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Biodegradation of a crude oil by three microbial consortia of different origins and metabolic capabilities

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Microbial consortia were obtained three by sequential enrichment using different oil products. Consortium F1AA was obtained on a heavily saturated fraction of a degraded crude oil; consortium TD, by enrichment on diesel and consortium AM, on a mixture of five polycyclic aromatic hydrocarbons [PAHs]. The three consortia were incubated with a crude oil in order to elucidate their metabolic capabilities and to investigate possible differences in the biodegradation of these complex hydrocarbon mixtures in relation to their origin. The efficiency of the three consortia in removing the saturated fraction was 60% (F1AA), 48% (TD) and 34% (AM), depending on the carbon sources used in the enrichment procedures. Consortia F1AA and TD removed 100% of *n*-alkanes and branched alkanes, whereas with consortium AM, 91% of branched alkanes remained. Efficiency on the polyaromatic fraction was 19% (AM), 11% (TD) and 7% (F1AA). The increase in aromaticity of the polyaromatic fraction during degradation of the crude oil by consortium F1AA suggested that this consortium metabolized the aromatic compounds primarily by oxidation of the alkylic chains. The 500-fold amplification of the inocula from the consortia by subculturing in rich media, necessary for use of the consortia in bioremediation experiments, showed no significant decrease in their degradation capability.
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Introduction

Microbial degradation by natural populations, the primary process in natural decontamination [22], can be enhanced by bioremediation technologies [3].

Petroleum products contain thousands of individual hydrocarbons and related compounds [23]. Their main components are usually subdivided into saturates (*n*- and branched-chain alkanes and cycloparaffins) and aromatics (mono-, di-, and polynuclear aromatic compounds [PAHs] containing alkyl side chains and/or fused cycloparaffin rings). The less abundant resins and asphaltenes consist of more polar compounds, containing heterocycles, oxygenated hydrocarbons and aggregates with high molecular weight.

Given the complexity of oil products, a combination of bacterial strains with broad enzymatic capabilities will be required to achieve extensive degradation. However, most of the crude oil degradation studies reported in the literature have been carried out with single or mixed bacterial strains isolated because of their ability to grow in mineral media with crude oil as the only carbon source [4,21,26]. Since alkanes are the most abundant compounds, these isolates are usually alkane degraders that, in some cases, are able to oxidize selectively the alkyl chains of certain alkylated PAHs, which are common in crude oils [11]. Our previous results using *Pseudomonas* sp. F21, isolated in a mineral medium with Arabian crude oil, corroborate this observation. Strain F21, which degrades all the *n*-alkanes and branched alkanes of low molecular weight, also causes selective depletion of methylated naphthalenes, phenanthrenes, chrysenes and pyrenes [4,26]. Nevertheless, since *Pseudomonas* sp. F21 is not able to utilize nonsubstituted PAHs (unpublished results, A.M. Solanas), depletion of this type of compound is probably due to oxidation of the alkylic chains, as described for other alkane degraders [11].

Oil-degrading mixed cultures can be constructed either by combining a number of strains with known complementary degradative capabilities (defined consortia) [13,14,19] or by direct enrichment procedures (nondefined consortia) [5,27,28]. The first approach is well defined and easily repeatable, but has certain disadvantages. First, since any one bacterial strain degrades only a limited number of crude oil components [20], in order to achieve wide and extensive crude oil biodegradation a consortium with many strains will be needed. Alkane degraders reported in the literature usually do not break aromatic rings, and PAH degraders do not grow on alkanes [12]. Recently, a few strains capable of degrading high molecular weight PAHs and alkanes have been reported [9], but this does not seem to be a general feature. Aromatic degraders use either monoaromatic hydrocarbons, hydrocarbons with two to three rings or hydrocarbons with three to four rings [15]. Furthermore, there is little information about strains that grow on alkylated PAHs with three or more rings [16,25], very common in crude oils. Degradation of the large portion of crude oil consisting of nonresolved nonidentified compounds has not been addressed. Another consideration in relation to defined consortia is the possible formation of toxic intermediate metabolites [7]. It has been reported that during the degradation of mixtures of hydrocarbons by selected strains, a number of intermediates of unknown persistence and toxicity may accumulate as a result of cometabolism [17].

Enrichment procedures with selected oil products or with some specific components can provide nondefined metabolically specialized microbial consortia. The result is a microbial population naturally selected by its metabolic cooperation in the degradation of

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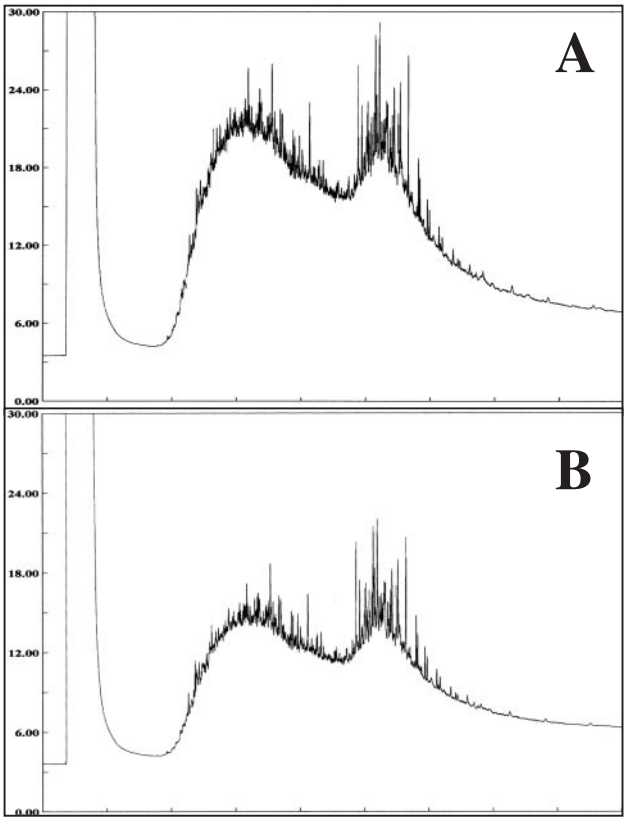

Figure 1 Chromatographic profiles (GC-FID) of the saturated fraction of Cantel Aptiano-Albiano crude oil after 30 days of incubation in uninoculated controls (A) and in cultures of consortium F1AA (B).

each mixture, with potentially higher efficiency in degrading identified and nonidentified components. In addition, degraders of dead-end products accumulated as a result of cometabolic processes [17] are likely to be selected. The prevention of the degradation of PAHs by dead-end products of their own metabolism has been reported. For example, it was demonstrated that the production of fluoren-9-one as a result of a nonproductive branch of the metabolism of fluorene by *Arthrobacter* sp. F101 inhibits the degradation of this PAH [7]. Mixed nondefined cultures can be maintained in the laboratory by continuous growth on the enrichment mixture. However, given that large amounts of inocula will be necessary for use in bioaugmentation technologies, these mixed cultures should be able to maintain their degradation capabilities after subculturing in rich media.

In this paper we describe the development of three specialized microbial consortia obtained by enrichment culture procedures on different hydrocarbon mixtures. A degraded crude oil was used to obtain a consortium specializing in degradation of saturated hydrocarbons of high molecular weight. A light derivative oil product, containing saturated hydrocarbons and PAHs with low molecular weight, was used to obtain a versatile consortium. Finally, a defined mixed of five PAHs, phenanthrene, anthracene, fluorene, fluoranthene and pyrene, was used in order to develop a consortium specializing in degradation of PAHs. After each 6-month period of subculturing, the consortia were subjected to metabolic characterization on their respective carbon sources.

The three consortia were incubated with a crude oil in order to elucidate their metabolic capabilities and to investigate possible differences in the biodegradation of these complex hydrocarbon mixtures in relation to their origin. Mineral medium with Casablanca crude oil was the sole source of carbon and energy. A rigorous chemical analysis of metabolic capability in relation to aliphatic and aromatic fractions was carried out. Since depletion of the aromatic hydrocarbon fraction is often due mainly to the oxidation of the alkylic chains of some PAHs and not to true ring fission, the aromaticity index described by Sugiura *et al* [27] was used to determine the catabolism caused by each consortium. Given that large amounts of inocula are necessary for bioaugmentation technologies, the biodegradation capabilities of these consortia were studied using direct inocula from the enrichment cultures and inocula from subcultures of these consortia in rich medium.

Materials and methods

Microbial consortia

The three microbial consortia were derived from soil samples from different hydrocarbon-contaminated sites near Barcelona (Spain). Microbial consortium F1AA was obtained from enrichment cultures established in mineral medium containing 0.5% (v/v) of the saturated fraction (F1) of Cantel Aptiano-Albiano oil, inoculated with a sandy soil contaminated with crude oil. Cantel Aptiano-Albiano is a Cuban crude oil from the Varadero field. A general characteristic of the crude oils from Varadero is that they are highly degraded, with no *n*-alkanes and acyclic isoprenoids in their saturated fraction. This fraction exhibits a profile of esteranes and hopanes over a noticeably unresolved complex mixture (UCM) in GC analyses [6] (Figure 1). The saturated fraction from Cantel Aptiano-Albiano oil was obtained as described below. Cultures were transferred monthly for 2 years.

Microbial consortium TD was obtained from enrichment cultures in mineral medium and diesel (0.1%, v/v; British Petroleum, Barcelona, Spain) inoculated with a sample of soil exposed to intermittent leaking from an diesel tank. Cultures were transferred twice weekly for 3 years.

Microbial consortium AM was obtained from a sandy soil contaminated with crude oil. Enrichment cultures were established in mineral medium containing a mixture of five PAHs (fluorene, phenanthrene, pyrene, anthracene, fluoranthene) at a final concentration of 0.1% (0.02% of each component). The consortium was maintained by twice-weekly transfers in fresh medium for 3 years. Degradation of the PAHs by the enrichment culture was determined by HPLC [24].

Bacterial counts from microbial consortia were performed using a miniaturized MPN method [29]. Total heterotrophs were counted with tryptone soy broth (Difco Laboratories, Detroit, MI); aliphatic degraders were counted in mineral medium containing 1 g/l of *n*-hexadecane; and aromatic hydrocarbon degraders were counted in mineral medium containing a mixture of phenanthrene (0.5 g/l),

Table 1 Characteristics of Casablanca crude oil

Composition[a] (%)	
Saturates (F1)	57
Monocyclic aromatics (F2)	11
Polycyclic aromatics (F3)	15
Polars (crude oil–F1–F2–F3)	17
n-C17/pristane	0.76

[a]Data obtained from gravimetric analyses.

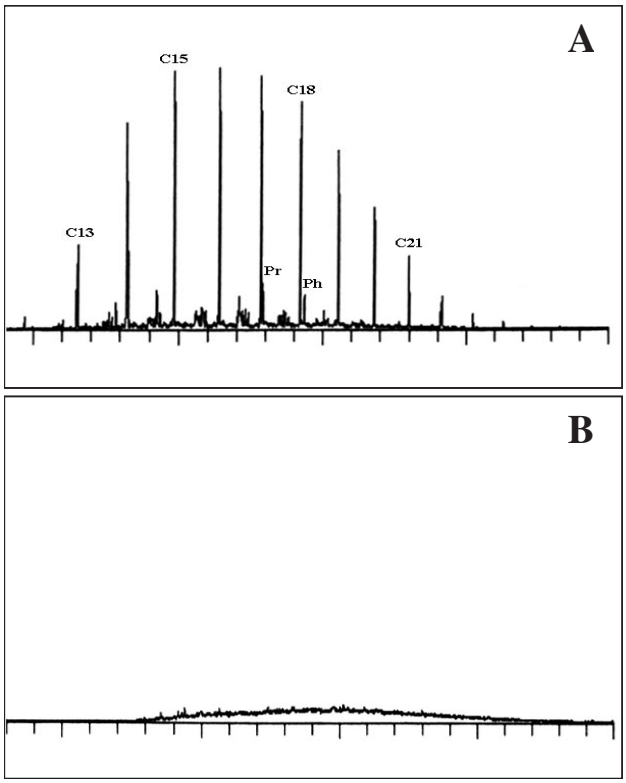

Figure 2 Total ion chromatographs (TIC) of organic extracts from 14 days noninoculated controls (A) and cultures of consortium TD in mineral media and diesel. (B) C13–C21, *n*-alkanes containing 13–21 carbons; Pr, pristane; Ph, phytane.

fluorene, anthracene and dibenzothiophene (each at a final concentration of 0.05 g/l).

Crude oil

Casablanca is a crude oil from the Tarragona Basin (Spanish Mediterranean off-shore). The composition analysis performed according to the methods below (Table 1) was consistent with that reported by Abaigés *et al* [2]. The oil sample was kindly supplied by Dr. J. Grimalt (CID, CSIC, Barcelona).

Media

The mineral medium used throughout this study was that described by Hareland *et al* [18]. The medium was sterilized before the addition of the crude oil. The oil was placed in an air-tight closed vial, sterilized at 121°C for 20 min and added to the mineral medium at a 0.5% (v/v) final concentration.

Biodegradation assays

Biodegradation assays were carried out in 250-ml Erlenmeyer flasks containing 50 ml of mineral medium and 250 μl (200 mg) of Casablanca crude oil as the sole source of carbon and energy. For each consortium two types of inocula were used, a direct inoculum obtained from the enrichment cultures in mineral media, and an inoculum containing cells from enrichment cultures subcultured in Luria-Bertani medium (LB).

The direct inoculum consisted of samples from transfers of the enrichment cultures incubated for 7 (TD and AM) or 15 days (F1AA). The inoculum pregrown in LB was obtained by subculturing 500 μl of the same transfers in 50 ml of LB. After overnight incubation, 1-ml samples of the LB cultures were washed and used to inoculate the mineral medium with Casablanca crude oil. For both types of inocula, the targeted final concentration of cells was approximately 10^7 cfu/ml.

Casablanca oil cultures were incubated for 10 and 20 days at 25°C with rotary shaking. Noninoculated flasks were included as controls for abiotic losses.

Analyses of crude oil samples

The residual crude oil from cultures and controls was extracted with dichloromethane (5×10 ml) in duplicate samples. Before extraction, anthracene-d_{10} was added to each flask as a surrogate internal standard to give a final concentration of 0.1 μg per μl (1 ml of a 5 mg/ml solution in acetone). The extracts were dried over Na_2SO_4 and concentrated in a rotary evaporator to a final volume of 50 ml. At this point, a 1-ml aliquot was separated for gas chromatography/mass spectrometry (GC-MS) analysis. The area of the peak for the surrogate internal standard was used to calculate a rate for recovery efficiency, which was later used to correct the quantitative data of the cultures. The remaining extract was evaporated to constant weight under a gentle nitrogen stream and analyzed as follows.

Gravimetric measurement: The amount of residual crude oil was determined by measuring the weight of the dry extract; and biodegradation was evaluated by comparison of its weight with a control sample.

Column chromatography: All the extracts were fractioned according to Aceves *et al* [1]. Each extract was resuspended in 1 ml of hexane and loaded in a glass column (30 cm×1 cm i.d.) filled with 8 g each of 5% water-deactivated alumina (70–230 mesh, Merck, Darmstadt, Germany) (top) and silica (70–230 mesh, Merck) (bottom). The following fractions were eluted: (F1), 20 ml of *n*-hexane (aliphatic hydrocarbons); (F2), 20 ml of 10% dichloromethane in *n*-hexane (monocyclic aromatic hydrocarbons); and (F3), 40 ml of 20% dichloromethane in *n*-hexane

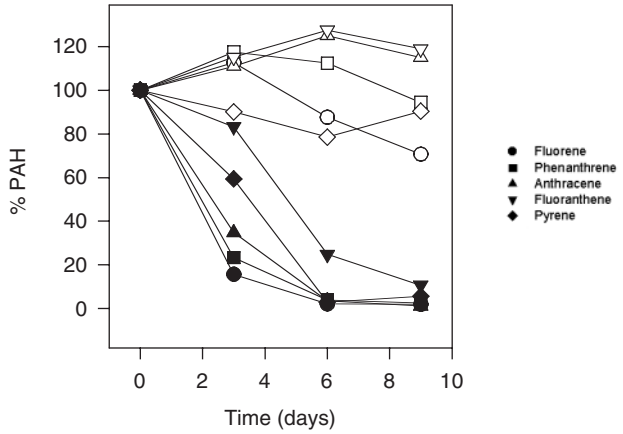

Figure 3 Degradation of a mixture of PAHs by consortium AM. The concentration of fluorene (● culture, ○ control), phenanthrene (■ culture, □ control), anthracene (▲ culture, △ control), fluoranthene (▼ culture, ▽ control) and pyrene (◆ culture, ◇ control) was determined by HPLC analyses of organic extracts.

Table 2 Composition of the enrichment cultures of each microbial consortium used to inoculate flasks in biodegradation experiments with Casablanca crude oil

	Heterotrophs[a]	Hydrocarbon degraders[a]	
		n-Hexadecane	PAHs
F1AA	$9.3\pm0.17^{b}\times10^{8}$	$1.4\pm0.17\times10^{6}$	$4.2\pm0.19\times10^{3}$
TD	$2.9\pm0.19\times10^{9}$	$1.8\pm0.18\times10^{9}$	$5.1\pm0.18\times10^{7}$
AM	$6.8\pm0.17\times10^{8}$	$7.8\pm0.17\times10^{7}$	$1.8\pm0.18\times10^{7}$

[a]MPN/ml.
[b]Standard error of $\log_{10}$(MPN).

(PAHs). These fractions were evaporated to dryness and submitted to gravimetric and GC-MS analyses.

GC-MS: GC-MS analyses were performed with a HP5890 series II gas chromatograph with a 5989A mass selective detector. Compounds were separated on a HP-5 capillary column (25 m×0.32 mm i.d.) with 0.25-μm film thickness and helium used as the carrier gas (10 psi). The column temperature was held at 35°C for 2 min and then programmed to 310°C at 4°C/min. This final temperature was held for 10 min. Injector, transfer line and analyzer temperatures were set at 280, 280 and 300°C, respectively. The injection was in splitless mode keeping the split valve closed for 30 s. Data were acquired in the electron impact mode (70 eV), scanning range m/z 50–650 at 1 s per decade. Naphthalene-d_8 was added to extracts in dichloromethane as internal standard.

The peak areas of *n*-alkanes, pristane and phytane in the total ion chromatogram for the aliphatic fraction (F1) were used to determine the biodegradation rate of these compounds through comparison of cultures and controls. Aromatic fraction (F3) analysis was done through GC-MS operating in selected ion monitoring (SIM) mode. Two windows were designed (0–27.6 and 27.6–70 min) and the targets for analysis were naphthalene, phenanthrene and their alkyl derivatives C1–C4; fluorene, dibenzothiophene and their alkyl derivatives C1–C3; and fluoranthene, pyrene and chrysene. To determine the biodegradation of each analyte, the corresponding reconstructed ion chromatograms were obtained and the areas of the compounds in cultures and in controls were compared.

^{13}C NMR: Spectra of the aromatic fractions (F3) were recorded in a Unity 300 spectrometer (Varian, Palo Alto, CA) at 300 MHz, the intensity of each chemical shift being determined relative to internal tetramethylsilane in $CDCl_3$. Aromaticity was defined as the ratio of aromatic carbon to total carbon. The amount of aromatic carbon was obtained from the integrated intensity of the peaks between 100 and 170 ppm, while the amount of aliphatic carbon was found from the integrated intensity of peaks between 8 and 58 ppm. The aromaticity index was calculated according to the equation: fa=(amount of aromatic carbon)/(amount of aromatic carbon+amount of aliphatic carbon) [27].

Results

Consortia

The three consortia were quite efficient in removing their hydrocarbon substrates. Although the GC-FID profile of Cantel Aptiano-Albiano corresponds to a highly degraded crude oil, consortium F1AA causes 39% depletion (GC-FID) of its aliphatic fraction in 30 days (Figure 1). This depletion is mainly due to the reduction of the UCM, while the profiles of the resolved esteranes and hopanes [6] of the control and the cultures are similar. Consortium TD caused the depletion of all the major GC-resolved components of diesel in 10 days (Figure 2). Consortium AM caused the complete depletion of four of the PAHs in the mixture (phenantrene, fluorene, pyrene and anthracene) in 6 days, while 90% of fluoranthene was removed in 9 days (Figure 3).

Similar results were maintained over time, which shows that their metabolic capabilities were sustained (data not shown). The microbial counts and the hydrocarbon-degrader composition of each consortium are shown in Table 2. Also, the counts were comparable during the period of the enrichments, which suggests that the mixed populations reached a certain stability.

Degradation of Casablanca crude oil by microbial consortia

For the biodegradation experiments with each consortium, one set of cultures was inoculated directly with cells from the enrichment cultures (direct inocula), and the other set was obtained by subculturing the enrichment cultures overnight in LB. Overnight incubation in LB led to an approximately 500-fold increase in population. The inocula from these LB cultures were then diluted in order to match the concentration of the direct inocula ($10^7\mu$l/ml). The purpose of using inocula pregrown in LB was to determine whether subculturing in rich medium, which would be necessary

Table 3 Percentages (%) of biodegradation of Casablanca crude oil by microbial consortia in 10 and 20 days of incubation

	F1AA				TD				AM			
	D		LB		D		LB		D		LB	
	10 days	20 days	10 days	20 days	10 days	20 days	10 days	20 days	10 days	20 days	10 days	20 days
Total[a]	26	30	14	35	20	22	15	37	15	23	22	27
F1[a]	53	60	25	57	49	48	25	54	28	34	29	44
F2[a]	29	42	12	28	13	14	12	23	15	27	18	19
F3[a]	0	0[b]	6	7	6	11	5	12	7	19	3	18
TPH[a] (F1+F2+F3)	38	46	21	44	37	37	16	42	22	30	23	36
n-alkanes[c]	100	100	100	100	100	100	100	100	90	98	88	98
Pr+Ph[c]	100	100	100	100	100	100	10	97	8	9	7	9

D, direct inoculum from enrichment cultures; LB, inoculum from subcultures in LB medium; Pr, pristane, Ph, phytane.
[a]Obtained by comparison of gravimetric data from cultures and from controls.
[b]The biodegradation rate was below the detection level.
[c]Obtained by comparison of chromatographic data from cultures to those of controls.

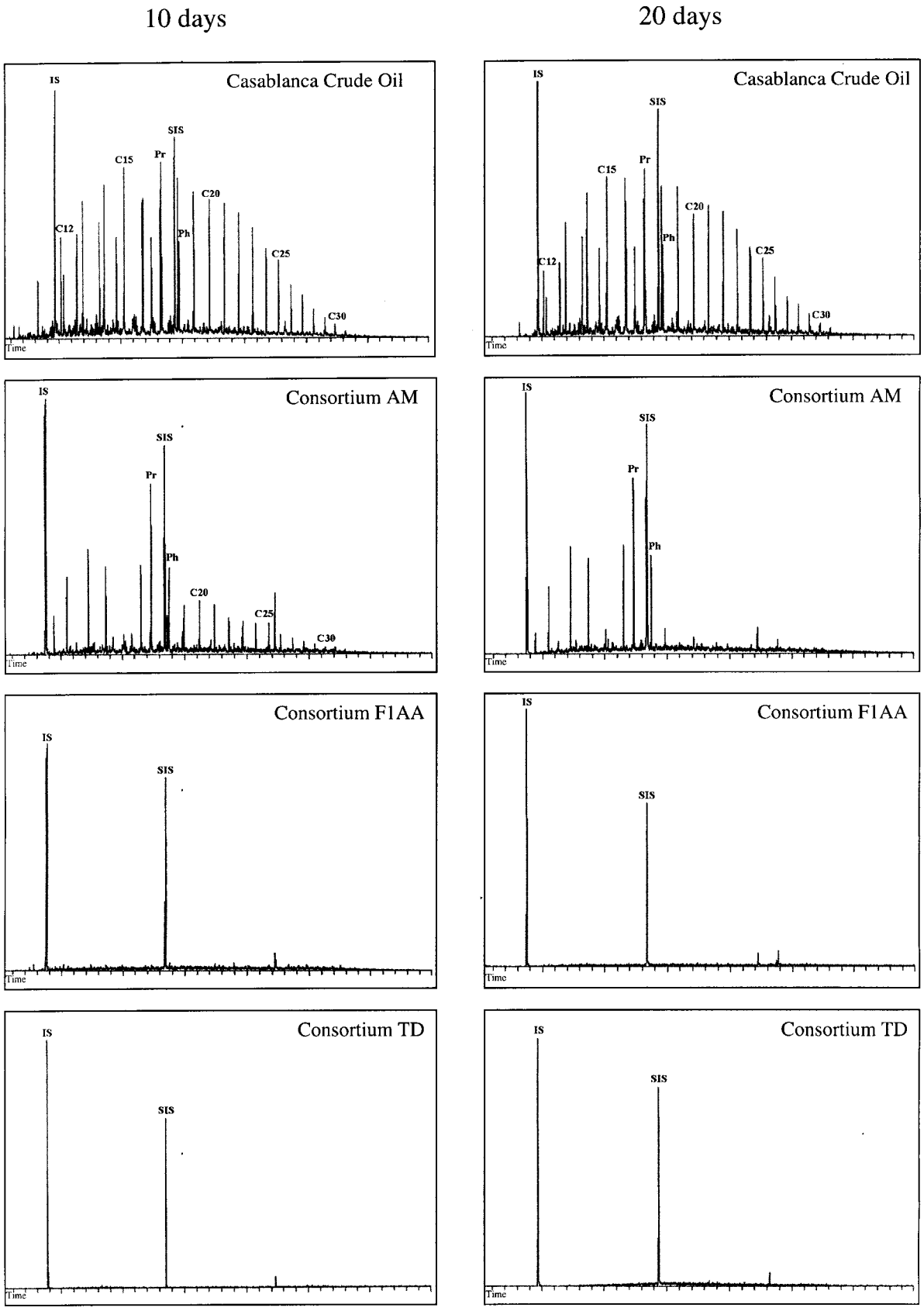

Figure 4 Total ion chromatographs (TIC) of organic extracts from 10 and 20 days noninoculated controls (A), and cultures of consortia F1AA (B), TD (C) and AM (D) in mineral media and Casablanca crude oil. C12–C30, *n*-alkanes containing 12–30 carbons; Pr, pristane; Ph, phytane; IS, internal standard; SIS, surrogate internal standard.

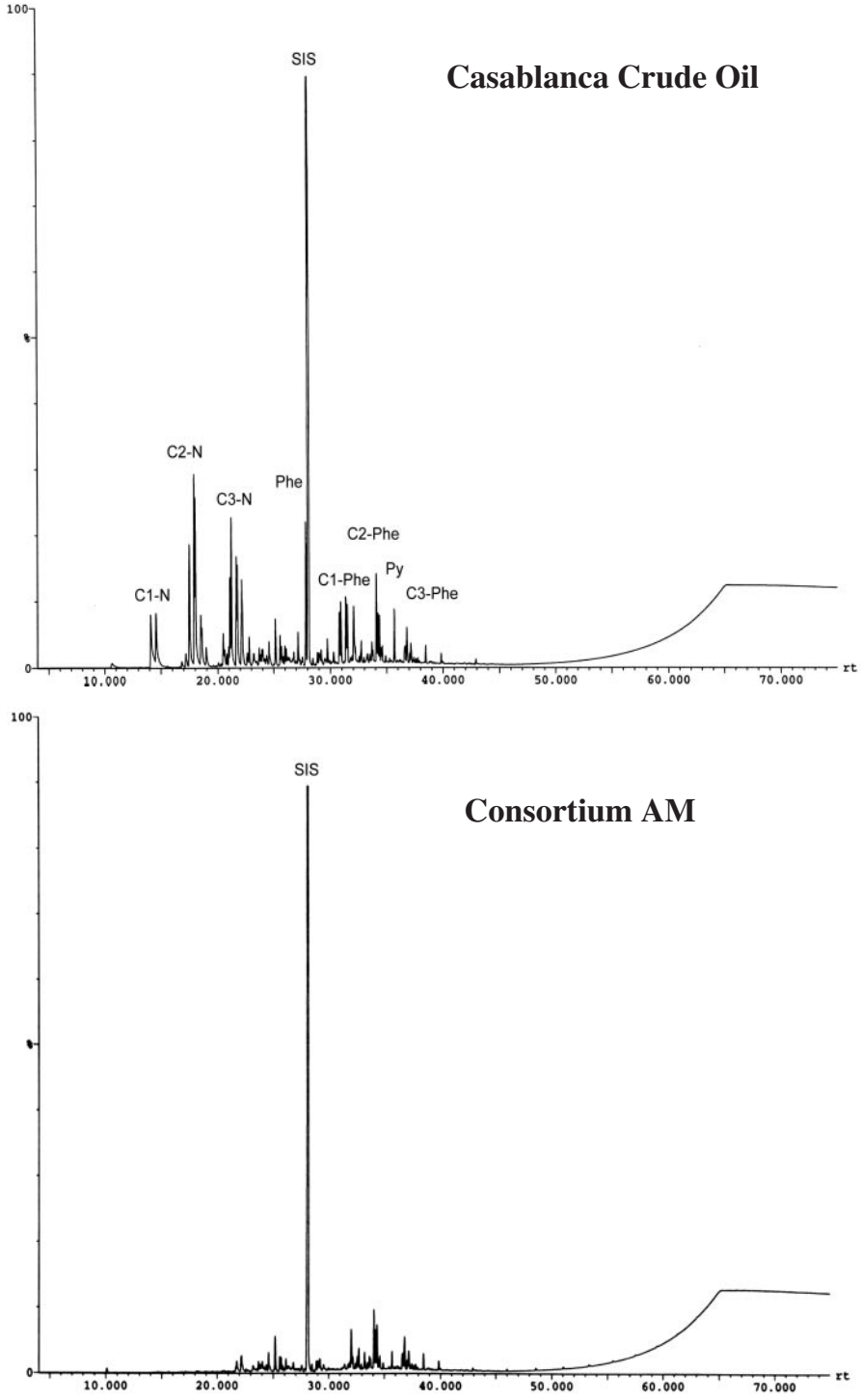

Figure 5 Chromatographic profile of the aromatic fraction of Casablanca crude oil using GC-MS operating in SIM mode. N, Phe, Py and SIS represent naphthalene, phenanthrene, pyrene and surrogate internal standard (anthracene-d_{10}), respectively. C1–C3 represent carbon numbers of the alkyl groups in alkylated PAHs.

for using these consortia as inocula in field bioremediation experiments, would modify their biodegradation capability.

Cultures incubated for 20 days showed a higher degree of degradation than the corresponding replicates incubated for 10 days. Therefore, we here report the degree of degradation at 20 days.

According to gravimetric analyses of the total extract, the direct cultures from consortia TD and AM caused 22% and 23% biodegradation of the crude oil in 20 days, while F1-AA degraded 30% (Table 3). Similar or higher percentages were obtained with the cultures inoculated with the overnight cultures in LB, indicating

Table 4 Percentage (%) degradation of targeted PAHs by microbial consortia

Molecule	F1-AA	TD	AM
Naphthalene (Naph)	100	100	100
C1-Naph	100	100	100
C2-Naph	100	100	100
C3-Naph	62	80	87
C4-Naph	59	68	11
Fluorene (Fl)	100	100	100
C1-Fl	97	100	87
C2-Fl	36	100	18
C3-Fl	0	94	9
Dibenzothiophene (DBT)	ND	ND	ND
C1-DBT	86	94	75
C2-DBT	5	96	40
C3-DBT	2	87	29
Phenanthrene (Phe)	97	100	100
C1-Phe	54	100	96
C2-Phe	54	100	48
C3-Phe	0	85	20
C4-Phe	0	45	35

C1–C4 represent carbon numbers of the alkyl groups in alkylated PAHs. Compounds Naph, C1-Naph, C2-Naph, C3-Naph, C4-Naph, Fl, C1-Fl, C2-Fl, C3-Fl, C1-DBT, C2-DBT, C3-DBT, Phe, C1-Phe, C2-Phe, C3-Phe and C4-Phe were characterized by the signals at m/z 128, 142, 156, 170, 184, 166, 180, 194, 208, 198, 212, 226, 178, 192, 206, 220 and 234, respectively. DBT was characterized by the signals at m/z 139+152. The percentage of biodegradation was calculated by comparing in cultures and controls the areas shown by the targeted compounds in reconstructed ion chromatograms from the F3. Naphthalene-d_8 was used as internal standard; anthracene-d_{10} was used as a surrogate internal standard.

that subculturing the consortia for a limited number of generations in liquid medium does not significantly modify their overall biodegradation potential.

GC-MS analyses of the total extracts show that both TD and F1AA caused a complete depletion of the major components, corresponding, as we discuss below, to *n*-alkanes and the branched alkanes. Consortium AM degraded all the *n*-alkanes, but most of the isoprenoid alkanes remained (Figure 4). In general, recovery of the surrogate internal standard after the extraction of the cultures was about 10% lower than for the respective controls (which represents 80–85% of the added crude oil). Although surrogate internal standards are of general use in environmental chemistry, the use of these compounds in quantifying biodegradation experiments is rare. Our results suggest that the lack of surrogate internal standards in studies may lead to overestimation of biodegradation.

The results observed with the GC-FID analyses of the total extracts were confirmed after they were fractioned by column chromatography. As shown in Table 3, consortium F1AA degraded a high percentage (60%) of the saturated fraction of Casablanca oil. This consortium also degraded the monoaromatic fraction (42%), but the polyaromatic fraction was not degraded (0%) by direct inoculum and only lightly degraded (7%) by LB inoculum. Gravimetric analyses of the fractions from the cultures of consortium TD showed lower, though still considerable, degradation of the saturated fraction (48%) and the monoaromatic fraction (14%), whereas the polyaromatic fraction was 11% degraded. Although consortium AM was obtained by sequential enrichment in nonalkylated PAHs, this mixed culture caused moderate depletion of saturates (F1) (34%). The degradation of the aromatic fractions was the most significant, 27% of F2 and 19% of F3 as expected on the basis of their origin. Figure 5 shows the chromatographic profiles of the aromatic fraction of Casablanca crude oil and the cultures inoculated by the AM consortium.

Degradation of total petroleum hydrocarbons (TPH=F1+F2+F3) was higher than that of the total extract, which can be explained by the lower degradability of resins and asphalthenes as well as the possible accumulation of partially oxidized metabolites extractable in the conditions of the study. In fact, the total amount of polars [total extract – (F1+F2+F3)] in the cultures of consortium F1AA increased by 60% over controls. Consortium TD had similar results, while consortium AM reduced the polars by 17%. TPH with the direct inocula was degraded similarly to TPH with the inocula subcultured in LB.

Degradation of targeted compounds did not always correlate with the gravimetric data, which is to be expected because of the many compounds not resolved in GC. GC-MS analyses showed that the *n*-alkanes in Casablanca crude oil were completely depleted by consortia F1AA and TD, and almost completely depleted by consortium AM (98%) in 20 days (Figure 4). The extent of this degradation of *n*-alkanes was very similar at 10 days (Table 3). Consortia F1AA and TD also caused the removal of branched alkanes: pristane and phytane were completely removed in 20 days. It should be noted that at 10 days of incubation, the TD culture inoculated with the overnight LB subculture had removed only 10% of pristane and phytane, indicating a greater delay than with the direct culture (Table 3). Consortium AM caused low degradation of branched alkanes (9% in 20 days).

The reconstructed ion chromatograms for targeted aromatic compounds demonstrated that the three consortia caused considerable degradation of the compounds containing two, three or even four aromatic rings. The results of the quantification of the selected compounds are shown in Table 4. Naphthalene, fluorene and phenanthrene were depleted to nondetectable levels by the three consortia, while the degradation of the alkylated derivatives generally decreased with the increase in the number of methyl groups. The highest degradation of targeted PAHs was achieved with consortia TD and AM. The results obtained with consortium F1AA were not expected because this consortium had not reduced significantly the weight (gravimetric measurement) of the polyaromatic fraction.

^{13}C NMR spectroscopy has been used in the analyses of the composition of various molecular species of petroleum [10]. More recently, Sugiura *et al* [27] used this technique to analyze biodegradation of Arabian crude oil. The aromaticity of the aromatic fraction in the controls was 0.225 and increased slightly to 0.253 after biodegradation with consortium F1AA (Table 5). Conversely, biodegradation with consortia TD and AM decreased the aromaticity of the aromatic fraction to 0.201 and 0.159, respectively.

Table 5 Aromaticity index obtained from the aromatic fraction (F3) of the extracts from control and cultures after 20 days of incubation

	Aromaticity
Control	0.225
F1AA	0.253
TD	0.201
AM	0.159

Aromaticity data showed no significant dispersion (lower than 1% in all cases).

Discussion

The three microbial consortia obtained show considerable efficiency in biodegradation of each class of hydrocarbons used as carbon source in their respective enrichment procedures. The extent of the attack on the different fractions of a crude oil by the different consortia was consistent with their origin. Consortium F1AA was more efficient in degradation of the saturated fraction; consortium TD attacked the aliphatic fraction to a high degree but also degraded the polyaromatic fraction; and consortium AM was the most efficient in the degradation of the polyaromatic fraction.

The amplification of the consortia 500-fold by subculturing in rich medium did not change significantly their degradative capability under the conditions of the study. These results support the use of these mixed cultures as inocula in bioremediation experiences.

It is difficult to compare gravimetric data with those reported by other authors. Biodegradation of crude oils has been studied extensively, but there is great variability in the concentration of crude oil used, the type of crude oil, the incubation time and in the methodologies used to quantify degradation. A number of different solvents, fractioning and analytical techniques have been used. Surrogate internal standards are seldom utilized and, according to our results, this results in overestimation of biodegradation. Also, as degradation cannot be evaluated with a single analytical parameter, gravimetric studies should be complemented by analytical methods to identify the major compounds and quantify targeted minor compounds.

Consortium F1AA showed the most extensive degradation of the total extract (30–35%) and TPHs (44–46%). The 60% biodegradation of the saturates is mainly due to the complete removal of *n*-alkanes, including compounds of high molecular weight with 20–33 carbons, and the disappearance of the branched alkanes. In a similar study, Sugiura *et al* [27] reported 19–34% degradation of the total extracts of different crude oils by a nondefined mixed culture (SM8) obtained by enrichment in artificially weathered crude oil, but the aliphatic fraction was only removed by 40–53%. Pristane and phytane were removed up to 75 to 100% depending on the crude oil. Palittapongarnpim *et al* [21] reported 26–63% biodegradation of Tapis crude oil TPHs by three different single bacterial isolates, and 39–87% by two strains of *Candida tropicalis*. However, these figures were based on depletion of the resolved peaks in GC chromatographic profiles. In fact, the chromatograms showing degradation of the total extract of Tapis crude oil by the best oil degrader (*C. tropicalis* MU15Y) show the persistence of some major peaks. Chhatre *et al* [8] reported a 70% degradation of the major peaks resolved in GC of Bombay high crude oil by a defined consortium constructed with four oil-degrading bacterial strains selected for their capability to degrade saturates and aromatics. This consortium also degraded more than 50% of the aliphatic and the aromatic fractions. However, these data were obtained by supplying concentrations of these fractions to the culture, and so cannot be compared with bulk crude oil degradation.

The high extent of biodegradation of the monoaromatic fraction by consortium F1AA may be due to degradation of alkyl chains of the major components, the alkylbenzenes. It has been demonstrated that the depletion of alkylbenzenes by alkane degraders may be due to partial degradation caused by the oxidation of the lateral linear chains [11].

Consortium F1AA did not cause a significant decrease in the weight of the aromatic fraction, although some targeted aromatic compounds were biodegraded. It should be noted that the target nonsubstituted PAHs are the most readily degradable and they are present in crude oil at very low concentrations. Therefore, they could have been attacked by the few aromatic degraders present in consortium F1AA (Table 2), without this having any detectable effect on the weight of the complex aromatic fraction. The increase in aromaticity of the F3 suggests that degradation of alkylated derivatives could be explained by cooxidation of the alkyl chains to produce partially oxidized PAHs. Similar results were reported by Sugiura *et al* [27]. The importance of cooxidation processes during the degradation of the crude oil by F1AA is also suggested by the increase in the polar fraction.

According to these results, consortium F1AA has potential for degradation of mineral oils. Consortium TD also showed extensive degradation of Casablanca crude oil. Although the total extract, TPHs, saturates and monoaromatics were attacked to a slightly lower extent than in consortium F1AA, the branched alkanes were also completely depleted. In addition, this consortium caused more than 10% degradation of the polyaromatic fraction and completely removed the targeted parent PAHs and their C1–C2 derivatives, reducing the C3- and C4- by more than 50% (except C4-Phe). The attack on targeted aromatics was higher than that reported in similar studies by other authors [5,27]. For example, in a detailed study on the degradation of alkylated HAPs from Arabian crude oils by a marine sediment community, Budzinsky *et al* [5] reported 30–70% degradation of C1- and C2-DBT, and 20–95% degradation of C1- and C2-Phe. Other studies reported higher degradation of F3 [8], but the different analytical techniques used and the lack of information about specific analytes make comparison of results difficult.

The results obtained with both direct inoculum and inoculum subcultured in rich medium suggest the potential of consortium TD for treating environmental samples polluted with crude oils and light derivatives such as diesel.

Consortium AM showed moderate degradation of the saturated fraction, with most of the branched alkanes remaining. The presence of alkane degraders in this consortium is indicated in Table 2. Whether those alkane degraders persist in enrichment after subculturing them in PAHs or are actually aromatic degraders that can degrade alkanes cannot be determined with the data available. Although most studies point out that alkane degradation and aromatic degradation seem to be exclusive properties [12], recent studies have reported some *Pseudomonas* and *Mycobacterium* strains that grow on both types of compounds [9]. The extent of the degradation of the aromatic fractions by these consortia was no higher than that shown by TD. These results could be explained by the lower concentration of nonsubstituted PAHs in crude oil than their alkylated derivatives and other families of compounds and by the complexity of the aromatic fraction. It is concluded that enrichment on selected PAHs does not make mixed populations more efficient in degradation of the aromatic fractions of crude oils than of crude oil fractions such as diesel. Work is in progress on the molecular taxonomic characterization of consortium AM and in the application of the three consortia in environmental samples contaminated with oil products such as mineral oils and creosote.

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