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Phospholipid fatty acids of a marine sedimentary microbial community in a laboratory microcosm: Responses to petroleum hydrocarbon contamination

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Abstract

Laboratory microcosm experiments were performed to evaluate petroleum hydrocarbon biodegradation and phospholipid fatty acid (PLFA) composition in either non-contaminated (NC) or contaminated (C) sedimentary microcosms with a crude oil. An analytical procedure was developed to extract both hydrocarbons and PLFAs from the same culture. PLFAs were analyzed over time during an aerobic microcosm experiment (0–21 days) to obtain a better understanding of the structural changes in bacterial sedimentary communities. We observed an increase in both hydrocarbon degraders and heterotrophic bacteria as reflected in PLFA concentrations. Inferred total biomass ranged from 4 to 11.3×10^9 and 22.1 to 199.2×10^9 bacterial cells kg^{-1} dry sediment for NC and C sediments, respectively. Total biomass was greater in C than NC sediment, increasing by about twice, 3×, 13× and 50× for 0, 2, 7 and 21 days, respectively. These modifications coincided with removal of petroleum hydrocarbons that reached 35% of total hydrocarbon and 64% of total *n*-alkanes after 21 days. Microbial community structure changes over time in the C and NC microcosms were shown with principal components analysis and a study of some characteristic PLFAs as biomarkers. Some modifications occurred after several days of petroleum exposure, but crude oil addition did not produce an obvious increase in the biomass of petroleum degraders.

1. Introduction

Numerous studies have demonstrated the importance of microorganisms for the remediation of marine sediments contaminated with petroleum hydrocarbons (Madsen, 1991; White et al., 1998). Bioremediation depends on numerous physicochemical factors, the availability of nutrients and the nature and activity of sedimentary microorganisms (Santas and Santas, 2000; Maki et al., 2003). In marine sediments, microbial communities are involved in many biochemical transformations of various xenobiotics, including the degradation of

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recalcitrant chemical compounds (Rajendran et al., 1994; Keith-Roach et al., 2002). Phospholipids (PLs) are found exclusively in cell membranes and not in other parts of the cell (Hill et al., 2000) and are rapidly metabolized after cell death (White et al., 1998). For these reasons, PLs can be used to characterize the community structure (Rajendran et al., 1993; White et al., 1996; Macnaughton et al., 1999). Moreover, PLFA analysis could provide insights into the nutritional status or physiological stress response of microorganisms (Weber et al., 1994; Heipieper et al., 1995; White et al., 1998). However, there are some limitations that should be highlighted. On one hand, the possibility that intact phospholipids in sediments may partly derive from membrane remnants cannot be excluded (Rütters et al., 2002a). On the other hand, the concepts "cell death" and "viability" in natural systems are both biologically and chemically unclear, mainly due to the various states of dormancy and starvation that bacteria can adopt (Kell et al., 1998; Barer and Harwood, 1999).

Identification of the organisms responsible for bioremediation or the determination of both biochemical and physiological hydrocarbon biodegradation mechanisms has been extensively studied with laboratory approaches (Weber et al., 1994; Heipieper et al., 1995; Doumenq et al., 1999; Doumenq et al., 2001; Aries et al., 2001b; Syakti et al., 2004). In the case of in situ approaches, comparable studies are more difficult because of the complex influence of many parameters related to the marine environment. For these reasons, we undertook a laboratory microcosm study to investigate bacterial responses over time following contamination by petroleum hydrocarbons. The principal contribution of this work is the use of microcosms as opposed to in vitro laboratory techniques to look at the dynamics of the interactions. We adapted a simple analytical procedure (Fang and Findlay, 1996) which allows simultaneous hydrocarbon and PLFA quantification for the same culture. Two series of microcosms experiments were conducted: either non-contaminated (NC) or contaminated (C) sediments with a crude oil ("Brut Arabian Light") topped at 250 °C (BAL 250). We analyzed both the residual petroleum hydrocarbon and PLFA contents of these microcosms at different stages (0, 3 h and 2, 7 and 21 days). Variation in PLFA profiles and statistical treatment (principal component analysis, PCA) of the data revealed the effects of petroleum hydrocarbon contamination on the marine sedimentary microbial community structure.

2. Experimental procedures

2.1. Chemicals and materials

Acetone, dichloromethane, heptane, methanol (chromasolv grade), diethyl ether (Puriss PA) and boron trifluoride in methanol (10% w/w) were obtained from Supelco (USA). Silica-gel and alumina (70–230 mesh) and thin layer chromatography (TLC) plates (Si 60 F_{254}), were obtained from Merck (Germany). Filters (GF/F, 47 mm ∅) and silica gel plus sep-pak™ cartridges were, respectively, obtained from Whatman (England) and Waters (Ireland). Dimethyl disulfide (99%), iodine (99.99%) and pyrrolidine (99%) were purchased from Sigma–Aldrich (Germany). Yeast extract and bact O peptone (Difco) were provided by BD biosciences (San Jose, CA, USA). Blend Arabian Light petroleum topped at 250 °C (BAL 250) was provided by Total (Lacq, France).

2.2. Sediment sampling

Sub-surface sediment (3 kg) was collected with PVC core samplers (20 cm length and 10 cm diameter) in April 2003 from an aquaculture zone (*Mytilus galloprovincialis*) in a semi-closed cove located at the western part of the Gulf of Fos, France (Carteau cove – 43°23′N, 4°53′E, 5 m depth; Fig. 1). The top of each core (0–2 cm) was extruded and sampled. Any debris and fragments of macrofauna were manually removed. The sediment was then successively sifted with 2 and 1 mm mesh sieves, immediately homogenized and divided into two equal parts. The first was used as a non-contaminated control (NC) and the second was contaminated (C) with a large amount of BAL 250 (about 16 g BAL 250 per kg of dry sediment).

2.3. Microcosm cultures

The contents of the microcosms were mixed (reciprocal shaker, 1.5 Hz) for 3 h before the first harvest, in order to homogenize the BAL 250 and sediment. Experiments were carried out in the dark at 20 °C in Erlenmeyer flasks containing 20 mL of synthetic seawater (SSW) and 100 g of wet sediment, on a reciprocal shaker (1.5 Hz). Each experiment was conducted in duplicate. The SSW was

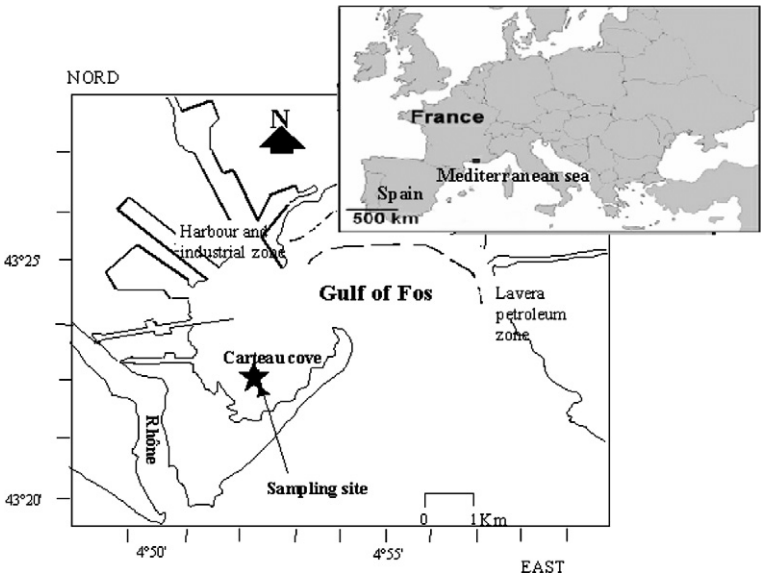

Fig. 1. Location of sediment sampling: Carteau Cove, France (43°23′N, 4°53′E, 5 m depth).

composed of 23 g L^{-1} of NaCl, 0.75 g L^{-1} of KCl, 2 g L^{-1} tris hydroxymethylaminomethane, 1 g L^{-1} NH_4Cl, 3.9 g L^{-1} $MgSO_4$, 5 g L^{-1} $MgCl_2 \cdot 6H_2O$, 1.5 g L^{-1} $CaCl_2$. The SSW pH was carefully adjusted to 7.8 with droplets of 2 M $HCl \cdot FeSO_4$ (0.1 mM), K_2HPO_4 (0.33 mM) and the V7 vitamin solution (0.5 mL L^{-1}) were added directly to the microcosm culture (Gilewicz et al., 1997). The experiments were harvested after 0 and 3 h and 2, 7 and 21 d. In addition, killed-cell controls were prepared by adding mercuric chloride (0.2 M in the 20 mL of SSW) to assess losses by abiotic processes.

2.4. Lipid and hydrocarbon extraction and fractionation

Sediments and SSW were extracted together using a modified Bligh and Dyer (1959) method at 4 °C for 24 h by agitation with a mixture of chloroform (100 mL) and methanol (200 mL). Carteau cove sediments contained about 60% of water (determined by freeze-drying assays), so additional water was not added. After centrifugation at 3500 *g* for 5 min, the mixture was filtered and separated into two phases by adding 80 mL of chloroform and 100 mL of distilled water. After settling for 12 h, the organic phase (bottom layer) was collected and the aqueous phase was twice re-extracted with 30 mL of chloroform. The organic phases were then combined and concentrated using rotary evaporation followed by blowdown under a gentle stream of nitrogen.

Lipid extracts were fractionated on columns (1.5 cm i.d., 30 cm length) packed with 13 g of silica gel 60 deactivated with 5% distilled water. Neutral lipids (saturated and polycyclic aromatic hydrocarbons) were eluted with 300 mL of chloroform (F_1), glycolipids with 150 mL of acetone (F_2) and the polar fraction (F_3), containing PLs and some non-phosphatidic polar lipids as impurities (Aries et al., 2001a) with 400 mL of methanol.

The first two fractions were combined and fractionated on columns (1 cm i.d., 40 cm length) packed with 8 g of silica gel 60 (lower layer) and 8 g of alumina (upper layer). Both silica and alumina had been deactivated earlier with 5% distilled water. Saturated hydrocarbons were eluted with 30 mL of *n*-heptane. Aromatic hydrocarbons were successively eluted with *n*-heptane/dichloromethane (20 mL, 90:10; v/v) and *n*-heptane/dichloromethane (40 mL, 80:20; v/v). Details of the gravimetric analysis of the total hydrocarbons can be found elsewhere (Le Dreau et al., 1997).

Preparative TLC was carried out on the F_3 fraction (silica gel F_{254} plates 20 cm × 20 cm × 0.2 μm) in order to isolate PLs from more polar compounds. Plates were developed with $CHCl_3$/MeOH/glacial acetic acid (65:25:8; v/v/v). The silica gel area corresponding to PLs ($0 < R_f < 0.85$) was revealed using Vaskovsky's reagent (Vaskovsky and Kotetsky, 1968).

Ester-linked PLFAs were transmethylated with BF_3/MeOH to produce fatty acid methyl esters (FAMEs; Morrison and Smith, 1964; Aries et al., 2001a,b). Double bond position and geometry of monounsaturated FAs were determined by forming dimethyl disulfide (DMDS) adducts (Nichols et al., 1986). Elucidation of the methyl branching position was achieved according to the method of Anderson and Holman (1975). The *N*-acylpyrrolidine derivatives were prepared by direct treatment of FAMEs and TLC purification on silica gel plates (heptane/diethyl ether 80:20 v/v) before gas chromatograph/mass spectroscopy analysis (GC/MS).

2.5. Saturated hydrocarbon, FAME and FAME derivative analysis

Saturated hydrocarbons, FAMEs and their derivatives were identified using GC/MS with a Hewlett–Packard 5890 series II gas chromatograph (HP, Geneva, Switzerland) equipped with a AT-5 MS capillary column (60 m, 0.25 mm, 0.25 μm) and coupled to a Hewlett–Packard 5898A MS Engine mass spectrometer. Chromatographic conditions were as follows: splitless injection (60 s) with He as carrier gas at a 1 mL min^{-1} constant flow rate. For the saturated hydrocarbon fractions, the oven programme was held at 30 °C for 1 min, then programmed at 50 °C min^{-1} to 120 °C and then 5 °C min^{-1} to 290 °C followed by a 25 min isothermal period. For FAMEs and DMDS derivatives, the temperature programme was 30 °C for 1 min, then 50 °C min^{-1} to 70 °C, 10 °C min^{-1} to 120 °C, 2 °C min^{-1} to 290 °C and finally held for 10 min. For pyrrolidide derivatives, the temperature programme was 30 °C for 1 min, then 50 °C min^{-1} to 100 °C, 20 °C min^{-1} to 200 °C, 2 °C min^{-1} to 290 °C and finally held for 20 min.

Electron ionization (70 eV) GC/MS was conducted both in full scan and in single ion monitoring (SIM) modes for qualitative and quantitative analyses, respectively. For quantitative hydrocarbon (*n*-alkanes) analysis in SIM mode (*m*/*z* 71 and *m*/*z* 99), squalane was used as internal standard. Full scan mode was used for the quantification of FAMEs and their derivatives. For FAME quantitative analysis, $n-21{:}0$ FA was used as internal standard.

2.6. Interpretation of PLFA analysis

The FA terminology used utilizes '*X*:*Y*ω*Z*' where '*X*' indicates the total number of carbons, '*Y*' the number of double bonds and 'ω' precedes '*Z*', the number of carbon atoms between the closest double bond and the aliphatic end of the molecule. Thus, 18:1ω9*c* designates an 18 carbon chain with a double bond between the 9th and 10th carbons. The *cis* or *trans* configuration is given after the position of the double bond by the letters *c* or *t*, respectively; 10Me18:0 refers to a methyl group on the 10th carbon from the carboxylic end of the fatty acid.

2.7. Statistical analysis

PCA was used to compare the evolution of the PLFA profiles between NC and C microcosm sediments. PCA was performed with *R*™ for Windows™. A sequence of n observations (the NC and C PLFA profiles from time 0 to 21 days) $\{x_i, i=1,\ldots,n\}$, characterized by p variables (each chromatographic peak from Table 1) such as $x_i=(f_{i1},\ldots,f_{ip})$ with:

$$\begin{cases} f_{ik} \geqslant 0, \quad k=1,\ldots,p, \ \ i=1,\ldots,n \\ \sum_{k=1}^{p} f_{ik}=1, \quad i=1,\ldots,n \end{cases}$$

are row-combined into a single matrix X.

The problem was to perform the PCA of X, i.e., to perform the eigenvalue decomposition of the variance matrix of X in order to determine the main directions of the observation space along which the data have the highest variability. It is well known that this problem requires the determination of the following eigen equation:

$$V\xi=\lambda\xi,$$

where $V=\frac{1}{n}X'_cX_c$ is the variance–covariance matrix of the observations calculated with X_c, the centered matrix of X, X'_c denotes the transposition of X and ξ is the p-eigenvector associated with the eigenvalue λ.

3. Results and discussion

In microbiological ecology studies of this type, one of the most important methodological problems relates to the extraction methods usually employed to analyze sedimentary petroleum hydrocarbons and the one used for sedimentary phospholipid PLFA extraction. The classical approach involves the use of two different extraction processes for hydrocarbons (mainly Soxhlet) and PLFAs (Bligh and Dyer, 1959). This could lead to inaccuracy (heterogeneity, partial recoveries) concerning the correlation between hydrocarbon uptake and the effects of petroleum on PLFA compositions. As suggested

Table 1
FA composition (% total PLFAs) of non-contaminated (NC) and contaminated (C) sediment microcosms from Carteau cove

PLFA	T0-NC	T3h-NC	T2-NC	T7-NC	T21-NC	T3h-C	T2-C	T7-C	T21-C
12:0	0.2	0.3	0.4	0.7	0.6	1.5		0.7	
*i*13:0	0.3	0.4	0.5	0.5	0.4	1.4	0.2	0.5	0.3
*i*14:0	1.2	1.2	0.8	0.9	1.1	1.7	0.8	0.6	0.3
*ai*14:0	0.7	0.8	0.9	0.7		0.7	0.8	1.0	0.2
14:0	5.1	5.2	3.9	2.4	1.2	6.2	4.4	2.8	1.7
4.8.12-Tri-Me-13:0	0.5	0.7	1.2	1.9	1.1	1.1	0.5	0.4	1.4
*i*15:0	5.8	5.4	5.9	4.7	3.9	3.4	3.6	3.5	3.5
*ai*15:0	7.4	7.3	3.9	6.5	4.9	2.4	5.1	5.0	1.5
15:0	1.8	1.7	1.4	1.4	1.6	5.8	3.2	2.1	1.8
*i*16:0	1.9	1.9	1.5	1.4	1.5	1.3	1.5	1.5	0.5
16:1ω7*c*	12.2	11.8	16.1	7.5	8.6	12.6	12.7	11.5	18.3
16:1ω5	1.6	1.6	2.0	2.6	2.2	2.2	1.0	0.6	2.2
16:0	22	22.4	18.9	17.4	23.4	17.5	20.8	34.4	27.5
x-Me-16:1	0.6	0.6	0.5	0.6	0.9	0.3	0.4	0.2	0.3
10-Me-16:0	0.9	0.9	0.8	1.0	1.0	0.4	0.7	0.7	0.2
*i*17:0	1.8	1.9	1.6	0.7	2.6	1.1	1.3	1.2	0.4
*ai*17:0	1.7	1.7	1.7	1.6	3.1	1.4	1.5	1.4	0.4
17:1ω8*c*	1.1	1.0	0.8	3.1	1.1	2.4	0.6	1.1	2.2
17:1ω6*c*	0.6	0.6	0.6	3.0	1.4	0.6	0.4	0.5	1.1
17:0	0.8	0.9	1.3	1.4	2.8	2.1	1.5	1.7	2.2
18:2ω6	0.7	0.9	0.8	0.8	2.3		0.4	0.3	1.5
18:2ω3	1.4	1.4	0.6				0.7	0.3	
18:1ω9*c*	4.3	4.0	4.9	5.3	7.1	8.3	6.2	3.8	3.1
18:1ω7*c*	16.1	16.5	16.5	21.9	13.6	13.3	19.1	17.0	24.2
18:1ω7*t*	0.5	0.5	0.2		1.1	0.7	0.2	0.2	0.1
18:0	4.8	4.4	4.7	5.6	7.5	1.4	3.8	3.2	2.4
9-Me-18:1	0.1	0.1	0.1	0.2	0.2	0.7	0.9	0.4	1.3
10-Me-18:0			0.2	0.2	0.8	3.2	3.4	0.9	0.1
19:1			0.4	0.8	2.8	1.0	0.2	0.3	0.3
19:0	1.3	1.3	1.5	0.8	0.7	1.0	0.2	0.3	0.1
20:4ω6			0.9			0.5	0.6		0.1
20:3			1.5			0.3	0.5		0.1
20:1	0.9	0.9	0.4	0.2	0.5	0.8	1.3	0.9	0.3
20:0	0.5	0.4	0.6	1.5		1.1	0.5	0.5	0.1
22:0	0.7	0.7	1.0	1.2		0.5	0.5	0.3	0.1
23:0	0.5	0.6	0.5			0.7	0.1		
24:0			0.6	1.4		0.4	0.4	0.2	0.1
25:0							0.1		0.1
$\sum$SFA[a]	59.9	60	53.7	53.9	58.3	56.2	55	63	44.9
$\sum$MUFA[b]	37.4	37.7	42.4	45.5	39.4	43	42.9	36.4	54.4
$\sum$PUFA[c]	2.3	2.3	3.8	0.6	2.3	0.8	2.2	0.6	1.6
PLFA concentration[d]	184.7	191.5	183.1	67.8	81.4	374.6	562.7	928.8	3376.3
Total biomass[e]	10.9	11.3	10.8	4	4.8	22.1	33.2	54.8	199.2

[a] SFA, saturated fatty acid.
[b] MUFA, monounsaturated fatty acid.
[c] PUFA, polyunsaturated fatty acid; *c* and *t*, respectively, correspond to *cis* and *trans* geometry of MUFA. *x*, is a non-identified branching position on a branched fatty acid.
[d] Total PLFA concentration in nmol kg^{-1} dry sediment.
[e] Total biomass in 10^9 bacteria kg^{-1} dry sediment. T2-21 = incubation times (2–21 days); T3h = incubation after 3 h. Each value is the mean of at least two experiments.

by Fang and Findlay (1996), a more accurate way should involve the analysis of both hydrocarbons and PLFAs extracted from the same sample. Consequently, based on a classic lipid extraction method (Bligh and Dyer, 1959), we adapted a previous methodology (Fang and Findlay, 1996) to our study.

In order to evaluate extraction recoveries with this method, we quantified gravimetrically total organic extracts from sedimentary microcosms

spiked with petroleum (hydrocarbon + synthetic sea water + sediment). The fractions F_1 and F_2 were recombined before the second fractionation (alumina/silica column) because F_2 could contain some hydrocarbons. Extraction yields were about $85 \pm 10\%$ ($n = 4$). Additionally, we performed the same test for aqueous cultures (without sediment), obtaining recoveries of about $90 \pm 5\%$ ($n = 4$). We considered that this analytical protocol is an improvement of Fang and Findlay's (1996) methodology since numerous interfering compounds were eliminated by alumina/silica purification of hydrocarbons and TLC clean-up of polar fraction containing PLs (Aries et al., 2001a). Lastly, another advantage of this method was the reduction in the time-consuming sample preparation related to the classical hydrocarbon extraction with solvent (e.g., Soxhlet).

3.1. PLFA composition of NC microcosms

The FA compositions (%) for the different experiments are given in Table 1. Assignments of such compositions to specific microbial groups have been documented (Findlay et al., 1990a,b). First, no significant differences were observed between T0-NC and T3h-NC in the PLFA chromatographic profiles. According to the work of White et al. (1998) and Hill et al. (2000), PLFA patterns for both T0-NC and T3h-NC indicated a contribution of aerobic Gram-negative bacteria as shown by the amounts of 16:1ω7c + 18:1ω7c + 18:1ω7t (about 29%).

In the same way, the high abundance of saturated fatty acids (SFAs), i.e., 14:0 (≈5%), 16:0 (≈22%) and 18:0 (≈4.5%) was attributed to the contribution of prokaryotic organisms (White et al., 1998). In addition, the presence of polyunsaturated fatty acids (PUFAs) is characteristic of microeukaryotes (Rajendran et al., 1993). The polyenoic 18:2ω6,9 (≈1%) is abundant in fungi but has also been reported to occur in algae and protozoa (Hill et al., 2000). Usually, a double bond at the ω6 position is attributed to an animal origin whereas ω3 is characteristic of either a plant or algal origin (White et al., 1996), although this is not without exceptions. In some in vitro studies, some authors have noted the occurrence of this fatty acid in certain bacteria (e.g., *Marinobacter hydrocarbonoclasticus* strain 617) grown on either pure *n*-alkanes or on *n*-alkanes mixture (Doumenq et al., 1999, 2001) and in a consortium composed of marine hydrocarbon-degrading bacteria grown on crude oil (Aries et al., 2001b). Psychrophilic and barophilic bacteria from Antarctic waters produce a wide range of PUFAs (Fang et al., 2002; Nichols and McMeekin, 2002). The various origins for polyenoic fatty acids show that such biomarkers should be used cautiously.

Additionally, we detected the occurrence of a mid-chain branched fatty acid 10Me16:0 (0.9%) which is considered as a sulfate-reducing bacteria (SRB) biomarker only when 10Me18:0 is absent (Parkes et al., 1992; Spring et al., 2000). For both T0-NC and T3h-NC, our observations were in agreement with these latter authors (i.e., absence of 10Me18:0). Therefore, we assume a small contribution of SRB to this sample, which could be due to the fact that we extruded sediment samples (0–2 cm layers) from a shellfish aquaculture zone (*Mytilus galloprovincialis*; Carteau cove; Fig. 1). At the same sampling site, Grossi et al. (2002) have shown that only the uppermost few mm of sediment (≈2–5 mm) was permanently oxygenated. Besides, we noticed the occurrence of the branched FA *ai*15:0 (4–7% of total PLFAs). This branched acid is characteristic of Gram-positive bacteria according to White et al. (1996). Lastly, only small amounts (≈1%) of PLFAs with more than 22 carbon atoms were observed. They are generally attributed to a contribution from terrestrial higher plants (de Leeuw et al., 1995), but in our case they were probably derived from *Zostera marina* (common eelgrass; Kharlamenko et al., 2001) leaves present in this zone.

3.2. Bioremediation in microcosms

Abiotic losses of hydrocarbons measured gravimetrically were $4.9 \pm 2\%$ (due to evaporation of some volatile organic compounds) and all data for the hydrocarbon removal with time in both NC and C sediment assays are shown in Table 2. Gravimetric analysis of total hydrocarbons (saturated + aromatic fractions) in the same sediment showed a concentration of about 220 mg kg^{-1} dry sediment (Table 2) for both T0-NC and T3h-NC. According to Volkman et al. (1992), the control sediment could be considered as being slightly contaminated. Fig. 2 shows a gas chromatogram for T0-NC, characterized by a distribution from *n*-C_{12} to *n*-C_{34}, with a small UCM (Unresolved Complex Mixture) centered at *n*-C_{29} and corresponding to the accumulation of branched and cyclic alkanes particularly resistant to degradation (Gough and Rowland, 1990). Such an UCM is generally related

Table 2
Biotransformation of total hydrocarbons (gravimetric) and *n*-alkanes in microcosms

Samples	Total HC (mg kg^{-1} dry sediment)[a]	Biotransformation of total HC (%)	Total *n*-alkanes (mg kg^{-1} dry sediment)	Biotransformation of *n*-alkanes (%)
T0-NC	222	–	5.6	–
T3h-NC	222	–	5.5	–
T2-NC	207	3.7	5	7.7
T7-NC	169	15	3.9	21.4
T21-NC	172	18.3	2.7	47.3
T0-C	10,033	–	545	–
T3h-C	10,007	–	541	–
T2-C	9238	1.5	438	14.2
T7-C	8262	9.8	381	23.2
T21-C	5636	37	154	64.1

[a] Total hydrocarbons, corresponding to sum of saturated- and aromatic hydrocarbon fraction. NC, non-contaminated; C, contaminated; T0-21 indicates incubation time (days).

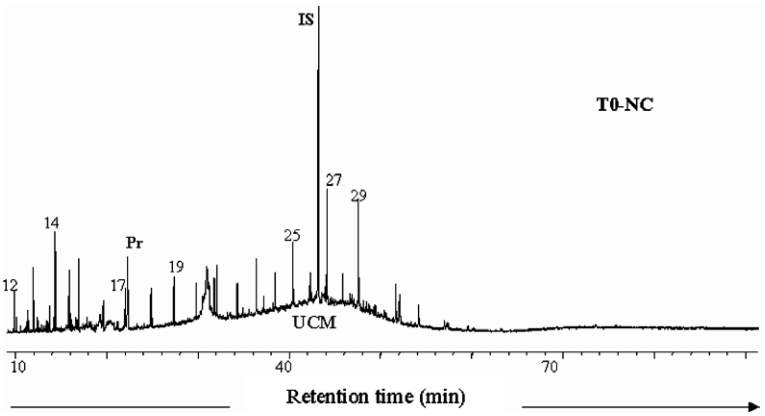

Fig. 2. GC/MS chromatogram (SIM mode) of saturated hydrocarbon fraction from initial non-contaminated sediment microcosm (T0-NC). Numbers indicate *n*-alkane carbon chain length; Pr, pristane; IS, internal standard (squalane).

to a weathered petroleum input and can be attributed to the important petrochemical activities in the area. Moreover, the values of two carbon preference indices $CPI_{15–25}$ and $CPI_{25–35}$ (Le Dreau et al., 1997) were, respectively, 1.9 and 2.7. Furthermore, $n\text{-}C_{29}/n\text{-}C_{17}$, $n\text{-}C_{17}$/Pr and $n\text{-}C_{18}$/Ph ratios were 2.3, 0.7, and 1.4 (Table 3), respectively. These values confirmed the preponderance of marine algae and terrestrial plant inputs (Le Dreau et al., 1997; Bourbonniere et al., 1997). In NC sediments (Table 2), a slight decrease in total hydrocarbons was found by the 21 day observation (from 222 to 17 mg per kg of dry sediment) with unchanged chromatographic profiles for saturated hydrocarbons (data not shown). This decrease could be due to both variability in the extraction method (~12% RSD), abiotic losses and biodegradation. Total hydrocarbon concentrations in T0-C and T3h-C microcosms were about 10 g kg^{-1}dry sediment (Table 2) and we did not notice any significant differences in the corresponding chromatographic profiles (data not shown).

Table 3
Indices based on *n*-alkane and acyclic isoprenoid abundance

Sample	$n\text{-}C_{29}/n\text{-}C_{17}$	$n\text{-}C_{17}$/Pr[a]	$n\text{-}C_{18}$/Ph[b]
T0-NC	2.3	0.7	1.4
T0-C	0.1	5.3	2.5
T3h-C	0.1	5.2	2.5
T2-C	0.1	4.8	2.2
T7-C	0.1	4.5	1.5
T21-C	0.1	3.7	1.1

[a] Pr, pristane.
[b] Ph, phytane.

In the contaminated microcosms, depletions of total hydrocarbons were 1.5%, 9.8% and 37% after 2, 7 and 21 days (Table 2), respectively. At the same time, *n*-alkane degradation increased from 14.2% to

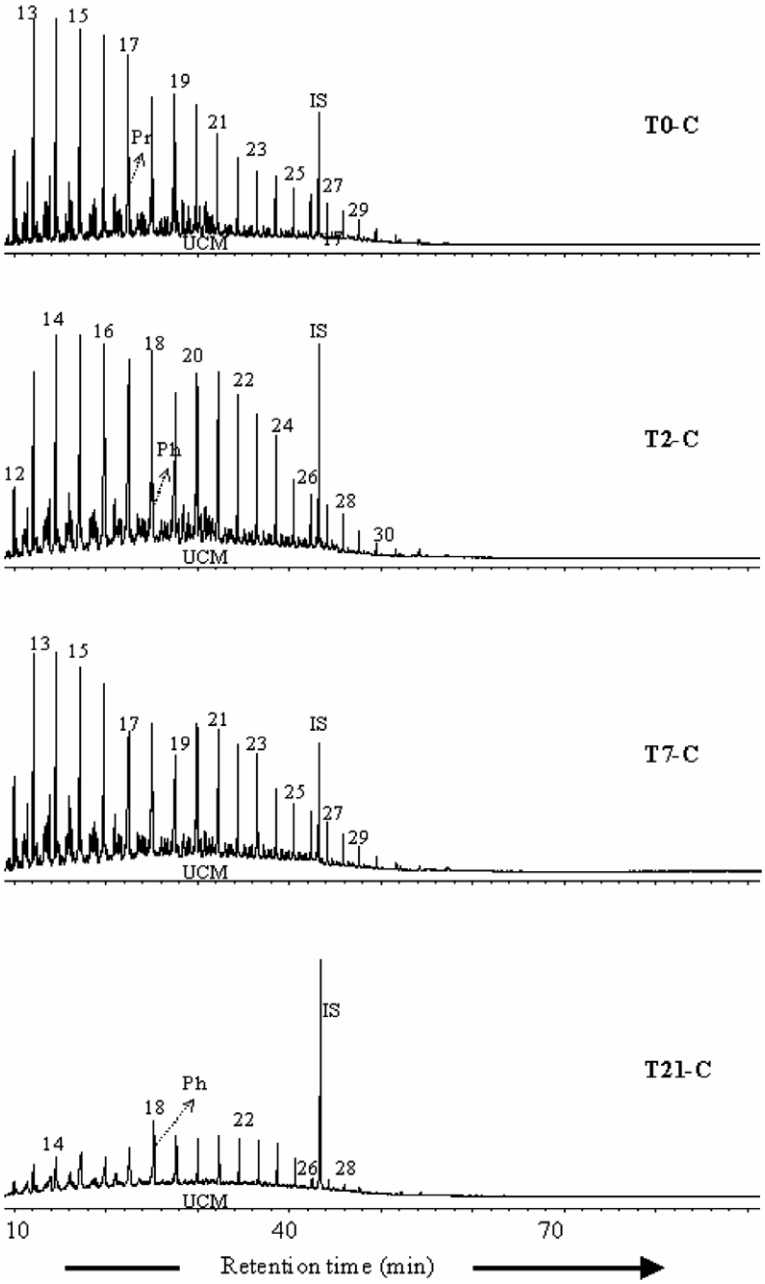

Fig. 3. GC/MS chromatograms (SIM mode) of saturated hydrocarbon fraction from contaminated sediment microcosms. Numbers indicate *n*-alkane chain length; Pr, pristane; Ph, phytane; and IS, internal standard (squalane). C, contaminated; T0-21, incubation time (from 0 to 21 days).

64.1% (Fig. 3 and Table 2) between T2-C and T21-C, with a corresponding decrease in the *n*-C_{17}/pristane and *n*-C_{18}/phytane ratios from 5.3 to 3.7 and from 2.5 to 1.1 (Table 3) indicating selective biodegradation of *n*-alkanes. Such an observation is in accord with previous studies of oil microbial degradation (e.g., de Jonge et al., 1997; Le Dreau et al., 1997).

3.3. Biomass changes in response to petroleum hydrocarbon contamination

Biomass estimations were calculated according to Smith et al. (1986) using the following conversion factor: 1 g of *Escherichia coli* contains 100 μmol of PLFAs and is equivalent to 5.9×10^{12} cells (dry

wt.). The initial viable cell density at T0-NC was thus 10.9×10^9 cell kg^{-1} dry sediment, and after 3 h of incubation, no significant change was found since the cell density was 11.3×10^9 cell kg^{-1} dry sediment (Table 1). In addition, the bacterial density of NC sediment remained relatively stable during the whole experiment (from 10.9 to 4.8×10^9 cell kg^{-1} dry sediment).

In the contaminated sediment (Table 1), there was an increase of about twofold (from 22.1 to 54.8×10^9 cell kg^{-1} dry sediment) over the first 7 days. The T21-C sample exhibited a significant biomass increase to 199.2×10^9 cell kg^{-1} dry sediment. The observed cell abundance enhancement in T21-C could be linked to the degradation of total HCs (5936 vs. 10,033 mg kg^{-1} dry sediment initially; Table 2) and especially to the important loss of total *n*-alkanes (154 vs. 545 mg kg^{-1} dry sediment initially; Table 2). However, it was not possible to determine if this increase in biomass was mainly due to the growth of hydrocarbon-degrading bacteria as compared to all heterotrophic microorganisms. For this purpose, we performed a detailed study of the PLFA composition changes over the time, supplemented with a statistical treatment of the data.

3.4. PLFA changes in response to petroleum hydrocarbon contamination

Differences in PLFA patterns occurred over time, reflecting changes in the microbial community structure. These changes for NC and C microcosms during the experiment were explored through PCA.

A plot of PLFA profiles is displayed on the first plane of the PCA (Fig. 4). The variability is highly structured and can be explained by displaying the first two eigenvectors. These scaled eigenvectors account for 67% of the total variance and their representation consists of positive or negative correlations of the different peaks with principal components 1 and 2. This plot shows an important difference between T7 and T21 C sediment (duplicate analysis), although the two replicates corresponding to T21 NC sediment revealed only a slight variation. The second axis (Fig. 4) corresponds to the second eigenvector in which particular FAs, i.e., *i*16:0, *i*17:0 and *ai*17:0, are the main contributors to the variation (Fig. 5). Such FAs are characteristic of Gram-positive prokaryotes (Rajendran et al., 1993, 1994). Consequently, the positive correlation of T21-NC according to the second axis

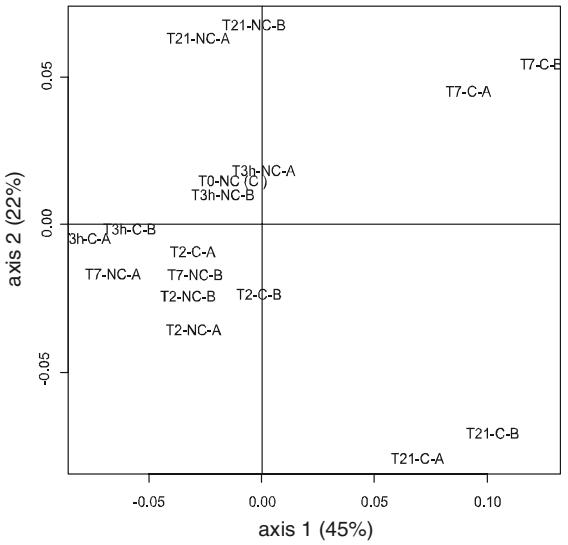

Fig. 4. Plot of PLFA profiles in the first plane of the PCA (67% of variability). NC, non-contaminated; C, contaminated; T0-21, incubation times (T0, 3 h, 2 days, 7 days and 21 days). A and B are replicates.

(Fig. 4) mainly suggests a time-dependent variation in the FA composition of the Gram-positive bacteria. Besides, if we look at the NC sediments (Table 1), we observe an increase in the abundance over time of some monoenoic compounds (e.g., 16:1ω5, 17:1ω6, 18:1ω7*t*, and 18:1ω9*c*). This might reflect an increase in the contribution of the Gram-negative bacteria (White et al., 1996). Similarly, we observed the disappearance of 18:2ω3 in the NC microcosm after 7 days, probably due to a lower abundance of microeukaryotes (microalgae; Findlay et al., 1990a). The associated occurrence of 18:2ω6 and 18:2ω9 in all NC samples might be due to the presence of hydrocarbonoclastic bacteria (Doumenq et al., 1999, 2001; Aries et al., 2001a) or fungal contribution (Bååth and Anderson, 2003). Finally, at T21-NC, the occurrence of 10Me18:0 (0.8%), which is characteristic of *Actinomycetes* (Kroppenstedt, 1985; Rütters et al., 2002b), might indicate a contribution from fungi.

In the contaminated microcosms, the PLFA compositions showed some noteworthy changes after 7 days. As reported by Bååth and Anderson (2003), several bacterial specific PLFAs can be considered indicators of bacterial biomass (i.e., *i*14:0, *i*15:0, *ai*15:0, 15:0, *i*16:0, 10Me16:0, *i*17:0, *ai*17:0, 17:0, 18:1ω7*t* and 10Me18:0). In this study, the petroleum hydrocarbon addition led to a decrease

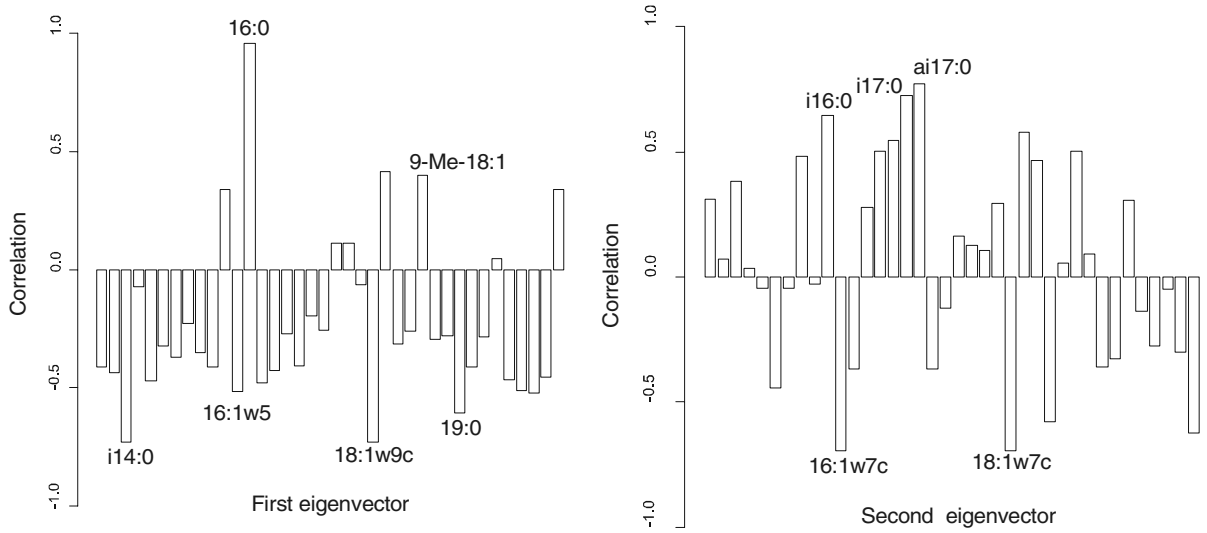

Fig. 5. Bar plot of first and second eigenvectors of the PCA. The height of each bar gives the correlation between a FA peak and the corresponding PCA axis. Labelled FAs correspond to those that most contributed to the variability in the PCA.

in most of these PLFAs (Table 1). Furthermore, we emphasize that 10Me16:0 and 10Me18:0 have been proposed as *n*-alkane-dependent FAs (Doumenq et al., 2001) and are related to a contribution from hydrocarbon-degrading communities (Aries et al., 2001b) under aerobic conditions. Previous in vitro studies (Aries et al., 2001b; Mazzella et al., 2005) have shown dramatic changes in the PLFA composition of hydrocarbon-degrading bacteria isolated from the same experimental site and cultured on petroleum hydrocarbons (pure cultures and reconstituted consortia). In fact, these authors noticed the occurrence of 10Me16:0 and 10Me18:0, several other branched mid-chain FAs and some odd numbered fatty FAs (e.g., 15:0 and 17:0) after a BAL 250 addition. In our case, we did not observe an increase in these specific FA classes. These observations suggest that the petroleum amendment did not induce an increase in hydrocarbon-degrader biomass. This is in accord with a previous in situ experiment (Mazzella et al., 2005). The authors proposed the following two explanations: first, the hydrocarbon degraders are able to degrade petroleum although they are present in low proportion within the sediments. Second, the bacterial communities in the NC sediment could be already adapted to petroleum and thus hydrocarbons were not an unusual carbon source.

Fig. 6 shows the PLFAs responsible for the time variability in either NC (b) or C sediments (c). The

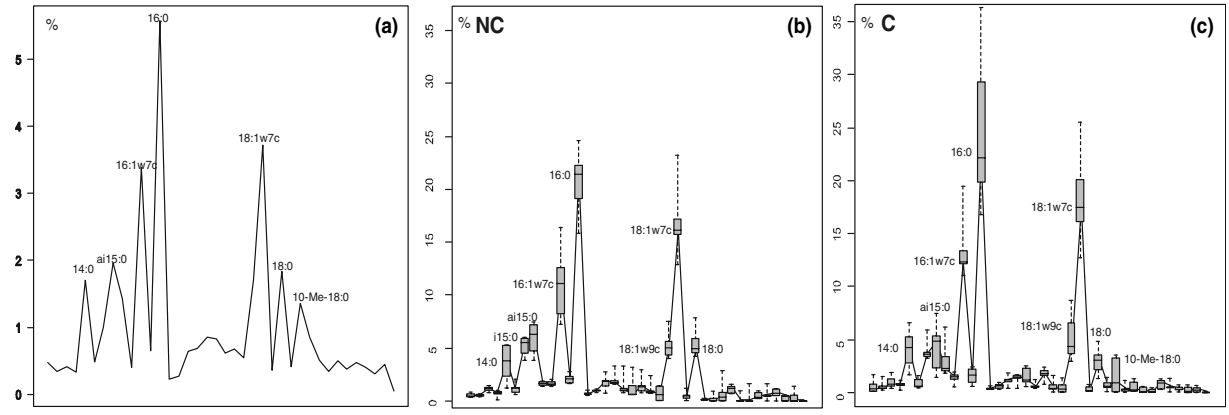

Fig. 6. (a) Total standard deviation (%) of entire set of PLFA profiles. Annotated peaks correspond to PLFAs that are most variable from experience. (b) Box plots of the non-contaminated cases (NC). (c) Box plots of the contaminated cases (C). The thin line links each median.

total variability graph (Fig. 6a) shows that the standard deviation is roughly proportional to the mean values of PLFAs. Most of the variability in NC and C sediments (Fig. 6b and c) was due to a few compounds: 16:0, 16:1ω7*c*, 18:1ω7*c*, 18:1ω9*c* and 18:0. These FAs are not particularly representative of hydrocarbon-degrading bacteria since there are commonly observed among the lipids of microorganisms (Lechevalier, 1977). Hence, the considerable variation in such PLFAs is in agreement with the fact that petroleum hydrocarbons affect all or most heterotrophic microorganisms and not only the hydrocarbon degraders.

In summary, the evolution of the microbial communities resulting from an artificial contamination by petroleum hydrocarbons (BAL 250) has been investigated using both PLFA analysis and statistical treatment of the data. Modifications of PLFA composition were found in concert with significant petroleum hydrocarbon degradation and indicate a non-specific increase in degraders in the microbial biomass. Such an approach might be useful for assessing in situ shifts in community structure in the case of chronic or accidental xenobiotic exposures. Such approaches might be also of interest for understanding "real-world" remediation and adaptation processes.

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