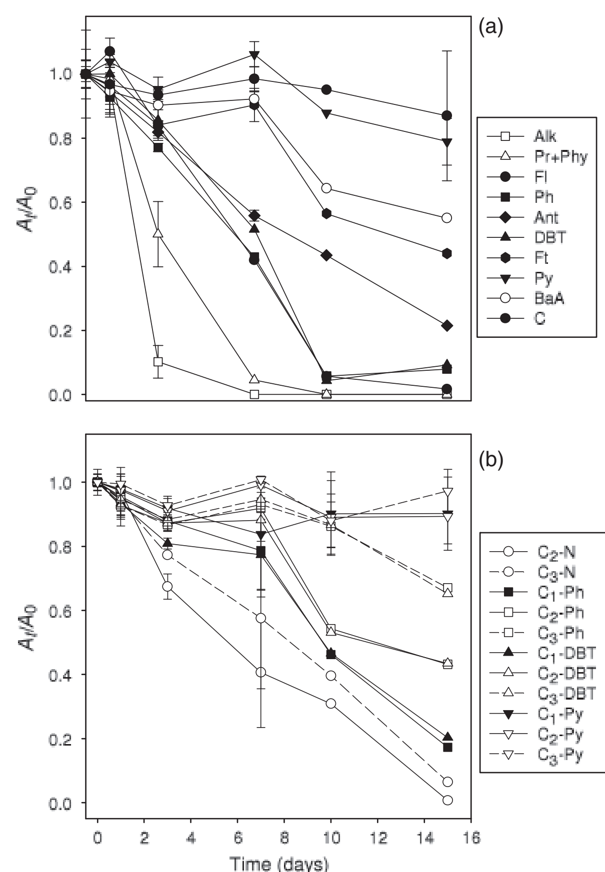

Fig. 4. Kinetics of degradation of the total linear alkanes (Alk), branched alkanes pristane and phytane (Pr+Phy), nonsubstituted PAHs (a), and of the alkyl PAHs (b) significantly depleted from the fuel by microbial consortium UBF in 2 weeks. The relative recovery (A_t/A_0) was calculated for the hopane normalized area of each analyte in respect to that found in the time 0 cultures. N, naphthalene; Fl, fluorene; Ph, phenanthrene; Ant, anthracene; DBT, dibenzothiophene; Ft, fluoranthene; Py, pyrene; BaA, benzo(a)anthracene; C, chrysene. C_1-, C_2- and C_3- indicate the number of methyl groups in each isomer family. Each data point represents the average of two independent replicate cultures.

Microbial community composition of subcultures enriched with fuel-oil fractions

The DGGE profile of a subculture of consortium UBF growing on the aliphatic fraction of the fuel (SF) presented two major bands (Fig. 5) already detected in the analysis of the parent consortium (Fig. 3). The most intense corresponded to B1 (*A. borkumensis*) while the other matched B8 (uncultured *Gammaproteobacteria*). Conversely, in a subculture growing in the aromatic fraction of the fuel (AF) all the bands detected were new (Fig. 5, Table 2). Band B9 was closely related to an uncultured *Bacteroidetes* from a marine microbial community study. Band B12, one of the most intense, comigrated with that of *A. borkumensis* in UBF but clone sequence analysis indicated a 94% similarity to the methylotrophic *Gammaproteobacteria*, *Methylophaga sulfidovorans*. Lastly, bands B16 and B21 corresponded to different members of *Marinobacter* (*Gammaproteobacteria*).

Microbial community composition of subcultures enriched with single PAHs

The DGGE fingerprints of UBF subcultures growing in single PAHs as the sole carbon source showed distinctive band patterns (Fig. 5). The most abundant population detected in the phenanthrene cultures (band B18) corresponded to a strain of *Marinobacter* sp. (Table 2); two additional bands were assigned to the *Alphaproteobacteria*, with sequences matching those reported for a *Tistrella mobilis* (B26, 99%), and an uncultured *Thalassospira* (B22). Members of *Tistrella* and *Thalassospira* have recently been described as phenanthrene degraders (Kodama *et al*., 2008; Zhao *et al*., 2008) and have been detected by molecular approaches in marine PAH-degrading microbial communities (Cui *et al*., 2008).

During the analysis of the anthracene-degrading subculture, only clones matching three of the five major bands in the DGGE profile (B24, B25 and B27) were recovered. Their

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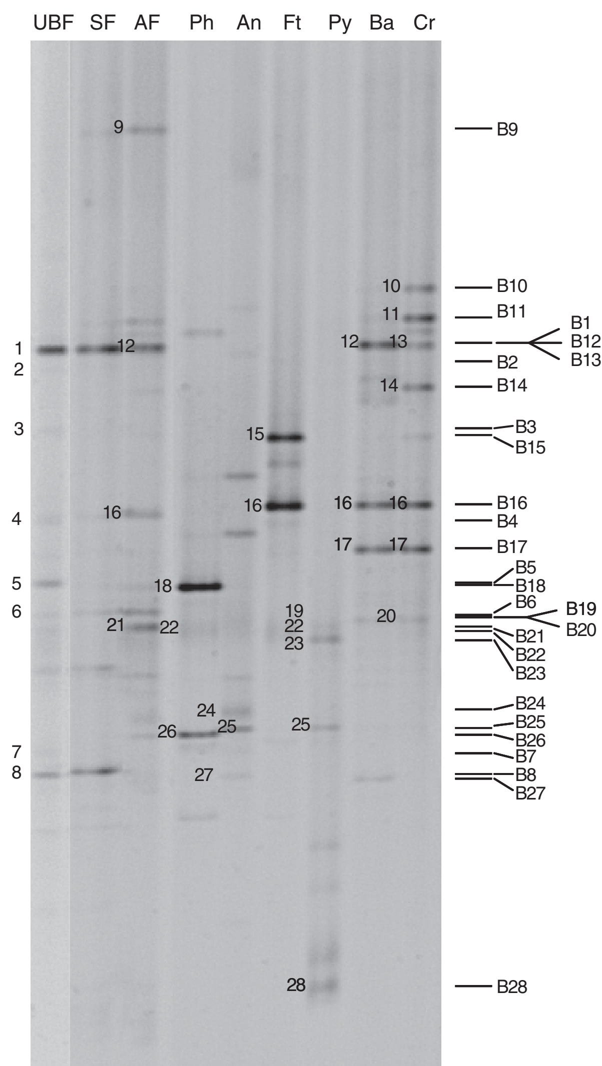

Fig. 5. DGGE profile of PCR-amplified 16S rRNA gene fragments from the microbial consortium UBF after 15 days of incubation, and from its subcultures in aliphatic fraction (SF), AF, phenanthrene (Ph), anthracene (An), fluoranthene (Ft), pyrene (Py), benzo(*a*)anthracene (Ba) and chrysene (Cr).

closest neighbors in the database were members of the *Alphaproteobacteria*, identified as *Stappia aggregata*, *Paracoccus* sp. and *Ochrobactrum* sp. This is consistent with a recent report of a *Paraccocus* sp. isolate able to use anthracene, and other three- and four-ring PAHs as the sole carbon sources (Zhang *et al*., 2004).

Of the two major bands detected in the fluoranthene culture analyses, one (B15) was assigned to the genus *Methylophaga*, whereas the other (B16) corresponded to a *Marinobacter* strain previously detected in the AF culture (Table 2). The pyrene culture showed the presence of members of *Thalassospira* (B22) and *Paracoccus* (B25) previously found in the phenanthrene and anthracene cultures, respectively. Three additional bands presented different degrees of similarity to a *Martelella mediterranea* strain (B19), an *Aurantimonas manganoxydans* strain (B23) and an uncultured *Gordonia* (B28). Therefore, the microbial populations found to be involved in pyrene degradation were similar to those found in the three-ring PAH enrichment cultures (*Alphaproteobacteria*), with the addition of an actinobacterial strain (*Gordonia*). This fits well with recent work describing the ability of some *Gordonia* sp. to degrade pyrene and other PAHs (Arenskötter *et al*., 2004), and the fact that the soil pyrene-degrading strains described until now are Actinobacteria.

In the benzo(*a*)anthracene culture we detected the members of *Methylophaga* (B12) and *Marinobacter* (B16) previously found in the AF and fluoranthene cultures. A newly detected band (B17) was also assigned to *Marinobacter*. The chrysene culture exhibited the most diverse DGGE banding profile, with seven bands all corresponding to *Gammaproteobacteria*. The sequences for five of the bands (B10, B13, B16, B17 and B20) matched *Marinobacter* to different degrees (94–99%), and the others (B11 and B14) were identical to those reported for two strains of *Methylophaga*.

Isolation and identification of bacterial strains

In total, 24 bacterial strains were isolated, of which nine (37%) presented 16S rRNA gene sequences previously found during the molecular analysis of the UBF microbial consortium or its subcultures (Table 3). Interestingly, five of the 10 LB isolates obtained from consortium UBF belonged to the *Cytophaga–Flexibacter–Bacteroides* (CFB) group, which was not observed during the molecular analysis; one was a *Gammaproteobacterium* (UBF8) and four were *Alphaproteobacterial* strains, with only one of these, *Roseovarius* sp. UBF10, having been detected during the molecular approach (B6). Hexadecane plates produced five isolates, three of which (UBF13–15) had been identified during the molecular analysis of the anthracene (B27, *Phyllobacteriaceae*; B25, *Paracoccus*) and the pyrene (B25 and B19, *M. mediterranea*) cultures. Two of those strains (*Martelella* sp. UBF14 and *Paracoccus* UBF15) were also recovered from pyrene plates, which produced a third isolate, *Marinobacter* UBF16 with a 16S rRNA gene sequence identical to those of isolates SF7 and AF1 from the aliphatic fraction and AF enrichment cultures, respectively, and to that of clone B21 from the AF enrichment culture.

All the aliphatic fraction culture isolates were recovered from LB plates, with only one of them, the *Marinobacter* sp. strain SF7 mentioned above, having also been purified from hexadecane plates. Strains SF1 and SF5, closely related to *Caulobacter* and *Stappia*, had been previously detected in the molecular analysis.

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Table 2. Sequence analysis of the clones selected from the subcultures established in artificial seawater with AF or single PAH substrates

Clone	Frequencies in clone libraries from enrichment cultures (%)*							Fragment length (bp)	Sim (%)	Closest relative in ARB-Silva 16S rRNA database (accession no.)	Phylogenetic group
	AF	Ph	An	Ft	Py	Ba	Cr				
B9	7							491	98	Uncultured *Bacteroidetes* bacterium (AF050545)	CFB
B10							21	496	95	Uncultured *Marinobacter* sp. (EF573927)	*Gammaproteobacteria*
B11							**7**	**492**	**100**	***Methylophaga marina*** **(**X95459**)**	***Gammaproteobacteria***
B12	7					30		492	94	*Methylophaga sulfidovorans* (X95461)	*Gammaproteobacteria*
B13							10	496	94	*Marinobacter vinifirmus* FB1 (DQ235263)	*Gammaproteobacteria*
B14							17	496	100	*Methylophaga* sp. DMS044 (DQ660929)	*Gammaproteobacteria*
B15				50				485	99	*Methylophaga* sp. V4.MO.19 (AJ244762)	*Gammaproteobacteria*
B16	22			30		15	17	458	98	*Marinobacter* sp. ws22 (AJ704789)	*Gammaproteobacteria*
B17						25	17	496	99	Uncultured *Marinobacter* sp. (EF573927)	*Gammaproteobacteria*
B18		78						492	100	Uncultured *Marinobacter* sp. (EF190071)	*Gammaproteobacteria*
B19					**7**			**1433**	**98**	***Martelella mediterranea*** **MACL11 (**AY649762**)**	***Alphaproteobacteria***
B20							3	496	95	*Marinobacter vinifirmus* FB1 (DQ235263)	*Gammaproteobacteria*
B21	**22**							**489**	**100**	**Uncultured *Marinobacter* sp. (**EF573927**)**	***Gammaproteobacteria***
B22		**11**			**33**			**451**	**99**	**Uncultured *Thalassospira* sp. (**AY922191**)**	***Alphaproteobacteria***
B23					38			1441	92	*Aurantimonas manganoxydans* SI85-9A1 (AAPJ01000003)	*Alphaproteobacteria*
B24			**57**					**441**	**100**	***Stappia aggregata*** **IAM 12614 (**D88520**)**	***Alphaproteobacteria***
B25			**29**		**4**			**441**	**99**	***Paracoccus*** **sp. 88/2-4 (**AJ313424**)**	***Alphaproteobacteria***
B26		11						454	99	*Tistrella mobilis* (AB071665)	*Alphaproteobacteria*
B27			**14**					**439**	**94**	***Ochrobactrum*** **sp. B2 (**AY661464**)**	***Alphaproteobacteria***
B28					15			1475	97	Uncultured *Gordonia* sp. (DQ4222886)	Actinobacteria

*Clone libraries obtained in AF (aromatics), Ph (phenanthrene), An (anthracene), Ft (fluoranthene), Py (pyrene), Ba (benzo(*a*)anthracene) and Cr (chrysene).
Components of the microbial communities that have been isolated are indicated in bold.

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Table 3. Bacterial isolates obtained from the microbial consortium UBF and its subcultures established in SF and AF

	Strains from enrichment cultures						
Phylogenetic group	UBF	SF	AF	Fragment length (bp)	Closest relative in ARB-Silva 16S rRNA gene database (accession no.)	Sim (%)	Clone‖
Alphaproteobacteria	UBF1*			903	*Roseovarius* sp. JC2138 (EU642856)	94	
	UBF4*			968	*Sphingopyxis* sp. DS15 (EF494193)	99	
	UBF6*			555	Marine *Rhodobacterales* bacteria (AY654838)	94	
	UBF10*		**AF4***	**998**	***Roseovarius pacificus* 81-2 (**DQ120726**)**	**98**	**B6**
	UBF11†			1029	*Ochrobactrum* sp. B2 (AY661464)	89	
	UBF13†			**860**	***Phyllobacteriaceae* bacterium D11-69 (**AM403234**)**	**99**	**B27**
	UBF14†,¶			**1062**	***Martelella mediterranea* MARC4H (**DQ768639**)**	**98**	**B19**
	UBF15†,¶			**1009**	***Paracoccus* sp. 88/2-4 (**AJ313424**)**	**98**	**B25**
		SF1*	**AF2***	**1132**	***Caulobacter halobacteroides* (**AJ007804**)**	**97**	**B5**
		SF4*		749	*Parvibaculum* sp. psc10 (EU930870)	97	
		SF5*		**658**	***Stappia* sp. SMB21 (**DQ868686**)**	**95**	**B24**
		SF6*		456	*Tetracoccus cechi* (Y09610)	94	
			AF5‡	**1027**	***Thalassospira lucentensis* P44 (**EU880514**)**	**97**	**B22**
Gammaproteobacteria	UBF8*			1070	*Idiomarina* sp. DF-B6 (EU515224)	98	
	UBF12†			909	*Pseudoalteromonas* sp. c7 (EU420060)	97	
	UBF16¶	**SF7*,†**	**AF1*,‡,§,¶**	**1037**	***Marinobacter* sp. DG1305 (**DQ486512**)**	**99**	**B21**
		SF3*		745	*Acinetobacter* ursingii LUH 4763 (AJ275039)	99	
			AF3*	**855**	***Methylophaga marina* (**X95459**)**	**96**	**B11**
CFB	UBF2*			439	*Bacteroidetes* bacterium strain A16s (AF300973)	97	
	UBF3*			594	*Flavobacteriaceae* bacterium LA8 (AF513435)	99	
	UBF5*			900	*Muricauda lutimaris* SMK-108 (EU156065)	98	
	UBF7*			377	*Flavobacteriaceae* bacterium (AM292402)	96	
	UBF9*			934	*Bacteroidetes* bacterium GMD38C4 (AY162087)	99	
Firmicutes		SF2*		782	*Staphylococcus* sp. IGCAR-7/07 (EF517954)	98	

Isolation media: artificial seawater supplemented with *LB, †hexadecane, ‡naphthalene, §phenanthrene or ¶pyrene.
The strains with 16S rRNA gene sequences identical to members of the microbial communities detected by molecular methods are given in bold.
‖Clones with identical 16S rRNA gene sequences to the obtained isolates.

The five isolates from the aromatic fraction culture had been detected previously during the molecular analyses, and three of them (AF1–2 and AF4) presented 16S rRNA gene sequences identical to different isolates from the SF and the UBF enrichment cultures (Table 3). Isolate AF5 (*Thalassospira*, isolated in naphthalene plates) was the only bacteria not recovered in LB and had been previously detected in the DGGE analysis of the phenanthrene and pyrene cultures (B22). Interestingly, isolate *Methylophaga* sp. AF3 had only been detected in the molecular analysis of the chrysene enrichment culture (B11). On the other hand, isolate AF1, identical to isolates UBF16 and SF7, was the only isolate that matched bands previously detected in the DGGE profile of the AF subculture (B21).

The isolation of *Caulobacter* sp. strain AF2 and *Roseovarius* sp. strain AF4, and the fact that they correspond to two important bands in the UBF microbial consortium profile at 15 days (B5 and B6, respectively), suggests that, as indicated by the microbial community dynamics and sequential degradation experiments, these bacterial strains are involved in the first steps of PAH degradation (low molecular-weight compounds). The fact that these strains were not detected in the DGGE profiles of the AF cultures (Fig. 5) could be due to the late incubation time at which those analyses were performed (30 days).

Discussion

The heavy fuel oil spilled by the *Prestige* had a high content of aromatic hydrocarbons (52%), presenting an interesting model to study the microbial degradation of PAHs and their alkyl derivatives within environmental petrogenic mixtures. The marine microbial consortium UBF, established by enrichment culture of natural populations from a beach affected by the *Prestige*, using this fuel as a sole carbon source, was highly efficient at removing a variety of fuel components. Both the aliphatic fraction and AF were extensively degraded, with a complete depletion of all the GC-resolved hydrocarbons after 15 days of incubation. It is noteworthy that the UCM, whose relative abundance usually increases in degraded oils (Wang *et al.*, 1998), were also substantially reduced, further evidence that the microbial populations in the UBF consortium combine remarkable degradation capabilities.

The PAHs containing three to five rings [including chrysene and benzo(*a*)pyrene] and their alkyl derivatives

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were seriously attacked (e.g. alkyl fluoranthenes/pyrenes 49–60% and alkyl chrysenes 47–80%). These biodegradation percentages are substantially higher than those reported in previous work on biodegradation of crude oils and derivatives, including a recent study on the degradation of the *Prestige* fuel oil by a highly specialized PAH-degrading microbial consortium (Díez *et al*., 2005) that showed a 22–30% degradation of alkyl fluoranthenes/pyrenes and a 22–25% degradation of alkyl chrysenes.

Microbial community analysis revealed a succession of populations associated to the progress of fuel component degradation. *Alcanivorax borkumensis* predominated during the first week of incubation, coinciding with the maximum rates of depletion of linear and branched alkanes. Populations of these *Gammaproteobacteria*, ubiquitous but usually occurring in small quantities in unpolluted marine waters, have been found to increase significantly in response to oil or aliphatic HC spiking in microcosm and mesocosm studies (Yakimov *et al*., 2005; Cappello *et al*., 2007; McKew *et al*., 2007) as well as in several natural marine environments (Kasai *et al*., 2001, 2002b; Röling *et al*., 2002). According to Syutsubo *et al*. (2001), this fast increment could be favored by the addition of inorganic nutrients (nitrogen and phosphorus) to the artificial seawater. The decrease in their frequency of detection after the first week is consistent with the general observation that *Alcanivorax* species are obligate hydrocarbon-degrading bacteria able to use only a few organic substrates for growth, namely, alkanes (up to C_{32}), long-chain isoprenoids and the alkyl groups of *n*-alkylbenzenes and *n*-alkylcycloalkanes (Yakimov *et al*., 1998; Dutta & Harayama, 2001). Furthermore, the key role of *Alcanivorax* in the degradation of aliphatic substrates within the UBF microbial consortium was confirmed by the analysis of the aliphatic fraction (SF) degrading subculture.

This first alkane biodegradation phase was followed by a progressive removal of PAHs and alkyl derivatives. Accordingly, the *Alcanivorax* populations were succeeded by members of the *Alphaproteobacteria* (including species of *Maricaulis* and *Roseovarius)*, and by nonidentified members of the *Gammaproteobacteria*, detected with lower frequency. Members of *Maricaulis* and their close relatives *Roseobacter* and *Roseovarius* have been previously associated to crude oil and PAH degradation in marine environments (Brakstad & Lodeng, 2005; McKew *et al*., 2007; Wang *et al*., 2008).

In general, PAH degradation proceeded as expected according to their molecular weights and water solubility. Fluoranthene and benzo(*a*)anthracene were the most easily and extensively degraded four-ring PAHs, presenting similar degradation kinetics. The slower degradation observed for pyrene could not be explained on the basis of lower substrate bioavailability, as its water solubility ($0.14\,mg\,L^{-1}$) is substantially higher that that of benzo(*a*)anthracene ($0.002\,mg\,L^{-1}$) (Cerniglia, 1992), suggesting that different degradation processes possibly associated with diverse microbial populations were involved in the degradation of these compounds.

The molecular analysis of the single PAH-degrading UBF subcultures gives a better understanding of the population dynamics associated with the degradation progress. Although the most frequently detected sequence in the phenanthrene subculture was that of the *Gammaproteobacteria* genus *Marinobacter*, most of the identified members of the three-ring PAH-degrading communities were *Alphaproteobacteria*. In contrast, all the microbial populations detected in the four-ring PAH-degrading cultures of fluoranthene, benzo(*a*)anthracene and chrysene, were *Gammaproteobacteria* (mainly *Marinobacter* and *Methylophaga*). This coincidence in the fluoranthene- and benzo(*a*)anthracene-degrading communities is in accordance with their analogous kinetics during the degradation of fuel by consortium UBF (Fig. 4).

Marinobacter has been found in several oil-degrading microbial communities (Deppe *et al*., 2005; Gerdes *et al*., 2005) and its ability to use alkanes and PAHs has been demonstrated (Gauthier *et al*., 1992; Hedlund *et al*., 2001). In contrast, several studies have reported the presence of members of the methylotrophic genus *Methylophaga* in oil-contaminated environments and oil-amended marine microcosms and seawater (Röling *et al*., 2002; Yakimov *et al*., 2005; Coulon *et al*., 2007); however, their ability to utilize hydrocarbons has not been demonstrated and they have never before been associated to high molecular-weight PAH-degrading communities.

The pyrene culture showed a distinctive community composition, with the detected populations showing a preponderance of *Alphaproteobacteria* (*Paracoccus*, *Martelella*, *Thalassospira* and *Aurantimonas*) together with one Actinobacteria (*Gordonia*). This is also consistent with the particular degradation kinetics observed for this compound during fuel degradation.

There are a number of studies on the composition of PAH-degrading marine microbial communities; however, the analysis of specific marine microbial communities involved in the utilization of benzo(*a*)anthracene and chrysene has not been addressed. On the other hand, a few recent studies have pointed out that the genus *Cycloclasticus* could play an important role in the degradation of PAHs in marine environments (Kasai *et al*., 2002a; Wang *et al*., 2008). Notably, these *Gammaproteobacteria* were not detected in the present work. In PAH-polluted soils, mycobacteria seem to play a major role in the degradation of pyrene (Kanaly & Harayama, 2000) and pyrene-degrading mycobacterial strains isolated from oil-contaminated beaches (Vila *et al*., 2001) have shown wide degradation capabilities toward a variety of fuel components in marine medium (Vila & Grifoll, 2009). In this study, the detection of an

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actinobacterial strain belonging to the genus *Gordonia* as an apparently major component of the pyrene-degrading community is consistent with a role of actinobacteria in PAH and specifically pyrene removal in coastal environments.

With the enrichment and isolation approaches used, it was possible to obtain nine pure cultures whose partial 16S rRNA gene sequence matched one or more of the 28 different clones, as well as 15 additional bacterial strains not previously detected. Work in progress involves further catabolic characterization of all the isolates to determine or confirm their potential role in the microbial community. In addition, the molecular data will be used to design new isolation culture media to further increase the effectiveness of the isolation protocols.

In summary, the analysis of the microbial community dynamics of the heavy fuel oil-degrading consortium UBF indicates a major role of *Alcanivorax* in the early removal of aliphatic components, followed by the removal of low molecular-weight PAHs by members of the *Alphaproteobacteria* group, and, thereafter, by *Gammaproteobacteria* such as *Marinobacter* and *Methylophaga*, responsible for the degradation of higher molecular-weight aromatic components. Our results also indicate that *Marinobacter* has the potential to play a role in the general removal of hydrocarbons from marine environments, as members of this genus were isolated from both the SF and AF degrading consortia, and grew on artificial seawater plates with either hexadecane or single PAHs as sole carbon source. This agrees with previous reports describing different strains of *Marinobacter* as alkane- or PAH-degrading marine organisms (Gauthier *et al.*, 1992; Hedlund *et al.*, 2001). Noteworthy, *Cycloclasticus*, recently proposed to be a major player in the removal of PAHs from marine environments, was not detected in this work.

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References

Albaigés J (1989) Marine pollution: an introduction. *Marine Pollution* (Albaigés J, ed), pp. 1–10. Hemisphere Publishing Corp., New York.

Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**: 3389–3402.

Arenskötter M, Bröker D & Steinbüchel A (2004) Biology of the metabolically diverse genus *Gordonia*. *Appl Environ Microb* **70**: 3195–3204.

Bragg JR, Prince RC, Harner EJ & Atlas RM (1994) Effectiveness of bioremediation for the Exxon Valdez oil spill. *Nature* **368**: 413–418.

Brakstad OG & Lodeng AGG (2005) Microbial diversity during biodegradation of crude oil in seawater from the North Sea. *Microbiol Ecol* **49**: 94–103.

Bruns A & Berthe-Corti L (1999) *Fundibacter jadensis* gen. nov., sp. nov., a new slightly halophilic bacterium, isolated from intertidal sediment. *Int J Syst Bacteriol* **49**: 441–448.

Cappello S, Denaro R, Genovese M, Giuliano L & Yakimov MM (2007) Predominant growth of *Alcanivorax* during experiments on 'oil spill bioremediation' in mesocosms. *Microbiol Res* **162**: 185–190.

Cerniglia CE (1992) Biodegradation of polycyclic aromatic hydrocarbons. *Biodegradation* **3**: 351–368.

Cole JR, Chai B, Marsh TL *et al.* (2003) The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. *Nucleic Acids Res* **31**: 442–443.

Coulon F, McKew BA, Osborn AM, McGenity TJ & Timmis KN (2007) Effects of temperature and biostimulation on oil-degrading microbial communities in temperate estuarine waters. *Environ Microbiol* **9**: 177–186.

Cui Z, Lai Q, Dong C & Shao Z (2008) Biodiversity of polycyclic aromatic hydrocarbon-degrading bacteria from deep sea sediments of the Middle Atlantic Ridge. *Environ Microbiol* **10**: 2138–2149.

Deppe U, Richnow HH, Michaelis W & Antranikian G (2005) Degradation of crude oil by an arctic microbial consortium. *Extremophiles* **9**: 461–470.

Díez S, Sabaté J, Viñas M, Bayona JM, Solanas AM & Albaigés J (2005) The Prestige oil spill. I. Biodegradation of a heavy fuel oil under simulated conditions. *Environ Toxicol Chem* **24**: 2203–2217.

Dutta TK & Harayama S (2001) Biodegradation of *n*-alkylcycloalkanes and *n*-alkylbenzenes via new pathways in *Alcanivorax* sp. strain MBIC 4326. *Appl Environ Microb* **67**: 1970–1974.

El Fantroussi S, Mahillon J, Naveau H & Agathos SN (1997) Introduction and PCR detection of *Desulfomonile tiedjei* in soil slurry microcosms. *Biodegradation* **8**: 125–133.

Fernández-Álvarez P, Vila J, Garrido-Fernández JM, Grifoll M & Lema JM (2006) Trials of bioremediation on a beach affected by the heavy oil spill of the Prestige. *J Hazard Mater* **B137**: 1523–1531.

Fernández-Álvarez P, Vila J, Garrido-Fernández JM, Grifoll M, Feijoo G & Lema JM (2007) Evaluation of biodiesel as bioremediation agent for the treatment of the shore affected by

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the heavy fuel oil spill of the Prestige. *J Hazard Mater* **147**: 914–922.

Floodgate GD (1995) Some environmental aspects of marine hydrocarbon bacteriology. *Aquat Microb Ecol* **9**: 3–11.

Gauthier MJ, Lafai B, Christen R, Fernandez L, Aquaviva 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.

Gerdes B, Brinkmeyer R, Dieckmann G & Helmke E (2005) Influence of crude oil on changes of bacterial communities in Arctic sea-ice. *FEMS Microbiol Ecol* **53**: 129–139.

Head IM & Swannell RPJ (1999) Bioremediation of petroleum hydrocarbon contaminants in marine habitats. *Curr Opin Biotech* **10**: 234–239.

Head IM, Jones DM & Röling WFM (2006) Marine microorganisms make a meal of oil. *Nat Rev Microbiol* **4**: 173–182.

Hedlund BP, Geiselbrecht AD, Bair TJ & Staley JT (1999) Polycyclic aromatic hydrocarbon degradation by a new marine bacterium. *Neptunomonas naphthovorans* gen. nov., sp. nov. *Appl Environ Microb* **65**: 251–259.

Hedlund BP, Geiselbrecht AD & Staley JT (2001) *Marinobacter* strain NCE312 has a *Pseudomonas*-like naphthalene dioxygenase. *FEMS Microbiol Lett* **201**: 47–51.

Kanaly RA & Harayama S (2000) Biodegradation of high-molecular-weight polycyclic aromatic hydrocarbons by bacteria. *J Bacteriol* **182**: 2059–2067.

Kasai Y, Kishira H, Syutsubo K & Harayama S (2001) Molecular detection of marine bacterial populations on beaches contaminated by *Nakhodka* tanker oil-spill accident. *Environ Microbiol* **3**: 1–10.

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

Kasai Y, Kishira H, Sasaki T, Syutsubo K, Watanabe K & Harayama S (2002b) Predominant growth of *Alcanivorax* strains in oil-contaminated and nutrient supplemented seawater. *Environ Microbiol* **4**: 141–147.

Kodama Y, Stiknowati LI, Ueki A, Ueki K & Watanabe K (2008) *Thalassospira tepidiphila* sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from seawater. *Int J Syst Evol Micr* **58**: 711–715.

Leahy JG & Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. *Microbiol Rev* **54**: 305–315.

McKew BA, Coulon F, Osborn AM, Timmis KN & McGenity TJ (2007) Determining the identity and roles of oil-metabolizing marine bacteria from the Thames Estuary, UK. *Environ Microbiol* **9**: 165–176.

Prince RC (1997) Bioremediation of marine oil spills. *Trends Biotechnol* **15**: 158–160.

Pruesse E, Quast C, Knittel K, Fuchs B, Ludwig W, Peplies J & Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucl Acids Res* **35**: 7188–7196.

Röling WFM, Milner MG, Jones DM, Lee K, Daniel F, Swannell RPJ & Head IM (2002) Robust hydrocarbon degradation and dynamics of bacterial communities during nutrient-enhanced oil spill bioremediation. *Appl Environ Microb* **68**: 5537–5548.

Swannell RPJ, Mitchell D, Lethbridge G *et al*. (1999) A field demonstration of the efficacy of bioremediation to treat oiled shorelines following the Sea Empress incident. *Environ Technol* **20**: 863–873.

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

Venosa AD, Suidan MT, Wrenn BA, Strohmeier KL, Haines JR, Eberhart BL, King D & Holder E (1996) Bioremediation of an experimental oil spill on the shoreline of Delaware Bay. *Environ Sci Technol* **30**: 1764–1775.

Vila J & Grifoll M (2009) Actions of *Mycobacterium* sp. strain AP1 on the saturated- and aromatic-hydrocarbon fractions of fuel oil in a marine medium. *Appl Environ Microb* **75**: 6232–6239.

Vila J, López Z, Sabaté J, Minguillón C, Solanas AM & Grifoll M (2001) Identification of a novel metabolite in the degradation of pyrene by *Mycobacterium* sp. strain AP1. Actions of the isolate on two- and three-ring polycyclic aromatic hydrocarbons. *Appl Environ Microb* **67**: 5497–5505.

Wang B, Lai Q, Cui Z, Tan T & Shao Z (2008) A pyrene-degrading consortium from deep-sea sediment of the West Pacific and its key member *Cycloclasticus* sp. P1. *Environ Microbiol* **10**: 1948–1963.

Wang Z, Fingas M & Sergy G (1995) Chemical characterization of crude oil residues from an arctic beach by GC/MS and GC/FID. *Environ Sci Technol* **29**: 2622–2631.

Wang Z, Fingas M, Blenkinsopp S, Sergy G, Landriault M, Sigouin L, Foght J, Semple K & Westlake DWS (1998) Comparison of oil composition changes due to biodegradation and physical weathering in different oils. *J Chromatogr A* **809**: 89–107.

Watson JS, Jones DM, Swanell RPJ & van Duin ACT (2002) Formation of carboxylic acids during aerobic biodegradation of crude oil and evidence of microbial oxidation of hopanes. *Org Geochem* **33**: 1153–1169.

Weisburg WG, Barnes SM, Pelleteir DA & Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* **173**: 697–703.

Yakimov MM, Golyshin PN, Lang S, Moore ERB, Abraham WR, Lunsdorf H & Timmis KN (1998) *Alcanivorax borkumensis* gen. nov., sp. nov., a new, hydrocarbon-degrading and surfactant-producing marine bacterium. *Int J Syst Bacteriol* **48**: 339–348.

Yakimov MM, Denaro R, Genovese M, Cappello S, D'Auria G, Chernikova TN, Timmis KN, Golyshin PN & Giuliano L (2005) Natural microbial diversity in superficial sediments of Milazzo Harbor (Sicily) and community successions during

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microcosm enrichment with various hydrocarbons. *Environ Microbiol* **7**: 1426–1441.
Yakimov MM, Timmis KN & Golyshin PN (2007) Obligate oil-degrading marine bacteria. *Curr Opin Biotech* **18**: 257–266.
Zhang H, Kallimanis A, Koukkou A & Drainas TC (2004) Isolation and characterization of novel bacteria degrading polycyclic aromatic hydrocarbons from polluted Greek soils. *Appl Microbiol Biot* **65**: 124–131.
Zhao HP, Wang L, Ren JR, Li Z, Li M & Gao HW (2008) Isolation and characterization of phenanthrene-degrading strains *Sphingomonas* sp. ZP1 and *Tistrella* sp. ZP5. *J Hazard Mater* **152**: 1293–1300.

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