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Natural attenuation and bioremediation of *Prestige* fuel oil along the Atlantic coast of Galicia (Spain)

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Abstract

Heavy fuel oil spilled from the oil tanker *Prestige* in November 2002 affected hundreds of km of Spanish shoreline. We carried out a two year study at two highly contaminated sites in order to monitor natural attenuation of the residues coating shore rocks and to test the effectiveness of bioremediation with an oleophilic fertilizer (S200). The methodology included an innovative approach for oil load calculation (based on image analysis techniques), the analysis of the fate of hydrocarbons by means of chemical biomarkers and different microbiological techniques for isolating and examining hydrocarbon degraders.

Considerable hydrocarbon depletion from shorelines (≈100% for light-medium linear alkanes and ≈35% for aromatics such as pyrene) was observed within the first months after the spill. However, this natural attenuation effect dramatically slowed down in the following year, although partial enhancement (especially for the aromatic fraction) was attained as a result of the application of S200 to stimulate indigenous microorganisms. In addition, one of the main outcomes of this work concerns the remarkable hydrocarbon depletion achieved at points where fresh water flowed through the shore rocks. The study of samples collected where this natural attenuation phenomenon took place made it possible to isolate heterotrophic bacteria and fungi that are likely to cooperate with cyanobacteria in hydrocarbon biodegradation. The overall results strongly advocate the implementation of new bioremediation approaches, including alternatives such as the use of natural fresh water to irrigate polluted areas.

1. Introduction

In November 2002, the oil tanker *Prestige* spilled an as yet undetermined amount of heavy fuel no. 6 that ended up coating hundreds of km of the Atlantic and Cantabrian coasts in Spain (Fig. 1). At many sites, mechanical removal of the fuel was

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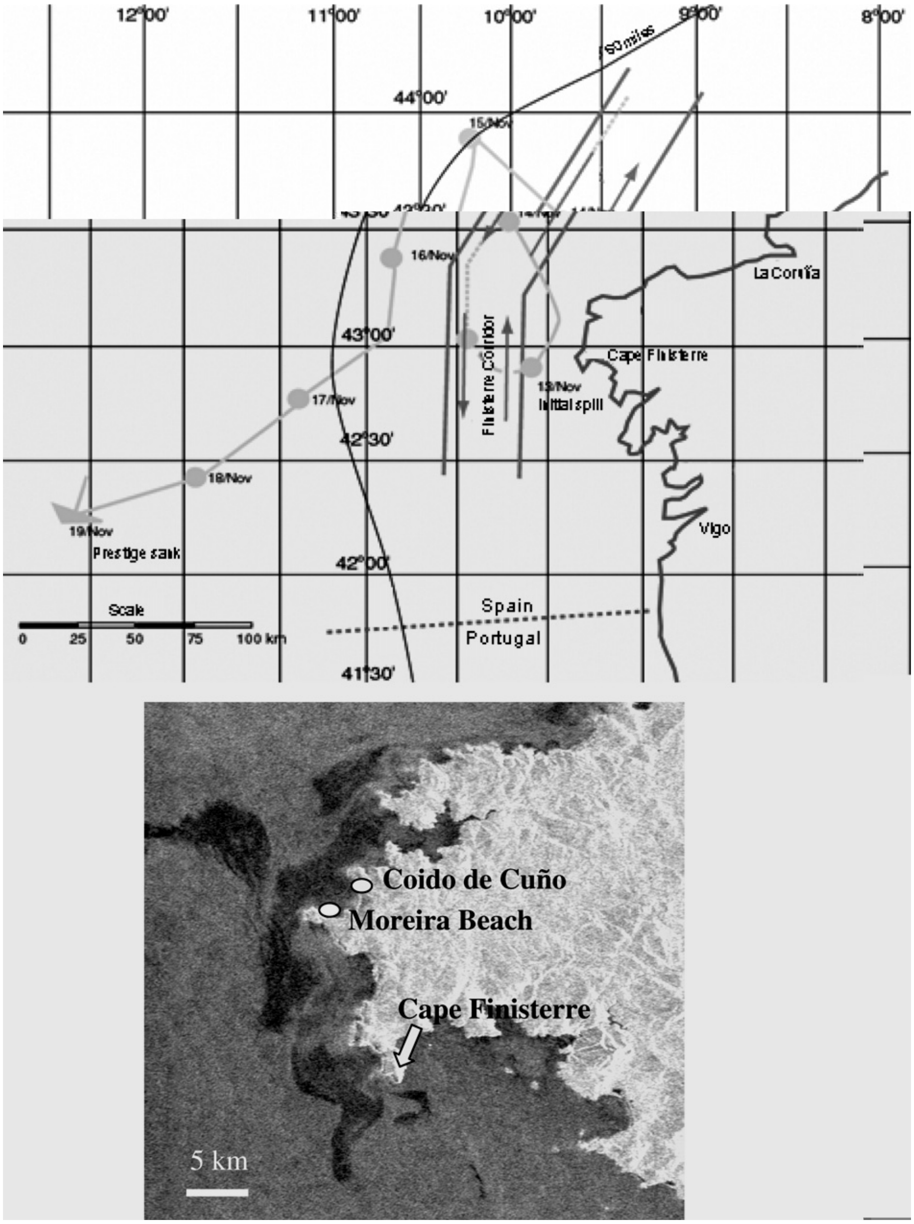

Fig. 1. Top-pathway followed by *Prestige* while spilling fuel oil no. 6 (13–19 November, 2002; the tanker finally sank 130 miles from Cape Finisterre, Spain). Bottom-ENVISAT radar image (European Space Agency) of the shorelines most affected by the arrival of *Prestige* fuel (black areas in the sea) and location of study sites.

undertaken manually on sandy beaches and using hot pressurized water washing wherever the fuel oil was firmly adhering to the surface of large boulders and man made structures. All these systems had limited effectiveness; however, at sites subject to special protection for environmental reasons, and along shorelines where the average grain size was pebble or cobble size, these techniques were not at all adequate. Therefore, alternatives based on bioremediation and/or monitoring of natural attenuation were recommended for certain selected locations.

After an initial process of emulsification, dispersion and partial dissolution while floating on marine water (Daling and Strøm, 1999; Moldestadt et al., 2004), the *Prestige* fuel oil reached shore, where it underwent a variety of natural attenuation (weathering) processes including evaporation, photochemical oxidation and biodegradation (Zhu et al., 2001). Thus, the study of specific areas of the polluted sites where environmental parameters (nutrients, humidity, oxygenation, etc.) are optimal for natural attenuation can provide useful information for the design of enhanced bioremediation techniques and, consequently, was one of main scopes of this work. However, the heterogeneity of the sediments involved and the variable spatial distribution of both fuel and hydrocarbon degraders further endorse the need to perform proper natural attenuation monitoring and pilot scale bioremediation experiments prior to full scale implementation of any biological treatment (Xu and Obbard, 2004). Hence, the very first problems to be addressed in these types of studies relate to defining parameters such as the local quantification of oil remaining after spillage (Bragg et al., 1994), which essentially requires appropriate sampling and analytical strategies. In this sense, the application of GC–MS techniques and biomarker determinations therefrom (Hostettler and Kvenvolden, 1994; Pollard et al., 1999; Page et al., 2002; Peters and Moldowan, 2004) is the most popular tool for evaluating changes in the chemical composition of oil derivatives spilled in natural environments.

As mentioned above, biodegradation is one of the main processes enhancing natural depletion of hydrocarbons. In this sense, the complex composition of the *Prestige* fuel oil (Alzaga et al., 2004; Fafet et al., 2004) implies that only microbial mixtures (generally termed “consortia”; Hurst, 2002) are capable of performing effective biodegradation in natural environments (Bouchez et al., 2000; Kanaly et al., 2000; Mishra et al., 2001). It is clear that individual microorganisms can metabolize only a limited range of hydrocarbon substrates (Wackett and Hershberger, 2001), so mixed populations with broad enzymatic capacities and synergistic/co-metabolic relationships are required to attack not only aliphatic and aromatic hydrocarbons, but also NSO (nitrogen, sulfur, oxygen) compounds and asphaltene fractions (Speight, 2001; Venosa and Zhu, 2003).

Bioremediation strategies include natural attenuation, as well as the acceleration of biodegradation by adding exogenous microbial populations (bioaugmentation) or by stimulating indigenous populations (biostimulation). Bioaugmentation approaches have not been successful at *Prestige* sites (Murado et al., 2004) and have generally been ruled out for marine oil spill remediation (Lee et al., 1997). On the other hand, full scale biostimulation in oil spills (Head and Swannell, 1999; Venosa and Zhu, 2003) was first used in Alaska with the Exxon Valdez spill, where the application of oleophilic and slow release fertilizers (Bragg et al., 1994; Zhu et al., 2001) played an important role. Since then, the benchmark of marine oil spill bioremediation is based on supplying exogenous nutrients (Oh et al., 2001; Maki et al., 2003). In particular, oleophilic fertilizers have been reported as the optimal alternative for bioremediating rocky areas in intertidal zones. Inipol EAP 22, designed by Elf Aquitaine after the Amoco Cadiz oil spill in France in 1978, was successfully applied in the remediation of some Exxon Valdez sites. After the Valdez experience (Pritchard et al., 1992), Inipol and similar products have been used in other oil spills with acceptable results (Santas et al., 1999). Hence, our first option was to use a commercially available oleophilic fertilizer – S200, while other possibilities, including the ongoing natural attenuation studies, were being completed.

2. Materials and methods

2.1. Site descriptions

Two sites belonging to one of the areas most severely affected by the *Prestige* fuel (Costa de la Muerte, La Coruña, Spain) were selected for this work and for bioremediation. Around the middle of November 2002, the spilled fuel oil reached “Coido da Cuño” and Moreira Beach (Fig. 1), both located on the Atlantic coast of Galicia (northwestern Spain), 80 km from La Coruña. Fuel continued to arrive at these sites in great quantities from November 2002 to January 2003, covering a considerable expanse of sediments at the upper tidal limit, coinciding with maximum high tide. The rate of waste arrival decreased dramatically thereafter and initial cleaning work began (access ways were improved, waste containers were installed, etc.). By February 2003, both sites were selected for research and bioremediation purposes because of their ecological and geological interest. In March, the present study commenced. At that point, the only clean-up procedure that had been implemented consisted of manually removing several tonnes of fuel covering the sediments.

Common aspects of both sites include the heterogeneity of rounded clastic sediment grains, ranging from a minimum of medium grain size sand

(< 0.5 mm diameter) at Moreira Beach up to huge granitic boulders (2 m diameter and larger) at the Cuño site. Remarkably, these sediments are distributed at both sites on top of thick strata of non permeable organic soil. Thus, both the high viscosity of the fuel and this geological substrate at these sites impede hydrocarbon penetration – an important difference with respect to the Exxon Valdez sites (Bragg et al., 1994). Consequently, our study focussed mainly on broad surface areas covered by cobbles (between 64 and 256 mm diameter) at the Moreira site and 'small' boulders (between 256 and 800 mm diameter at the Cuño site). As a final common characteristic, small meandering freshwater streams flow on the western side of the Moreira beach and on the southern side of the Cuño site.

The northern area of Moreira Beach is comprised of a thin layer of medium grain siliceous sand on a considerable slope that remains completely under water during high tide. In the southern, eastern and western areas, the sand is covered by a regular layer of porphydic and granitic pebbles, cobbles and boulders derived from geological substrate meteorization. These shore rocks occupy an irregular surface area of ca. 2000 m^2. Furthermore, the effects of the strong winds that are typical of the area generated a small, emerging dune system along the top of the slope where some of the rocks are partially buried in the sand.

The Coido da Cuño site is located 5 km north of Moreira Beach and opens on to the sea on the west. Geologically it is very similar to Moreira, except that the grain size of the sediments ranges from gravel particles (2 mm minimum on the northern side of the beach) up to larger boulders on the southern side, which finally ends in a 20-m vertical granite cliff. Both the cliff and the area where the boulders are located lay on interbedded coluvial Quaternary sediments, mixed with the above mentioned organic substrate and granitic pebbles. Shore rocks (pebbles, cobbles and boulders) cover an approximate surface area measuring 400 m long by 20 m wide at low tide (8000 m^2).

2.2. Fuel load quantification

In the casuistry described above, fuel quantification is preferably expressed as fuel load per sediment surface rather than in terms of fuel load per sediment mass (this type of expression should be reserved only for sandy sediments). Additionally, the selection of proper C:N:P ratios for biostimulation design requires that fuel load be calculated in order to estimate available organic carbon. Thus, two parameters were needed to quantify the total amount of fuel remaining on the shore rocks (regardless of grain size): (i) total surface area covered by fuel and (ii) total fuel load per rock surface.

To determine the total surface area covered by the fuel, special image analysis techniques were applied: the affected sites were divided into sub-areas of 200 m^2, then two spheres of known surface area were randomly included in each sub-area and repeated digital photographs of these sub-areas of each site were taken to complete the total surface of the sites affected. Afterwards, the *Split*® (Split Engineering 2001, Arizona, USA) software was used for digital analysis of the images taken. This programme calculates grain size distribution curves assuming that each rock could be assimilated into an ellipsoid having the same area and factoring in the known area of the spheres that appear in each image. Successive calculations of all the sub-areas of the boulder–cobble–pebble fields estimate an approximation to the total surface area of fuel-covered rocks.

A statistical measurement of the fuel layer thicknesses was then needed to finally determine fuel load per unit of rock surface. This second parameter was calculated by averaging the weights of fuel-sampling increments taken by scraping the rocks: twenty different operators scraped duplicates of 4 cm^2 (2 cm side squares) of fuel from ten rocks randomly distributed at the Cuño site; the same operation was performed at the Moreira site. The weights of the increments were averaged; this design thereby considerably minimized standard deviation. Similar procedures were used when necessary to specify results in certain site sub-areas.

2.3. Sampling methodology and strategies

Systematic sampling of the sub-areas was conducted following the general procedure described above for fuel-covered rocks, consisting of scraping duplicate composite samples. Each composite sample comprised ten increments of 4 cm^2 of fuel geometrically distributed at sampling stations, corresponding to ten rocks (pebbles, cobbles, or boulders depending on the sub-area sampled). The increments were taken by scraping the fuel covering 4 cm^2 of rock using appropriate metal tools, avoiding the collection of rock fragments, algae, or any other impurity as far as possible. For oily sandy sediments at the Moreira site and oily gravel sediments at the Cuño site respectively, the strategy followed was the same as described for the rocks (except for sample weight); therefore, approximately 100 g for sandy sediments were weighed while 15 g were totally taken from fuel covering shore rocks. The samples were then placed in containers which were sealed and stored in the dark at 4 °C. When samples were needed for microbiological determinations; they were taken and stored in sterile conditions. Some modifications to these general protocols were applied to specific sampling stations and are described below.

2.4. Hydrocarbon analysis

The analysis of the fuel samples scraped from the rocks was performed as follows: The hydrocarbons were extracted from a 1 g sample with CH_2Cl_2 in a 50 ml Soxhlet apparatus for 24 h (EPA Method 3540 C). After solvent evaporation and redilution in dichloromethane, 2 ml extracts were purified using liquid chromatography with *n*-hexane as sole solvent (EPA Method 3630 C). Purified extracts (containing only aliphatic and aromatic fractions) were injected into a GC system (HP 6890 Series chromatograph) equipped with a capillary column (AT5 Alltech; 25 m × 0.25 mm i.d.); the oven temperature was raised from 60 °C to 300 °C at 6 °C/min. The injector temperature was 275 °C. This equipment was attached to a MSD HP 5973 Series mass detector, which allowed peak area determinations in selective ion monitoring (SIM) mode using the Wiley 275 database (EPA Method 8270 C). Analysis of sandy sediments differed only with regard to the weight of the initial sub-sample (20 g) extracted in the Soxhlet apparatus.

Different ratios were calculated from the data in GC–MS chromatograms to measure weathering and biodegradation. They were calculated after carefully studying the samples taken during the first months of fieldwork and are described below.

2.5. Microbiological methods

Microbial counts were performed as follows: a representative aliquot (1 g) of each collected fuel sample was added to 10 ml of a sterile 0.1% $Na_2HPO_4 \cdot 12\,H_2O$ solution and mixed (vortex stirrer) vigorously for 10 min according to standard procedures for soil samples (Gallego et al., 2001). An aliquot (1 ml) of the supernatant suspension (also used as inoculum for selective enrichment; see below) was taken and 0.1 ml of successive dilutions of the culture were spread in triplicate TSB (tryptic soy broth, Merck) agar plates, supplemented with 2% NaCl and 0.1% yeast extract and cultivated at 30 °C for 48 h in aerobic conditions (note that this medium and others described below may cause in some cases a reduced cell count due to unusual growth conditions). For fungi growth, duplicates were spread in potato agar medium (Oxoid) supplemented with 2% NaCl. The predominant strains were also isolated in TSB medium and tested in diesel agar synthetic medium (DS): 0.13% NH_4NO_3; 0.05% $MgSO_4 \cdot H_2O$; 0.02% $CaCl_2 \cdot 2H_2O$; 0.5% KH_2PO_4; 0.5% K_2HPO_4 and 2% filter-sterilized diesel fuel as sole carbon source.

Selective enrichment techniques were conducted in liquid synthetic medium having the same composition as DS solid medium, except that the diesel was substituted for 1% *Prestige* fuel as sole carbon source; 2 ml of the inocula obtained as above were added to 100 ml of medium and incubated in a rotary shaker at 250 rpm and 30 °C for 5 d. Then, 0.1 ml of the suspension was used for plate counting in TSB-agar medium and 2 ml were used as a second inoculum for another flask containing the same medium. The most abundant strains after three cultivation steps were isolated and preserved by freezing at −70 °C in 1.7% trehalose and 25% glycerol medium.

Basic morphological data regarding bacteria, fungi and cyanobacteria and their behaviour in natural fuel-containing media were obtained by means of phase contrast microscopy (Nikon Eclipse E-200). Bacterial viability was analyzed by staining the cells with propidium iodide (PI) and SYTO 9 green fluorescent nucleic acid stain (LIVE/DEAD Bac-Light Bacterial Viability Kit, Molecular Probes). Samples were examined under a Leica TCS-SP2-AOBS confocal laser-scanning microscope (CLSM) at the appropriate wavelength, and the images were mixed using the Leica Confocal Software.

2.6. Biostimulation experiments with oleophilic fertilizer S-200

The oleophilic fertiliser S200 (IEP Europe) is listed in the NCP (National Oil and Hazardous Substances Pollution Contingency Plan, USA) as a bioremediation agent. S200 (Díez et al., 2005) is a microemulsion of a saturated solution of urea (N source) in oleic acid containing phosphate esters (P source). To test its effectiveness for bioremediation purposes, three separate 200 m^2 sub-areas of Moreira Beach were selected and subdivided; half received S-200 fertilizer and half remained as untreated controls. S-200 was applied monthly with a mechanical sprayer three times during the summer of 2003, in total dosages according to the desirable C:N:P ratio of 100:10:1 (Head and Swannell, 1999; Zhu et al., 2001) and based on the previously calculated fuel load affecting the shorelines.

3. Results and discussion

3.1. Fuel characterization and definition of depletion indexes

The original fuel oil has been repeatedly analysed by different authors; for instance, SARA (saturate, aromatic, resins and asphaltene) fractionation revealed concentrations of approximately 25% saturated hydrocarbons, 35% aromatics, 20% resins and 20% asphalthenes (Alzaga et al., 2004; Fafet et al., 2004). This approach revealed that we were dealing with a heavy residue from atmospheric distillation, usually classified as fuel n°6. Moreover, our initial GC–MS studies of fuel taken directly from the ship at the beginning of the spill showed that alkylated PAHs and heavier linear and branched alkanes (C_{12}–C_{40}) were the most abundant families in the saturate and aromatic fractions (as previously reported by Alzaga et al. (2004), Benoit and Haeseler (2004)).

For the present study, internal standards such as isoprenoids (pristane and phytane) and hopanes needed to be determined. Pristane and phytane are useful in the initial stages of the degradation processes to monitor *n*-alkane depletion; however, as

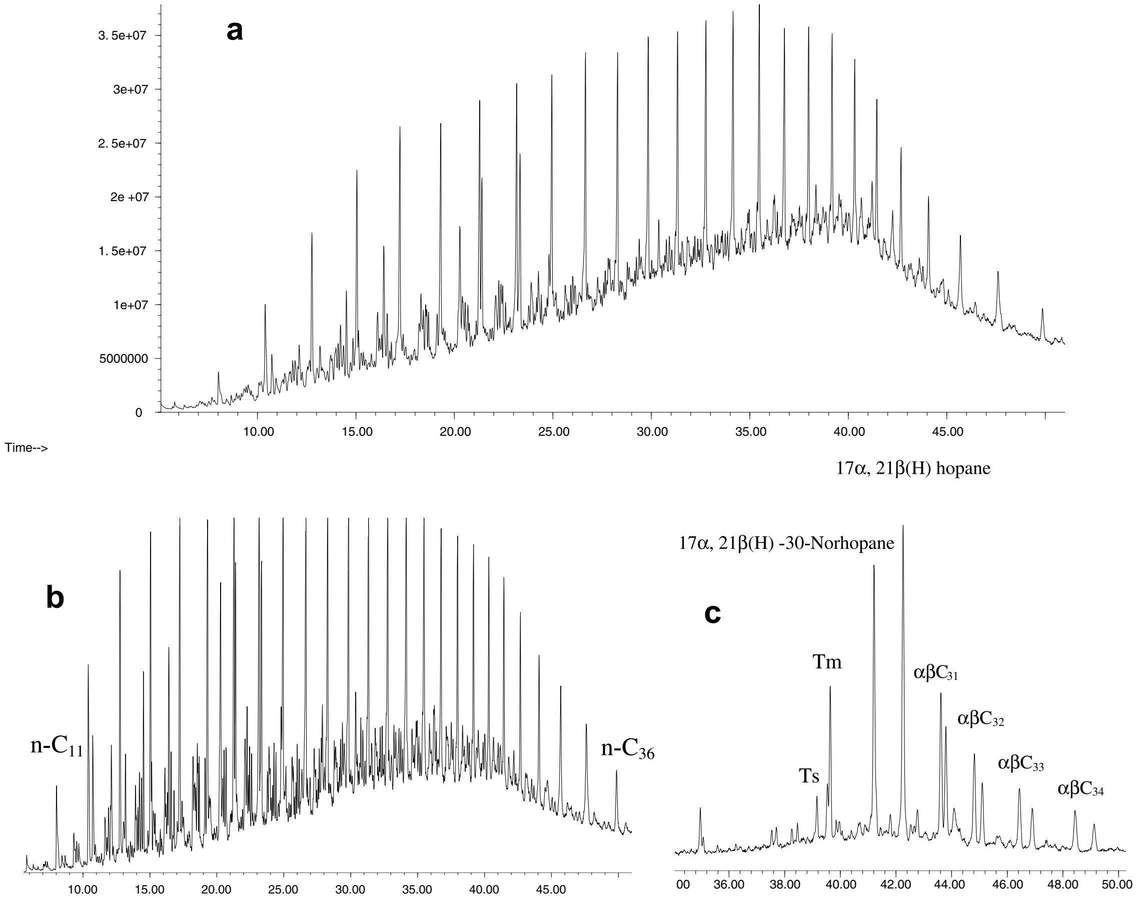

Fig. 2. GC–MS chromatograms of initial it *Prestige* fuel oil: (a) total ion chromatogram (TIC) mode, (b) selective ion mode (SIM, *m*/*z* 57) showing *n*-alkanes, (c) *m*/*z* 191 SIM showing hopane fingerprinting. (Ts: 18α(H)-22,29,30-trisnorhopane; Tm:17α(H)-22,29,30-trisnorhopane; 31αβ, 32 αβ, 33 αβ and 34 αβ are pairs of R and S isomers of 17α(H), 21β(H), 22-homohopane, 17α(H), 21β(H), 22-bishomohopane, 17α(H), 21β(H), 22-trishomohopane and 17α(H), 21β(H), 22-tetrakishomohopane respectively).

reported previously (Sasaki et al., 1998; Wang and Fingas, 2003) and also demonstrated below, they are often degraded more quickly than is generally thought. On the other hand, normalization to hopane has been reported as the best method for quantifying hydrocarbon depletion and therefore biodegradation, specifically the use of 17α(H), 21β(H)-hopane as an internal conservative standard (Bragg et al., 1994; Prince et al., 1994; Pollard et al., 1999; Peters and Moldowan, 2004). Furthermore, 17α(H), 21β(H)- hopane was clearly the most abundant terpane in the *Prestige* fuel oil, followed by 17α(H) 21β(H)-30-norhopane (Fig. 2), and its extremely low solubility, biodegradability and volatility guarantee that it would persist in the residual fuel on shoreline sediments. Consequently, it was selected as an indicator of the progress made with bioremediation treatment resulting from the Exxon Valdez oil spill in Alaska (Prince et al., 1994). It has since been used to quantify the extent of biodegradation in many other studies. Hopane degradation has been described (Bost et al., 2001) to occur in highly specific laboratory conditions and ancient oil reservoirs, although never in oil spills similar to the *Prestige* one. In any event, even if hopane were slightly degraded during biotransformation, the effects would only slightly underestimate the actual oil depletion.

Initially, comparison of the ratios of several individual compounds or groups of compounds was chosen to define depletion ratios. Selection criteria consisted of abundance in *Prestige* fuel, ready location and identification in SIM chromatograms and representivity of some of the most important families in fuel oil (Speight, 2001). The list included linear aliphatic hydrocarbons of light-medium molecular weight (heptadecane and octadecane), waxes ranging from triacontane to tritriacontane, isoprenoids (pristane and phytane), three ring alkyl PAHs (methyl and dimethyl anthracenes and phenanthrenes – C1-APh and C2-APh), pyrene (four ring PAH), alkyl derivatives of the most abundant sulfur compounds (dimethyl dibenzothiophenes – C2-DBT) and finally, 17α(H), 21β(H) hopane; all have been widely used for establishing depletion ratios and for other similar purposes (Peters and Moldowan, 2004).

It is clear that an important spatial variability in the analyses would be expected (Page et al., 2002). At the *Prestige* sites, this is accounted for by several factors including tidal reach, different daily hours of sun (promoting or depleting volatilisation of light compounds and/or photochemical oxidation of aromatics), grain size, presence or absence of sandy or gravel sediments coating the polluted rocks, existence of areas with preferential circulation of rain waters, etc. Therefore, for cross site ratio variability, a sampling procedure was performed by selecting several 200 m^2 sub-areas at the Cuño site. Three replicate samples were taken from five of these sub-areas, comprising 10 geometrically distributed fuel sub-samples, scraped from 4 cm^2 surfaces as explained above. Thus, a total of 15 samples was taken and analysed using GC–MS (Table 1). The ratios of different compounds in the fuel oil were calculated by averaging

Table 1
Depletion ratios obtained by averaging results of 15 samples taken simultaneously at Cuño site in July 2003

Ratio		Mean value ± std. error	Relative std. error (%)
Isoprenoid normalization	C17/pristane	0.41 ± 0.24	59.06
	C18/phytane	0.45 ± 0.20	46.64
	C17 + C18/pristane + phytane	0.43 ± 0.22	50.07
	C30 + C31 + C32 + C33/Pristane + phytane	2.28 ± 1.12	49.17
Hopane normalization	C17 + C18/17α21β hopane	1.52 ± 0.48	31.58
	C30 + C31 + C32 + C33/17α21β hopane	8.12 ± 1.41	17.37
	Pristane + phytane /17α21β hopane	3.75 ± 0.63	16.86
	$\sum$(C_1-APh + C_2-APh)/17α21β hopane	15.36 ± 2.91	18.93
	$\sum C_2$-DBT/17α21β hopane	3.71 ± 0.73	19.57
	Pyrene/17α21β hopane	1.13 ± 0.20	17.81
Others	Pristane/phytane	0.78 ± 0.07	9.05
	$\sum C_1$-APh/$\sum C_2$-APh	0.82 ± 0.07	8.68
	$\sum C_2$-APh/$\sum C_2$-DBT	2.35 ± 0.42	18.06

C_1-APh: methyl anthracenes and phenanthrenes; C_2-APh: dimethyl anthracenes and phenanthrenes; C_2-DBT: dimethyldibenzothiophenes.

the GC–MS data after normalization with the internal standards. Results showed significant heterogeneity, indicating that different weathering processes were taking place in an apparently homogeneous scenario. However, local variations in the sub-areas were considerably smaller than those obtained for the overall site. Thereby, specific sub-areas were selected for monitoring the changes in fuel composition over time. Note that, as reported in Table 1, the ratios with greater degrees of uncertainty are those that include light and easy degradable hydrocarbons such as n-C_{17} and n-C_{18} alkanes. In addition, it seems that the values obtained using pristane and phytane as hypothetical non-degradable standards fluctuated much more (relative standard error $> 45\%$) than those obtained with 17α(H), 21β(H) hopane (relative standard error 16.86% to 50.07%); hence, the former were ruled out and the latter selected for fuel evolution analysis. It would be reasonable to consider these 'hopane ratios' to be synonymous with biotransformation ratios, assuming that, at the beginning of our study (March 2003), physical weathering (vaporization and dissolution) of the *Prestige* fuel oil was almost negligible in most sites. The same assumption was discussed in bioremediation studies for North Slope crude oil at the Exxon Valdez sites (Bragg et al., 1994). In any case, the complex processes implied in natural attenuation, the difficulties in detection of pathway intermediates and the non-feasibility of performing abiotic experiments at field sites, suggest to us the use of the term "depletion ratios" instead of "biotransformation ratios".

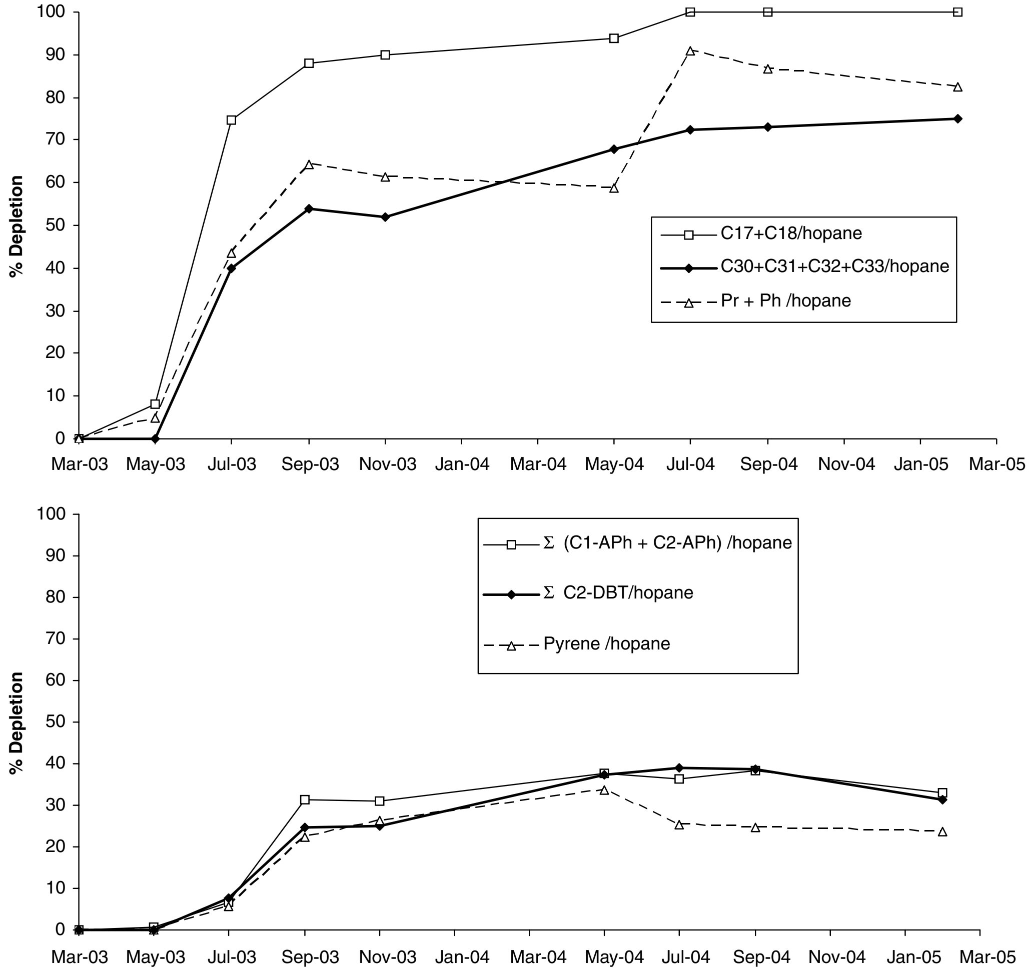

Fig. 3. Temporal fate of depletion indexes calculated for samples taken at Cuño site over a 2 year period. Errors are within % values in Table 1. (Pr: pristane, Ph: phytane; C_1-APh: methyl anthracenes and phenanthrenes; C_2-APh: dimethyl anthracenes and phenanthrenes; C_2-DBT: dimethyldibenzothiophenes; hopane: 17α(H), 21β(H), hopane).

Finally, and to facilitate understanding, depletion ratios were modified to provide depletion indexes (Pollard et al., 1999; Whittaker et al., 1999) as a percentage of depletion for the different families of compounds. For example, the term 'pyrene depletion index' is expressed in terms of concentration of pyrene and 17α(H), 21β(H) hopane as:

$$\text{Pyrene depletion index}(\%) = 100 - \left(\frac{(\text{pyrene}/17\alpha21\beta\ \text{hopane})_{\text{sample}}}{(\text{pyrene}/17\alpha21\beta\ \text{hopane})_{\text{reference}}}\cdot 100\right)$$

3.2. Evolution of fuel residues

Fuel residue fate at the Cuño site was investigated over a two year period by means of periodic analysis of triplicate samples taken at three 200 m^2 areas of polluted shore rocks (the rest of the site was used for several on-going bioremediation experiments). Depletion indexes were averaged to obtain representative values applicable to the whole site. The results are summarized in Fig. 3, where substantial degradation of all the hydrocarbon families studied during the first summer after the spillage is clearly seen. The depletion was particularly evident not only in the case of the lighter *n*-alkanes, as expected in natural environments (Ghazali et al., 2004), but also in the aromatic fraction. However, the process slowed down and more than one year after the end of the summer of 2003, the levels of the studied compounds in the aromatic fraction remained stable, whereas aliphatics were degraded somewhat further. It is interesting to point out that the rate of degradation of heavier *n*-alkanes is similar to or even less than that for isoprenoids. This result emphasizes the differences between in situ biodegradation and controlled ex situ treatment and laboratory studies, in which a *n*-alkane (irrespective of chain length) is generally assumed to be more biodegradable than an isoprenoid. On the other hand, some of the fluctuations in indices for aromatics and branched alkanes (seen in Fig. 3), may be attributable to the standard errors shown in Table 1, although interference from resin and asphaltene fractions might also be a possible explanation. In this sense, the presumed appearance of saturates and aromatics as a result of asphaltene photobiooxidation processes (Pineda-Flores et al., 2004) and the possible growth of the resin fraction via aromatic photooxidation (Jézéquel et al., 2003) should be considered.

Table 2
Depletion ratios for Cuño site from triplicate samples taken at 3200 m^2 areas of polluted shore rocks during a 2-year period

Ratio	Pr/Ph	$\sum$C1-APh/$\sum$C2-APh	$\sum$C2-APh/$\sum$C2-DBT
Mar-03	0.82	0.84	2.23
May-03	0.78	0.81	2.12
Jul-03	0.70	0.82	2.35
Sep-03	0.63	0.62	2.25
Nov-03	0.67	0.67	2.21
May-04	0.59	0.70	2.48
Jul-04	0.44	0.58	2.28
Sep-04	0.43	0.58	2.60
Feb-05	0.37	0.61	2.37

Furthermore, the information in Table 2 completes the data and reports faster degradation for pristane vs. phytane and for C_1-PAHs vs. C_2-PAHs, whereas C_2-dibenzothiophenes and C_2-PAHs have similar degradation rates (N.B. pristane/phytane and $\sum C_1$-APh/$\sum C_2$-APh ratios diminish over time, whereas $\sum C_2$-APh /$\sum C_2$-DBT shows only slight fluctuation). Be that as it may and considering the overall data (Fig. 3 and Table 2), a classification of depletion susceptibility for compounds in the *Prestige* fuel oil at natural sites can be proposed:

Table 3
Depletion ratios for a 2 cm deep fuel core taken at a sampling point in the most highly polluted zone (granite cliff) at Cuño site (Sept., 2003)

Fuel compound	External section	Intermediate section	Internal section
C17 + C18/17α21β hopane	3.32	5.67	6.30
C30 + C31 + C32 + C33/17α21β hopane	5.08	8.58	9.89
pristane + phytane /17α21β hopane	3.05	4.64	4.67
$\sum C_1$-APh + C_2-APh /17α21β hopane	19.45	31.32	32.82
$\sum C_2$-DBT/17α21β hopane	4.51	6.92	7.65
pyrene/17α21β hopane	1.28	1.78	1.75
pristane/phytane	0.77	0.86	0.92
$\sum C_1$-APh/$\sum C_2$-APh	0.86	0.95	0.99

light-medium *n*-alkanes ≫ branched alkanes (pristane > phytane) > heavy linear alkanes (waxes) > C_1-three ring PAHs > C_2-three ring PAHs ∼C_2-dibenzothiophenes > four ring PAHs.

All of this suggests that the microorganisms at the polluted sites could potentially degrade aliphatics extensively while at the same time slowly degrading the aromatic fraction. These results are somewhat consistent with those obtained by Medina-Bellver et al. (2005) who demonstrated hydrocarbon depletion at *Prestige* sites by means of isotopic fractionation. They also isolated several strains capable of degrading aliphatics and some aromatics. Nevertheless, the hostile environment of shore rocks exposed to strong winds, sea tides, low nutrient levels and other harsh conditions may conceivably hinder or even impede the biodegradation of targeted compounds. Thus, the existence of important amounts of still fresh, light hydrocarbons, together with the high temperatures to reduce fuel viscosity, favouring microbial growth, facilitated biodegradation only during the first summer. Additionally, at many locations, the considerable thickness of fuel coating did not allow sufficient penetration of oxygen, nutrients, water and microorganisms; consequently only the superficial layers were effectively weathered. This was verified by sampling and analysing the external, intermediate and internal sections of a 2 cm deep fuel core extracted from the vertical granite cliff at the Cuño site; the results clearly showed a gradient of decreasing effect of degradation (Table 3).

3.3. Natural attenuation vs. bioremediation with S200

Using the same approach as for the Cuño site, several parcels were selected at Moreira Beach to monitor natural attenuation of periodically sampled

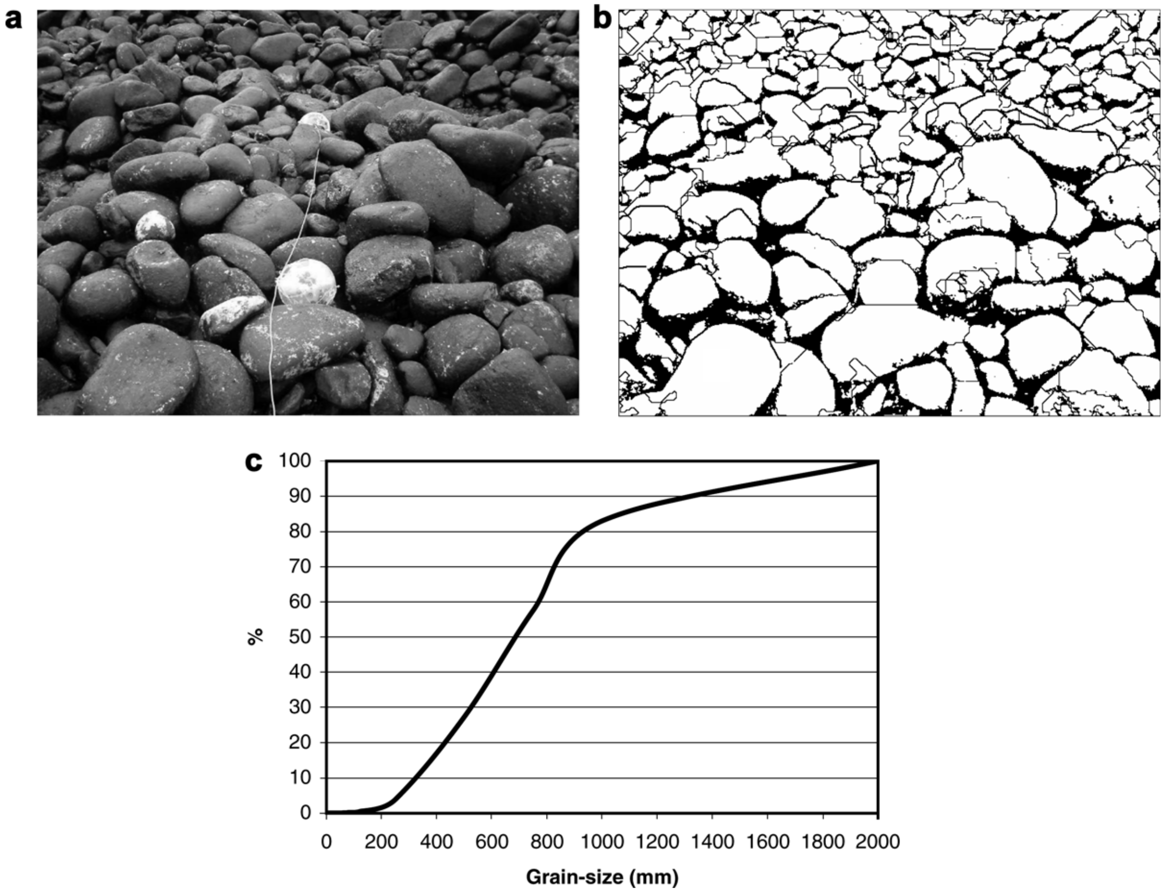

Fig. 4. (a) Photograph of one of polluted sub-areas at Cuño site; all the cobbles and boulders are coated with fuel (black), two white spheres were placed in the scenario. (b) Image analysis performed with the Split Program. (c) Grain size distribution corresponding to initial photograph.

fuel residues. The fate of hydrocarbons revealed very similar patterns to those at Cuño site (Fig. 5, rapid depletion in the first months and then a slowing down of the process). In addition, we focussed on a 1 year comparison between parcels that had undergone natural attenuation vs. those for which S200 was applied. Before field application of the product, laboratory testing was performed which confirmed that S200 was appropriate for use in accelerating hydrocarbon biodegradation, as recently reported (Díez et al., 2005; Gallego et al., 2005). At the field site, fuel load determined the optimum amount of fertiliser for biostimulation; hence, calculations were made as explained above (an example of image analysis and grain size determination is shown in Fig. 4). Results revealed a thickness of fuel coatings ranging from about 0.1 mm to 2 cm, so an approximate fuel load of 3.5 kg/m^2 at the Moreira site (S200 was dosed in accordance with these data -see above).

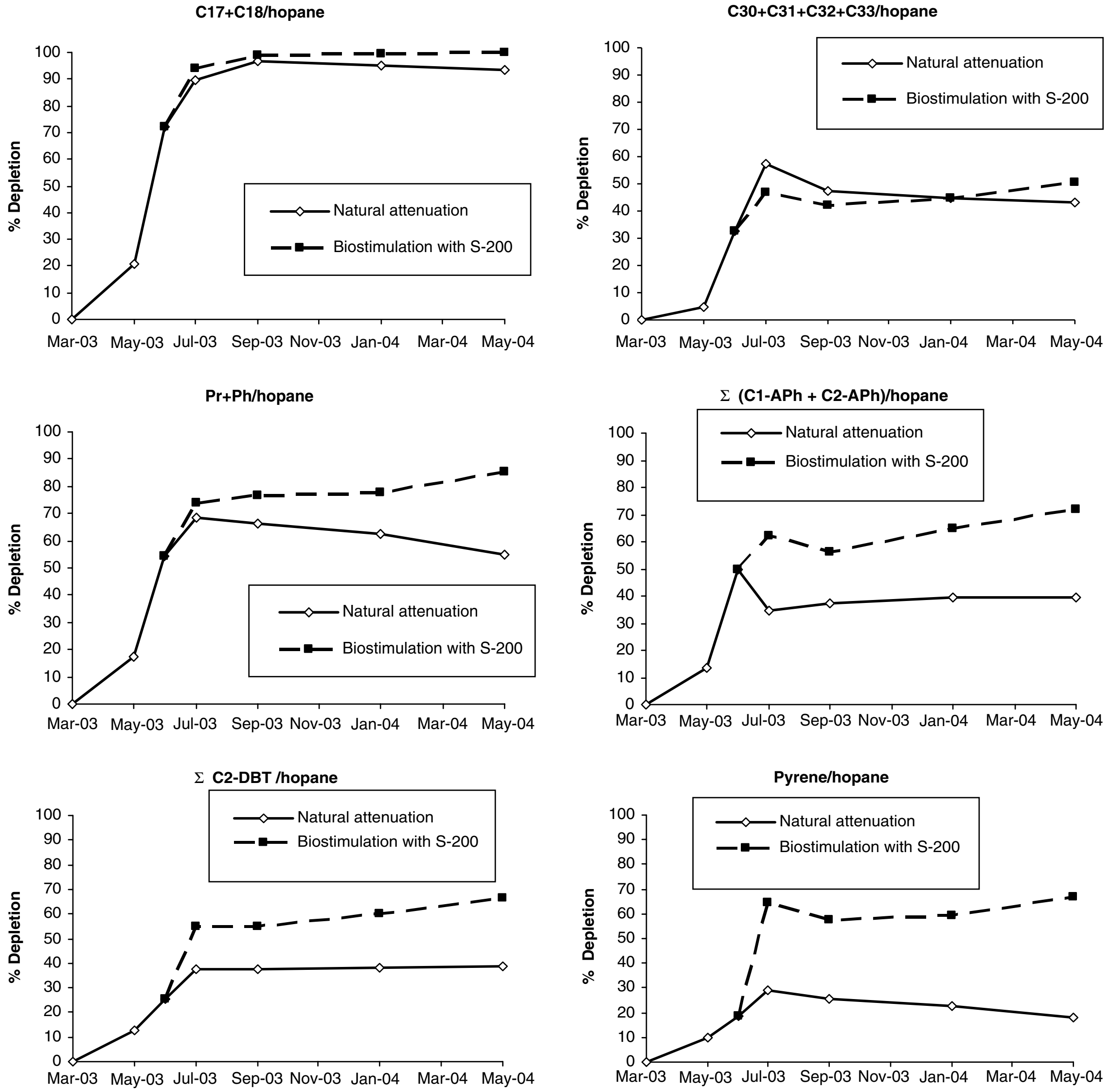

Fig. 5. Depletion indices measured at Moreira site (see text) during a 1 year sampling period. N.B. in June 2003, half of the parcels studied at the beach were biostimulated with S 200; application was repeated in July and August 2003. Results are averages of duplicate samples taken at the S200-treated and untreated sub-areas, respectively. Errors are within % in Table 1. (Pr: pristane, Ph: phytane; C_1-APh: methyl anthracenes and phenanthrenes; C_2-APh: dimethyl anthracenes and phenanthrenes; C_2-DBT: dimethyldibenzothiophenes; hopane: 17α(H), 21β(H), hopane).

The results (Fig. 5) revealed that the product achieved an additional hydrocarbon depletion ranging from 10% up to 30% depending on the fuel compounds, in comparison with natural attenuation ratios; the only exception was waxes (long chain *n*-alkanes) that probably interfere in some way with the long chain oleic compounds in the fertilizer. Furthermore, it is interesting to point out that microbial counts in TSB medium averaged 10^6 CFU/g fuel before S200 application and did not increase significatively during the following months; however, the proportion of those microorganisms capable of growing in the selective DS medium was doubled from 20% to 40%, indicating an enrichment in the hydrocarbon degrading community, which likely improved depletion indices. In fact, the efficiency of oleophilic fertilizers is based on their resistance to physical wave action and their hydrophobic and emulsifying properties that facilitates microbial access to hydrocarbons (Santas et al., 1999; Murado et al., 2004), thereby promoting growth of hydrocarbon-degrading bacteria. However, at the sites studied in this work, and despite initial successful results, the product effect did not persist through the following winter and spring, so non-cost effective successive applications would have been needed.

The overall results of these experiments proved that N and P contents are not the only limiting parameters that impeded biodegradation. Conversely, two factors strongly hindered the progress of biodegradation; first, the above mentioned hostile microenvironments of fuel-coated shore rocks, combined with the fuel thickness and, second, the high content of heavy – albeit non-toxic – oil fractions such as resins and asphaltenes (especially if we consider the relative increase in these fractions after the rapid initial depletion in light-medium aliphatics and aromatics). Detailed ecotoxicological studies should evaluate whether the product remaining along the shorelines poses an environmental risk or can be considered an inert bituminous residue.

3.4. Enhanced natural attenuation by fresh water

During the first months of the study reported here, fuel layers were visibly thinner and turned brownish or were even covered with greenish mats in areas where fresh water from local streams flowed through the polluted shore rocks. At first sight, the physical and chemical effects of this water appeared to be responsible for the changes observed in fuel structure and colour. Nevertheless, further analysis at these “hot spots” revealed both depletion in biodegradable hydrocarbons (Fig. 6) and microbial activities promoting biodegradation (Fig. 7). The microbial study revealed a considerable increase in biomass compared to that obtained from polluted shore rocks not affected by fresh water flow (10^7–10^8 CFU/g vs. 10^5 CFU/g fuel). Subsequent sampling and selective enrichment of cultures (see Materials and methods) made it possible to collect >100 bacterial and approximately 30 fungal species from the fuel samples at different stages of biodegradation [these microorganisms are currently being grouped and analysed using restriction fragment length polymorphism (RFLP) and 16S rDNA sequencing methods].

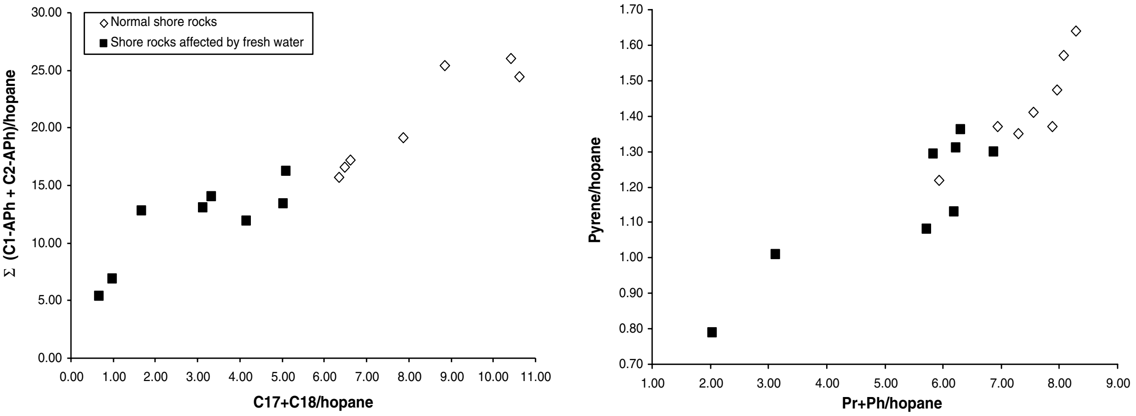

Fig. 6. Representative depletion ratios of fuel samples taken at shore rocks located in the stream flow and others taken at areas close to the former, but without the influence of fresh water. (Pr: pristane, Ph: phytane; C_1-APh: methyl anthracenes and phenanthrenes; C_2-APh: dimethyl anthracenes and phenanthrenes; hopane: 17α(H), 21β(H), hopane).

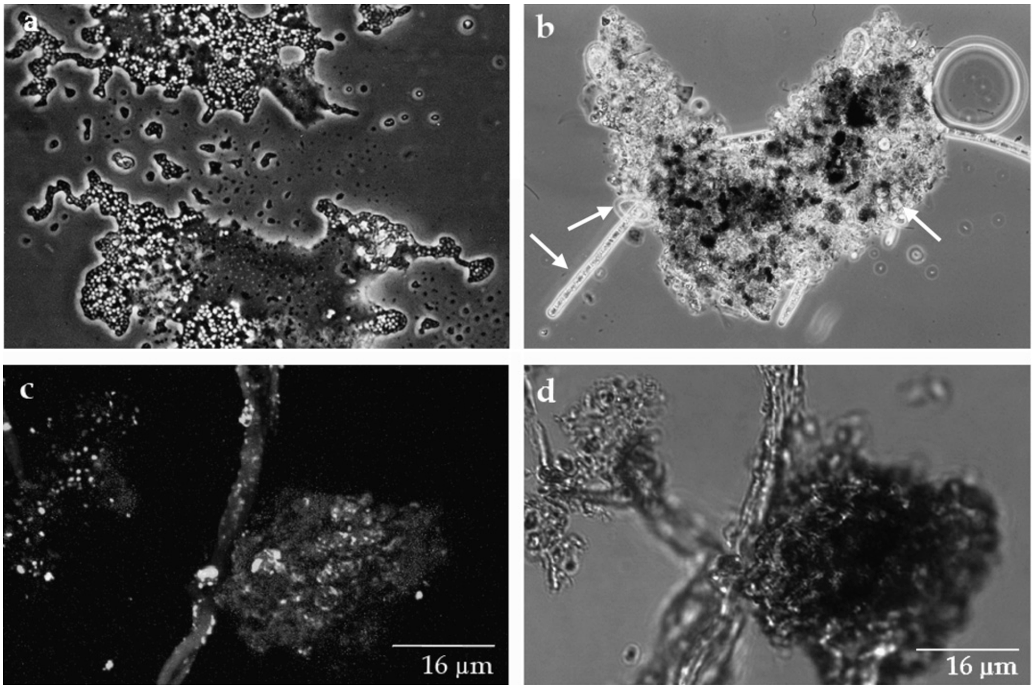

Fig. 7. (a) Phase contrast (1000×) image showing bacterial colonization of fuel hydrocarbon aggregates while growing in selective enrichment experiments in liquid synthetic medium with *Prestige* fuel oil as sole carbon source. (b) Phase contrast microscopy (1000×) of a sample of a microbial mat at Cuño site. At least three different cyanobacteria can be distinguished (arrows), one of which is filamentous (*Hormogoneae* group). (c) Confocal laser scanning microscopy (CLSM) of a sample from a polluted area with evident oil degradation activity (see text). Samples were stained with propidium iodide (PI) and SYTO 9. Live bacteria appear in green. Oil masses appear as loose (left) or compact (right) reddish conglomerates. Green filament in the centre is probably an alga. (d) The same field observed under differential interference contrast mode.

Bacteria appeared to be viable and actively degrading the hydrocarbon mass when observed under a confocal laser scanning microscope (Fig. 7c and d). Moreover, microbial observations enabled detection of microbial consortia consisting of different species of bacteria and cyanobacteria (Fig. 7b) that are likely to have promoted fuel degradation. Detailed examination of cyanobacteria showed the presence of different morphologies and groups (*Anabaena*, *Oscillatoria* and others).

Evidence of the cooperation of mixed cultures in natural environments for biodegradation has been reported (Bounchan et al., 2000). In this context, cometabolic and synergistic reactions (Alexander, 1999; Knapp and Bromley-Challoner, 2003) probably play a special role in the degradation of highly complex mixtures such as the *Prestige* fuel. At the sites studied, the presence of organic substrates (see Site description), natural fresh water and the variety of coastal macrobiotic life directly implies the existence of highly varied microbial life, as was the case. Also, the historically frequent arrival of small oil spills, tar balls, and other hydrocarbon sources to the area, led to the establishment of previously adapted microbial populations capable of rapidly responding to large spills. In this case, it seems that cyanobacteria, possibly fungi, and associated aerobic bacteria appear to form a consortium that fosters fuel biodegradation (Bender and Phillips, 2004) and, under optimal conditions, would be extremely effective, as revealed by the increasing biodegradation effects visible in the chromatograms (Fig. 8).

In fact, there is no doubt that cyanobacteria play an important role in these microbial mats by establishing oxygen gradients and/or supplying nutrients for heterotrophic bacteria (Al-Hasan et al., 1998; Cohen, 2002). Nevertheless, given that there is no scientific certainty that points to a direct role of cyanobacteria in degradation pathways (Abed and Köster, 2005), we suggest that, as previously proposed (Cohen, 2002), heterotrophic aerobic bacteria are responsible for the hydrocarbon degradation at the sites. In this context, the oil-insensitive

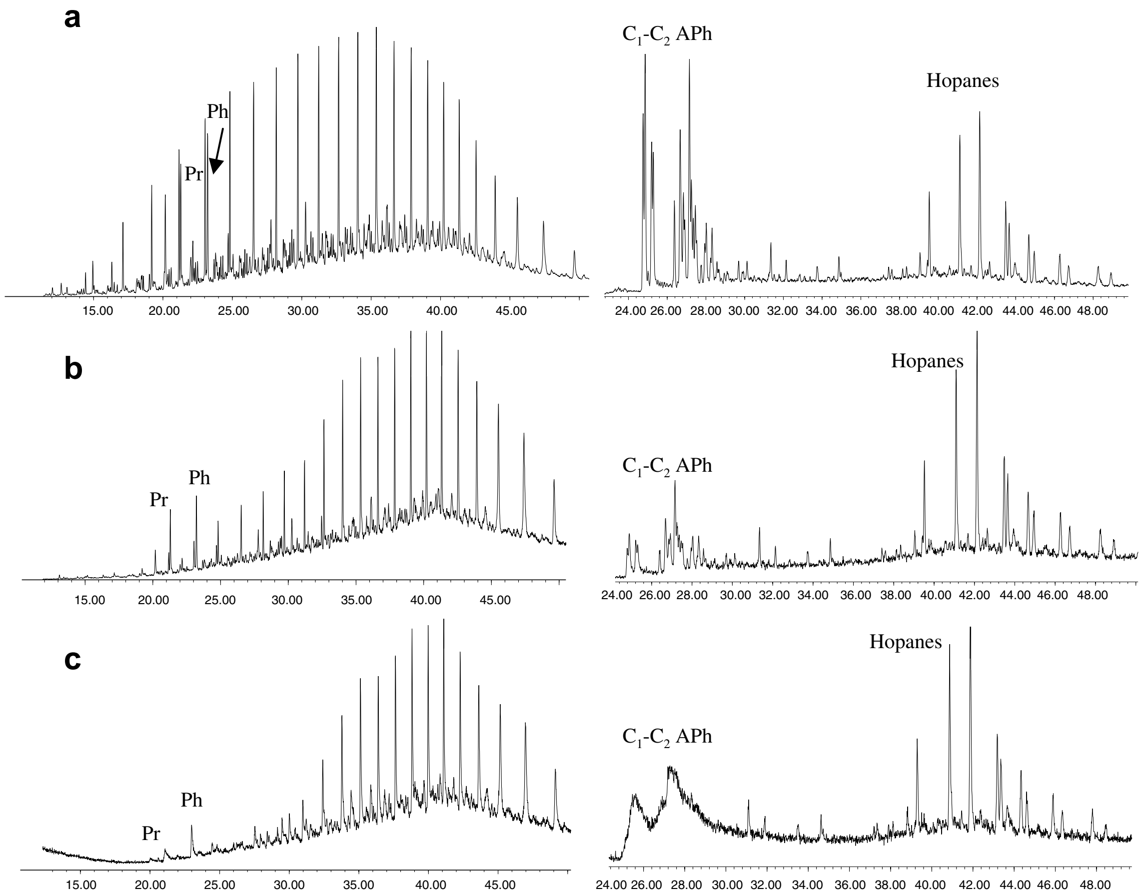

Fig. 8. SIM chromatograms (*m*/*z* 57, *m*/*z* 191 right) after sampling a fuel-polluted boulder partially submerged in fresh water at Cuño site. (a) corresponds to the fuel scraped from upper part of the rock (never submerged) where the fuel was black; (b) is result of GC–MS analysis of fuel scraped from the intermediate part where it was intermittently submerged in fresh water and appeared brownish; (c) was obtained after analysis of fuel that was always submerged in fresh water and covered by a microbial mat. (Pr: pristane; Ph: phytane; C_1–C_2 APh: methyl and dimethyl anthracenes and phenanthrenes).

cyanobacteria would provide the molecular oxygen for aerobic metabolism, as well as cooperating in the physical desegregation of the fuel, whereas aerobic bacteria would make use of typical mechanisms, i.e. biosurfactant and bioemulsifier production (Fig. 7a), to facilitate their own access to hydrocarbons (Gallego et al., 2001; Ron and Rosenberg, 2001).

As explained above, this microbial potential is enhanced only in specific areas where humidity, nutrient levels and a variety of microorganisms are maintained by the gentle flow of fresh water. More importantly, this freshwater flow weakens fuel adhesion and so increases bioavailability, dramatically enhancing biodegradation. On the other hand, wherever a flow of fresh water is absent, the inhospitable microenvironment on the shore rock surfaces, combined with the physicochemical characteristics of the fuel (heavy and viscous, and high resin and asphaltene contents) all but impede microbial activity. To overcome this problem, new bioremediation engineering strategies based on irrigation of shore rocks are needed (Gallego et al., unpublished results).

4. Conclusions

1. The fate of hydrocarbons at shorelines affected by oil spills is highly conditioned by the significant heterogeneity of the oil load, weathering processes and microbial activity, among other factors. Therefore, rigorous sampling protocols should be implemented to quantify fuel loads on shore rocks and geochemical changes should be systematically monitored by using reliable analytical procedures. In this work, novel meth-

ods of image analysis have been developed to meet the first objective and will be useful in future comparable studies. In addition, the demonstrated usefulness of hopane biomarkers, especially 17α(H), 21β(H) hopane, enabled us to effectively monitor the evolution of different hydrocarbon families.
2. The *Prestige* fuel oil that reached the Spanish coasts had chemical characteristics (high proportion of asphaltenes and resins) that hindered rapid weathering, specifically biodegradation, suggesting that the bioavailability of heavy fractions was extremely low at most of the sites. More than two years after the spill, the sites where no remediation treatment was performed still maintain >50% of the initial amount of aromatic compounds; however, light and medium *n*-alkanes were almost totally degraded in the immediate months following the spill.
3. The hydrocarbon depletion was enhanced at subareas where the oleophilic fertilizer S200 was applied. Moreover, microbiological analysis revealed an important growth of hydrocarbon degrading populations wherever S200 was applied. As a consequence, this product can be considered a reasonable option for bioremediation of marine oil spills.
4. Hydrocarbon-degrading microorganisms were widely distributed at the polluted sites, revealing rapid adaptation to the presence of large amounts of fuel and ruling out any necessity for applying bioaugmentation. Highly complex consortia of eukaryotic and prokaryotic microbes (cyanobacteria, fungi and bacteria) conceivably enabled degradation of fuel to take place when parameters such as humidity, dissolved oxygen and nutrient availability were optimal and fuel adhesion was physically weakened. However, these conditions were present and so only favourable in certain, small areas of the contaminated sites where fresh water flowed through the shore rocks. This conclusion suggests further novel bioremediation approaches based on irrigation with fresh water flowing into the contaminated sites.

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