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Microbial Community Response to a Simulated Hydrocarbon Spill in Mangrove Sediments

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In this study, we examined the hypothesis that the microbial communities in mangrove sediments with different chemical and historical characteristics respond differently to the disturbance of a hydrocarbon spill. Two different mangrove sediments were sampled, one close to an oil refinery that had suffered a recent oil spill and another that had not been in contact with oil. Based on the sampled sediment, two sets of mesocosms were built, and oil was added to one of them. They were subjected to mimicked mangrove conditions and monitored for 75 days. Archaeal and bacterial communities were evaluated through PCR-DGGE. Both communities showed the emergence of small numbers of novel bands in response to oil pollution. 16S rRNA gene clone libraries were constructed from both mesocosms before the addition of oil and at day 75 after oil addition. LIBSHUFF analysis showed that both mangrove-based mesocosms contained similar communities at the start of the experiment and that they were different from the initial one, as well as from each other, after 75 days. These results hint at a role of environmental history that is not obvious from community diversity indicators, but is apparent from the response to the applied stress.

***Keywords*:** mangrove sediments, petroleum pollution, bacterial diversity, community dynamics, mangrove microbial populations, culture independent methods

Mangroves are highly productive coastal ecosystems (Jennerjahn and Ittekkot, 2002) located on transition zones between terrestrial, freshwater and oceanic environments. The tidal action creates large changes in salinity and water temperature during the day, and these changes lead to a very characteristic flora and fauna. Mangroves comprise 60-75% of the world's tropical and subtropical coastlines (Holguin *et al.*, 2001), and they participate in many functions of adjacent environments due to their high productivity and nutrient exportation (Wieder and Lang, 1982). Hence, this environment is of major importance to human population because of its role in the feeding and reproduction of many economically important marine animals. Given their importance, mangrove habitats have been pinpointed as one of the most critical (threatened) environments that need urgent attention and conservation (Al-Sayed *et al.*, 2005; Duke *et al.*, 2007). With regard to oil pollution, mangroves are specially affected (Lewis, 1983). Since most of the oil spilled in the sea converges to the shore, when this pollutant reaches a mangrove forest, it persists there due to the limited wash by tidal action as a result of the vegetation density and the low microbial decomposition that take place in the anaerobic sediments (Lewis, 1983).

Despite the importance of mangrove forests, only in the past few years have researchers tried to elucidate the structure of mangrove microbial communities (Holguin *et al.*, 1992; Holmboe *et al.*, 2001; El-Tarabily, 2002; Brito *et al.*, 2006; Gonzalez-Acosta *et al.*, 2006; Sousa *et al.*, 2006; Yan *et al.*, 2006; Gomes *et al.*, 2007, 2008; Liang *et al.*, 2007). Although some of these studies have evaluated the diversity of uncultured microorganisms (Sousa *et al.*, 2006; Yan *et al.*, 2006; Gomes *et al.*, 2007, 2008; Liang *et al.*, 2007; Taketani *et al.*, 2009), not many have been performed in oil-polluted mangrove forests (Gomes *et al.*, 2007, 2008; Taketani *et al.*, 2009).

In this study, we compared the dynamics of the prokaryote (bacteria and archaea) communities of two different mangrove sediment systems. One system had been subjected to an oil spill in 2002, which destroyed most of the mangrove forest, whereas the other was still pristine. Starting from the premise that two different samples belonging to the same habitat type (mangrove forest) had been sampled, we hypothesised that the microbial communities of both mangroves would behave in different ways when challenged with oil as a result of the previous contact with this pollutant.

Materials and Methods

Sampling

Sediments for mesocosm construction were collected from two different mangroves located in the 'Todos os Santos' bay at the northeast coast of Brazil. The sediments were obtained from locations outside the mangrove forest to avoid the effects of rhizospheric bacterial populations in the mesocosm. When accessible, spatial variation was evaluated by pH measurements at different sites to determine a suitable sampling location that reflected most of the mangrove (Table 1). The choice of pH as an indicator of spatial variation was based on the fact that it has been pinpointed as the main

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Table 1. Sediment physical and chemical characteristics

	Na[a]	Ca[a]	Mq[a]	Al[a]	S[a]	pH[a]	Corg (%)	Total P[b]	K[b]	Fe[c]	Cu[c]	Zn[c]	Mn[c]
Polluted mangrove	32.32	41.5	14.9	2.9	91.1	4.6-7.2[d]	8.3	120	913	840	37.8	100.8	39.9
Pristine mangrove	54.27	38.3	25.1	2.6	120.7	3.6-7.5[e]	5.9	42	1177	1025	23.1	21.0	96.6

[a] meq/100 ml; [b] mg/L; [c] mg/kg; [d] n=9; [e] n=4

factor driving spatial variation in microbial communities (Fierer and Jackson, 2006). Although some variation existed inside each mangrove, they were not significantly different based on an F-test with 99.6% confidence. From the selected mangrove areas, 20 kg of the water-saturated top sediment layer (0-10 cm) was collected from three different sites (within a 5-meter circumference) and homogenised. All samples were kept cold (4°C) until arrival in the laboratory. The pristine sample was collected at the following coordinates, S12°43′46.7″ W38°30′09.1″, and the polluted sample was collected at S12°42′38.1″, W38°33′49.9″. The linear distance between the sampling stations was 7.0 km. Physical and chemical characteristics of both sediments were measured and are presented in Table 1.

Mesocosm design

Four mesocosms were set up to allow the application of two different treatments. Each mesocosm consisted of a 20-L aquarium containing 10 L of wet sediment (1.2 kg/L dry wt). In order to mimic tidal action, two pumps were connected to the mesocosms, one of which was used to pump artificial sea water (Natural Sea Salt Mix, Oceanic Systems, USA) into the aquarium (roughly 5 L each time), while the other removed the water after 6 h. This produced an artificial tide that kept the sediment under 5 cm of water for 6 h each time. This procedure was repeated every 6 h. The artificial seawater (20 L) was stored in a 20-L bottle, and 10 L of that water was refreshed every 5 days. A light:dark regime of 12 h of light and 12 h of darkness was used to mimic the light period of the 'Todos os Santos' bay latitude through the use of timers and three 100 W tungsten light sources hung approximately 30 cm above the mesocosms. After setting up each mesocosm, these were left functioning for 3 d in order to stabilise and mimic the stratification found in this environment before the onset of the experiment.

One mesocosm of each mangrove sediment received 2% (v/v) heavy crude oil, while the other was left without the addition of oil (control). Samples were collected before the addition of oil (T-0), as well as on days one (T-1), seven (T-7), 15 (T-15), 27 (T-27), 45 (T-45), 65 (T-65), and 75 (T-75) after oil pollution. At each sampling, 10 g of wet sediment was taken from three points at the mesocosm surface (0-5 cm) during the low tide, and these samples were homogenised prior to DNA extraction.

DNA extraction

Extraction of total DNA from sediment samples was carried out using the FastDNA® Spin kit for Soil (BIO101, USA) following the manufacturer's protocol. DNA samples were purified using the Wizard® Genomic DNA Purification System (Promega, The Netherlands). The quality and quantity of DNA were checked using eletrophoresis on agarose gels. DNA was, on average, 20 kb in size, and yields ranged from 25-50 μg/g of sediment material (dry wt).

PCR-DGGE

To obtain bacterial community profiles, all DNA from sediment samples was amplified with primers F968-GC and 1378R (Heuer and Smalla, 1997) to obtain the 16S rRNA gene profile. PCR was performed in a GeneAmp PCR System 9700 (Applied Biosystems, USA). The 50 μl PCR mix consisted of 5 μl 10× Stoffel buffer (Roche, Switzerland), 200 μM of each deoxynucleoside triphosphate, 400 nM of each primer, 1% formamide, 0.5 U Stoffel Taq DNA polymerase (Roche), 31 μl H_2O and 1 μl (about 20 ng) of template DNA. The following touchdown PCR program was used: 94°C for 3 min; 94°C for 1 min, 65°C (decrease per cycle 1°C) for 1 min, 72°C for 3 min (10 cycles); 94°C for 1 min, 55°C for 1 min, 72°C for 3 min (35 cycles); 72°C for 30 min. To obtain an Archaeal 16S rRNA gene community fingerprint, we utilised primers 344f and 517r as described previously (Bano *et al.*, 2004). PCR products were checked for quantity and quality by electrophoresis in 1% agarose gels.

DGGE (denaturing gradient gel electrophoresis) analyses were performed on a PhorU2 system (Ingeny International, The Netherlands) according to the manufacturer's protocol, using a gradient (Muyzer *et al*., 1993) of 40-60% denaturants (urea/ formamide) for bacterial profiles and 40-55% for Archaeal profiles (100% denaturants is 8 M urea plus 40% formamide), a buffer temperature of 60°C and 110 for 18 h. Following electrophoresis, the polyacrylamide gels were stained using silver staining. The digitised images of DGGE gels were analyzed by Image Quant ver. 5.2 (Pharmacia, USA) to generate densitometric profiles. Bands were considered valid when the peak height relative to total peak height exceeded 1%, as described previously (Iwamoto *et al.*, 2000). Any band passing this threshold was considered as a single phylotype. To perform multivariate tests on the DGGE data, the statistical software PRIMER (version 5) was used. Non-metric multidimensional scaling (MDS) was carried out based on Bray-Curtis similarities (10 restarts, 999 interactions each). Analysis of similarity (ANOSIM) tests were performed using 999 permutations to evaluate statistically significant differences in community assemblages (Clarke, 1993).

Cloning and sequencing

Four samples were selected for 16S ribosomal RNA gene based clone library analysis. These were Polluted+oil, T-0 (Poll T-0); Polluted+oil, T-75 (Poll T-75); Pristine+oil, T-0 (Prist T-0); and Pristine+oil, T-75 (Prist T-75). Bacterial 16S rRNA genes were amplified by using the B8F (5′-AGAGTTTGATCMTGGCTCAG-3′) forward primer (Edwards *et al.*, 1989) and the universal U1406R (5′-ACGGGCGGTGTGTRC-3′) reverse primer (Lane, 1991). PCR mixtures (25 μl) contained 10.2 mM Tris buffer, 2.3 mM $MgCl_2$, 50 mM KCl, 2% DMSO, 5 μg of bovine serum albumin, 0.2 mM of each dNTP, 0.2 μM of each primer, and 0.5 U of Taq DNA polymerase. PCR was performed in a Perkin-Elmer GeneAmp PCR System 9700 (Perkin-Elmer Applied Biosystems, Nieuwerkerk a/d IJsel, the Netherlands) using the following program: 95°C for 5 min; 35 cycles of 94°C for 1 min, 57.5°C for 30 sec, 72°C for 4 min, with a final elongation step of 72°C for 7 min. These PCR products were ligated into the pGEM-T easy vector (Promega, USA) according to the manufacturer's protocol. The ligation products were introduced into *Escherichia coli* MM294 competent cells (Sylphium Life Sciences, The Netherlands) by transformation according to the supplier's protocol, after which cells were plated onto selective media (Sambrook and Russell, 2001). Following growth at 37°C, white

colonies were randomly picked, and plasmids with inserts of the correct size were isolated using the Cetyl Trimethyl Ammonium Bromide (CTAB) isolation method (Sambrook and Russell, 2001). From a total of 400 clones obtained for each library, 150 were randomly selected for sequencing. Sequencing reactions were performed on the plasmid material according to the Perkin-Elmer ABI Prism protocol (Applied Biosystems, USA) using primer 1406r to avoid vector contamination. Sequence runs were done on an ABI377 DNA sequencer (Applied Biosystems).

Sequence analysis

All chromatograms were analyzed for sequence quality using Bioedit (Hall, 1999). Sequences containing ambiguities were not further analyzed. Sequences were searched for vector contamination using NCBI's VecScreen (http://www.ncbi.nlm.nih.gov/VecScreen/VecScreen.html), and contaminant sequences were omitted. The sequences were then checked for the presence of chimeras using the chimera-check tool of Greengenes (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi; DeSantis *et al.*, 2006).

The sequences that passed the above checks (Table 2) were classified using the "classification tool" of Greengenes. This tool classifies any 16S rRNA gene sequence based on several different taxonomic classification systems. For the current study, we selected the so-called "Hugenholtz" systematics (Hugenholtz and Pace, 1996), which allows the classification of sequences that are considered unclassifiable by other systematics. The sequences obtained were aligned using CLUSTAL X (Thompson *et al.*, 1997), and the output file was used in Phylip 3.66 to construct a distance matrix using the Jukes and Cantor distance model (Jukes and Cantor, 1969). These were then used in the software DOTUR (Schloss and Handelsman, 2005) to identify operational taxonomic units (OTUs) and generate estimations of richness and diversity, as well as rarefaction curves. Sequences with 97% or higher similarity (approximately the level that serves to delineate species) were considered to represent single OTUs. Pairwise alignments of libraries and distance matrices were made as described above. These were used for library comparisons using web-based LIBSHUFF (http://LIBSHUFF.mib.uga.edu; Singleton *et al.*, 2001). We decided that since we aimed to address pairwise differences and not the absolute significance of these differences, no Boferoni correction of *P* would be performed based on these comparisons. The sequences reported in this study were submitted to GenBank with accession numbers EU420182-EU420589, EU420591-EU420597, and EU420599-EU420603.

Results

Bacterial PCR-DGGE

The bacterial community profiles observed in the mesocosms from both origins were highly complex (Fig. 1), revealing a high number of phylotypes. The number of bacterial phylotypes observed ranged from 13 (Prist+oil T0) to 51 (Poll T75). The average number of phylotypes that met the >1% threshold was 33.3±7.5 (Mean±SD), 34.3±3.9, 24.0±2.9, and 21.3±4.2 in the mesocosms Poll, Poll+oil, Prist, and Prist+oil, respectively. A visual inspection of the community pattern, however, showed a greater number of phylotypes. This indicated that most of the communities present in the samples were formed by rare phylotypes.

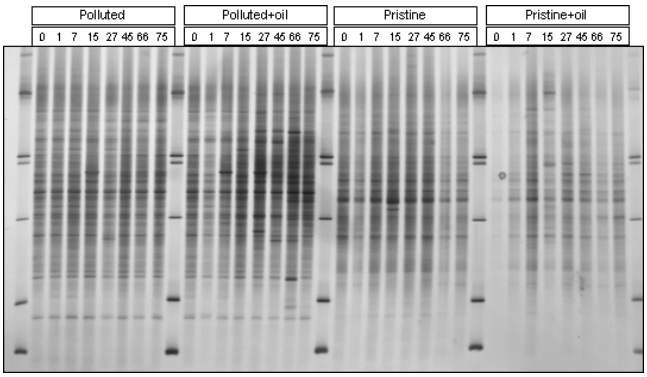

Fig. 1. DGGE profiles of bacterial 16S rRNA gene fragments from all four mesocosms. The numbers above each lane represents the day of sampling.

Figure 2A shows MDS ordination using Bray-Curtis similarities of the samples. The samples could be assigned to two distinct groups, one formed by samples from the two pristine mesocosms and the other by samples from the polluted samples. Additionally, inside the group formed by the pristine samples (squares), the samples from the mesocosms that received oil were separated from the ones that did not receive oil. This separation was not apparent in the mesocosms constructed from the mangrove samples with a history of contamination. Based on the spread of the samples in the MDS plot, it can be inferred that bacterial communities from the different mesocosms showed different behaviours during the course of the experiment. The ANOSIM test showed that these differences were indeed statistically significant (data not shown), with the only exception being the pairwise comparison between the mesocosms Poll and Poll+oil, which showed some overlap between the groups (pairwise *R* of 0.477 and a significance level of 0.1%).

Table 2. Diversity and richness indices calculated for the four clone libraries[a]

	Size[b]	OTUs[c] sampled	Shannon's H'	Simpson's 1/D	Chao1 estimator	ACE
Pristine T0	81	61	394.40 (374.89; 413.92)[d]	0.012	326.2 (167.45; 1721.69)	338.28 (184.12; 685.44)
Pristine T75	114	89	438.95 (424.29; 453.60)	0.005	231.05 (162.36; 364.07)	289.46 (184.91; 507.96)
Polluted T0	96	73	417.53 (401.32; 433.75)	0.007	223.27 (143.70; 392.38)	245.30 (156.84; 427.09)
Polluted T75	126	86	430.23 (416.05; 444.41)	0.008	208.00 (146.43; 332.29)	207.50 (150.73; 314.02)

[a] Values calculated on DOTUR using 0.97 similarity cut-off
[b] Presented as the number of clones sequenced.
[c] Operational Taxonomic Units
[d] Values in parenthesis represent the 95% confidence intervals

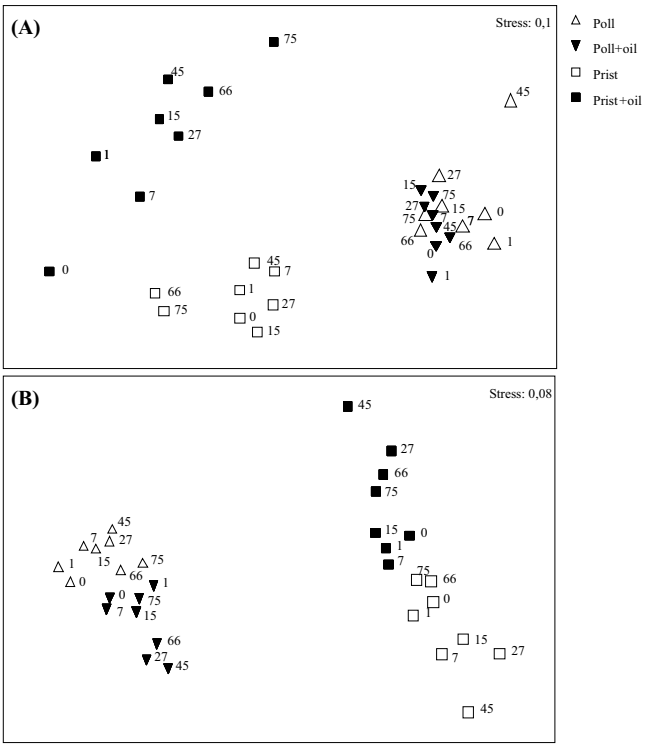

Fig. 2. MDS ordination of Bray-Curtis similarities of DGGE profiles from mangrove sediment mesocosms. (A) Bacterial community profile MDS analysis, (B) Archaeal community profile MDS analysis.

Archaeal PCR-DGGE

The pattern observed in the archaeal community DGGE (Fig. 3) also revealed the existence of complex communities in all mangrove sediment mesocosms. Moreover, high stability of community structure throughout the experiment was noted. The richness of phylotypes observed ranged from 21 (Prist T7) to 31 (Poll T27), with averages of 25.3±2.2, 27.3±1.8, 28.1±1.5, and 26.75±1.3 in the Prist, Prist+oil, Poll, and Poll+oil mesocosms, respectively. This indicates that, as observed for the bacterial DGGE profile, most of the visually counted phylotypes were below the 1.0% threshold set and could be considered rare phylotypes. Most of the bands observed in all four mesocosms showed no change throughout the experiment. One major band appeared in both communities from samples with oil addition (Fig. 3, arrows), and this band had the same position, indicating a similar phylotype. The band was also present in the polluted mangrove without the addition of oil.

Figure 2B shows MDS ordination using Bray-Curtis similarities of the samples. The samples were nominally assigned to two distinct groups, one formed by patterns derived from the two pristine mesocosms and the other formed by those from the polluted mesocosms. Additionally, inside these groups the samples from the mesocosms that received oil were separated from the ones that did not receive oil. These differences were confirmed by the ANOSIM test to be statistically significant (data not shown).

Bacterial 16S rRNA gene clone libraries

In order to further examine the microbial community

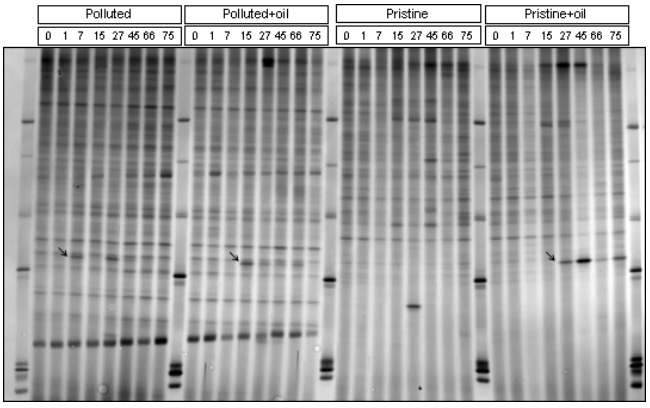

Fig. 3. DGGE profiles of archaeal 16S rRNA gene fragments from all four mesocosms. The numbers above each lane represents the day of sampling. Arrows indicate the most dominant band to appear after treatment.

dynamics in the sediments under the conditions created in this study, we constructed four bacterial 16S rRNA gene clone libraries. Rarefaction analysis (Fig. 4) of the sequences obtained in the clone libraries showed that all libraries had captured roughly the same level of the extant diversity. In fact, all curves remained far from reaching the theoretical upper (asymptotic) limit of the curve. This result was in accordance with that observed for the diversity estimates.

The libraries prepared from Prist T0, Prist T75, Poll T0, and Poll T75 contained 81, 114, 96, and 129 clones, respectively, giving a total of 420 clones (Table 2). The number of observed OTUs (defined as sequences with 0.97 similarity) found in each library was 61, 89, 73, and 86, respectively (Table 2). When analyzing the data using DOTUR, the number of estimated OTUs (Chao1 estimator of richness) found in the library Prist T0 was 326, which was higher than that estimated for the other libraries (ranging from 208 to 231). The estimated richness, however, was found to be not significantly different among the libraries. The ACE richness estimator and the Simpson index showed similar patterns (Table 2).

Based on the four libraries, we then determined the diversity captured from the samples, as indicated by the Shannon index (Table 2). The analysis showed that the diversity in the four

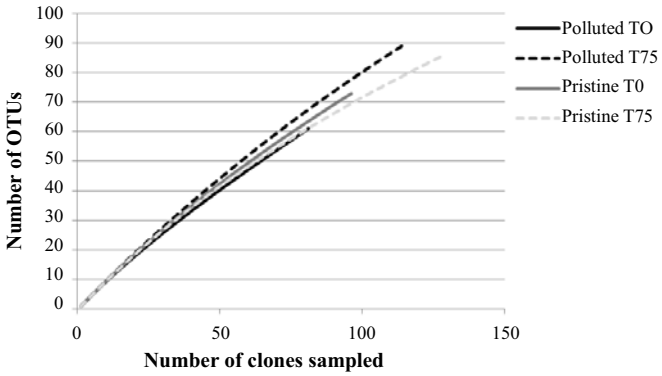

Fig. 4. Rarefaction analysis for all four libraries. Curves represent (grey line) Pristine T-0; (dashed grey line) Pristine T-75; (black line) Polluted T-0; (dashed black line) Polluted T-75.

Table 3. Delta-C and p-values from the LIBSHUFF analysis[a]

	Pristine T0	Pristine T75	Polluted T0	Polluted T75
Pristine T0	–	0.884 (0.001)	0.074 (0.350)	–
Pristine T75	0.763 (0.001)	–	–	0.373 (0.001)
Polluted T0	0.138 (0.151)	–	–	0.619 (0.002)
Polluted T75	–	0.799 (0.001)	0.081 (0.467)	–

[a] The table is organised to represent the X (column) coverage by Y (rows); p-values are presented in parenthesis.

libraries was not different across the libraries, even though the Shannon index value was slightly lower for the Prist T0 library, indicating a less diverse community.

Classification of the sequences by the Greengenes classifycation tool (Fig. 5) showed that the major classes found in all four libraries were similar. Alphaproteobacteria, Deltaproteobacteria, and Anaerolineae were the most abundant classes found in all libraries. These classes together represented 41, 53, 43, and 66% of the clones in libraries Prist T0, Prist T75, Poll T0, and Poll T75, respectively. Proteobacteria represented the most commonly found (37.5 to 57.9% of the sequences retrieved) phylum in all the libraries. After oil addition, there was a decline in the percentage of Anaerolineae, which were quite abundant at the onset of the experiment, and an increase in the abundance of Deltaproteobacteria. Specifically, the Anaerolinea initially occurred at around 29.6 and 23.9% of the total community (Prist T0 and Poll T0, respectively), but the relative abundance of clones of this group had changed to 8.7 and 12.4% after 75 days. With respect to the Deltaproteobacteria, these percentages were initially 14.8 and 11.4% but reached 23.6 and 18.3% at 75 days. The response to oil was greater in the libraries from the sediments constructed with polluted mangrove sediment than in the pristine ones. Overall, based on the classification of the clones, the bacterial communities at the start of the experiment were more similar to each other than to their counterparts after 75 days.

In addition, we compared the libraries using LIBSHUFF (Table 3). This analysis showed that the two T-0 libraries were not different from each other (lower p-value 0.151). The divergence incurred from the initial community and between the communities at the end of the experiment, however, were clear. The lower p-value for the comparison of the two libraries obtained from the pristine mangrove sediments was 0.001. Hence, there was a significant change in the community over the timecourse of the experiment. The same change (p-value of 0.001) was observed when comparing the two libraries prepared for the sediments that received oil, although this analysis showed that the Poll T-75 library was a subset of the Poll T-0 library. The low p-value of the comparison between the two libraries at the end of the experiment shows that the divergence between the two communities in the two mesocosms was indeed significant.

Discussion

In this study, we examined the role of habitat history (previous contact with oil) on the microbial community response to stress exerted by oil pollution. We expected that the contamination suffered in the past and the geographical separation (creating the differences in sediment chemistry observed in Table 1) would have led the communities of both mangroves to diverge, as was seen previously (Labbe *et al.*, 2007).

Initially, PCR-DGGE analyses of the control mesocosms (uncontaminated) showed a very stable community over time for both the Bacteria and the Archaea. Hence, we predicted that a robust inference of treatment effects could be obtained from any differences observed in the oil-treated mesocosms, despite the lack of replicates. The addition of oil to both mesocosms indeed led the communities (both archaeal and bacterial) to diverge from the uncontaminated communities. This divergence was greater in the samples from the pristine

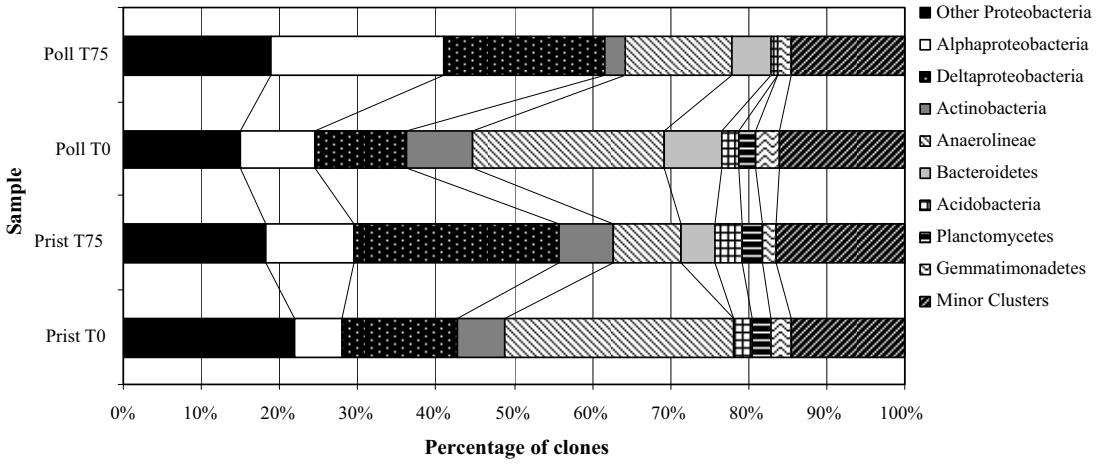

Fig. 5. Classification chart of the four libraries. Values shown as percentages of the total number of clones.

mangrove, indicating that the phylotypes present in the polluted mangrove were more resistant to change caused by contact with oil, while the pristine community had to adapt in response to the challenge presented by the oil (Osborn *et al.*, 2000; Margesin *et al.*, 2003; Perez-Jimenez and Kerkhof, 2005; Labbe *et al.*, 2007; Taketani *et al.*, 2009).

This result indicates that the bacterial community present in the mangrove previously exposed to oil might have been primed by previous exposure to hydrocarbons in this sediment, including the preselection of possibly minority populations capable of responding quickly to the addition of oil.

The small effect caused by the addition of oil observed in the archaeal DGGE profiles is not unexpected. In sediments (both marine and freshwater) that are often anaerobic, the majority of the microbial activity can be attributed to sulphate reducing bacteria (SRB), archaeal methanogens, and archaeal anaerobic methane oxidisers (Jorgensen, 1982). So far, it is known that the two latter groups are not able to use complex hydrocarbons (as present in oil) as a carbon source (Muyzer and Stams, 2008). This niche separation, thus, would explain the small alteration observed. On the other hand, since syntrophic anaerobic methane oxidation is carried out by SRBs and archaeal populations (Muyzer and Stams, 2008), the influence of the addition of hydrocarbons on the SRB populations (discussed below) could explain the minor changes observed in the archaeal community.

We found only small numbers of estimated OTUs in this study; however, this may in fact be a serious underestimation caused by the fact that the number of OTUs estimated is affected (and limited) by library size. In the libraries we obtained, a major portion of the clones were affiliated with groups that encompassed anaerobic bacteria. In both the T0 libraries, the *Anaerolineae* were abundant. This group is formed by strictly anaerobic bacteria (Yamada *et al.*, 2006, 2007). Moreover, in the T75 libraries, members of the *Deltaproteobacteria* abounded (represented in the libraries by the genera *Desulfobacteraceae*, *Desulfobulbaceae*, *Desulfuromonadales*, *Desulfobacterium*). These genera encompass major sulphate reducing bacteria (SRB), which are anaerobic bacteria commonly found in freshwater and marine anoxic sediments (Rees and Patel, 2001; Klepac-Ceraj *et al.*, 2004; Asami *et al.*, 2005; Mussmann *et al.*, 2005; Da Silva *et al.*, 2007; Foti *et al.*, 2007; Kjeldsen *et al.*, 2007; Kondo *et al.*, 2007; Miletto *et al.*, 2007; Suzuki *et al.*, 2007a, 2007b). SRB are frequently associated with the anaerobic hydrocarbon degradation process (Perez-Jimenez and Kerkhof, 2005; Kniemeyer *et al.*, 2007; Miralles *et al.*, 2007a, 2007b).

The community composition of our mangrove sediment was not similar to that reported in a recent study on an Asian subtropical mangrove (Liang *et al.*, 2007). In Liang's study, it was shown that gammaproteobacteria formed the major class in the sediment. We observed large numbers of unclassified Proteobacteria and several deltaproteobacteria, but only small numbers of gammaproteobacteria. This difference could be due to actual differences in physico-chemical characteristics of the mangrove sediment, leading to different community compositions. Methodological factors, however, might also explain these divergent observations. Liang *et al*. (2007) sampled shallower sediment. Since marine sediments usually present a steep decline in oxygenation after a few millimeters from the surface, the populations found in slightly deeper sediments may vary greatly from those observed in the upper part of the sediment (Wilms *et al.*, 2006). Moreover, other differences in methodology, such as the DNA extraction method and PCR primer system used could explain the differences between the communities.

It is clear that the libraries presented in this study do not represent the full extent of the diversity present in the samples; however, phylogenetically-based tests such as LIBSHUFF have enough power to enable the discernment of differences in community composition despite the size of the sample (Oline, 2006). LIBSHUFF showed that the communities present in the two T0 libraries environments were not different. This result was contrary to what was observed in the PCR-DGGE experiments. This discrepancy in the results can be explained by the differences in the resolution of both techniques and possibly the different biases intrinsic to each of them. One major difference between the analyses is phylogenetic resolution: while DGGE is only able to explore a single level (phylotype=band), clone library sequencing coupled with LIBSHUFF analysis compares the samples at several levels (from species to domain). Hence, the later analytical method is more robust that the previous one.

The similarity observed between both T0 libraries (despite the differences in environment characteristics) indicates that the type of environment has (in this case) a stronger effect on community composition than the characteristics presented in Table 1. The lack of an apparent effect of the past oil contact could be explained by the low amount of hydrocarbons found in the polluted mangrove at the time of sampling and its composition. Peixoto *et al*. (unpublished data) observed that the polluted mangrove contained up to tenfold total hydrocarbon levels and that the hydrocarbons found were very resilient compared to the non-polluted mangrove. Hence, such low hydrocarbon levels would not impact large populations and would support virtually no growth as a result of the recalcitrance of the matter.

On the other hand, as evidenced by the results of both DGGE and the clone libraries, the changes brought about by the addition of oil to the mesocosms in all samples were different from one another, resulting in significantly differing libraries after 75 days. This indicates that even at virtually indiscernible current differences, the history of mangrove sediment in terms of previous (oil) stressors may have important implications for the potential to respond to a similar stress. In soil, a major effect of habitat history on community composition and the response to changes in land use have been reported previously (Salles *et al.*, 2004; Buckley *et al.*, 2006; Hamamura *et al.*, 2006; Oline, 2006; Salles *et al.*, 2006).

Differences between habitats with and without a history of hydrocarbon contamination in terms of the ability of the community to degrade the hydrocarbon pollutants have been observed (Olivera *et al.*, 1997; Johnsen and Karlson, 2005; Polymenakou *et al.*, 2006). The degradation of hydrocarbons in previously polluted environments may lead the community to be more efficient in assimilating these pollutants when compared to their pristine counterparts. This has been related to a higher count of hydrocarbonoclastic bacteria (Polymenakou *et al.*, 2006) and a greater resistance to hydrocarbon stress

(Margesin *et al.*, 2003; Labbe *et al.*, 2007). Since mobile genetic elements are very often carriers of hydrocarbon degradation genes, the spread of these elements can be another factor that accounts for the rapid adaptation (Diaz, 2004; Ma *et al.*, 2007; Marri *et al.*, 2007).

We hypothesise that the apparently different capacities to diversify and respond to oil stress that we observed might be due to the previous selection of populations or genes that allow the survival and growth of bacteria in the oil-stressed environment. At the onset of the experiment, such populations might have been dormant or present in such small numbers that they could not be identified. With the onset of the contamination; however, these initially minor populations might have been responsible for the response to the stress. In the pristine sediment following oil addition, populations that might never have been challenged with the pollutant should go through a longer process of succession to yield a community similar to the one seen in the oil-treated sediment with oil history. We cannot affirm which underlying factors might have been responsible (e.g., horizontal gene transfer, immigration of alien populations), but speculate that a combination of immigration and adaptation events may have been the underlying cause of the changed communities.

Specific population-level events are hard to discern when analyzing microbial communities as a whole, and hence, a "silent" potential to thrive or, at least, to survive under conditions of hydrocarbon pressure may build up inside a community that has a history of challenge(s) with hydrocarbon. When again challenged, such a community may respond differently from one with no history of exposure to the stress factor, although both of them hold similar communities before this challenge.

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