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How a Bacterial Community Originating from a Contaminated Coastal Sediment Responds to an Oil Input

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Abstract Bacterial communities inhabiting coastal sediments are subjected to oil spills. In order to examine the early structural response of a complex bacterial community to oil pollution, a kinetic study of the crude oil impact on bacterial communities inhabiting sediments from the contaminated Etang-de-Berre lagoon was performed. The sediments were maintained in slurries in presence or absence of crude oil and the kinetic study was carried out 14 days. During this period, 54% of crude oil was biodegraded showing the importance of the early degradation step. The metabolically active community (16S rRNA transcript analysis) was immediately impacted by the oil input, observed as an apparent decrease of species richness in the first hour of incubation. Nevertheless, this shift was quickly reversed, highlighting a fast, adaptative and efficient response of the metabolically active bacterial population. The high proportion of sequences related to hydrocarbonoclastic strains or petroleum-associated clones in active oiled community was consistent with significant increasing numbers of cultivable hydrocarbonoclastic bacteria at the end of the experiment. We concluded that "Etang-de-Berre" bacterial communities inhabiting oiled sediments for decades adopted a specific structure depending on oil presence and were able to face hydrocarbon contamination quickly and efficiently.

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Introduction

Oil pollutes by entering ecosystems naturally by seepage from reservoirs and through anthropogenic activities including the extraction of oil from reservoirs, transportation, processing in refineries and handling and disposal by end users. Oil pollution of the sea and coastlines is difficult to contain and causes significant ecological perturbations leading to negative impacts on local fishing, aquaculture, and tourist industries [10]. Some oil components accumulate and biomagnify through the food chain, and those that are mutagenic or carcinogenic are prejudicial to the long term health of marine organisms and their predators, including humans [15]. Fortunately, a substantial proportion of the oil entering marine habitats is degraded by indigenous microorganisms and their use in bioremediation processes has proven to be an effective method for cleaning up residual oil in a variety of coastal environments [5, 13, 27].

A great deal of research on the diversity and the metabolic activities of complex microbial communities able to cope with oil compounds in environment has been carried out, but many unknowns persist and more work is needed to elucidate and understand their complexity. The impact of oil on complex bacterial community structures and the bacterial capacity to degrade hydrocarbons has been mainly examined under mineral nutrient and/or bioremediation agent treatments. Although some studies showed that these treatments

improved significantly the oil biodegradation [7, 27], other studies demonstrated either no effect of nutrient addition [20] or that the nutrients have a greater impact on the bacterial community than the oil alone [8]. Moreover, the bioremediation agents produce residues that showed a relatively high toxicity [7]. In addition, oil impact on the bacterial community depends on the history of the site pollution. Most of the pristine communities exposed to petroleum contamination presented a decrease in diversity associated with the selection of tolerant populations and then a resilience phenomenon [2, 8, 27]. In contrast, the response of indigenous communities previously exposed to petroleum compounds, dominated by organisms able to utilise and/or survive to toxic hydrocarbons, is expected to occur faster and more specifically [4, 14]. The bacterial community diversity may also be influenced by the complexity of chemical mixtures present and the length of the exposure time. Recent works showed that the study of the metabolically active populations provides new insights for the characterization of the bacterial community response to oil contamination [2, 21]. However, information on the early response (within the first hours and days) of a complex bacterial community to oil exposure is still scarce. Gaining insights into the bacterial abundance, phylogenetic structure changes and degradation activity occurring in the first stage of oil contamination would be of great interest since it is hypothesised that the hydrocarbonoclastic populations are established within 2 weeks in the community related to fast biodegradation events [14].

The aim of the present study was to determine the short- and mid-term modifications occurring on the pre-established bacterial communities from coastal sediment in response to a simulated oil spill. The Etang-de-Berre, a brackish lagoon bordering the Mediterranean Sea in the South of France, is characterised by important industrial activities and receives important amounts of hydrocarbons from refineries, petrochemical plants and transportation systems in addition to biogenic inputs [16]. The bacterial communities inhabiting these sediments being well-adapted to the oil presence seemed to carry all the metabolic machinery for coping with hydrocarbon exposure. Thus these bacterial communities constitute an interesting environmental model to gain a better understanding of the effectiveness of biodegradation in contaminated coastal environments. Thus, combining gas chromatography-mass spectrometry (GC-MS) analysis, culture-dependant and molecular approaches (terminal-restriction fragment length polymorphism (T-RFLP), 16S rRNA gene and transcript libraries), the response of whole microbial community and its active members was screened throughout a slurry experiment during the first hours and days after crude oil input.

Materials and Methods

Sediment Sampling

The subsurface sediments (0–2 cm) were collected from the station EDB1 (43°29′05″N; 5°11′17″E) of the Berre brackish lagoon bordering the Mediterranean Sea on October 2005 [24]. These shallow sediments (water column height around 30 cm) have been chronically subjected to hydrocarbon contamination for several decades, leading to hydrocarbon concentration of around 100 μg/g dry sediment. In order to assess the impact of the experimental design on the original bacterial community structure, a subsample (0.5 g) of homogenised sediments was immediately frozen in liquid nitrogen and stored at −80°C until the analysis of in situ DNA bacterial community structure (IS). The remaining sediments were maintained at room temperature during the transport and the slurry experiment.

Slurry Set-up

The sediment samples were homogenised through a 3 mm sieve and maintained for 4 weeks under daylight condition and at room temperature in order to remove low-molecular weight hydrocarbons (boiling point c.a. 50 and <200°C). A sediment slurry was then prepared with 40% of wet sediment and 60% of sterile synthetic water (SSW) at a salinity of 20 PSU (KCl 0.428 g/l; $CaCl_2.2H_2O$ 0.84 g/l; NH_4Cl 1.514 mg/l; $MgSO_4.7H_2O$ 3.794 g/l; $MgCl_2.6H_2O$ 3.017 g/l; NaCl 15.14 g/l; Na_2CO_3 0.15 g/l; trace element solution SL12 [23] without EDTA; potassium phosphate buffer 10^{-5} M at pH 7), and stabilised at 150 rpm agitation for 1 week. Triplicate microcosms were then set up in 100 ml sterile vial bottles containing 16 ml of the stabilised slurry and incubated for 2 days at room temperature in daylight prior oil addition. Half of the slurry bottles were amended with 274±28 mg Vic Bilh oil (Vic Bilh field in South East of France) and which represented the biotic-spilled oil condition (B+OIL). This level of contamination was chosen to simulate an oil spill that is likely to induce a perturbation in the bacterial communities. The main characteristics of Vic Bihl oil are as follows: density 926.71 kg/l at 15°C, viscosity 30 Cst at 50°C, API: 21. The remaining slurries were defined as the biotic-unamended condition (B−OIL). Abiotic spilled oil condition (AB+OIL) were set up using SSW, sterile Fontainebleau sand and Vic Bilh oil (Fig. 1). Before autoclaving, ultrasonic treatment in dichloromethane (DCM) (3×15′) was applied to the Fontainebleau sand and the DCM was evaporated during 24 h under airflow. Slurry microcosms were incubated under aerobic conditions at room temperature in daylight and with continuous agitation on an orbital shaker (150 rpm). Triplicate micro-

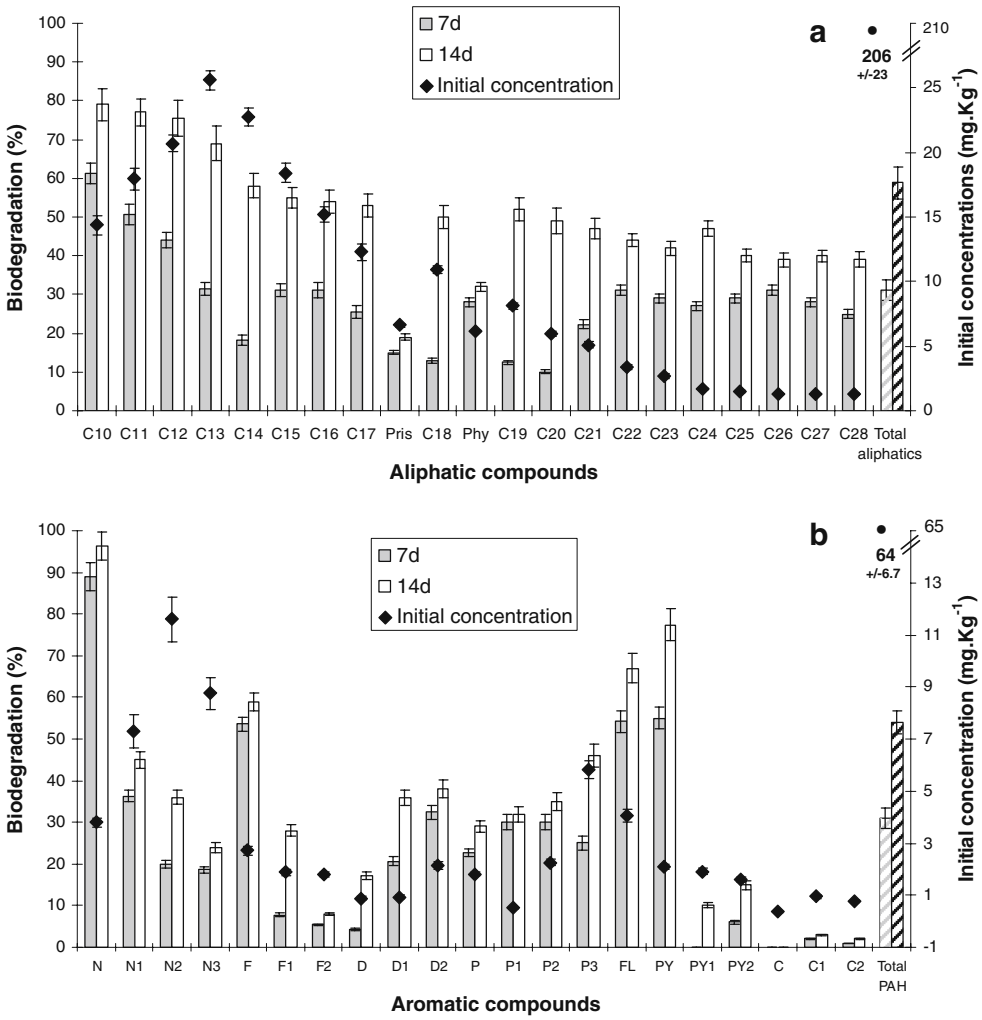

Figure 1 Concentration of the aliphatic (from C_{10} to C_{28}) (**a**) and aromatic (**b**) fractions in the oiled slurry at t0 and % of biodegradation at t7 and t14 days. C_{29} to C_{39} aliphatic compounds were also found representing around 1% of the total aliphatics (Table S1, Electronic supplementary material). *N* naphthalene, *F* fluorene, *D* dibenzothiophene, *P* phenanthrene, *FL* fluoranthene, *PY* pyrene, *C* chrysene (C1 to C3 represent carbon number of alkyl groups in alkylated PAH homologues). Each percentage represents the mean of hydrocarbon fractions from replicate samples

cosms were then used for analysis after 0, 1, 2, 3, 6, 12 h and 1, 2, 3, 4, 5, 7, 9, 12 and 14 days of incubation. For chemical analysis, microcosms were immediately frozen and stored at −80°C. For molecular analysis, the slurry samples were centrifuged at 8,000×*g* for 5 min at 4°C. The pellets were immediately frozen in liquid nitrogen and conserved at −80°C until DNA and RNA extraction. Only RNase-free certified plastic wares were used. All molecular analyses except libraries were carried out in triplicates.

Chemical Analysis

Total petroleum hydrocarbon (TPH) extraction from chemically dried sediment samples (5±0.05 g) was performed using a sequential ultrasonic solvent extraction as described by Risdon et al. [26]. Deuterated alkanes and polycyclic aromatic hydrocarbons (PAHs) including dodecane-d^{22}, nonadecane-d^{40}, triacontane-d^{62}, naphthalene d^{8}, phenanthracene-d^{10}, chrysene-d^{12} and perylene-d^{12} (purchased from Sigma Aldrich, Dorset, UK) were added to the extracts at 0.5 and 0.4 gml^{-1}. TPH, aliphatic and aromatic fractions were identified and quantified by gas chromatography-mass spectrometry using a Perkin Elmer AutoSystem XL gas chromatograph coupled to a Turbomass Gold mass spectrometer operated at 70 eV in positive ion mode. The column used was a Restek fused silica capillary column (30×0.25 mm internal diameter) coated with RTX®-5MS (0.25 μm film thickness). Splitless injection with a sample volume of 1 μl was applied. The oven temperature was increased from 60°C to 220°C at 20°C/min^{-1} then to 310°C at 6°C/min and held at this temperature for 15 min. The mass spectrometer was operated using the full scan mode (range *m/z* 50–500) for quantitative analysis of target alkanes and PAHs. For each compound, quantification was performed by integrating the peak at specific *m/z*. External multilevel calibrations were carried out for both aliphatic and aromatic fractions, ranging from 0.5 to 2500 μgml^{-1} and from 1 to 5 μgml^{-1}, respectively. For quality control, a 500 μgml^{-1} diesel standard and mineral oil were checked every ten samples. The percent depletion of all hydrocarbons were normalised to chrysene that is considered as one of the more persistent PAHs and calculated using the equation:

$\%\text{loss} = [((A_0/H_0) - (A_s/H_s))/(A_0/H_0)] \times 100$ as previously described by Prince et al. [25]. A_s and H_s are the concentrations of the target compound and chrysene in the oil sample, respectively, and A_0 and H_0 are the initial concentrations in the crude oil.

The significance of hydrocarbon degradation relative to controls was determined by repeated measures of analysis of variance followed by the post-hoc Tukey's multiple comparison test when $P<0.05$. All tests were performed using GraphPad InStat version 3.0a (GraphPadSoftware, San Diego California USA, www.graphpad.com).

Hydrocarbon-growing Bacteria Enumeration

Hydrocarbon-growing bacteria enumeration of the B−OIL and B+OIL microcosms was performed by the plate-count method in triplicates after 0, 7 and 14 days. Plates were prepared with washed agar (20%) and sterile synthetic seawater, supplemented or not with hexadecane or naphthalene as sole carbon source. Hexadecane (750 µl) was distributed on a sterilised filter paper placed in the Petri dish lid and in contact with agar surface to allow agar covering by capillarity. One hundred microlitres of a solution of naphthalene (31.25 mg/ml) was directly spread on agar plates. Naphthalene was dissolved in diethyl ether for easy distribution of the substrate and quick evaporation of the solvent. One hundred microlitres of appropriate dilutions of slurry in sterile synthetic seawater were surface spread onto plates. Plates were incubated for 47 days at room temperature. The non-parametric U test of Mann and Whitney was applied to CFU counts in order to determine whether the differences between the different incubation time and slurry conditions were significant.

Thirty colonies from 0, 7 and 14 days enumeration plates were selected for their different morphology and isolated. Their capacity to grow on hexadecane or naphthalene was checked by successive subcultures in liquid or solid medium containing hexadecane or naphthalene as sole carbon source. Identification of hydrocarbonoclastic bacteria was carried out by 16S rRNA gene sequencing as described below.

Molecular Analysis

DNA and RNA Extraction

Total genomic DNA extractions from the IS and the slurry microcosms were performed in triplicates at day 0, 7 and 14 using the alternative lysis method of the UltraClean™ Soil DNA isolation kit (MOBIO Laboratories Inc., USA). The manufacturer instructions were followed except for the initial step of lysis in which each sediment sample were vortexed horizontally during 20 min. Purified DNA was suspended in 50 µl of sterile water and examined by agarose gel electrophoresis. All extracted genomic DNA samples were stored at −20°C until further processing.

Total RNA extraction of the slurry microcosms were performed in triplicates using the FastRNA Pro Soil-Direct kit (Q-Biogen) following manufacturer's instructions after a first step of lysis (three times for 1 min at 30 Hz) in a bead beater (TissueLyser, Qiagen) and without a matrix purification step. RNA pellets were suspended in 100 µl sterile water and controlled on 1.2% formaldehyde agarose gel. To remove any genomic DNA contamination, the samples were treated with Turbo DNase (Ambion). The quality of RNA extract was checked using a RNA 6000 Nano Chip kit and Agilent 2100 Bioanalyser. All RNA extracts were stored at −80°C until further processing.

Reverse Transcription

DNase-treated RNA were reverse transcribed with Moloney murine leukaemia virus reverse transcriptase (M-MLV RT, USB Corporation) as follows: the random hexamers (5 µl at 0.1 U $A_{260,}$ Roche), the RNase OUT (40 U) and the RNA extract (2 µl) were denatured 5 min at 65°C and chilled 5 min at 4°C. The 5× reaction buffer, dNTP mix (0.5 µmol) and M-MLV RT (240 U) were then added to a final volume of 50 µl. The reaction was incubated 2 h at 42°C. RT products were used immediately for polymerase chain reaction (PCR) amplification and the remaining products were stored at −20°C. Possible DNA contamination of RNA templates was checked by PCR amplification of aliquots of RNA without reverse transcription step. No DNA was detected in these reactions.

PCR Amplification and T-RFLP Analysis

The bacterial 16S rRNA gene was amplified by PCR using the primers (*Escherichia coli* numbering) 8F-TET (5′-tetrachlorofluorescein phosphoramidite-5′ AGAGTTTGATCCTGG CTCAG-3′; [19]) and unlabelled 1489R (5′-TACCTTGT-TACGACTTCA-3′; [30]), giving a product of approximately 1,480 bp. The PCR and T-RFLP analysis were carried out as described by Bordenave et al. [2] using Eurobio *Taq* polymerase. The fluorescent PCR products were cleaned with GFX DNA and Gel Band Purification kit (GE Healthcare) and 100 ng of purified product digested separately with 3 U of enzyme *Hae*III and *Hinf*I in a final volume of 10 µl for 3 h at 37°C (New England Biolabs). A 1 µl of restriction digests were then mixed with 20 µl of deionized formamide and 0.5 µl of a TAMRA labelled Genescan 500 bp internal size standard (Applied Biosystems), denatured for 5 min at 95°C, and immediately transferred to ice. Samples were loaded onto an ABI PRISM 310 automated genetic analyser (Applied Biosystems). T-RFLP profiles were aligned by

identifying and grouping homologous fragments, and normalised by calculating relative abundances of each terminal restriction fragments (T-RFs) using height fluorescence intensity. Combined data from each restriction enzyme, normalised T-RFLP profiles were compared. For statistical analysis, the averages of the T-RFs heights detected in triplicates with each endonuclease (*Hae*III and *Hinf*I) were used. In order to identify the impact of oil on the bacterial community structure at DNA and RNA levels, clustering analysis coupled to two-dimensional non-metric multidimensional scaling ordination (2D-nMDS) based on Bray Curtis similarity taking into account the average of T-RF relative abundances was carried out with Primer6 (Plymouth Routines In Multivariate Ecological Research, version 6.1.6).

DNA and RNA Clone Library Analyses

One DNA (0h B-OIL DNA) and five cDNA libraries (0h B−OIL cDNA, 9d B−OIL cDNA, 12d B−OIL cDNA, 9d B+OIL cDNA, and 12d B+OIL cDNA) were constructed and analysed. Purified PCR or RT-PCR products amplified with the 8F and 1489R unlabelled primers were cloned into *E. coli* TOP10F' using the pCR2.1 Topo TA cloning kit (Invitrogen, Inc). Clones were selected randomly. Inserts were amplified using M13 primers (Eurogentec) surrounding the cloning site and analysed by RFLP (Restriction Fragment Length Polymorphism) with the same endonucleases used for T-RFLP analysis using the same conditions. Identical clones on the basis of their RFLP profiles were clustered and one representative clone of each cluster was sequenced, except for 0h B−OIL DNA library where singletons were not sequenced. Sequences were analysed as previously described by Païssé et al. [24].

Selected inserts were sequenced using M13 primers (Eurogentec) and the Big Dye® Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences were first analysed using the CHECK CHIMERA programme on the RDP Database Project website (http://rdp8.cme.msu.edu/html/) [6]. Then sequences were compared with the GenBank nucleotide database library by BLAST on-line searches [1]. Multiple sequence alignment of clones was performed by using CLUSTALX v1.83 [29] and PROcessor of SEQuences v2.91 [9]. Phylogenies were constructed with the Molecular Evolutionary Genetics Analysis v3.0 programme [18] using Kimura two parameters model and Neighbour-joining algorithm. The significance of the branching order was determined by bootstrap analysis with 1000 resampled data sets. Sequences with similarity over 97% were considered to represent one phylotype and were grouped for statistical analysis. PAST (PAleontological Statistics v1.60) software from http://folk.uio.no/ohammer/past/ website was used to perform rarefaction analysis and calculate diversity indices for each clone library [11]. In order to determine the significance of differences between the clone libraries, the LIBSHUFF method was applied [28].

Nucleotide Sequence Accession Numbers

The sequences determined in this study have been submitted to the EMBL database and assigned Accession Nos. FM242199 to FM242479.

Results

Hydrocarbon Analysis

The total hydrocarbon concentration of the sediment slurries before Vic Bilh oil addition was 5 mg/kg of dried weight sediment. The oil fingerprint analysis showed a clear dominance of polycyclic aromatic hydrocarbons (97%) over aliphatic hydrocarbons (3%) (Table S1, supplementary data). Once Vic Bilh crude oil was added to the slurries, as expected the oil fraction mass balance was reverted to a dominance of aliphatic hydrocarbons (76%) over aromatics (24%) (Fig.1). The most dominant hydrocarbon compounds were *n*-alkanes ranging from C_{10} to C_{20} constituting 90% of the aliphatics and the 2- and 3-ring PAHs including naphthalene, dibenzothiophene, phenanthrene and their alkylated forms as well as fluorene and fluoranthene (88 % of PAHs) for the aromatics.

Oil emulsification was observed in all biotic-oil amended slurries (B+OIL) after 2 days of experiment, suggesting biological degradation of crude oil [22]. GC-MS analysis of the B+OIL slurries revealed significant degradation of aliphatic ($F=7.548$, $p<0.001$) and aromatic ($F=57.488$, $p<0.001$) hydrocarbons after 14 days (Fig. 1). The total extractable hydrocarbons were reduced to 69% and 46% of their initial amount after 7 and 14 days, respectively. Tukey's multiple comparisons test showed that biodegradation of the aliphatics was, however, not significant between 7 and 14 days ($q=2.472$, $p>0.05$). It further showed that biodegradation of the aromatic hydrocarbons was significant between T0 and 14 days ($q=0.3683$, $p<0.05$). More than 60% of the most toxic PAH compounds, including naphthalene, fluorene, fluoranthene and pyrene were degraded in 14 days (Fig. 1).

Hydrocarbon-degrading Bacteria Count

Hydrocarbon-degrading bacteria in the microcosms were estimated to be 1.8×10^7 CFU.ml^{-1} prior to oil addition and remained stable all through the experiment for the unoiled slurries ($p>0.05$). For the oiled slurry, the same trend was

observed for the beginning of the experiment, but the number of oil-degraders increased significantly (1×10^8 to 1.67×10^8 CFU/ml) after 14 days of incubation. At the end of the experience, significant differences between the cultivable bacteria of B+OIL (1×10^8 to 1.67×10^8 CFU/ml) and B−OIL slurries (1×10^7 to 3.3×10^7 CFU/ml) were observed (Mann–Whitney T=2.5, $n_1=n_2=9$, $p<0.001$) (Fig. 2).

After successive cultures of the 30 strains isolated from agar plate to liquid medium, two were able to grow quickly on hexadecane as sole carbon source. Analysis of the 16 S rRNA gene sequence (~1100 pb) of the two strains showed 99% identity with the 16S rRNA gene sequence of *Alcanivorax borkumensis* SK2.

Total and Active Bacterial Community Dynamics

The experiment set-up has been developed to explore the response of the metabolically active bacterial populations from homogenous and stable DNA community. Since experimental settings could induce changes in the bacterial community structure, the influence of sediment manipulation has been assessed by comparing the T-RFLP profiles obtained from 16S rRNA gene analysis of the Etang-de-Berre sediments (IS) and 0h B−OIL samples (Fig. 3). The low similarity (37%) between these two community profiles based on the relative abundance of terminal-restriction fragments (T-RFs) confirmed the structure modifications induced by the experimental procedures. However, the difference observed in the bacterial community structure of IS and 0h B−OIL samples was not the consequence of a diversity decrease since the OTUs specific richness were nearly identical (data not shown). Qualitative changes (population shift) occurred in the disturbed and weathered sediment. The similarities (Bray-Curtis index) between

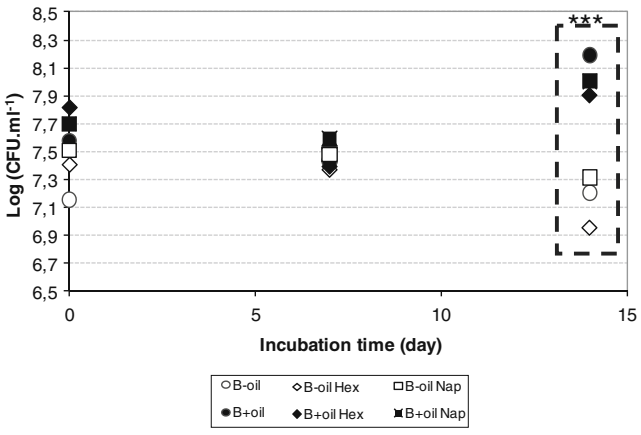

Figure 2 Distribution of hydrocarbonoclastic cultivable bacteria grown on minimum agar plates supplemented or not with hydrocarbon. *CFU.ml*$^{-1}$ Colony forming units, *Hex* hexadecane, *Nap* naphthalene. *Asterisks* high significant difference between sample

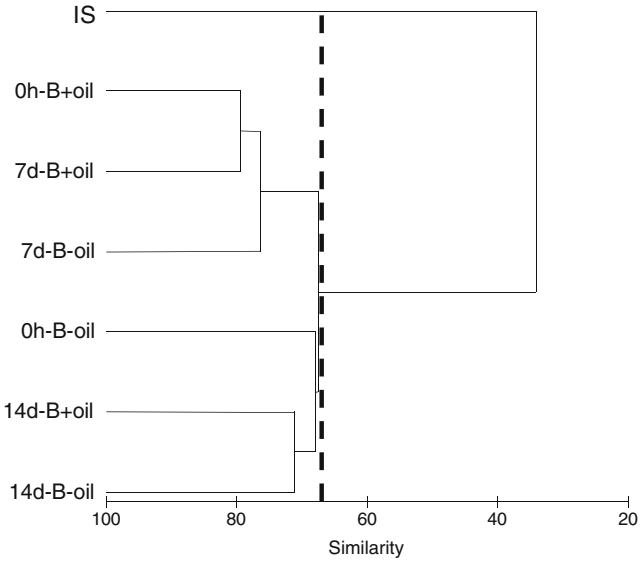

Figure 3 Similarity analysis of the DNA bacterial community structures based on T-RFLP of 16S rRNA gene from in situ (IS) and slurry experimental samples. The UPGMA cluster analysis was performed using square root transformation of T-RFLP data and the Bray Curtis similarity index. Dashed line represents 67% of similarity index. Microcosm triplicate averages were used for the cluster analysis

microcosm communities were higher than 67% which were similar to those obtained between replicates (up to 70%). Therefore, the DNA based analysis did not show clear modifications in the total bacterial communities structure related to the incubation conditions (B+OIL and B−OIL) over the time (days 0, 7 and 14) (Fig. 3).

The changes in metabolically active bacterial community structure in oil amended and unamended microcosms were examined by T-RFLP analysis followed by cluster analyses and a two-dimensional non-metric multidimensional scaling ordination (Fig. 4). The similarity between microcosm triplicates was always higher than 65% and reached up to 80%. A temporal modification is observed in both microcosms that were related to the well known bottle effect. Oil addition induced an immediate shift (within 1 h) in the metabolically active bacterial community structure (Fig. 4a). This difference is characterised by an important decrease in the OTUs specific richness (R) in the B+OIL slurry (8 and 12 T-RFs in B+OIL slurry vs. 23 and 18 in B−OIL slurry with *Hae*III and *Hinf*I profiles, respectively) (Fig. 5). However, the metabolically active community was restored 1 h later. Following this first step of adaptation, the communities were stable during 3 days. Two bacterial community structures showing differences between the contaminated and uncontaminated slurries were observed after 3 days of incubation (Fig. 4b). These structures were maintained until the ninth day (clusters B and C). After 12 days of incubation the differences between the communities increased (clusters D and E) (Fig. 4b). Thus the T-RFLP analysis showed five main clusters, the first cluster A

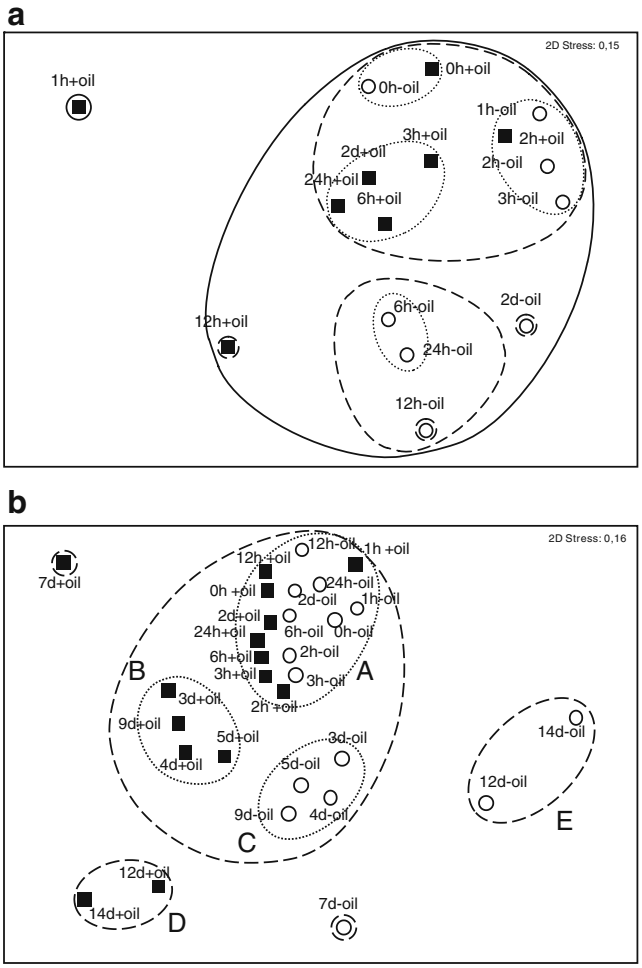

Figure 4 Changes in metabolically active bacterial community structures based on T-RFLP analysis of 16S rRNA transcripts. The 2D-MDS plot analyses were performed using square-root transformation of T-RFLP data and the Bray Curtis similarity index. *Circles* and *squares* represent average of microcosm triplicates (**a** MDS-plot analysis of 0 h to 2 days samples; *black lines*, *dashed bold line* and *dashed thin line* represent, respectively, similarity index 70, 65 and 59. **b** MDS-plot analysis of 0 h to 14 days samples of oiled and unoiled conditions. Five main clusters are mentioned (*A*, *B*, *C*, *D* and *E*) representing the specific bacterial community structure patterns. *Dashed bold line* and *dashed thin line* represent, respectively, similarity index of 53 and 45 for the MDS-plot analysis)

including the communities from day 0 to 2 days, clusters B and C corresponding to communities from 3 to 9 days, of oiled and unoiled, respectively. The two last clusters included the communities from day 12 to 14, cluster D was formed by communities from oiled microcosms while the cluster E contained the communities from unoiled microcosms.

Bacterial Community Composition and Dynamics

In order to further characterise the metabolically active communities, 16S rRNA transcript libraries were constructed for a representative sample of each cluster identified by T-RFLP. The analysis of the five libraries, 0h B−OIL (cluster A), 9d B+OIL (cluster B), 9d B−OIL (cluster C), 12d B+OIL (cluster D) and 12d B−OIL (cluster E), allowed the identification of the dominant metabolically active populations associated to the presence of crude oil. As expected, the Shannon diversity indices indicated a higher diversity at DNA level than RNA (Table 1).

The RFLP analysis of the initial DNA community library revealed that 84% of the clones analysed were singletons. Sequences from patterns with more than one clone were mainly related to *Gamma-* (49%) and *Deltaproteobacteria* (35%), followed by Diatom chloroplast (11%), *Alpha-* (2%), *Epsilonproteobacteria* (2%) and *Acidobacteria* (2%) sequences. The *Gammaproteobacteria* group was also dominant in all the RNA libraries throughout the experiment. The comparison of the cDNA clone libraries with the LIBSHUFF method revealed that 12d B−OIL and 12d B+OIL libraries were composed of significantly different phylotypes (XY p value=0.004; YX p value=0.021) while no difference could be observed between the 9d B−OIL and 9d B+OIL cDNA clone libraries (XY p value=0.034; YX p value=0.174), confirming the T-RFLP results (Fig. 5). Changes in the relative abundance of the main groups composing the metabolically active communities were observed. The *Deltaproteobacteria* subclass maintained the same relative abundance from 0 h to 12 days in the B-OIL condition (around 20%) whilst its abundance decreased until 11% and 14% at respectively 9 and 12 days of incubation in the B+OIL slurry (Table 2). In contrast, the *Gammaproteobacteria* group reached 58% of the active members of the oiled slurry at the end of the experiment while its abundance decreased significantly in the unoiled slurry (Table 3). The relative abundance of *Alphaproteobacteria* sequence increased during the experiment period from 4% at 0 h to respectively 14% and to 23% in oiled and unoiled conditions at 12 days (Table 2).

Around 50% of total sequences detected in the DNA library (Tables 2 and 3) have highest Blastn hits with sequences originated from hydrocarbon-degrading or hydrocarbon-associated strains belonging to *Gamma-* and *Deltaproteobacteria*. This important proportion of sequences related to highly specialised bacteria in the community studied was also observed in the metabolically active bacterial community. Indeed, the hydrocarbon-degrading associated sequences in 0h B−OIL, 9d B−OIL, 12d B−OIL, 9d B+OIL and 12d B+OIL cDNA libraries represent respectively 27%, 25%, 40%, 37% and 39% of total sequences (Tables 2 and 3). The proportion of hydrocarbon-degrading bacteria sequences was relatively similar between the B−OIL and B+OIL conditions. However *Gammaproteobacteria* sequences belonging to *Alcanivorax* and *Cycloclasticus* genera or hydrocarbon-associated clones were dominant in the oiled slurries, while

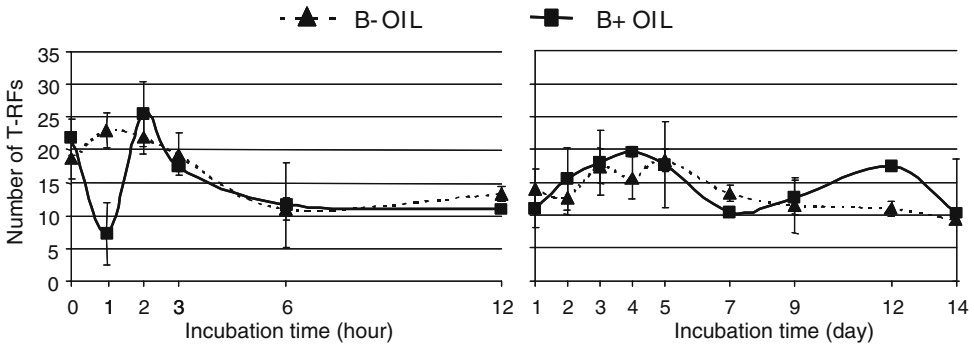

Figure 5 Number of OTUs in metabolically active bacterial communities during the incubation period monitored by T-RFLP analysis. The profiles obtained using *Hae*III are presented here. *Triangles* and *squares* represent the microcosm triplicate average

sequences related to *Deltaproteobacteria* belonging to benzene mineralizing consortium remained dominant in the unoiled microcosms, reflecting the 0 h DNA library sequences proportion.

Discussion

Heavily petroleum-contaminated sediment was chosen for this study since the community inhabiting these sediments is adapted to the presence of oil [24] and consequently its bacterial community presents more potential abilities than those from pristine environments to cope with oil hydrocarbons. These abilities include not only biodegradation capacities [3], but also a higher resistance potential to the toxic effect of the pollutant. In order to reduce the low molecular-weight hydrocarbon content of sediment and to adapt the bacterial communities to laboratory conditions, the collected sediments were weathered. Thus, after this treatment the observed bacterial community response would be specifically linked with the crude oil addition. The slurry setting also promotes aerobic metabolisms that are well-known to be more efficient in hydrocarbon biodegradation process, nevertheless because the oxygenation was exclusively performed by air diffusion we assume that anoxic niches cannot be excluded. During the slurry experiment, no significant change in the total bacterial community structure was observed in response to oil input despite the fact that an important part of the hydrocarbon compounds was biodegraded in 7 days. Thus the short incubation period performed in this study allowed to describe the fast response of the community present in the slurry by analysing the active community (rRNA transcripts analysis). Indeed, the metabolically active bacterial communities changed quickly and differentially depending on the incubation conditions. For instance, the unoiled community was rapidly modified during the experiment probably due to the depletion of hydrocarbon compounds, mainly alkanes and low molecular weight PAHs, and the ageing conditions of the microcosms whereas the B+OIL community structure was maintained closer to the original community due to the oil addition. Previous studies performed at DNA level on pristine or low contaminated communities indicated that after an important lag period [2, 12] oil contamination induced drastic changes in the bacterial community structure associated with a decrease of diversity [27, 31]. These modifications were either attributed to a strong selection for petroleum-degrading bacteria [13, 17, 31], or to toxic effect of oil on the growth of certain microorganisms [12]. In our case (i.e. a bacterial community inhabiting heavily contaminated sediments with a long history of contamination), oil appeared necessary for maintaining the metabolically active populations. Oil addition induced a qualitative shift (specific richness decrease) in the metabolically active community after 1 h. Nevertheless, this shift was reversed after 1 h, highlighting a fast, adaptative and efficient response of the metabolically active bacterial population. Thus this study reinforces the concept that important processes in response to oil contamination occur within few hours following an oil

Table 1 Characteristics of the 0h B−OIL DNA and 0h B−OIL, 9d B−OIL, 9d B+OIL, 12d B−OIL, 12d B+OIL cDNA clone libraries

Sample	Total no. of clones	No. of taxa	No. of singletons	Coverage	Shannon index
0h B−OIL DNA	364	285	240[a]	0.34	5.511
0h B−OIL cDNA	86	61	46	0.45	3.946
9d B−OIL cDNA	79	57	46	0.42	3.838
12d B−OIL cDNA	54	47	41	0.24	3.800
9d B+OIL cDNA	60	44	36	0.36	3.625
12d B+OIL cDNA	57	46	37	0.35	3.757

[a] Singletons not sequenced

Table 2 Identification of phylogenetic groups from DNA (0h B−OIL) and cDNA clone sequences (0h B−OIL, 9d B−OIL, 9d B+OIL, 12d B−OIL and 12d B+OIL) and their relative abundance

Phylogenetic groups	Main closest sequence (EMBL accession no., % of identity with the closest clone[a]	No. and % of clones in each bacterial division					
		DNA[a]	cDNA				
		0h B−OIL	0h B−OIL	9d B−OIL	12d B−OIL	9d B+OIL	12d B+OIL
Eukaryotic plastids							
Bacillariophyta	*Melosira varians* p107 (AJ536464), 98.7 *Phaeodactylum tricornutum* (EF067920), 97.9 *Bacillaria paxillifer* p73 (AJ536452), 97.8	10 (11.4)	11 (14.1)	2 (2.5)	1 (1.8)	11 (20)	3 (5.3)
Cyanobacteria							
Oscillatoriales	*Planktotricoides raciborskii* NSLA3 (AB045962), 99.3 *Arthrospira platensis* Sp-10 (DQ279767), 95.8 Uncultured cyanobacterium (DQ446127), 94.9	–	2 (2.6)	2 (2.5)	3 (5.5)	2 (3.6)	–
Chloroflexi	*Dehalococcoides* sp. BHI80-15 (AJ431246), 87.8 Uncultured bacterium clone (DQ154828), 96.2	–	1 (1.3)	1 (1.3)	3 (5.5)	1 (1.8)	1 (1.8)
Verrucomicrobium group	*Opitutus* sp. SA-9 (AY695840), 91.3 Uncultured Verrucomicrobia Artic95D-9 (AY028220), 95.8	–	1 (1.3)	3 (3.8)	2 (3.6)	1 (1.8)	1 (1.8)
Spirochaetes	Uncultured spirochete (AY605171), 97.7 *Spirochaeta bajacaliforniensis* DSM16054T (AJ698859), 90	–	2 (2.6)	–	–	2 (3.6)	–
Fusobacteria	*Leptotrichia* sp. Oral clone EI022 (AF385572), 87.4	–	1 (1.3)	–	–	–	–
Acidobacteria group	Uncultured Acidobacteriaceae (DQ394979), 97.9	2 (2.3)	–	–	–	–	2 (3.5)
Actinobacteria	*Solirubrobacter* sp. (AB245336), 90.8 Candidatus *Microthrix parvicella* (DDQ147287), 91.7	–	1 (1.3)	–	1 (1.8)	–	–
Nitrospirae	*Nitrospira marina* (X82559), 99.7	–	–	1 (1.3)	–	–	–
Unknown sequence			4 (5.1)		1 (1.8)		
Epsilonproteobacteria	*Sulfurimonas paralvinellae* (AB252048), 92.7	2 (2.3)	–	–	–	–	–
Betaproteobacteria	*Ralstonia detusculanense* (AF280433), 99	–	–	–	–	–	1 (1.8)
Deltaproteobacteria							
Myxococcales	*Polyangium thaxteri* Pl t3 (AJ233943), 92.5 *Nannocystis exedens* Na e571 (AJ233947), 94.5 *Enhygromixa salina* SMK-1-3 (AB097591), 97.9	–	3 (3.8)	6 (7.6)	–	2 (3.6)	1 (1.8)
Bdellovibrionales	*Bacteriovorax* sp. (EF092444), 98.3 *Bdellovibrio* sp. (AF084855), 89	–	5 (6.4)	1 (1.3)	–	1 (1.8)	1 (1.8)
Desufobacterales	Benzene mineralizing consortium (AF029047), 99.3 *Olavius algarvensis* endosymbiont (AF328857), 98.5 *Desulfobacterium cetonicum* DSM 7267 (AJ237603, 99.3	31 (35.2)	4 (5.1)	9 (11.4)	10 (18.2)	3 (5.5)	6 (10.5)
Alphaproteobacteria							
Rhodobacterales	*Thalassobius mediterraneus* CECT 5383 (AJ878874), 96.2 *Caulobacter halobacteroïdes* (AB008849), 97 *Pedomicrobium manganicum* (X97691), 94.1 *Roseobacter* sp. B11 (DQ659411), 99.7	2 (2.3)	1 (1.3)	1 (1.3)	3 (5.5)	2 (3.6)	1 (1.8)
Parvularculales	Uncultured sludge bacterium H9 (AF234706), 94	–	–	1 (1.3)	–	–	–
Rhizobiales	*Parvibaculum lavamentivorans* DS-1 (AY387398), 98.3	–	–	–	5 (9)	–	4 (7)
Kopriimonodales	*Koprimonas byunsanensis* KOPRI 13522 (DQ167245), 88	–	–	1 (1.3)	1 (1.8)	–	–
Rhodospirillales	Uncultured bacterium clone (EF208679), 98.7 *Rhodovibrio* sp. 2MB1 (AY987846), 90.2 *Defluvicoccus* Ben114 (AF179678), 92.7	–	2 (2.6)	2 (2.5)	4 (7.3)	4 (7.3)	2 (3.5)
Uncultured	*Olavius loisae* endosymbiont (AF104473), 93.9	–	–	2 (2.5)	–	–	1 (1.8)

This table summarises all phylogenetic groups examined except *Gammaproteobacteria* division (see Table 3)

[a] An 84% of singletons of the library have been not sequenced and not considered here. The percentage includes only the patterns with more than one clone

Table 3 Identification of *Gammaproteobacteria* sequences from phylogenetic analysis of DNA (0h B−OIL) and cDNA clone sequences (0h B−OIL, 9d B−OIL, 9d B+OIL, 12d B−OIL, and 12d B+OIL) and their relative abundance

Phylogenetic groups	Main closest sequence (EMBL accession no.), % of identity with the closest clone[a]	No. and % of clones in each bacterial division					
		DNA[a]	cDNA				
		0h B−OIL	0h B−OIL	9d B−OIL	12d B−OIL	9d B+OIL	12d B+OIL
Chromatiales							
Ectothiorhodospiraceae	*Thioalkalivibrio thiocyanodenitrificans* ARhD (AY360060), 93.7	2 (2.3)	2 (2.6)	–	–	–	–
Chromatiaceae	*Chromatium* sp. EP 2204 (Y12299), 97.5	12 (13.6)	8 (10.3)	3 (3.8)	–	1 (1.8)	1 (1.8)
	Allochromatium palmeri BH-24 (AJ871279), 99.5						
	Marichromatium indicum JA100 (AJ543328), 96.4						
Thiotrichales							
Piscirichettsiaceae	*Cycloclasticus* sp. (AF093004), 100 *Thiovibrio halophilus* HL5 (DQ390450), 96.6	6 (6.8)	6 (7.7)	–	–	1 (1.8)	–
	Dibenzofuran-degrading bacteria DBF-MAK (AB086228), 100						
Francissella	*Francissella philominagia* 1951 (DQ813266), 98.7	2 (2.3)	1 (1.3)	–	–	–	–
Oceanospirillales							
Oceanospirillaceae	Uncultured gamma bacteria (AY622252), 95.9 *Oceanobacter* sp. RED65 (AY136131), 90.4	2 (2.3)	5 (6.4)	12 (15.2)	4 (7.3)	3 (5.5)	10 (17.5)
Alcanivoraceae	*Alcanivorax diesolei* NO1A (AY683531), 95 *Alcanivorax borkumensis* (Y12579), 100	2 (2.3)	4 (5.1)	2 (2.5)	4 (7.3)	7 (12.7)	6 (10.5)
Oleiphilaceae	*Oleiphilus messinensis* ME102 (AJ295154), 94.7	–	1 (1.3)	–	1 (1.8)	–	–
Uncultured clone							
Hydrocarbon associated	Uncultured soil bacterium clone M54-Pitesti (DQ378269), 100 Uncultured soil bacterium clone PYR10d3 (DQ123668), 99.2	5 (5.7)	6 (7.7)	9 (11.4)	7 (12.7)	9 (16.4)	10 (17.5)
Unaffiliated Gammaproteobacteria	Unknown marine gammaproteobacteria NO5 (AY007676), 99.7 Gammaproteobacterium (AY386332), 97.1	10 (11.4)	7 (9)	15 (19)	3 (5.5)	5 (9.1)	5 (8.8)
	Gammaproteobacterium (AB212803), 93.6						
	Uncultured bacterium clone HB2-32-21 (DQ334627), 99.7						
Sulfur-oxidising symbiont	*Vestimentiferan endosymbiont* (AF165907), 94.5 *Lucina nassula* gill symbiont (X95229), 94.7	–	–	6 (7.6)	2 (3.6)	–	1 (1.8)

[a] An 84% of singletons of the library have been not sequenced and not considered here. The percentage includes only the patterns with more than one clone

spill event. These processes lead to an efficient biodegradation which probably include many other biological processes, such as remarkable bacterial resilience to toxicity and physico-chemical modifications of the oil degrading matrix.

The significant increase in numbers of hydrocarbon-degrading bacteria, the high proportion of sequences affiliated to hydrocarbon-degrading strains in the initial community and the high abundance of highly specialised metabolically active oil-degrading populations confirmed the adaptation of this community to the oil presence. Although increases of the proportion of hydrocarbon–degraders in response to hydrocarbon contamination have been previously observed [4], such a high proportion of hydrocarbon-degrading organisms detected within sediment bacterial community have not previously been described. The sediments of the retention basin of the Berre lagoon represent an exceptional environment associated to crude oil pollution. Both oil amended and unamended microcosms showed a high proportion of active bacteria related to hydrocarbon degraders, as expected since these specialised bacteria were found dominant in the community present in the slurry. Nevertheless, it is important to note that both communities are significantly different since, the gammaproteobacteria responded faster to the

hydrocarbon input suggesting that they have probably an important role in hydrocarbon depletion.

Interestingly, the relative abundance of *Alphaproteobacteria* increased during the course of the experiment, irrespective of the slurry condition. This finding is in agreement with other studies that suggest that these bacterial groups succeed to *Gammaproteobacteria* in the process of bioremediation [2, 27]. This is further supported by the higher relative abundance of *Alphaproteobacteria* observed in the unoiled slurry compared with the oiled slurry, since the bioremediation process in the unoiled slurry was ahead compared to the oiled slurry.

To the best of our knowledge, the present work is the first study reporting the succession of metabolically active bacterial populations occurring during the early stage of the response to oil contamination. The T-RFLP fingerprints revealed an important modification of the metabolically active bacterial community structure within the first hours following oil addition. This observation suggests that essential mechanisms take place during the early period, however further investigations are requested to understand and characterise them. The bacterial communities from the sediment of the Etang de Berre ecosystem exhibited several properties that are suitable for studying the early response at the functional level to fully understand the metabolic strategy developed by the whole community to face crude oil contamination.

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