

Polyphasic approach for assessing changes in an autochthonous marine bacterial community in the presence of *Prestige* fuel oil and its biodegradation potential

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Abstract A laboratory experiment was conducted to identify key hydrocarbon degraders from a marine oil spill sample (*Prestige* fuel oil), to ascertain their role in the degradation of different hydrocarbons, and to assess their biodegradation potential for this complex heavy oil. After a 17-month enrichment in weathered fuel, the bacterial community, initially consisting mainly of *Methylophaga* species, underwent a major selective pressure in favor of obligate hydrocarbonoclastic microorganisms, such as *Alcanivorax* and *Marinobacter* spp. and other hydrocarbon-degrading taxa (*Thalassospira* and *Alcaligenes*), and showed strong biodegradation potential. This ranged from >99% for all low- and medium-molecular-weight alkanes (C₁₅–C₂₇) and polycyclic aromatic hydrocarbons (C₀- to C₂- naphthalene, anthracene, phenanthrene, dibenzothiophene, and carbazole), to 75–98% for higher molecular-weight alkanes (C₂₈–C₄₀) and to 55–80% for the C₃ derivatives of tricyclic and tetracyclic polycyclic aromatic hydrocarbons (PAHs) (e.g., C₃-chrysenes), in 60 days. The numbers of total heterotrophs and of *n*-alkane-, aliphatic-, and PAH degraders, as well as the

structures of these populations, were monitored throughout the biodegradation process. The salinity of the counting medium affects the counts of PAH degraders, while the carbon source (*n*-hexadecane vs. a mixture of aliphatic hydrocarbons) is a key factor when counting aliphatic degraders. These limitations notwithstanding, some bacterial genera associated with hydrocarbon degradation (mainly belonging to α - and γ -Proteobacteria, including the hydrocarbonoclastic *Alcanivorax* and *Marinobacter*) were identified. We conclude that *Thalassospira* and *Roseobacter* contribute to the degradation of aliphatic hydrocarbons, whereas *Mesorhizobium* and *Muricauda* participate in the degradation of PAHs.

Keywords Consortium · Heavy fuel oil · Hydrocarbon biodegradation · Hydrocarbonoclastic bacteria · Microbial community · *Prestige* oil spill

Introduction

In November 2002, the oil tanker *Prestige* sank about 240 km off Cape Finisterre (NW Spanish coast) to a depth of 3,500 m. Before it sank, it released around 60,000 tonnes of a Russian heavy fuel oil (type M-100), containing 22% of aliphatic hydrocarbons, 50% of aromatics, and 28% of resins and asphaltenes. Consequently, the microbial communities in Galician coastal waters were confronted with a highly recalcitrant product. As biodegradation plays a major role in oil depletion, a detailed understanding of the biodegradation processes and the bacterial communities involved is required in order to foresee the environmental damage and to develop effective remediation strategies.

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Chronically hydrocarbon-polluted sites, such as the Spanish NW coasts, are likely to harbor autochthonous microbial communities that can biodegrade oil (Harayama et al. 1999). Hence, intrinsic biodegradation was detected after the *Prestige* oil spill (Medina-Bellver et al. 2005). These communities are necessarily highly heterogeneous as oils are complex mixtures containing hundreds, even thousands, of different compounds and most hydrocarbon-degrading bacteria usually degrade a limited range of hydrocarbons (McKew et al. 2007a).

In previous studies, we characterized microbial communities of oil-polluted sites and compared them to those from non-polluted sites (Alonso-Gutiérrez et al. 2008). In addition, we conducted a successful bioremediation experiment in a polluted beach (Jiménez et al. 2006, 2007), which showed the high biodegradation potential of autochthonous microbiota. However, the evolution of hydrocarbon-degrading populations and the role of specific taxa throughout the degradation of this fuel are yet to be clarified.

This paper evaluates a 60-day biodegradation process carried out by an autochthonous marine bacterial community obtained by enrichment in fuel oil. The inoculum was a sample of oil–water emulsion “chocolate mousse” recovered from the Galician coast a few days after the oil spill. Changes in the total eubacterial community and in the predominant hydrocarbon-degrading [aliphatic-, *n*-alkane, and polycyclic aromatic hydrocarbon (PAH) degrading] populations were tracked by denaturing gradient gel electrophoresis (DGGE) and by a most-probable number method. In addition, some hydrocarbon degradation-related organisms were identified.

Materials and methods

Inoculum and enrichment procedure The emulsified fuel oil sample was collected from the Ría de Ortigueira (A Coruña, Spain) in May 2003. The microbial consortium was obtained from this sample by enrichment in autoclave-sterilized 250-mL Erlenmeyer flasks that contained 50 mL of artificial seawater, approximately 50 mg of the sample and inorganic nutrients (5 mM NH_4NO_3 and 0.35 mM K_2HPO_3 ; Díez et al. 2005). Flasks were shaken at 150 rpm and 25°C. The culture (500 μL) was transferred into fresh flasks every third week for 17 months.

Biodegradation assessment The biodegradation experiment was conducted under the same conditions as the enrichment. In this case, as the 48 flasks contained 100 mg of original (not weathered) fuel oil, the volatile alkane and PAH contents were much higher. Microcosms (33) were inoculated with 500 μL of growing culture, and 15 more

were kept as abiotic controls. Samples were taken at 0, 5, 10, 20, and 60 days for chemical analysis (three inoculated and three control each time) and at 0, 5, 10, 20, 40, and 60 days for microbial analysis (three flasks each time).

Chemical analysis Aliquots of 5–10 mg of dehydrated fuel were dissolved in hexane and fractionated in a previously conditioned cartridge (6 mL hexane, Merck), containing cyanopropyl-silica solid phase ($\text{SiO}_2/\text{C}_3\text{-CN}$, 1.0/0.5 g) (Interchim, Montluçon, France; Alzaga et al. 2004). The aliphatic and aromatic fractions were obtained by eluting with 4.0 mL of hexane (FI) and 5.0 mL of hexane-dichloromethane (1:1) (FII), respectively. Both fractions were analyzed by Gas chromatography–mass spectrometry (Jiménez et al. 2006). The weathering or extent of biodegradation of each compound was measured using $17\alpha(\text{H}),21\beta(\text{-H})$ -hopane (m/z 191) as an internal conservative molecular marker (Prince et al. 1994). The peak areas of the target analytes were measured in the reconstructed ion chromatograms at m/z 85 for aliphatics and at the corresponding molecular ion ratio for the aromatics.

Counting of total heterotrophic and hydrocarbon-degrading microbial populations Bacterial counts of total heterotrophs *n*-hexadecane and PAH degraders were performed by a miniaturized most-probable number (MPN) method in 96-well microtiter plates with eight replicate wells per dilution, as described elsewhere (Alonso-Gutiérrez et al. 2009). The media were corrected to reach 3% NaCl. DNA was extracted from the highest dilute for further DGGE analysis, to provide an image of the predominant hydrocarbon-degrading taxa.

Isolation of culturable strains Total culturable heterotrophs were isolated on artificial seawater agar (Scharlau Chemie SA, Barcelona), supplemented with tenfold diluted Luria–Bertani, and on tryptic soy agar corrected to reach 3% NaCl (Alonso-Gutiérrez et al. 2009). They were incubated at 20°C. *n*-Hexadecane degraders were isolated on mineral agar (BMTM agar, 3% NaCl), supplemented with *n*-hexadecane in the vapor phase (Sei et al. 2003). All isolated strains were stored at -80°C in 20% (v/v) glycerol for subsequent analysis.

Screening of the hydrocarbon-degrading capability of strains To assess hydrocarbon-degrading capacity on solid media, we used artificial seawater agar supplemented with *n*-hexadecane and a mixture of PAHs containing phenanthrene, anthracene, fluorene, and dibenzothiophene (Wrenn and Venosa 1996). Microtiter plates with 200 μL of artificial seawater per well were used in liquid screenings. The carbon sources were *n*-tetradecane, *n*-hexadecane, *n*-heptadecane, *n*-octadecane, pristane and phenanthrene,

dibenzothiophene, pyrene or fluorene, and yeast extract (Alonso-Gutiérrez et al. 2008).

DNA extraction Total community DNA was extracted from the original marine sample, the microcosms with fuel oil and the MPN plates using a PowerSoil DNA extraction kit (MoBio Laboratories, Inc., Solano Beach, CA, USA), following the manufacturer's instructions. Suitable yields of high-molecular weight DNA were obtained (5–20 $\mu\text{g g}^{-1}$ fuel paste), and no further purification was required.

16S rRNA gene clone library Two clone libraries were developed: one from the original sample (before enrichment) and the other from the consortium (after enrichment). Almost complete 16S rRNA genes were PCR-amplified from genomic DNA, using primers F27 and R1492 and Takara Ex Taq DNA polymerase (Takara Bio, Otsu, Shiga, Japan) in 50 μL of reaction medium. Each reaction mixture contained 2.5 U of polymerase, 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM MgCl_2 , 200 μM of each deoxynucleoside triphosphate, 0.5 μM of each primer, and 100 ng of template DNA quantified by a Low DNA Mass Ladder (Gibco BRL, Rockville, MD, USA). The mixtures were subjected to an initial denaturation and enzyme activation step (5 min at 95°C), 40 cycles of 30 s at 96°C, 30 s at 50°C and 2 min at 72°C, followed by an extension step of 10 min at 72°C. All PCR reactions were performed with a Mastercycler (Eppendorf, Hamburg, Germany).

PCR products were ligated in pGEM-T Easy Vector and transformed in competent *Escherichia coli* DH5 α cells, following the manufacturer's protocol. A restriction analysis of clones was performed to identify those that contained a suitable fragment. The PCR products were digested with 5 U of *Eco*RI (Roche) for 2.5 h at 37°C. Fifty recombinant plasmids were extracted with a Wizard Plus SV Minipreps DNA Purification System (Promega) and then reamplified with primers F27 and R1492.

Denaturing gradient gel electrophoresis The V3–V5 variable regions of the 16S rRNA gene were amplified using F341-GC and R907 primers (Yu and Morrison 2004). The F341-GC primer included a GC clamp at the 5' end (5'-CGCCCCGCGCGCCCCGCGCCCGTCCCGCCGCCCCGCCCCG-3'). Each reaction contained the mixture detailed above. After 9 min of initial denaturation and enzyme activation at 94°C, a touchdown thermal profile protocol was executed, and the annealing temperature was decreased by 1°C per cycle from 65°C to 55°C, followed by 20 additional cycles of 1 min denaturation at 94°C, 30 s of primer annealing at 55°C, 1 min of primer extension at 72°C, and a 10-min final primer extension.

Approximately 400 ng of purified PCR product was loaded onto a 6% (w/v) polyacrylamide gel (0.75 mm thick), with denaturing gradients ranging from 40% to 80% (100% denaturant contains 7 M urea and 40% formamide). DGGE was performed in 1 \times Tris–acetate–EDTA (TAE) buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4), using a DGGE-2001 System (CBS Scientific, Del Mar, CA, USA) at 100 V and 60°C for 16 h. DGGE gels were stained for 45 min in 1 \times TAE buffer containing SybrGold (Molecular Probes, Eugene, OR, USA) and scanned by a Bio-Rad molecular imager FX Pro Plus multi-imaging system (Bio-Rad Laboratories, Hercules, CA, USA). DGGE images were processed using Quantity One version 4.5.1 image analysis software (Bio-Rad Laboratories), as reported elsewhere (Jiménez et al. 2007). Predominant DGGE bands were excised with a sterile razor blade, resuspended in 50 μL of sterilized Milli-Q water, stored at 4°C overnight, PCR-reamplified with primers F341 and R907, and cloned (when necessary) in pGEM-T.

Analysis of DGGE images Bacterial diversity analysis and principal component analysis (PCA) of band types were performed, and the relative peak areas were calculated as previously described (Viñas et al. 2005) for the different DGGE profiles to consider possible shifts in the composition of the microbial populations.

Sequencing and phylogenetic analysis Sequencing was accomplished with the ABI PRISM™ Big Dye Terminator Cycle Sequencing Ready Reaction Kit (version 3.1) and an ABI PRISM™ 3700 automated sequencer (Applied Biosystems, Foster City, CA, USA), following the manufacturer's instructions. Sequences were edited and assembled with BioEdit 7.0.9 software (Hall 1999), inspected for the presence of ambiguous base assignments and subjected to the Check Chimera program of the Ribosomal Database Project (Maidak et al. 2000). Subsequently, they were examined and compared with the BLAST alignment tool comparison software (Altschul et al. 1990) and the classifier tool of RDP (Maidak et al. 2000). Sequences were aligned with reference ones obtained from GenBank using ClustalW (Thompson et al. 1994), prior to phylogenetic analysis by Mega4 software (Tamura et al. 2007).

Nucleotide sequence accession numbers The nucleotide sequences identified in this study were deposited in the GenBank database under accession numbers HM171160 to HM171208 (clones from the original marine sample), HM171209 to HM171258 (clones from the consortium), HM171259 to HM171269 (isolates, M81.SA to M81.SK), and JF264441 to JF264462 (DGGE bands).

Results

Adaptation of the microbial community to the presence of a heavy fuel oil

In the original marine sample, the clones belonged mainly to γ -Proteobacteria genera, particularly *Methylophaga* (54%) (Fig. 1). Less abundant were the α -Proteobacteria and Bacteroidetes. However, after enrichment, the consortium was composed mainly of *Alcanivorax* spp. (66%), although *Marinobacter* and *Methylophaga* were also present (Fig. 1).

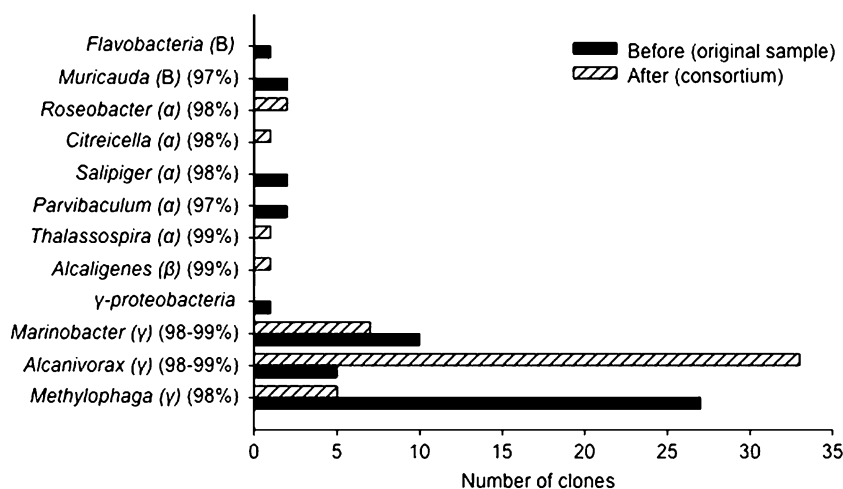
Biodegrading capabilities

The selected consortium caused a rapid and intense degradation of the resolved compounds and the unresolved complex mixture of both fractions. At day 60, ~50% of aliphatics and 40% of aromatics were depleted, compared with the abiotic controls. The *n*-alkanes up to *n*-C₂₇ were fully degraded (Table 1) and the higher molecular-weight alkanes (C₂₈–C₄₀) significantly depleted (75–98%), following well-established patterns for marine oil biodegradation. In the aromatic fraction, biodegradation mainly affected the lighter PAHs (including heteroatomic compounds, e.g., >99% of naphthalenes, phenanthrenes, dibenzothiophenes, and carbazoles), the extent and rates decreasing with alkylation (Table 1), although highly recalcitrant tetra- and penta-cyclic aromatic hydrocarbons (and even their alkyl-derivatives) were also significantly degraded. The degradation of C₃-pyrenes and C₃-chrysenes was 75% and ~60%, respectively.

Growth of the total heterotrophic and hydrocarbon-degrading populations in a biodegradation process

Total heterotrophic populations increased rapidly at the beginning of the incubation from 10⁶ to 10⁸ MPN per milliliter in 10 days and remained stable until the 40th day.

Fig. 1 Distribution of the clones in the 16S rDNA libraries corresponding to the original sample (inoculum) and the M81 consortium. Percent id relates to the identity to the closest related sequence in the GenBank database determined via Blast analysis of ~1,500 bp sequences (*E. coli* position F27-R1492). α , β , and γ represent alpha-, beta-, and gammaproteobacteria, respectively, and B Bacteroidetes



Aliphatic hydrocarbon degraders Two distinct microbial populations were counted: *n*-alkane degraders (grown using *n*-hexadecane as a sole source of carbon and energy) and aliphatic degraders (grown with the aliphatic fraction of a crude oil). The growth of both populations is shown in Fig. 2. At the early stages of incubation, their counts were similar (10⁵ to 10⁸ MPN per milliliter in 10 days). However, at the end of the experiment, whereas the aliphatic-degrading population remained the same size as the total heterotrophs (10⁷), the *n*-alkane degraders were a logarithmic unit lower.

PAH degraders The aromatic-degrading population grew more slowly, and the corresponding counts were significantly lower (10⁴ to 10⁷ MPN per milliliter in about 15 days). To check the suitability of the culture medium used to count this population, the same medium with a 1% NaCl was assayed with several culture samples (Fig. 3).

Changes in the composition of the bacterial community structure throughout the biodegradation process

In the total eubacterial community, 29 different bands were detected (21 sequenced; Fig. 4, Table 2). Profiles of triplicate microcosms were similar at the beginning, with Pearson product-moment correlation coefficients ranging from 0.95±0.02 to 0.978±0.009. At day 60, the variability was much higher (0.84±0.09). α - and γ -Proteobacteria were the predominant taxonomic groups in all the sampling periods (Table 2). Whereas *Thalassospira* sp., *Marinobacter hydrocarbonoclasticus*, *Roseobacter*, *Parvibaculum*, and *Alcaligenes* were detected throughout the incubation, *Alcanivorax* was only present until day 20 and *Lutibacterium* and *Muricauda* only at the final stages. Microbial diversity (*H'*) and richness decreased significantly at the beginning of the incubation (in 5 days;

Table 1 Biodegradation half-lives ($t_{1/2}$) of target alkanes and PAHs and percentages of degradation ($n=3$) at 60 days of incubation

Alkane	$t_{1/2}$ (days)	R^2	Percent degradation	PAH ^a	$t_{1/2}$ (days)	R^2	Percent degradation
C15	6.8	0.905	100	N	2.4	0.742	100
C16	6.3	0.948	100	N1	6.1	0.525	100
C17	6.0	0.947	100	N2	5.4	0.705	100
Pr	7.6	0.903	100	N3	8.2	0.649	93
C18	9.0	0.934	100	P	6.0	0.642	100
Ph	9.6	0.880	100	A	9.0	0.599	100
C19	7.3	0.883	100	P1	8.1	0.845	100
C20	8.4	0.914	100	P2	11.0	0.891	99
C21	8.5	0.721	100	P3	28.8	0.549	89
C22	7.9	0.876	100	D	1.7	0.966	100
C23	8.1	0.812	99	D1	13.0	0.618	98
C24	8.2	0.877	100	D2	21.3	0.622	93
C25	8.3	0.881	99	D3	27.4	0.662	85
C26	8.3	0.886	99	Carb	6.3	0.686	100
C27	9.0	0.921	99	Carb1	7.7	0.814	100
C28	9.0	0.811	98	Carb2	11.0	0.663	97
C29	12.4	0.865	97	Carb3	17.3	0.756	96
C30	13.2	0.848	96	Bcarb	8.7	0.845	100
C31	13.6	0.849	95	Fl	17.2	0.524	93
C32	14.5	0.891	95	Py	18.4	0.498	96
C33	11.9	0.950	97	Py1	29.6	0.637	81
C34	12.4	0.938	96	Py2	30.0	0.525	77
C35	14.7	0.986	93	Py3	38.3	0.597	75
C36	18.4	0.982	88	BA	50.2	0.885	60
C37	21.2	0.839	80	C	29.1	0.825	77
C38	28.3	0.626	76	C1	50.6	0.716	64
C39	33.8	0.636	71	C2	45.3	0.755	65
C40	30.3	0.629	75	C3	53.3	0.662	61

^a This include the following PAHs: *N* naphthalenes; *P* phenanthrenes; *Fl/Py* fluoranthenes/pyrenes; *BA* benz[a]anthracene; *C* chrysenes, and the following heteroaromatic compounds: *D* dibenzothiophenes; *Carb* carbazoles; *Bcarb* benzocarbazoles, with different alkylation levels

Table 3) and then fluctuated slightly until the end of the experiment.

Aliphatic degraders The DGGE profiles of the main aliphatic hydrocarbon-degrading (FI) and *n*-alkane-degrading (C16) populations are shown in Fig. 5. In the FI profiles, 15 distinct bands were detected and sequenced, and matched one band or another of the total community profiles (Table 2). Most of them belonged to α -Proteobacteria, with *Thalassospira* sp. and *Parvibaculum* sp. dominating. *Alcanivorax borkumensis* was only detectable at day 10. By this day, there was a biodiversity peak in both indexes (Table 3), after which they gradually returned to the initial levels. The C16 profiles were much less diverse (seven bands). Biodiversity was extremely low at the beginning (only two different bands, *Thalassospira* sp. and *Alcaligenes* sp.), but it increased by day 20.

Unexpectedly, *Alcanivorax* spp. was not detected in any of the profiles.

PAH degraders Twenty-two different bands were detected (15 sequenced) in the PAH degraders profiles (Fig. 6), one of which was new (P16, related to *Mesorhizobium* sp.). Bands P8 (*Thalassospira tepidiphila*), P9 (*Roseobacter* sp.), and P11 (*Muricauda aquimarina*) were not present in any of the FI or C16 profiles. Richness in microbial diversity increased at the beginning of the experiment. The Shannon–Weaver index decreased, but by the 60th day, it was higher than at the beginning.

Principal component analysis Figure 7 shows the scatter plot for the first two components (PC1 and PC2, 47.5% of variability). The loadings for each band in PC1 and PC2 are shown in Table 2.

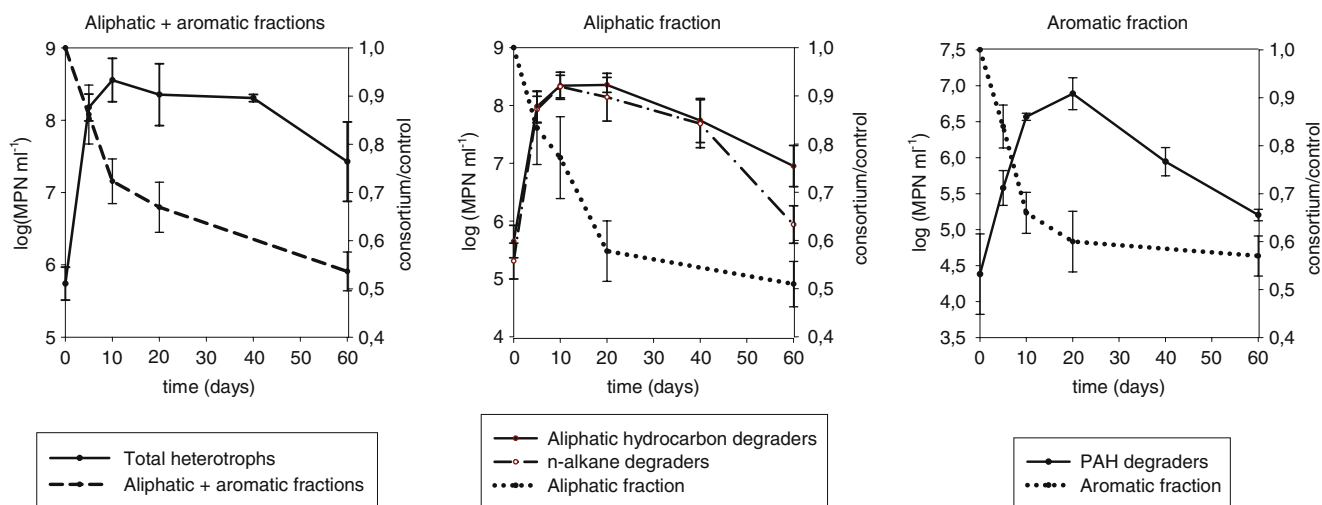


Fig. 2 Growth of the heterotrophic, *n*-alkane-, aliphatic-, and PAH-degrading populations and relative depletion of the aliphatic and aromatic fractions of oil in the inoculated microcosms with respect to

the sterile controls over the 60 days of incubation. Error bars indicate 95% confidence interval and SD, respectively ($n=3$)

Isolation and identification of cultivable organisms

Eleven bacterial strains were isolated in different cultivation media; they were closely related to: *Paracoccus yeei*, *Marteella mediterranea* and *Mesorhizobium* sp., *Alcanivorax* spp., four strains; *Marinobacter*, two strains; *Muricauda aquamarina*, and *Staphylococcus pasteurii*. Almost all of them (*Marteella mediterranea* and *Mesorhizobium* were not tested) grew using *n*-alkanes as the sole carbon and energy sources, but only the *Alcanivorax* species grew on the isoprenoid pristane. Five strains, two of *Alcanivorax* and the ones affiliated to *Paracoccus*, *Muricauda*, and *Staphylococcus*, transformed the PAHs tested.

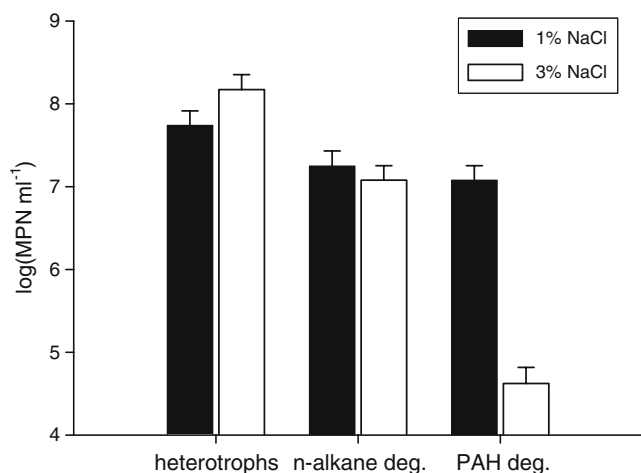


Fig. 3 Effect of salinity in the growth of total heterotrophic, *n*-alkane- and PAH-degrading bacteria. Error bars correspond to the 95% confidence interval ($n=3$)

Discussion

Changes in the community structure within the enrichment process

The prevalence of genus *Methylophaga* in the original sample could be due to the presence in the ocean of C_1 or

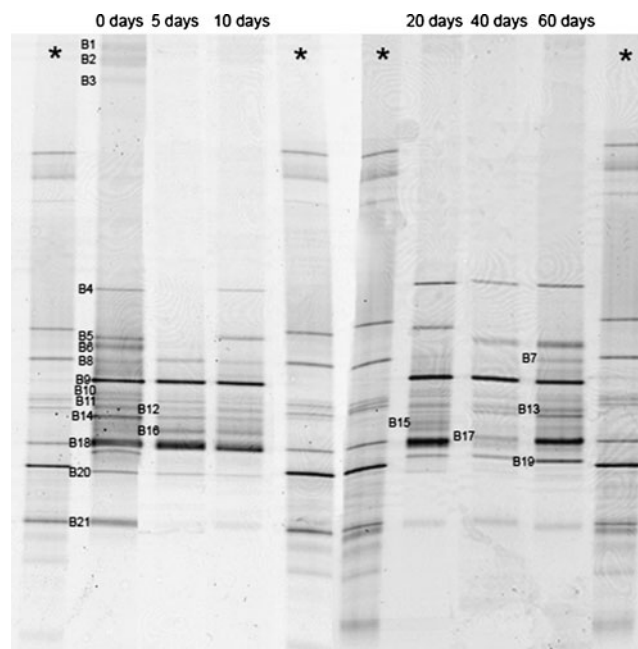


Fig. 4 DGGE profiles of PCR-amplified 16S rRNA genes from microcosms samples showing the changes in the composition of the bacterial community at different sampling periods. Lanes marked with asterisks correspond to the DGGE markers. The codes (B1–B21) indicate predominant bands that were excised and sequenced (~500 bp; see Table 2 for the description). Bacterial diversity was calculated (Table 3), and a PCA analysis was performed to assess possible shifts in its composition (Fig. 7)

Table 2 Properties of DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms in the GenBank database determined via BLAST analysis of ~500 bp sequences (*E. coli* positions F341–R907)

PCA ^a band	PC1 loading	PC2 loading	Accession number	Closest organism in GenBank database (accession no) (% similarity)	Phylogenetic group ^b	Total DNA gel band ^c	Aliphatic deg. gel band ^c	PAH degraders gel band ^c
1	0.868	-0.116	JF264441	Rhodobacteraceae bacterium p1rb1 (FN811297) (98%)	Rhodobacteraceae (α)	B1 (0, 5)	–	–
2	0.858	-0.077	JF264442	Rhodobacteraceae bacterium p1rb1 (FN811297) (99%)	Rhodobacteraceae (α)	B3 (0)	–	–
3	0.618	-0.143	JF264443	Uncultured Rhodobacteraceae bacterium (DQ870539) (98%)	Rhodobacteraceae (α)	B4 (0)	–	–
5	0.051	0.730	JF264444	Sphingobacteriales bacterium Kimo37 (AB260041) (98%)	Sphingobacteriales (Bacteroidetes)	B4 (0, 10, 20, 40, 60)	A1 (FI 10)	P1 (all)
6	0.209	0.087	JF264445	<i>Alcanivorax borkumensis</i> (EU603451) (99%)	Alcanivoraceae (γ)	B5 (0, 10, 20)	A2 (FI 10)	P2 (all)
7	0.310	0.735	JF264446	<i>Lutibacterium amuloderans</i> (AY026916) (98%)	Sphingomonadaceae (α)	B6 (0, 20, 40, 60)	A3 (FI 10)	P3 (all)
8	-0.152	0.558	JF264447	<i>Alpha proteobacterium</i> 36 G5 (EF033448) (97%)	Rhodobacteraceae (α)	B7 (20, 40, 60)	A4 (FI 10)	P4 (all)
9	0.789	-0.276	JF264448	<i>Roseobacter</i> sp. RED68 (AY136132) (98%)	Rhodobacteraceae (α)	B8 (0, 5, 10)	A5 (FI 10)	–
11	0.262	0.643	JF264449	<i>Marinobacter hydrocarbonoclasticus</i> BBD-216-4 g (GQ901059) (98%)	Alteromonadaceae (γ)	B9 (all)	A6 (FI 10, C16 60)	P5 (all)
12	-0.113	-0.151	JF264450	Uncultured Rhodobacteraceae bacterium (FJ425647) (99%)	Rhodobacteraceae (α)	B10 (0, 10, 20)	A7 (FI 10, C16 20, 60)	P6 (all)
13	0.340	0.431	JF264451	Uncultured alpha proteobacterium (AB367473) (99%)	Rhodobacteraceae (α)	B11 (all)	A8 (FI 10)	P7 (10, 20, 60)
14	0.695	-0.366	JF264452	<i>Thalassospira</i> sp. QDHT-16 (FJ210811) (100%)	Rhodospirillaceae (α)	B12 (0, 5, 10)	A9 (FI 10, 20, C16 60)	–
15	-0.409	0.045	JF264453	<i>Sphingopyxis flavimaris</i> (FR691421) (99%)	Sphingomonadaceae (α)	B13 (20, 40, 60)	A10 (FI 0, 10, 20, C16 20, 60)	–
16	0.717	0.206	JF264454	<i>Thalassospira tepidiphila</i> (AB265822) (99%)	Rhodospirillaceae (α)	B14 (all)	–	P8 (all)
17	0.344	0.678	JF264455	<i>Roseobacter</i> sp. AP12 (EU374898) (98%)	Rhodobacteraceae (α)	B15 (0, 10, 20, 40, 60)	–	P9 (all)
18	0.119	-0.326	JF264456	Uncultured Rhodospirillales (EU361429) (98%)	Rhodospirillaceae (α)	B16 (0, 5, 10)	A11 (FI 20)	P10 (10, 20, 60)
19	-0.174	0.516	JF264457	<i>Muricauda aquimarina</i> (EU440979) (99%)	Flavobacteriaceae (Bacteroidetes)	B17 (40, 60)	–	P11 (10, 20, 60)
20	-0.186	-0.636	JF264458	<i>Thalassospira</i> sp. 1-1B (AB265822) (99%)	Rhodospirillaceae (α)	B18 (all)	A12 (all)	P12 (0, 20, 60)
21	-0.240	-0.178	JF264459	<i>Alcaligenes</i> sp. isolate CCUG 36768 (AJ133493) (99%)	Alcaligenaceae (β)	B19 (all)	A13 (all)	P13 (10, 60)
22	0.045	0.086	JF264460	Uncultured <i>Rhodobacteraceae</i> bacterium (DQ870525) (97%)	Rhodobacteraceae (α)	B20 (0, 5, 10, 20, 40)	A14 (FI 10, C16 20)	P14 (0, 10)
23	-0.313	-0.297	JF264461	<i>Parvibaculum</i> sp. Psc10 (EU930870) (99%)	Phyllobacteriaceae (α)	B21 (all)	A15 (FI 0, 10, 20, 60, C16 20, 60)	P15 (10, 20, 60)
24	-0.107	0.838	JF264462	<i>Mesorhizobium</i> sp. 8.98 (AY870682) (99%)	Phyllobacteriaceae (α)	–	–	P16 (all)

^a PCA bands 4 and 10 were used for PCA analysis, but they could not be sequenced. Nevertheless, they did not contribute strongly to any of the first two components, as their loadings were ~0.

^b Sequences were matched with the closest relative from the Ribosomal Database Project. B corresponds to Bacteroidetes and α, β, and γ to gamma-, beta-, and alphaproteobacteria, respectively.

^c All the bands in the degraders DGGE profiles matched one or another of the total DNA profiled, except P16. In brackets are profiles in which the band has been detected

Table 3 Shannon–Weaver diversity indexes calculated for DGGE profiles and numbers of DGGE bands detected

	Day	Shannon–Weaver diversity index (H')	Number of DGGE bands (richness)
Total DNA	0	2.67±0.02	22
	5	1.99±0.02	11
	10	2.35±0.07	15
	20	2.00±0.05	14
	40	2.00±0.09	11
	60	2.17±0.07	13
Aliphatic hydrocarbon degraders	0	1.26	4
	10	2.48	15
	20	1.45	6
	60	1.03	4
<i>n</i> -Alkane degraders	0	0.67	2
	10	0.58	2
	20	1.43	6
	60	1.65	7
PAH degraders	0	2.26	13
	10	1.92	20
	20	2.08	17
	60	2.32	20

For the total DNA, H' represents the mean value±the SD ($n=3$)

C₂ compounds, like methanol and methylamine (Gibb and Hatton 2004), which are carbon sources for this genus (Neufeld et al. 2007). Nevertheless, this microorganism has been reported in several hydrocarbon-polluted sites (Röling et al. 2002, Schwermer et al. 2008). Thus, its potential role in the degradation of the fuel has to be considered, particularly because this fuel has a high sulfur content (2.28%), and species like *Methylophaga sulfidivorans* use dimethyl sulfur as carbon source (de Zwart et al. 1996).

According to previous studies (Kasai et al. 2002; McKew et al. 2007b), during the enrichment process, the community evolved toward the predominance of two hydrocarbonoclastic genera (*Alcanivorax* and *Marinobacter*), considered to be early colonizers after an oil spill, as primary degraders of the most biodegradable fractions (Röling et al. 2002). They were thus found in the Japan Sea just after the *Nakhodka* spill (Kasai et al. 2002) and have been related to hydrocarbon biodegradation in other studies (Schleheck et al. 2004; Alonso-Gutiérrez et al. 2008). In addition, *Alcanivorax* predominates in oil-degrading communities when nutrients are abundant (Yakimov et al. 2007) and was one of the main microorganisms found just after the spill in the Ría de Vigo (Alonso-Gutiérrez et al. 2008), which has large concentrations of dissolved N and P (around 0.6 and 0.06 mg L⁻¹, respectively; Nogueira et al. 1997).

Another interesting change was the detection (which may reflect an increase) of other genera related to hydrocarbon degradation (*Thalassospira*, *Alcaligenes*, *Citricella*, or *Roseobacter*).

Biodegrading capabilities of the consortium

The *Prestige's* fuel oil was a complex mixture. Thus, by enriching the original sample, we obtained a rich and highly efficient oil-degrading bacterial consortium, able to cause an exceptionally intense biodegradation in the aliphatic and aromatic fractions. Particularly remarkable was the degradation the alkylated PAHs, including C₃-chrysenes (a recalcitrant group of compounds). In addition, the consortium degraded the carbazole compounds extensively, at similar rates than phenanthrenes and dibenzothiophenes. These heterocyclic hydrocarbons have carcinogenic and toxic properties (Tsuda et al. 1982), and their water solubility may make them bioavailable. However, their biodegradation has been only poorly assessed. Nevertheless, carbazole and alkylcarbazoles may supply nitrogen for the degrading microbiota (Huang et al. 2003).

Recently, only minor biodegradation was described for the strain API of *Mycobacterium* sp., a well-known aromatic hydrocarbon degrader, growing on the same fuel in the same incubation time (Vila and Grifoll, 2009). Nevertheless, the potential capacity of a consortium is usually higher than that of a pure strain. In this respect, Gallego et al. (2007) combined bacteria with different metabolic capabilities in order to degrade a heavy oil refinery tank bottom sludge. They degraded 43% of tricyclic PAHs in 10 days, whereas our enrichment consortium degraded 70–75% of C₃-phenanthrenes and

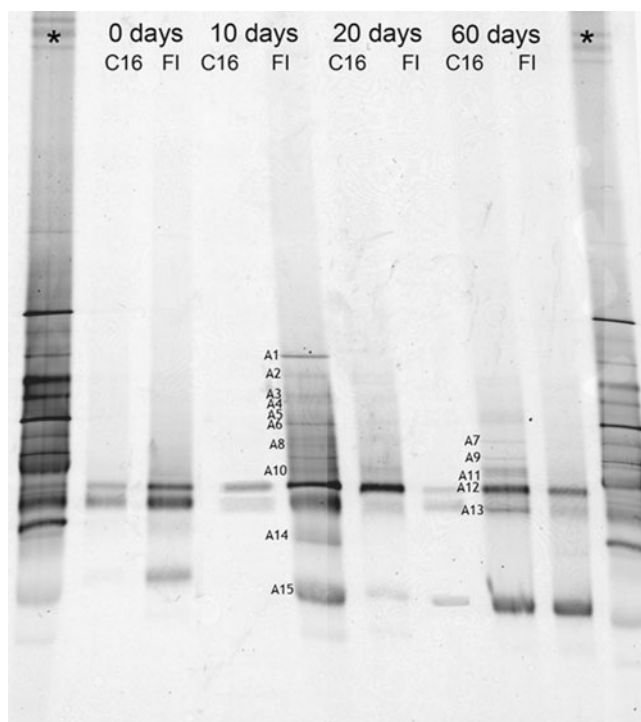


Fig. 5 DGGE profiles of PCR-amplified 16s rRNA genes from microcosms samples showing the changes in the composition of the predominant *n*-alkane-degraders (C16) and aliphatic-degraders (FI) at different sampling periods. Lanes marked with asterisks correspond to the DGGE markers. The codes (A1–A13) indicate predominant bands that were excised and sequenced (~500 bp; see Table 2 for the description). Bacterial diversity was calculated (Table 3), and a PCA analysis was performed to assess possible shifts in its composition (Fig. 7)

C₃-dibenzothiophenes, ~60 C₃-pyrenes and ~40% C₃-chrysenes in the same time (results not shown).

Enrichment procedures can provide a microbial community with potentially higher efficiency in degrading identified and non-identified components. In addition, degraders of dead-end products accumulated in co-metabolism processes (Casellas et al. 1998) are likely to be selected, too, which can help avoid inhibitory effects that might prevent further PAH biodegradation. In this respect, in the above-mentioned study with *Mycobacterium* sp., several intermediate metabolites were detected.

Microbial community dynamics

The predominance of α -Proteobacteria and γ -Proteobacteria groups through the biodegradation experiment is consistent with previous results from coasts affected by the *Prestige* oil spill (Jiménez et al. 2007; Alonso-Gutiérrez et al. 2009). Røling et al. (2002) reported that in artificial media the γ -Proteobacteria subclass is replaced by α -Proteobacteria. Nonetheless, the relative importance of α -Proteobacteria decreased at the end of the incubation period.

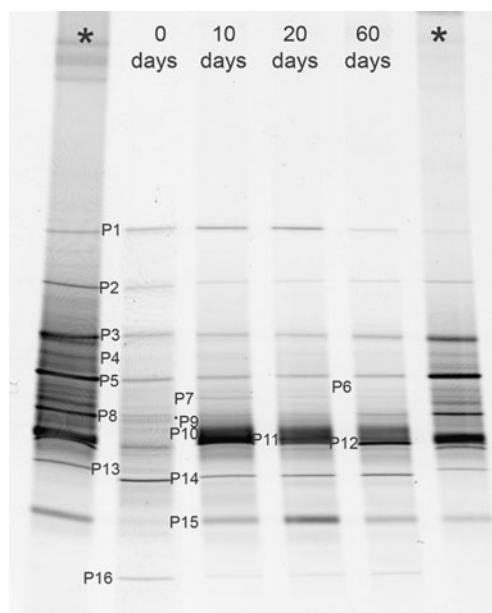


Fig. 6 DGGE profiles of PCR-amplified 16s rRNA genes from microcosms samples showing the evolution of the structure of the predominant PAH-degraders at different sampling periods. Lanes marked with asterisks correspond to the DGGE markers. The codes (P1–P16) indicate predominant bands that were excised and sequenced (~500 bp; see Table 2 for the description). Bacterial diversity was calculated (Table 3), and a PCA analysis was performed to assess possible shifts in its composition (Fig. 7)

The PCA revealed changes in the microbial structure throughout the incubation, which reflect the changes in oil composition: from a labile mixture, rich in *n*-alkanes and low molecular weight PAHs, to a weathered product, enriched in recalcitrant compounds. In PC1, samples were ordered and clustered according to the incubation time (Fig. 7). The bands with the highest loadings corresponded

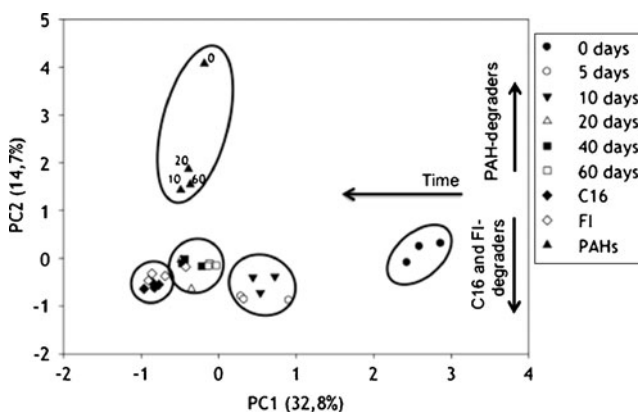


Fig. 7 Principal component analysis scatter plot for the first two components (PC1, 32.8%; PC2, 14.7% of variability) of DGGE profiles of PCR-amplified 16S rDNA, representing the differences in the structures of the total bacterial community and the *n*-alkane-(C16), aliphatic-(FI), and PAH-degrading (PAHs) populations throughout the biodegradation process. The samples for total DNA at day 0 were significantly different ($P < 0.05$) from the rest

to the *Rhodobacteraceae* group and *Thalassospira* (Table 2). These microorganisms may thus play a predominant role at the beginning of the degradation process. The most negative loadings were those of bands corresponding to *Sphingopyxis*, *Parvibaculum*, and *Alcaligenes*, which were more important in the final stages.

In addition, a microorganism related to *Alcanivorax borkumensis* was mainly detected in the first 20 days, in which the maximum depletion of the most labile components takes place. The ability to produce biosurfactants (Abraham et al. 1998) might help *Alcanivorax* being a first colonizer after a spill. However an enhancement of bioavailability (caused by oil emulsification) could promote the growth of other efficient degrading taxa (Gertler et al. 2009). This might explain the detection of these bacteria just after the *Prestige* oil spill took place (Alonso-Gutiérrez et al. 2009) and its absence in the later surveys (Jiménez et al. 2007).

Worthy of note is the absence of the Gram-positive *Rhodococcus*, present in various locations affected by the *Prestige* oil spill (Jiménez et al. 2007; Alonso-Gutiérrez et al. 2009). Nevertheless, it is not found among the prevailing bacteria in recent hydrocarbon-polluted environments (Margesin et al. 2003), and, in vitro, it is outcompeted by fast-growing taxa such as *Alcanivorax* or *Thalassospira*.

Microorganisms relevant to biodegradation

The community had a wide variety of hydrocarbon-degrading bacteria able to grow in aliphatic and aromatic hydrocarbons as the sole carbon and energy sources, most of which grew in all of the microtiter plates used for MPN counts: *Marinobacter*, *Alcanivorax*, *Thalassospira*, *Lutibacterium*, *Parvibaculum*, *Roseobacter*, and *Alcaligenes* (Gauthier et al. 1992; Chung and King 2001; Dutta and Harayama 2001; Schleheck et al. 2004; McKew et al. 2007a; Kodama et al. 2008).

In this respect, whereas salinity does not seem to affect the growth of total heterotrophs and aliphatic degraders (Fig. 3), it strongly affects the counting of PAH degraders, which was unexpected, since the consortium is a marine community. Nevertheless, several studies have reported a repression of PAH degradation with increasing salinity (Kästner et al. 1998, Tam et al. 2002).

In addition, two of the isolated *Alcanivorax* spp. not only grew in alkanes as the sole carbon and energy sources but also in PAHs. Hence, these strains are highly adapted to the degradation of oil or complex mixtures in which both aliphatic and aromatic hydrocarbons are abundant.

Genera *Thalassospira* and *Roseobacter* were detected in all the profiles, although they have been related to *n*-alkanes degradation (Hara et al. 2003; McKew et al. 2007a;

Kodama et al. 2008). In this respect, PCA analysis associated band 20 (*Thalassospira* sp., with a low loading for PC2) to aliphatic degradation.

In relation to aliphatic hydrocarbon degradation, the FI profiles contained not only all the bands of C16 profiles but also some new bands. Thus, we highly recommend the replacement of *n*-hexadecane by a complex substrate such as the aliphatic fraction as sole source of carbon and energy for counting the alkane degraders. One of these bands corresponded to *Alcanivorax borkumensis*. Interestingly, although *Alcanivorax* species (including all of our isolates) degrade *n*-hexadecane, no bands corresponding to this genus were found in the C16 profiles. McKew et al. (2007a) suggested that in single-substrate *n*-alkane microcosms (with *n*-tetradecane or *n*-hexadecane), *Alcanivorax* could be out-competed by other *n*-alkane-degrading taxa. However, this genus is more efficient in branched alkanes (such as pristane or phytane) degradation (Hara et al. 2003, McKew et al. 2007a). In fact, among all the isolates obtained in the present study, only the ones belonging to *Alcanivorax* spp. grew in pristane. This could explain its major occurrence in oil-enriched communities.

Mesorhizobium and *Muricauda aquimarina* were only detected in the PAH's MPN medium. However, both microorganisms were isolated, and *Muricauda* not only transformed all the PAHs tested but also grew with all of the short *n*-alkanes (up to *n*-C₁₈). The relationship of *Muricauda aquimarina* to hydrocarbon biodegradation had not been proven before, unlike *Mesorhizobium* sp., which was recently related to the degradation of phenanthrene and fluorene (Wang et al. 2008). In this respect, according to their loadings in PC2, *Mesorhizobium*, *Lutibacterium anuloderans*, and a *Sphingobacteriales* might be related to PAH degradation.

Our results suggest that α and γ -Proteobacteria are the predominant oil-degrading taxonomic groups. In addition, key microorganisms involved in the degradation of a heavy fuel oil were identified and the hypothesis that *Alcanivorax* plays an important role in branched alkane degradation is reinforced. The *Thalassospira* and *Roseobacter* genera may be important to aliphatic hydrocarbon degradation, whereas *Mesorhizobium* and *Muricauda* are related to PAH degradation. In conclusion, we strongly recommend the review of established methods for the counting of aliphatic and aromatic hydrocarbon-degrading bacteria, as the carbon source is a key factor when counting aliphatic hydrocarbon degraders and PAH-degrader counts are significantly affected by salinity.

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References

- Abraham WR, Meyer H, Yakimov M (1998) Novel glycine containing glucolipids from the alkane using bacterium *Alcanivorax borkumensis*. *Biochim Biophys Acta* 1393:57–62
- Alonso-Gutiérrez J, Costa MM, Figueras A, Albaigés J, Viñas M, Solanas AM, Novoa B (2008) *Alcanivorax* strain detected among the cultured bacterial community from sediments affected by the 'Prestige' oil spill. *Mar Ecol Prog Ser* 362:25–36
- Alonso-Gutiérrez J, Figueras A, Albaigés J, Jiménez N, Viñas M, Solanas AM, Novoa B (2009) Bacterial communities from shoreline environments (Costa da Morte, Northwestern Spain) affected by the Prestige oil spill. *Appl Environ Microbiol* 75(11):3407–3418
- Altschul S, Gish W, Miller W, Myers E, Lipman J (1990) Basic local alignment search tool. *J Mol Biol* 215(3):403–410
- Alzaga R, Montuori P, Ortiz L, Bayona JM, Albaigés J (2004) (2003) Fast solid-phase extraction–gas chromatography–mass spectrometry procedure for oil fingerprinting application to the Prestige oil spill. *J Chromatogr A* 1025:133–138
- Casellas M, Grifoll M, Sabaté J, Solanas AM (1998) Isolation and characterization of a 9-fluorenone-degrading bacterial strain and its role in synergistic degradation of fluorene by a consortium. *Can J Microbiol* 44(8):734–742
- Chung W, King G (2001) Isolation, characterization, and polyaromatic hydrocarbon degradation potential of aerobic bacteria from marine macrofaunal burrow sediments and description of *Lutibacterium anuloderans* gen. nov., sp. nov., and *Cycloclasticus spirillensus* sp. nov. *Appl Environ Microbiol* 67(12):5585–5592
- de Zwart J, Nelisse P, Kuenen J (1996) Isolation and characterization of *Methylophaga sulfidovorans* sp. nov.: an obligately methylotrophic, aerobic, dimethylsulfide oxidizing bacterium from a microbial mat. *FEMS Microbiol Ecol* 20(4):261–270
- Diez 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(9):2203–2217
- Dutta T, Harayama S (2001) Biodegradation of n-alkylcycloalkanes and n-alkylbenzenes via new pathways in *Alcanivorax* sp. strain MBIC 4326. *Appl Environ Microbiol* 67(4):1970–1974
- Gallego JLR, García-Martínez MJ, Llamas JF, Belloch C, Peláez AI, Sánchez J (2007) Biodegradation of oil tank bottom sludge using microbial consortia. *Biodegradation* 18:269–281
- Gauthier MJ, Lafay B, Christen R, Fernandez L, Acquaviva M, Bonin P, Bertrand JC (1992) *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int J Syst Evol Microbiol* 42:568–576
- Gertler C, Gerdtz G, Timmis KN, Yakimov MM, Golyshin PN (2009) Populations of heavy fuel oil-degrading marine microbial community in presence of oil sorbent materials. *J Appl Microbiol* 107(2):590–605
- Gibb S, Hatton A (2004) The occurrence and distribution of trimethylamine-N-oxide in Antarctic coastal waters. *Mar Chem* 91(1–4):65–75
- Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* 41:95–98
- Hara A, Syutsubo K, Harayama S (2003) *Alcanivorax* which prevails in oil-contaminated seawater exhibits broad substrate specificity for alkane degradation. *Environ Microbiol* 5(9):746–753
- Harayama S, Kishira H, Kasai Y, Shutsubo K (1999) Petroleum biodegradation in marine environments. *J Molec Microbiol Biotechnol* 1(1):63–70
- Huang H, Bowler BFJ, Zhang Z, Oldenburg TBP, Larter SR (2003) Influence of biodegradation on carbazole and benzocarbazole distributions in oil columns from the Liaohe basin, NE China. *Org Geochem* 34:951–969
- Jiménez N, Viñas M, Bayona JM, Albaigés J, Solanas AM (2007) The Prestige oil spill: bacterial community dynamics during a field biostimulation assay. *Appl Microbiol Biotechnol* 77:935–945
- Jiménez N, Viñas M, Sabaté J, Diez S, Bayona JM, Solanas AM, Albaigés J (2006) The Prestige oil spill. 2. Enhanced biodegradation of a heavy fuel oil under field conditions by the use of an oleophilic fertilizer. *Environ Sci Technol* 40:2578–2585
- Kasai Y, Kishira H, Sasaki T, Syutsubo K, Watanabe K, Harayama S (2002) Predominant growth of *Alcanivorax* strains in oil-contaminated and nutrient-supplemented sea water. *Environ Microbiol* 4(3):141–147
- Kästner M, Breuer-Jammali M, Mahro B (1998) Impact of inoculation protocols, salinity, and pH on the degradation of polycyclic aromatic hydrocarbons (PAHs) and survival of PAH-degrading bacteria introduced into soil. *Appl Environ Microbiol* 64(1):359
- Kodama Y, Sutiknowati L, Ueki A, Watanabe K (2008) *Thalassospira tepidiphila* sp. nov., a polycyclic aromatic hydrocarbon-degrading bacterium isolated from seawater. *Int J Syst Evol Microbiol* 58:711–715
- Maidak BL, Cole JR, Lilburn TG, Parker CT, Saxman PR, Stredwick JM, Garrity GM, Li B, Olsen GJ, Pramanik S, Schmidt TM, Tiedje JM (2000) The RDP (ribosomal database project) continues. *Nucleic Acids Res* 28(1):173–174
- Margesin R, Labbé D, Schinner F, Greer C, Whyte L (2003) Characterization of hydrocarbon-degrading microbial populations in contaminated and pristine alpine soils. *Appl Environ Microbiol* 69(6):3085–3092
- McKew BA, Coulon F, Osborn A, Timmis KN, McGenity TJ (2007a) Determining the identity and roles of oil-metabolizing marine bacteria from the Thames estuary, UK. *Environ Microbiol* 9(1):165–176
- McKew BA, Coulon F, Yakimov MM, Denaro R, Genovese M, Smith CJ, Osborn AM, Timmis KN, McGenity TJ (2007b) Efficacy of intervention strategies for bioremediation of crude oil in marine systems and effects on indigenous hydrocarbonoclastic bacteria. *Environ Microbiol* 9(6):1562–1571
- Medina-Bellver JI, Marin P, Delgado A, Rodríguez-Sánchez A, Reyes E, Ramos JL, Marqués S (2005) Evidence for in situ crude oil biodegradation after the Prestige oil spill. *Environ Microbiol* 7(6):773–779
- Neufeld J, Schäfer H, Cox M, Boden R, McDonald I, Murrell J (2007) Stable-isotope probing implicates *Methylophaga* spp and novel Gammaproteobacteria in marine methanol and methylamine metabolism. *ISME J* 1:480–491
- Nogueira E, Pérez F, Ríos A (1997) Seasonal patterns and long-term trends in an estuarine upwelling ecosystem (Ría de Vigo, NW Spain). *Estuar Coast Shelf Sci* 44(3):285–300
- Prince RC, Elmendorf DL, Lute JR, Hsu CS, Haith CE, Senius JD, Dechert GJ, Douglas GS, Butler EL (1994) 17 α (H)-21 β (H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. *Environ Sci Technol* 28(1):142–145
- Röling WFM, Milner MG, Jones DM, Lee K, Daniel F, Swannell RPJ, Head IM (2002) Robust hydrocarbon degradation and dynamics of bacterial communities using nutrient-enhanced oil spill bioremediation. *Appl Environ Microbiol* 68(11):5537–5548
- Schleheck D, Tindall B, Rosselló-Mora R, Cook A (2004) *Parvibaculum lavamentivorans* gen. nov., sp. nov., a novel heterotroph that initiates catabolism of linear alkylbenzenesulfonate. *Int J Syst Evol Microbiol* 54:1489–1497
- Schwermer CU, Lavik G, Abed RMM, Dunsmore B, Ferdelman TG, Stoodley P, Gieseke A, de Beer D (2008) Impact of nitrate on the structure and function of bacterial biofilm communities in

- pipelines used for injection of seawater into oil fields. *Appl Environ Microbiol* 74(9):2841–2851
- Sei K, Sugimoto Y, Mori K, Maki H, Kohno T (2003) Monitoring of alkane-degrading bacteria in a sea-water microcosm during crude oil degradation by polymerase chain reaction based on alkane-catabolic genes. *Environ Microbiol* 5(6):517–522
- Tam N, Guo C, Yau W, Wong Y (2002) Preliminary study on biodegradation of phenanthrene by bacteria isolated from mangrove sediments in Hong Kong. *Mar Pollut Bull* 45(1–12):316–324
- Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol Biol Evol* 24(8):1596–1599
- Thompson J, Higgins D, Gibson T (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix. *Nucleic Acids Res* 22:4673–4680
- Tsuda H, Hagiwara A, Shibata M, Ito N (1982) Carcinogenic effect of carbazole in the liver of a (C57BL/6NxC3H/HeN)F1 mice. *J Natl Cancer Inst* 69:1389–1393
- 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 Microbiol* 75(19):6232–6239
- Viñas M, Sabaté J, Espuny M, Solanas AM (2005) Bacterial community dynamics and polycyclic aromatic hydrocarbon degradation during bioremediation of heavily creosote-contaminated soil. *Appl Environ Microbiol* 71(11):7008–7018
- 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(8):1948–1963
- Wrenn B, Venosa A (1996) Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. *Can J Microbiol* 42:252–258
- Yakimov MM, Timmis KN, Golyshin P (2007) Obligate oil-degrading marine bacteria. *Curr Opin Biotechnol* 18(3):257–266
- Yu Z, Morrison M (2004) Comparisons of different hypervariable regions of *rrs* genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 70(8):4800–4806