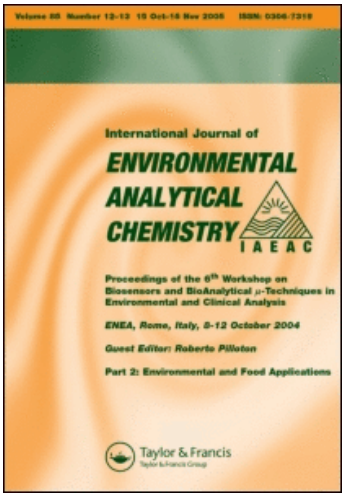

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Selective Aerobic Degradation of Methyl-Substituted Polycyclic Aromatic Hydrocarbons in Petroleum by Pure Microbial Culturest

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Studies of laboratory degradation of Arabian light crude oil (b.p.>200°C) by pure cultures of bacterial strains *Pseudomonas* sp. isolated from tanker ballast waters showed that biodegradation of alkyl-substituted polycyclic aromatic hydrocarbons strongly depends on the number, position, and the type of the substituents.

Biodegradation of alkylphenanthrenes and dibenzothiophenes decreased with increasing number of substitutents. Biodegradation of methylphenanthrenes (P), diben-

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zothiophenes (DBT), pyrenes (Py) and chrysenes (C) showed a pattern similar to that previously observed for the methylnapthalenes; compounds containing unsubstituted α and β adjacent positions were more readily degraded. Thus, 2- and 3-MP, 2- and 3-MDBT, 1-MPy and 2- and 3-MC were shown to be less resistant to degradation within these families. Cycloalkylaromatics, such as partially aromatized steranes, hopanes and 8,14-secohopanes were generally resistant to degradation by the cultures employed.

KEY WORDS: Aromatic hydrocarbons, petroleum biodegradation, *Pseudomonas* sp, Shpolskii spectrometry.

INTRODUCTION

Microbial degradation is assumed to be one of the main processes, accounting for the ultimate fate of petroleum in the aquatic environment.[1] Because of the interest in understanding the mechanisms causing the disappearance of oil from estuarine and marine waters the ability of freshwater and marine bacteria to degrade petroleum, either in the field or in the laboratory by pure or mixed cultures, has been studied extensively.

The rates of biodegradation of petroleum hydrocarbon families,[2-6] the relationships between degradation pathways and crude oil composition[7,8] and the role of environmental conditions[3,9-11] are some of the aspects that have received major attention in these studies. As a result, a fair agreement exists about the relative rates of aerobic degradation of the different hydrocarbon families.[2-4,12] Howeverer, little reference has been made to individual components within each family, except for the *n*-alkanes and some particular aromatics (e.g. naphthalene and methylnapthalenes) whose degradation is easily recognizable by gas chromatography[3,4,13,14] and for some geochemical markers (e.g. steranes, hopanes and aromatized steranes) which are readily characterized by mass fragmentography.[15-17]

The application of signature patterns of alkylsubstituted polycyclic aromatic hydrocarbons (PAHs) in the study of petroleum maturation in the reservoirs[18] and in source recognition in the environment[19,20] has prompted an interest in the effects of natural processes such as biodegradation on the homologous and isomeric distributions of these components. In addition it is known that

biological activity of PAHs and their methylderivatives is dependant on their isomeric structures,[21] so that the persistence or degradation of specific isomers in the environment is a question of major concern.

In the present paper we report the aerobic biodegradation in the laboratory of an Arabian light crude oil resiude (b.p. > 200°C), with emphasis on the resulting changes in the distributions of alkylphenanthrenes and dibenzothiophenes as well as in the isomeric methylphenanthrenes, dibenzothiophenes, pyrenes and chrysenes. The degradation was carried out using a pure culture of *Pseudomonas*, a genus which has been widely used in laboratory studies of petroleum degradation.[22-25]

EXPERIMENTAL

Biodegradation experiments

An Arabian light crude oil residue (b.p. > 200°C) was added at concentrations of 0.4% to 100 ml of artificial sea water supplemented with NaH_2PO_4 (3.5×10^{-4} M) and KNO_3 (10^{-2} M). This mixture was inoculated with a pure bacterial isolate (*Pseudonomas* sp.) and incubated at 22°C in an orbital shaker at 120 rpm during 20 days as described previously.[13] After this period, the degradation was stopped by adding 10 ml of CCl_4 and keeping the mixture at 4°C until analysis. In each experiment, sterile flasks were used as controls, and were submitted to the same conditions without inoculation.

Hydrocarbon analysis

The biodegraded crude oil was extracted from the culture medium with 3×15 ml of CCl_4. The extract was dried over anhydrous Na_2SO_4 and vacuum evaporated to near dryness. The residue was diluted in 1 ml of *n*-hexane and fractionated by column chromatography using deactivated silica (8 g) and alumina (8 g) (5% H_2O), as described elsewhere.[26] The saturated fraction was eluted with *n*-hexane (20 ml); two more fractions were eluted with 10% methylene chloride in *n*-hexane (20 ml; monocyclic aromatic hydrocarbons) and 20% methylene chloride in *n*-hexane (40 ml; PAHs).

a) GC and COM-GC-MS analyses The PAHs fraction was further fractionated into alkylnapthalenes (Rf 0.37) and alkylphenanthrenes (Rf 0.27) by silica-gel thin layer chromatography after two developments with *n*-hexane. The latter fraction was analyzed by GC-FID-FPD (Perkin Elmer 990-Tracor) using 30 m DB-5 fused silica columns (J&W Scientific), temperature programmed from 60 to 280°C at 2°C min. Methylphenanthrenes and methyldibenzothiophenes were evident from the GC profiles. Individual isomers were confirmed by COM-GC-MS (Hewlett-Packard 5995-9825) and published retention data.[27] Cyclic scanning was performed at 690 amu per second and temperatures were held at 300, 200 and 230°C for the GC-MS interphase, the ion source and the analyzer, respectively. The chromatographic conditions were those previously indicated for the GC analysis. The alkyl homologue distributions were calculated from the mass fragmentograms using a desk computer (HP 86) with a digitalizer (HP 9111A).

b) Low temperature emission spectroscopy Alternatively, the PAH fraction was resolved according to ring number and alkyl substituents by normal and reversed phase HPLC as described elsewhere.[28] Each collected fraction was dissolved in a suitable solvent (*n*-hexane, *n*-octane) at low concentration ($c \approx 10^{-6}$ M) and analyzed by low temperature emission spectroscopy.[29] Fused silica tubes containing the solutions were attached to the cold head of a closed cycle cryogenerator (CTI Cryodine) operating at a temperature of 15 K. Excitation was provided by the light of a Xenon lamp (450 W) dispersed by an H 20 Jobin–Yvon monochromator (bandpass: 4 nm). Lumnescence emission was observed through an HR 1000 Jobin–Yvon monochromator (bandpass: 0.80 nm). Detection was provided by a photomultiplier (EMI 9789) coupled to a microcomputer (IBM).

RESULTS AND DISCUSSION

Gross compositional changes

The microbial strain selected for this study (F21) was the most active degrador of hydrocarbons among a set of seven strains of *Pseudomonas* isolated from tanker ballast waters.[30] The pattern of utilization of saturates and aromatics in the Arabian light crude oil is

TABLE I

Results of a 20 days degradation of an Arabian light crude oil residue (b.p. > 200°C) by *Pseudomonas* (strain F-21) at different conditions

Crude oil concentration in water (%)	Incubation temp. (°C)	% of crude oil degradation[a]			
		Saturates	Aromatics		
			Monocyclic	Polycyclic	S. containing
0.4	20	70	61	48(44)[b]	36
0.4	40	78	67	54	—
0.1	20	88	82	66	58

[a]Measured by GC.

[b]Percentage of degradation by inoculation of the pure fraction.

illustrated in Table I, where the percentage of degradation under different conditions is reported, based on the quantitation of resolved and unresolved components in the gas chromatogram of the corresponding fractions. The sulfur-containing PAHs (S-PAHs) were monitored by GC-FPD.

Temperature and time have only a moderate influence. When the incubation was performed at 40 days instead of 20 days only a 10% increase in degradation was achieved both for the saturate and the aromatic fraction. A similar small increase was observed when the time was doubled from 20 to 40 days. However, the concentration of oil in water, which relates to the substrate/nutrient ratio, had a significant effect, particularly for the S-PAHs, which achieve a 60% increase of degradation (Table I).

In general, the ranges of degradation for the different fractions are comparable or even higher than those reported by other authors using pure[17] or mixed cultures.[4,31] The relative rates also corresponds to those established for aerobic conditions: saturates > monocyclic > polycyclic > S-PAHs.[2,4,12] Although several authors have described a preferential degradation of aromatics under conditions of non-supplementation,[3] we have adopted those conditions (see the experimental part) which are more common in coastal environments.

Another question was the ability of these microorganisms to grow in pure fraction of PAHs which is also illustrated in Table I. Only a slight decrease of activity (from 48 to 44%) was observed when the

incubation was performed on the crude oil or on the previously isolated PAH fraction, so that cooxidation, although possible, is not strictly necessary for explaining the observed PAH degradation.

The severity of the degradation is exemplified in Figure 1. As shown, all the *n*-alkanes and most of the isoprenoids and low molecular weight iso+cyclic alkanes have disappeared from the saturated fraction. However, the polycyclic alkanes, namely the triterpanoid hopanes (I), are still preserved. This indicates moderate to extensive degradation (levels 4–5) according to the scale established by Volkman *et al.*[14]

At these levels mono and polycyclic aromatic hydrocarbons are also removed or reduced. Fedorak and Westlake[3] and Oudot[4] already noticed that simple aromatics were more readily degraded than certain alkanes. However, monoaromatic hydrocarbons attached to naphthene rings are less susceptible to degradation.[4,15,31] In this way, series of trace components such as the recently discovered benzophanes, which contain a benzene ring attached to the pentacyclic hopane structure (II) and the partially aromatized 8, 14 secohopanes (III)[32] are clearly observed in the chromatogram of the aromatic fraction (Figure 2). Other geochemical markers such as the partially aromatized steranes still require the use of mass-fragmentography, although the resistance of these markers to degradation provide a useful tool for correlation studies of biodegraded oils.

Conditions under which alkanes have not been totally metabolized, may nevertheless permit partial degradation of PAHs, with the particularity that attachment of alkyl substituents to the aromatic rings decreases the susceptibility to degradation. This degradation pattern has been observed among alkylnapthalenes in both laboratory[3,13] and field studies.[33,34] The marked differences, however, in vapor pressures and water solubilities among this homologous series[35] may suggest that losses are due to a combination of physicochemical processes rather than to biodegradation. In the present study the same trend has been observed and quantified for higher PAHs, namely alkyl-Ph and DBT (Figure 3). This appears therefore to be a common degradation pathway for alkylaromatics, although sulfur heterocycles are slightly more resistant to degradation. This pattern is consistent with the qualitative observations of Atlas *et al.*[36] following the Amoco Cadiz oil spill and must be taken into account in the use of homologue distributions for PAH source identification.

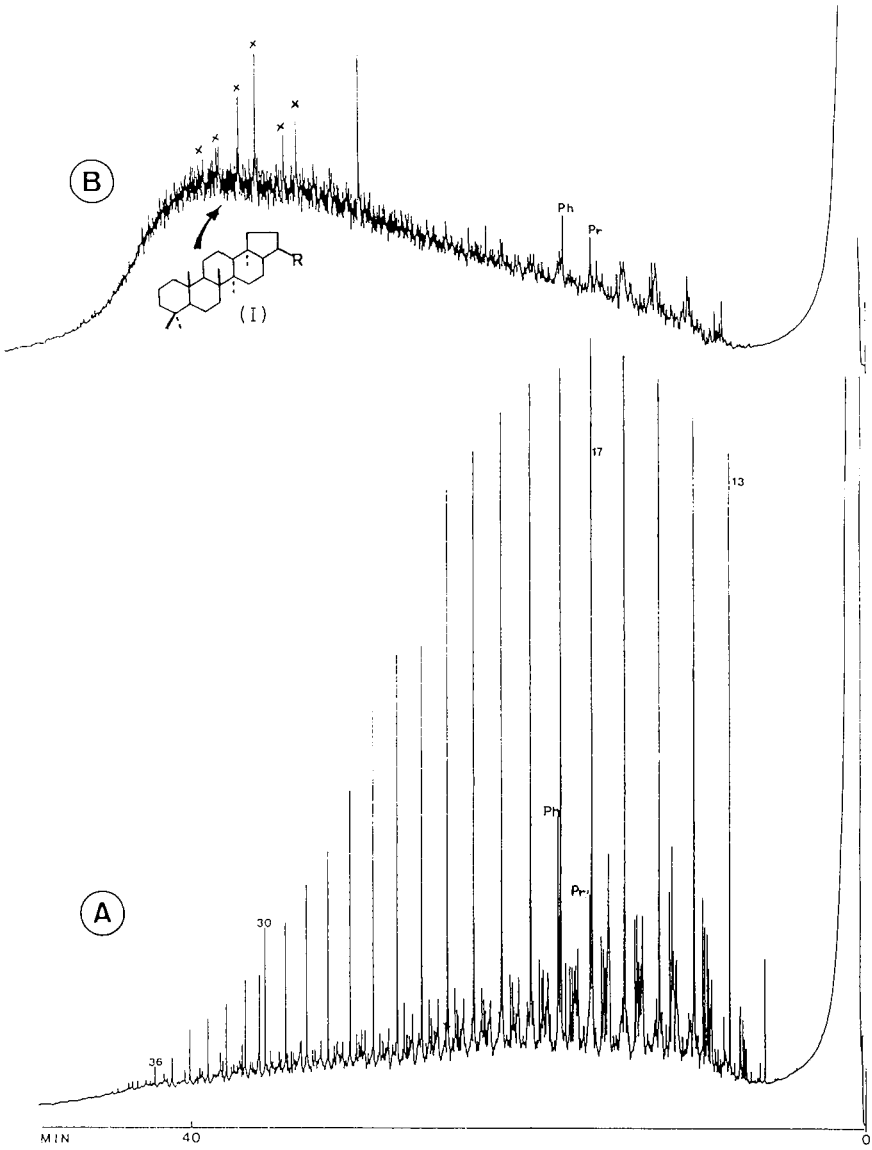

FIGURE 1 Gas chromatograms of the saturated fractions of Arabian light crude oil residue before (A) and after (B) degradation. Numbers indicate the series of *n*-alkanes. Pr = pristane, Ph = phytane, *x*: 17α(H), 21β(H)-hopanes.

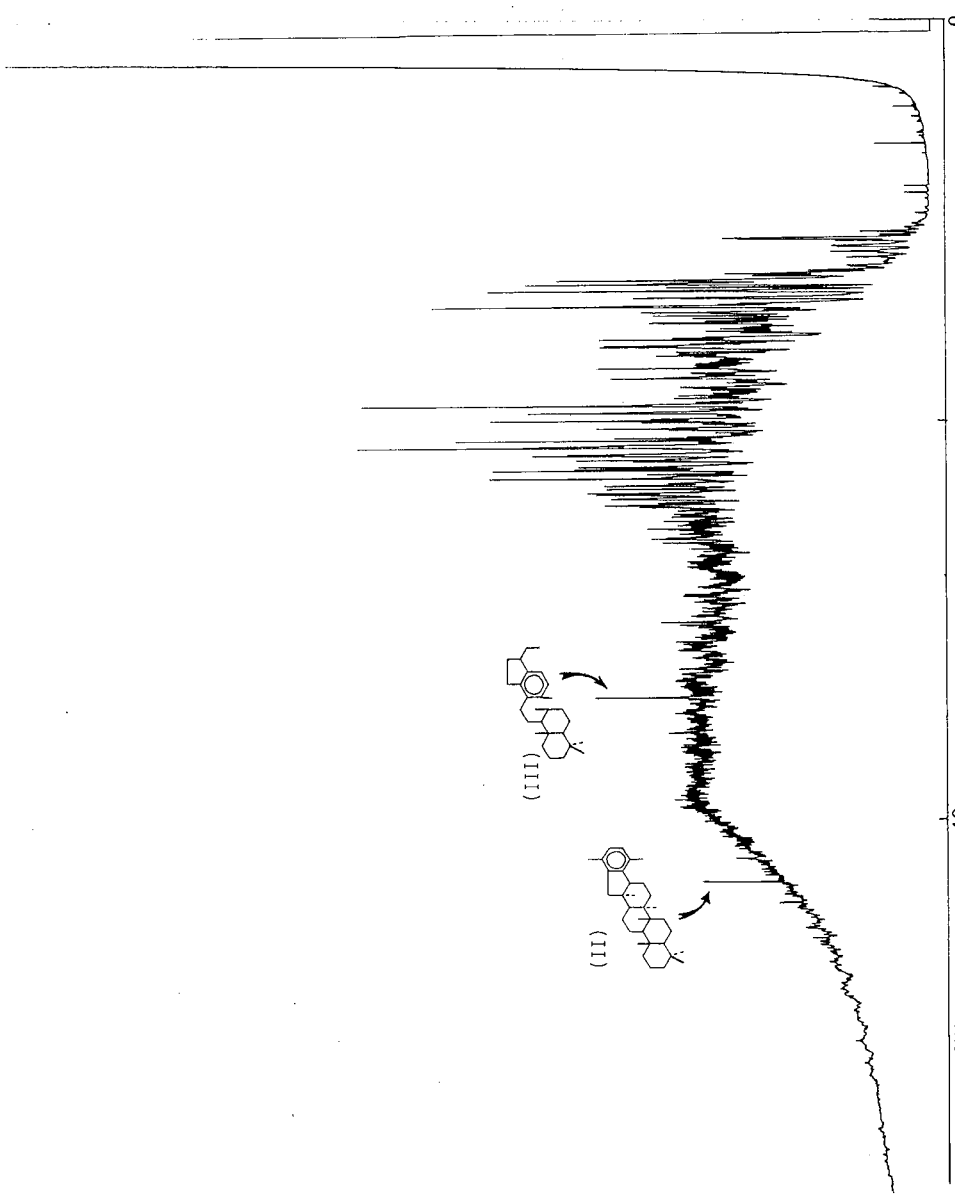

FIGURE 2 Gas chromatogram of the laboratory degraded aromatic fraction of Arabian light crude oil. II: benzophanes. III: 8, 14-secohopanoids.

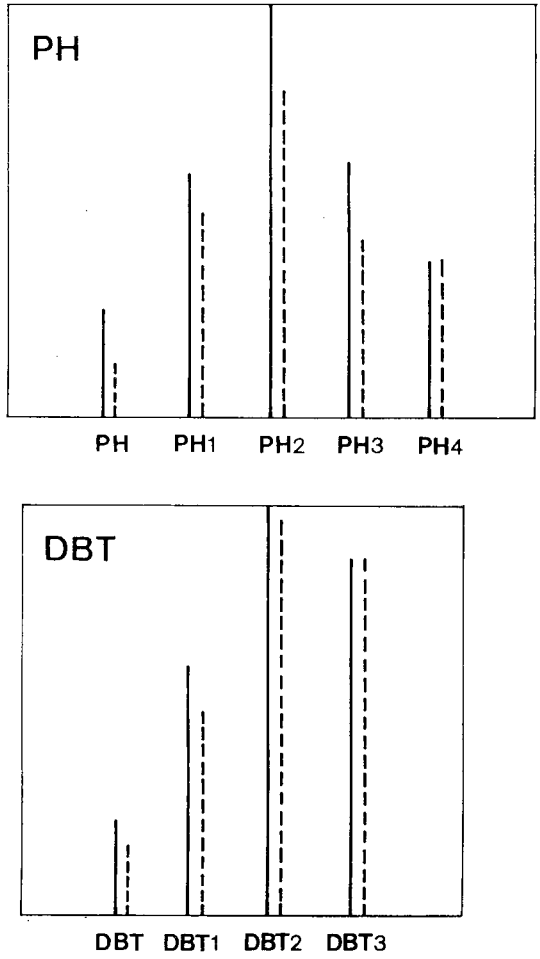

FIGURE 3 Homologous distributions of alkylphenanthrenes (PH) and dibenzothiophenes (DBT) in Arabian light crude oil before (solid bars) and after (dotted lines) degradation. Numbers indicate the number of carbon atoms in side chains.

Selective biodegradation of methyl-PAH isomers

Current information on the bacterial degradation of PAHs has been extensively reviewed;[22-25] the degradative pathways of parent PAHs have been shown to be very similar. Early studies demon-

strated that they are initially converted to dihydrodiols, ortho to each other and subsequently to hydroxycarboxylate derivatives by enzymatic cleavage of the ring.[22] The stereochemistry of the initial oxidation was later proved to be *cis* and preferably in α and β positions, by contrast with a mammalian system where it occurred in *trans*.[24]

Much less attention has been paid to the alkylated PAHs, either as pure compounds or within petroleum, probably due to a lack of pure reference compounds and of adequate techniques for analysis of the complex mixtures of petroleum hydrocarbons. Oxidation of alkyl-PAHs may take place in the aromatic ring as indicated above, or in the alkyl side chains, depending on the micro-organism strain, nature of the substrate and position of the substituents. Thus, toluene and ethylbenzene were both oxidized in the ring in α and β positions by *Pseudomonas* sp. and hydroxylated in the side chain by *Nocardia* sp.[22] Studies on xylene metabolism have shown that m- and p-xylene will support the growth of pure cultures through ring oxidation, but attempts to isolate organisms which will grow on o-xylene as the sole carbon source have been unsuccessful.[22] Nevertheless, in this case partial oxidation of methyl substituents can be obtained during growth of bacteria on other hydrocarbons, e.g. *n*-alkanes. This process, called cooxidation,[37] is believed to play an important role in the degradation of petroleum.

Either in this situation or in the direct oxidation of the aromatic nucleus, the position of methylsubstituents might be expected to have a definite influence on the degradation rates of different isomers.

In this respect, in a previous investigation of degradation of Arabian crude oil by *Pseudomonas*,[13] we have shown by that dimethylnaphthalane (DMN) isomers containing unsubstituted adjacent α and β positions are preferentially degraded; the degradation is enhanced when a methyl group is present in a contiguous position. Thus, 2,6- and 2,7-DMN are the more easily depleted isomers. Previously, Raymond *et al.*[38] found that *Nocardia* sp. cooxidizes preferentially the β methyl substituents among the DMN. No other laboratories have apparently addressed the question of the regioselectivity in the metabolism of alkylated-PAHs.

In order to gain further insight into this problem we have extended the investigation to higher methyl-PAH derivatives, such as

phenanthrenes (P), dibenzothiophenes (DBT), pyrenes (Py) and chrysenes (C) (Figure 4).

High resolution gas chromatography resolves many components of the aromatic fractions of petroleum (Figure 2) but leaves a multitude of unresolved or barely separated hydrocarbons. The trace quantities of the hydrocarbons of interest in the Arabian light crude oil required the use of alternative analytical techniques. Thus, methyl-P and DBT were identified by mass fragmentography (COM-GS-MS), the latter being confirmed by GC-FPD. On the other hand, methyl-Py and C isomers were monitored by high resolution emission spectroscopy (Shpolskii effect), which offers a promising potential for identifying isomeric PAHs in complex mixtures[39] (Figure 5).

Among the methyl-P, the 9-methyl isomer appears to be the most refractory to degradation (Figure 4A). This would suggest that microbial oxidation of *P* takes place in positions 9 (and 10) which are now occupied. Studies of localization of molecular energies have shown that these positions (*K*-region) exhibit higher reactivity;[40] they have, however, been found to be the major site of metabolism only in mammals.[41]

Gibson[24] isolated *cis*-3,4-dihydrodiol in bacterial oxidation of *P* and Cerniglia and Yang[42] identified *trans*-1,2 and 3,4-dihydrodiols as major metabolites in fungal oxidation. We do not know whether the lack of enzymatic attack at the *K*-region of *P* is a general feature for *Pseudomonas* or there is a preferent cooxidation of methyl substituents situated in ring A. An interesting feature is that 9-MP is the most resistant isomer during biodegradation of crude oils in geological reservoirs (Albaigés *et al.*, unpublished results). This is significant from the geochemical standpoint, because the ratio 2 $-$MP$+$3$-$MP/P$+$1$-$MP$+$9MP has been proposed as a maturity parameter for petroleum.[18] The similarity of *in vitro* and *in situ* degradation pathways affords complementary evidence of aerobic degradation of petroleum in reservoirs.[14]

The degradation of S-PAHs and particularly alkyl-DBT has been shown to proceed less efficiently than for the related PAHs, either *in vitro* (Figure 3)[5] or in field studies.[33,36] However, degradation of MDBT exhibit similar stereoselectivity. Isomers with methyl substituents in β position or with positions α and β unoccupied (2- and 3-MDBT) are easily degraded (Figure 4B). This feature must be taken into consideration when the isomeric distribution of MBDT is used

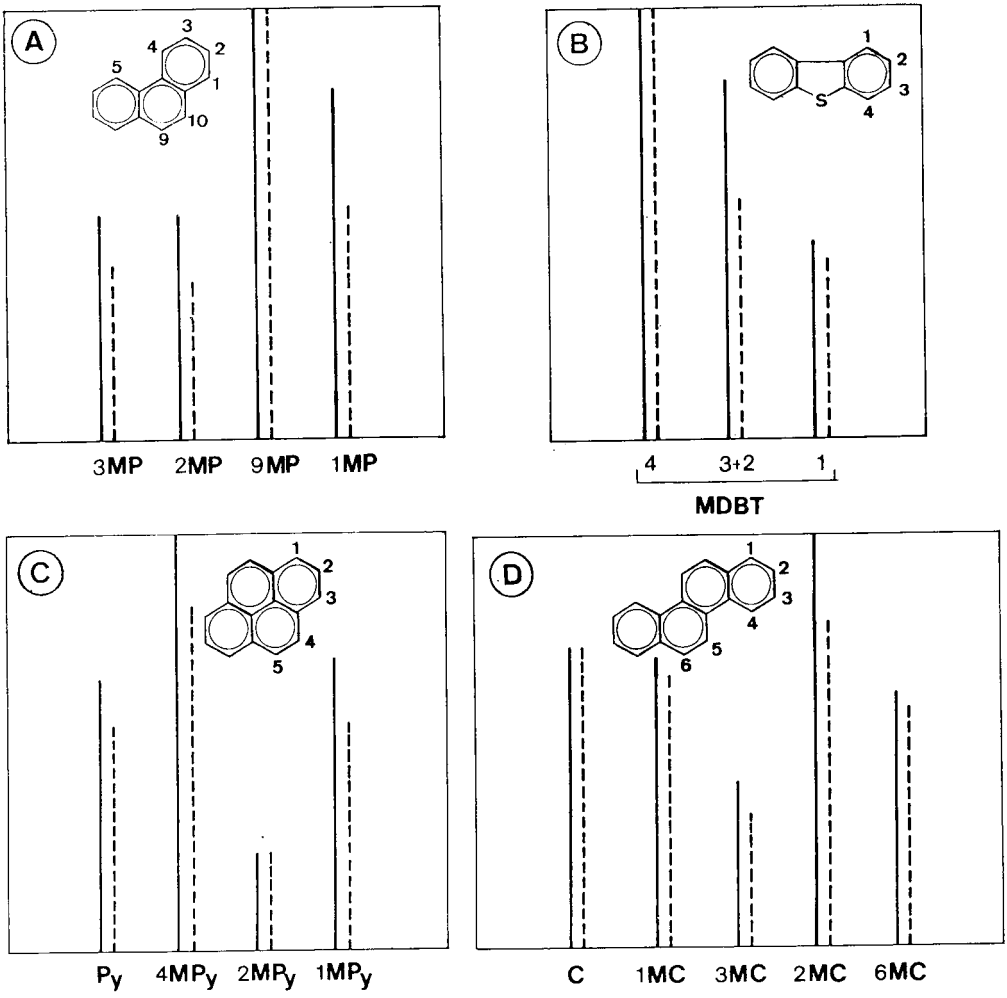

FIGURE 4 Isomeric distributions of methylphenanthrenes (A), dibenzothiophenes (B), pyrenes (C) and chrysenes (D) in Arabian light crude oil, before (solid lines) and after (dotted lines) degradation. Numbers indicate the methyl position in the molecule.

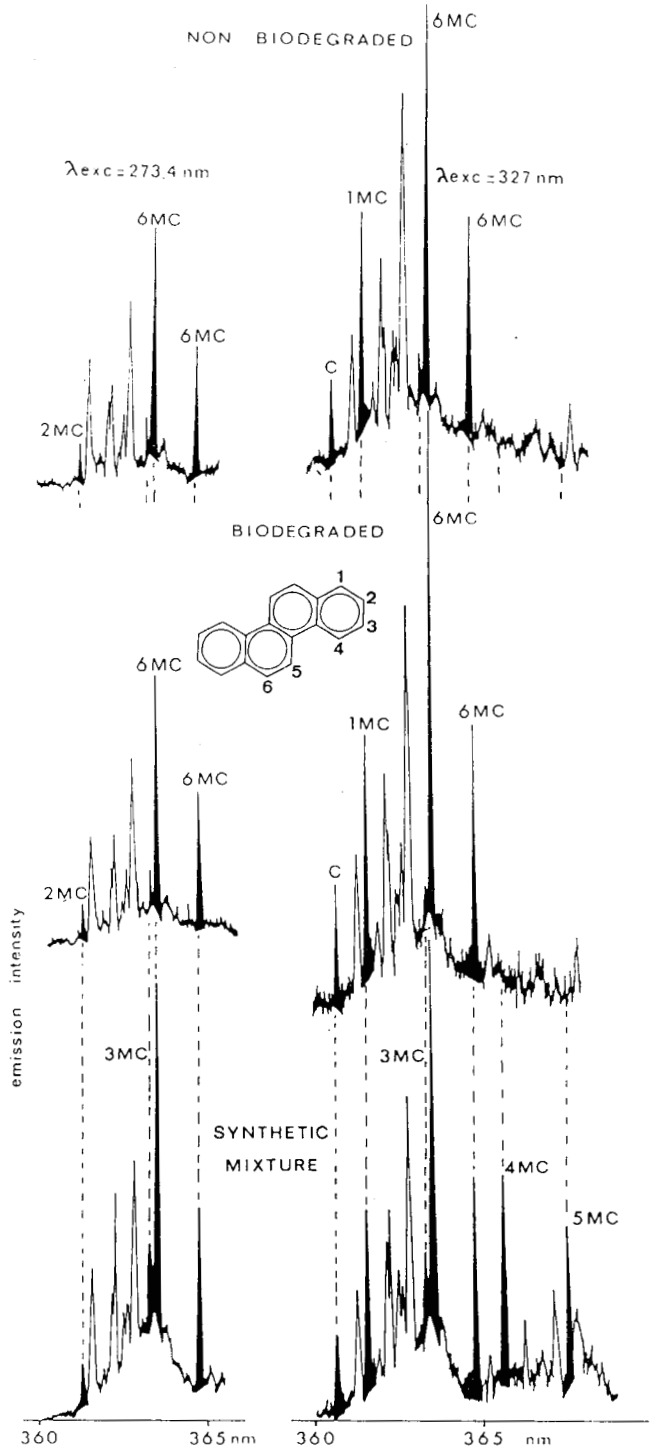

FIGURE 5 High resolution emission spectra of chrysene fractions of Arabian light crude oil, before and after degradation and a reference mixture.

for assessing sources of oil pollutants in the marine environment, as has been proposed.[43]

The degradation of M-Py and C, monitored by HR emission spectroscopy (Figure 5), followed similar trends. MC isomers containing free $\alpha\beta$ positions or methyl substituents in β (2- and 3-MC) are more susceptible to degradation (Figure 4D). However, in the case of Py the two criteria are not equivalent and then the 2-MPy (methyl in β position) is the more resistant (Figure 4C). It is difficult in this study to prove, without identifying metabolites, whether direct PAH oxidation or cooxidation processes occur. However, the last result, together with the easier degradation of the less substituted PAHs and the ability of strain F-21 to grow in a pure PAH fraction cannot exclude the possibility of a direct oxidation of the aromatic ring. In any case we have shown that the stereoselectivity of degradation of methyl-PAHs is a common process that may have significant geochemical and environmental implications.

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