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Degradation of the *"Erika"* oil

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Abstract – Since March 2001, samples of the remaining oil from the wreck of the "*Erika*" have been collected along the Atlantic coastline in order to assess the natural degradation rate. Four years after the sinking of the tanker, chemical analyses of the oil revealed the influence of environmental parameters on the degradation kinetics. Among the diverse parameters controlling the fate of oil in the environment, biodegradation by microorganisms is known to play an important role. To investigate the role of microorganisms on "*Erika*" oil degradation, microbial mats from the Guérande salt marches were maintained in slurries containing the pollutant. From these slurries experiments, a low biodegradation rate of the "*Erika*" oil was detected indicating the degradation capacities of microbial mats. Biodiversity studies were conducted to further understand the biodegradation processes. Microbial mats from the Guérande salterns were maintained in microcosms to evaluate the impact of "*Erika*" oil on bacterial communities. Molecular analysis based on 16S rRNA and *pufM* encoding genes allowed fingerprinting of the bacterial and purple anoxygenic bacterial (PAB) communities respectively. These studies revealed bacterial diversity and communities changes showing the adaptation of microorganisms to the "*Erika*".

Key words: Microbial mat / Biodiversity analysis / Heavy fuel oil / PAH / T-RFLP

Résumé – Dégradation du pétrole de l'*Erika*. Depuis mars 2001, des échantillons de pétrole résiduel présent sur la côte Atlantique, suite au naufrage de l'« *Erika* », ont été collectés afin d'évaluer les taux de dégradation naturelle. Quatre ans après la marée noire, les analyses chimiques de ce pétrole résiduel révèlent l'influence des paramètres environnementaux sur les cinétiques de dégradation. Parmi les différents paramètres influençant le devenir du pétrole dans l'environnement, la biodégradation par les micro-organismes joue un rôle important. Afin d'étudier le rôle des micro-organismes sur la dégradation du pétrole de l'« *Erika* », des tapis microbiens des salines de Guérande ont été incubés en présence du polluant. Bien que de faibles taux de biodégradation ont été mesurés, ces analyses ont mis en évidence la capacité de dégradation des tapis microbiens. Afin de comprendre les mécanismes de biodégradation et d'évaluer l'impact du pétrole de l'« *Erika* » sur les communautés bactériennes, des tapis microbiens des salines de Guérande ont été maintenus en microcosmes. Les études moléculaires basées sur l'analyse des gènes codant pour l'ARN ribosomique 16S et le gène *pufM* ont permis d'obtenir des empreintes des eubactéries et des bactéries pourpres anoxygéniques (PAB) respectivement. Ces études ont révélé des modifications de la diversité et de la structure des communautés bactériennes montrant l'adaptation des micro-organismes à la présence de pétrole.

1 Introduction

In late December 1999, the northern French Atlantic coast was impacted by an oil spill following the wreck of the Maltese oil tanker *Erika*. Crude oil is composed mainly by hydrocarbons, such as saturated hydrocarbons, aromatics, resins and asphaltenes (Harayama et al. 1999) which are highly noxious compounds as they exhibit toxic, mutagenic, and carcinogenic properties (Churchill et al. 1999; Coates et al. 1997). Previous studies on the impact of oil spills report persistent effects lasting from five to more than ten years (Jones et al. 1998; Southward 1978). According to its composition of 90% of heavy residues and 10% of a light fraction, the *Erika* heavy fuel oil was found to be more resistant to natural degradation processes than light crude oils (Oudot 2000). After the contamination, the fate of petroleum hydrocarbon compounds in the environment depends on both physico-chemical and biological environmental variables that control different

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mechanisms such as photo and chemical oxidations, evaporation, dilution, sedimentation, and microbial activities. Among these processes, biodegradation by microorganisms is known to play an important role (Leahy and Colwell 1990; Swannell et al. 1996). Hydrocarbons differ in their susceptibility to microbial attack and generally degrade in the following order of decreasing susceptibilities: *n*-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Leahy and Colwell 1990). For efficient oil bioremediation after a catastrophic oil spill, there is a need to evaluate the kinetics and relative rates of natural degradation and estimate the active role played by microorganisms. For this purpose, it is important to understand the behaviour of microbial populations responsible for degrading crude oil. In coastal zones, photosynthetic microbial mats develop at the water-sediment interface (Caumette et al. 1994; Van Gemerden 1993). Since these vertically laminated structures exhibit important bacterial diversity that offer high metabolic potential for hydrocarbons degradation, they can be considered as relevant models to study the impact of crude oil on bacterial communities (Abed et al. 2002).

The specific aims of this research were: to assess the kinetics of natural degradation processes of the *Erika* fuel oil with respect to the prevailing environmental parameters by monitoring *in situ* the fate of hydrocarbon compounds, to evaluate the degradation capacities of bacterial communities present in microbial mats (Guérande, Brittany, France) using slurry experiments, and to estimate the impact of the *Erika* oil on these microbial communities by culture independent molecular analysis.

2 Materials and methods

2.1 Monitoring natural degradation of *Erika* fuel oil

To assess the natural degradation of the *Erika* fuel oil according to the environmental conditions, *CEDRE* employees involved in the clean-up operations were asked to identify polluted areas satisfying two main criteria: (1) areas where no human cleaning operations were performed and (2) areas where sufficient oil was present to allow sampling for several years. More than 75 sites were suggested for this study. After a survey of these sites, 21 were selected and samples collected. For each site, samples were collected in triplicate, in various locations according to sun – wave exposure and composition of the beach substratum. Samples were then frozen and stored at −20 °C until analysis.

Sample extraction

For oil-sediment sample extraction, approximate 50 mg of the samples were weighed, dissolved in methylene chloride and sonicated for 10 min. The solvent was filtered through sodium sulfate then evaporated using a rotavapor. For mud sample extraction, a preliminary step before oil extraction, was to evaporate the water by heating at 40 °C for 4 hours. The same procedure as cited above was then used. For sand samples extraction, approximate 150 ml of sand was mixed with sodium sulfate. Oil was then extracted using a soxhlet with methylene chloride for 4 hours.

Hydrocarbon composition analysis

Approximate 30 mg of the samples were purified through low-pressure liquid chromatography on an open silica-alumina column (saturates and aromatics were eluted simultaneously with a mixture pentane/methylene chloride 80/20). Solvent was then evaporated to 2 ml and the sample analysed by gas chromatography equipped with a mass spectrometer detector (GC/MS).The GC was an HP 6890N (Hewlett-Packard, Palo Alto, CA, USA) equipped with a split/splitless injector (Pulsed Splitless time: 1 min, flow 50 ml min^{-1}). The injector temperature was maintained at 270 °C. The interface temperature was 290 °C and the GC temperature programmed from 50 °C (1 min) to 300 °C (20 min) at 5 °C min^{-1}. The carrier gas was Helium at a constant flow of 1 ml min^{-1}. The capillary column used was an HP 5 MS (Hewlett-Packard, Palo Alto, CA, USA): 60 m × 0.25 mm ID × 0.25 μm film thickness. The GC was coupled to an HP 5973 Mass Selective Detector (MSD) (Electronic Impact: 70 eV, voltage: 1200 V). Saturates and PAHs were quantified using Single Ion Monitoring mode with the molecular ion of each compound at 1.4 cycles s^{-1}.

Quantification was performed with respect to an internal reference, 17α(H), 21β(H)-hopane (m/z = 191). This "biomarker" (non-biodegradable, non-photooxidisable, non-volatile and non-water-soluble) is widely used as a conserved internal marker within the oil to follow the disappearance of other components within the oil (Jezequel et al. 2003; Peters and Moldowan 1993; Prince et al. 1994).

2.2 Microbial mat sampling and microcosms experiment set up

Guérande microbial mats were collected from a salt march near La Baule (Brittany, France). Salt marches provide environments of various salinities, ranging from the salt concentration of sea-water up to those at which NaCl precipitates. The mean daytime temperature in the area is 11 °C to 12 °C, with rainfall of 800 to 900 mm per year (Giani et al. 1989). A freshly taken microbial mat sample was cut into pieces of equal size (20 cm^2) and placed in glass tubes without water. Half of the mat samples were not contaminated (controls) whilst the remainder mat were contaminated with *Erika* fuel oil. Approximately equal amounts of oil (200 to 300 mg) were added on the surfaces of contaminated mats before adding synthetic seawater (salinity 70 g L^{-1}). Microbial mats microcosms were maintained at room temperature under a light regime of 16 H day light/8 H dark. Samples were taken every 15 days for 3 months. At each time, one contaminated microcosm and one control microcosm taken randomly, were sub-sampled using Pasteur micropipettes and then stored immediately at −80 °C. Biodiversity was estimated by the T-RFLP method on two mixed sub-samples.

2.3 Genomic DNA extraction

Mixed community DNA was extracted from microbial mat fractions with the Ultraclean soil DNA isolation kit (Mo Bio Laboratories, Solana Beach, California), by using the recommended protocol with minor modifications as previously described (Precigou et al. 2001).

2.4 PCR amplification and purification

Optimisation of the polymerase chain reaction (PCR) was performed for each sample by adjusting the amount of genomic DNA extract used to obtain a strong band on an agarose gel, without visible non-specific product. PCR was performed using a reaction mixture of 200 μM each desoxynucleoside triphosphate (Qiagen), 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase (Qiagen), 1X PCR Buffer (Qiagen), and 0.2 μM each primer. The first primers used were the eubacterial primer 8-27F (Lane 1991), and the universal primer 1472-1489R (Muyzer et al. 1995) amplifying specifically the 16S ribosomal gene. To assess specifically purple bacteria, *pufM* gene coding for the photosynthetic centre was targeted with the primers: PB557F and PB750R (Madigan et al. 2000). Forward primers, 8-27F and PB557F were hexachlorofluorescein labelled. PCR (50 μl) was performed in a MJ Research PTC-100 thermocycler by using an initial denaturation step of 95 °C for 15 min, followed by 35 cycles of a program consisting of denaturation at 95 °C for 30 s, primer annealing at "52 °C (for 16S rDNA) and 54 °C (for *pufM*)" for 30 s, and extension at 72 °C for 30 s. A final extension at 72 °C for 7 min was performed after the programmed number of cycles were completed.

PCR products were then purified by using the GFX DNA and Gel Band Purification kit (Amersham Biosciences) as directed by the supplier.

2.5 Restriction enzyme digestion

After a quantification of the purified PCR product by the "dots method", 300 ng of amplified DNA mixed in a final volume of 30 μl of restriction enzyme mix containing 10 U of restriction enzyme (HaeIII) in 1X reaction buffer (New England BioLabs), were incubated for 3 h at 37 °C.

2.6 T-RFLP analysis

Three microliters of the digested PCR product was mixed with 1 μl of TAMRA500 size standard (Applied Biosystems Instruments, Foster City, California). DNA fragments were separated by size by capillary electrophoresis at 15 000 volts for 30 min on an ABI PRISM 310 Genetic Analyzer (Applied Biosystems). The 5′ terminal fragments were visualized by excitation of the hexachlorofluorescein molecule (hex) attached to the forward primer. The gel image was captured and analyzed by using Genescan version 3.1 analysis software (ABI). Negative controls (no genomic DNA) were conducted with every PCR and run on Genescan gels.

Data sets were constructed using peaks where fluorescence was higher than 100 for at least one sample. Statistical analyses were done using hierarchical cluster analysis, the unweighted-pair group method using arithmetic averages (UPGMA). These statistical analyses were done with MVSP software (Multi-Variate Statistical Package 3.1, Kovach Computing Services).

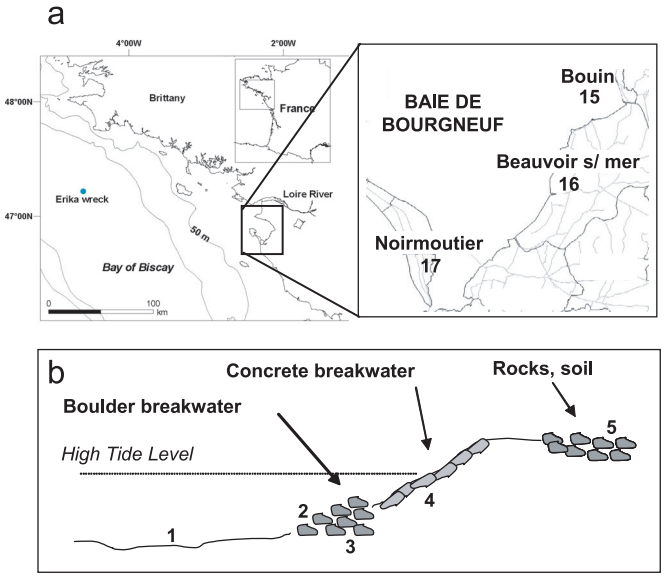

Fig. 1. Location of sites sampled in "Baie de Bourgneuf" (a) and schematic representation of the sampling areas indicated by numbers (b).

2.7 In vitro *Erika* fuel oil biodegradation

To estimate the biodegradation potential of Guérande microbial mats, slurry experiments were conducted. Slurries consisted of ground-up microbial mats maintained in synthetic sea water contaminated with *Erika* fuel oil (0.16–0.24 g L^{-1}). Heat sterilized microbial mats served as controls. Slurries were incubated in aerobic conditions at 30 °C with shaking at 180 rpm under continuous light exposure. Samples were collected at selected time intervals over the three months incubation period.

Hydrocarbons composition of *Erika* fuel oil was analysed as previously described (Mazeas and Budzinski 2001).

3 Results

3.1 Monitoring the natural degradation of *Erika* fuel oil

The results presented in this study were from "Baie de Bourgneuf" (Vendée-France) (Fig. 1a). Samples were collected from various sites according to sun-wave exposure and composition of the beach substratum (Fig. 1b). Table 1 shows the characteristics of the sampling sites. For each sample collected since March 2001, relative abundances (Hopane Unit) of *n*-alcanes (Fig. 2a), *n*-C30 (Fig. 2b), benzothiophenes (parent and alkyl homologues) (Fig. 2c) and chrysenes (parent and alkyl homologues) (Fig. 2d) were obtained.

In comparison with the other samples covered by thin layer of oil, less *n*-alkane degradation was observed for boulder breakwater samples (thick patches of oil at the surface and under rocks). Moreover, *n*-alkanes and *n*-C30 abundances increased in these samples. *n*-alkanes were degraded at a similar rate for samples 1, 4 and 5 despite the different level at which they were collected. Degradation of light PAHs (benzothiophene) (Fig. 2c) was similar for all the samples. Heavier PAHs such as chrysenes (Fig. 2d) were degraded at different rates

Table 1. Environmental conditions and locations of samples collected from "Baie de Bourgneuf".

Samples	Location	Substrate nature	Solar radiation	Layer (mm) (*)	Tidal level
1	16	Mud	Exposed	5	Intertidal
2	15	Rocks (surface)	Exposed	>10	Intertidal
3	15	Rocks (shade)	Non exposed	>10	Intertidal
4	17	Concrete	Exposed	2	Supra-tidal
5	15	Rocks, soil	Exposed	5	Supra-tidal

(*) pollutant layer was estimated visually at each sampling round.

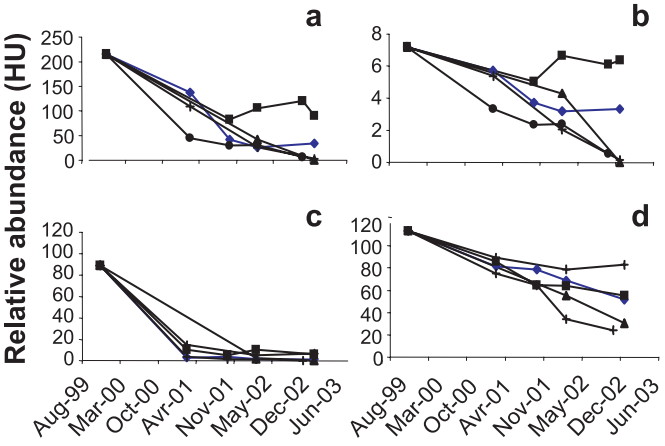

Fig. 2. Relative abundance (HU) of *n*-alkanes (a), *n*-C30 (b), benzothiophenes (parent and alkyl homologues) (c) and chrysenes (parent and alkyl homologues) (d) for samples collected since March 2000 in the areas 1 (+), 2 (■), 3 (♦), 4 (•), and 5 (▲) presented in Figure 1b.

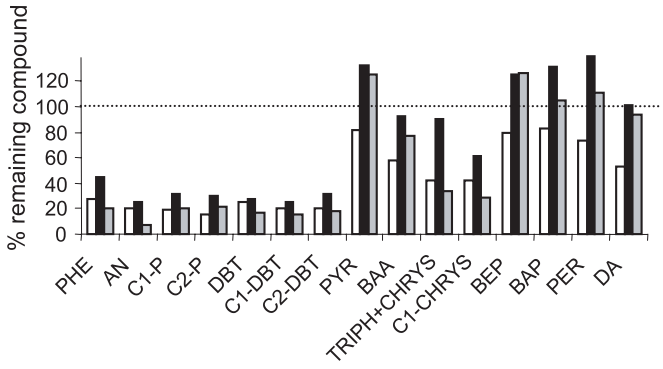

Fig. 3. Degradation of aromatic compounds of *Erika* oil by Guérande microbial mats. % remaining compound corresponds to the average of duplicates measured at t30 (white), t60 (black), and t90 (grey). Dotted line corresponds to 100% compound (t0). PHE: phenanthrene, AN: anthracene, C1-P: methylphenanthrene, C2-P: dimethylphenanthrene, DBT: dibenzothiophene, C1-DBT: methyldibenzothiophene, C2-DBT: dimethyldibenzothiophene, PYR: pyrene, BAA: Benz(a)anthracene, TRIPH+CHRYS: trimethylphenanthrene + chrysene, C1-CHRYS: methylchrysene, BEP: benz(e)pyrene, BAP: Benz(a)pyrene, PER: perylene, DA: dibenz(a,h)anthracene.

according to the environmental conditions: supra-tidal samples were more readily degraded than intertidal ones. Figure 2d shows that the depletion of chrysenes was more important for the samples collected at a supra-tidal level (samples 4 and 5) compared to intertidal samples.

3.2 Evaluation of *Erika* fuel oil degradation capacities of Guérande microbial mats

Over the three months incubation period, changes in *Erika* fuel oil composition were observed under both biotic and abiotic conditions. However, although the results showed an important variation, a biotic degradation could be suspected since methylated compounds were less degraded. The degradation of aromatic compounds shows an important modification of the petroleum composition within the first month (Fig. 3). Furthermore, phenanthrene and dibenzothiophene derivatives were among the most biodegraded hydrocarbons. High molecular weight compounds were less biodegraded.

3.3 Impact of *Erika* fuel oil on bacterial communities of Guérande microbial mats

T-RFLP analysis using specific primers targeting eubacterial 16S rRNA encoding genes allowed fingerprinting of 22 operational taxonomic units (OTUs) revealing an important diversity. Although the bacterial communities structure changed through the experimental period in both contaminated and uncontaminated microcosms, the comparison between the different conditions showed that the evolution was different depending upon the presence of oil. Cluster analysis from the T-RFLP data showed that control samples from the 30th to the 75th day formed a separate cluster to the other samples (Fig. 4).

Purple anoxygenic community structure changes were analysed by T-RFLP targeting *pufM* genes specific for these bacterial populations. T-RFLP fingerprints showed an abundant bacterial diversity characterised by the detection of 26 OTUs (Fig. 5). As observed with eubacterial community structure analysis, biodiversity changes were observed in both contaminated and uncontaminated microcosms during the experimental period. However the evolution of some bacterial populations was found to be different according to the experimental conditions. Between the 15th and the 45th days, OTU with length of 132 base pairs (bp) was less developed in the presence of *Erika* fuel oil than in the controls. In contrast during the same period OTU with length of 200 bp increased in the contaminated microcosms while no changes were observed in the uncontaminated controls. From the 60th day to the end of

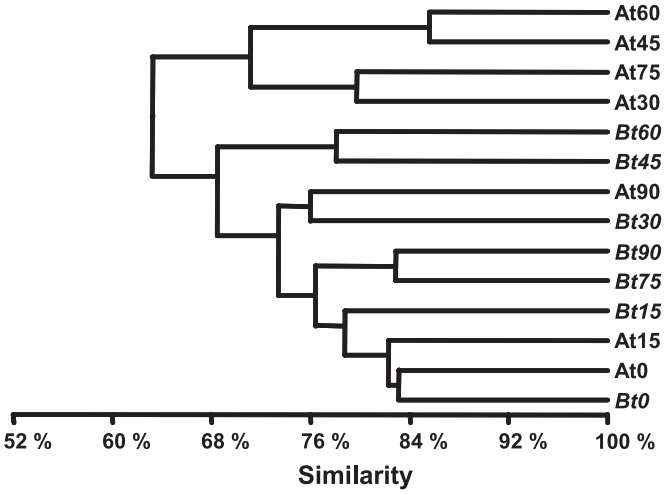

Fig. 4. Clustering of eubacterial communities in uncontaminated (A samples) and contaminated (*B* samples) microbial mats microcosms during 90 days incubation period (from t0 to t90). The analysis was obtained from T-RFLP fingerprints of *16S rRNA* genes.

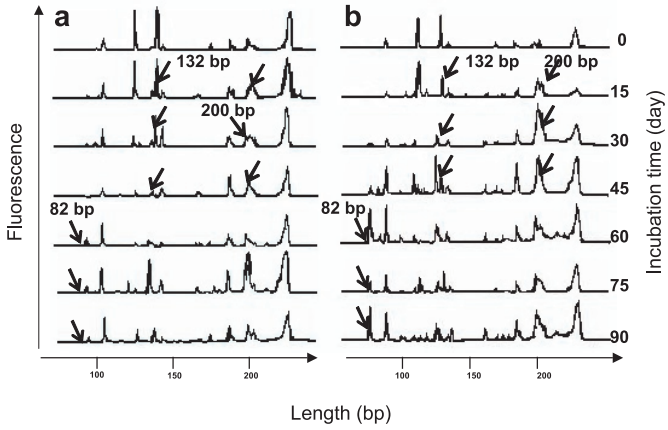

Fig. 5. Terminal restriction fragment length polymorphisms (T-RFLP) fingerprints of purple anoxygenic communities in uncontaminated (a) and contaminated (b) microbial mats microcosms during 90 days incubation period. 5′ terminal restriction fragments were obtained after *pufM* gene enzymatic digestion by HaeIII. Arrows show operational taxonomic units (OTUs) discussed in the text. bp: base pairs.

the experiment (90th day), an OTU with a length of 82 bp also increased in the polluted mats. Comparison between the community fingerprints was obtained by cluster analysis (Fig. 6). Two main clusters were observed, cluster I with initial samples (from t0 to t15) and cluster II with samples of the rest of experiment. In cluster I contaminated sample Bt15 was separated from the other three samples. Similarly, contaminated sample Bt30 was found to be isolated in cluster II.

4 Discussion

The *in situ* monitoring of the fate of *Erika* fuel oil indicated that *n*−alkanes were less degraded in thick patches of oil at the surface and under rocks than in samples characterised by thin layers of pollutant. Thus these results suggested that *n*-alkanes degradation may be related to the thickness of the pollutant layer. Moreover, the surprising "increases" of *n*-alkanes and n-C30 abundances for samples from thick patches of oil (sample 2) were due to their heterogeneity since only the first μm of these pollutant layers is liable to be degraded by all the processes of natural degradation. The fact that samples characterised by a thin layer of pollutant showed that *n*-alkanes were degraded at similar rates whatever the level at which they were collected suggest that biodegradation was probably the most significant process in the degradation of these compounds. Degradation of light PAHs (benzothiophene), was related to evaporation, dissolution, biodegradation processes which are known to be effective for all light compounds (Wang and Fingas 1995). Heaviest PAHs such as chrysenes (Fig. 2d), known to be photosensitive compounds (Garrett et al. 1998), were degraded at different rates according to the environmental light conditions.

Thus monitoring analysis revealed that the natural degradation was dependent of both the pollutant thickness and the level from the seawater at which the samples were collected. The degradation rate increase as the pollutant layer decreases and supra-tidal samples appears to be the most degraded. Nevertheless, although the assessment of natural degradation by using relative abundance of *n*-alkanes and PAH could be useful to establish which natural degradation processes occur during oil weathering, it does not show the "total" degradation of the pollutant since *n*-alkanes and PAH represent less than 4% of the *Erika* fuel oil (Oudot et al. 2002).

Clustering analysis of T-RFLP fingerprints of eubacterial communities indicated that contaminated communities and uncontaminated communities evolved differently, suggesting that *Erika* fuel oil has an impact on the evolution of bacterial communities structure. Similar results have been previously reported showing the impact of toxic compounds to bacterial communities (Abed et al. 2002; MacNaughton et al. 1999). To further analysis the impact of the *Erika* fuel oil on microbial mats, we focus our investigation on purple anoxygenic bacterial (PAB) communities that play an important metabolic role in microbial mats (Van Gemerden 1993). Among this PAB community, T-RFLP fingerprints showed that a PAB population was sensitive to pollutant, i.e., OTU 132 bp related by predictive digestion of *pufM* genes to some purple non sulfur bacteria such as *Rhodoplanes elegans* or *Rubrivivax gelatinosus*. Other PAB populations (OTUs 82 and 200 bp) were capable to survive and/or utilize toxic contamination; predictive digestions of *pufM* genes could relate OTU 200 bp to two different bacterial types: *Erythrobacter* sp, or the purple sulfur bacteria *Halochromatium salexigens* SG3201. Therefore more *pufM* gene sequence data are required to improve the characterisation of PAB populations by T-RFLP analysis. Furthermore, the impact on these OTUs was observed at different periods indicating that the microbial mats adapted in several steps by a succession of different communities. These observations supported by statistical analysis showing two clusters revealed three main periods. Firstly, from t0 to t15 an initial hydrocarbon impact was observed characterised by a high divergence between contaminated and uncontaminated communities at t15 and t30. This first impact

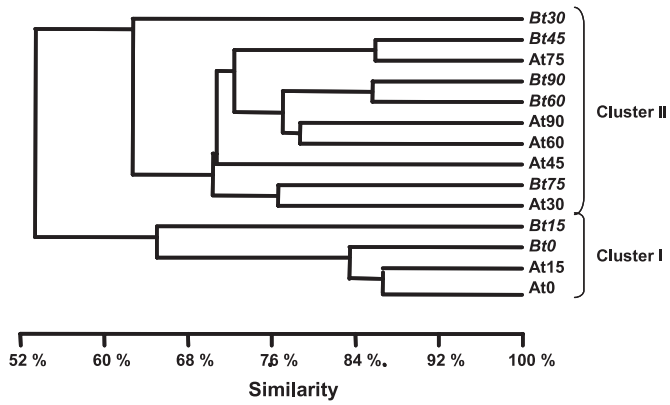

Fig. 6. Clustering of purple anoxygenic communities in uncontaminated (A samples) and contaminated (*B* samples) microbial mats microcosms during 90 days incubation period (from t0 to t90). The analysis was obtained from T-RFLP fingerprints of *pufM* genes.

on the PAB community would probably due to both hydrocarbon toxicity and opaque character of the *Erika* fuel oil. Secondly, between t15 and t30 a strong bacterial communities divergence was observed what ever the conditions (contaminated or not) suggesting an experimental conditions impact. Finally, for the rest of the experiment similarity between contaminated and uncontaminated PAB communities was found to increase progressively. In accordance with the appearance of the contaminated microcosms and the degradation results, these observations suggest that opaqueness and toxicity due to *Erika* fuel oil were less important. The observed *Erika* fuel oil modification can be explained by both abiotic and/or biotic processes. Our degradation results indicate that biodegradation by microorganisms may be occurring. These results are consistent with preliminary observation demonstrating the low biodegradability of the *Erika* fuel oil (Oudot 2000). However previous studies demonstrated that microorganisms produce surfactants that facilitate hydrocarbon solubilization and degradation (Desai and Banat 1997; Hua et al. 2003; Van Dyke et al. 1991). It has also been demonstrated that microbial mats from different origins were found to be rich in microorganisms with high biodegradation potential (Abed et al. 2002; Hoffmann 1996). To further understand biodegradation by microbial mats, it will be interesting to isolate key microorganisms and fully explore their potential to degrade hydrocarbons.

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