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Detection by denaturing gradient gel electrophoresis of ammonia-oxidizing bacteria in microcosms of crude oil-contaminated mangrove sediments

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ABSTRACT. Currently, the effect of crude oil on ammonia-oxidizing bacterium communities from mangrove sediments is little understood. We studied the diversity of ammonia-oxidizing bacteria in mangrove microcosm experiments using mangrove sediments contaminated with 0.1, 0.5, 1, 2, and 5% crude oil as well as non-contaminated control and landfarm soil from near an oil refinery in Camamu Bay in Bahia, Brazil. The evolution of CO_2 production in all crude oil-contaminated microcosms showed potential for mineralization. Cluster analysis of denaturing gradient gel electrophoresis-derived samples generated with primers for gene amoA, which encodes the functional enzyme ammonia monooxygenase, showed differences in the sample contaminated with 5% compared to the other samples. Principal component analysis showed divergence of the non-contaminated samples from the 5% crude oil-contaminated sediment. A Venn diagram generated from the banding pattern of PCR-denaturing gradient gel electrophoresis was used to look for operational taxonomic units (OTUs) in common. Eight OTUs were

found in non-contaminated sediments and in samples contaminated with 0.5, 1, or 2% crude oil. A Jaccard similarity index of 50% was found for samples contaminated with 0.1, 0.5, 1, and 2% crude oil. This is the first study that focuses on the impact of crude oil on the ammonia-oxidizing bacterium community in mangrove sediments from Camamu Bay.

Key words: Impacted mangrove sediment; PCR-DGGE; amoA; Ammonia-oxidizing bacteria

INTRODUCTION

Camamu Bay is the third largest bay in Brazil, considering the volume of water. It includes biomes of unquestionable diversity, such as the Atlantic rain forest, coral reefs and one the biggest and most exuberant still well-preserved mangrove in the country. The mangrove ecosystem is considered to be a permanent preservation area (Brazil, 1965; Brazil Federal Law No. 4771, 15/09/65) and biological reserve (CONAMA, 1985; No. 004, 18/09/85). However, due to their location, mangroves are regions exposed to the risk of contamination that derives from urban development, industrial exploitation, mining, ports, fisheries, and tourism (Kathiresan and Bingham, 2001). Contamination by oil and its derivatives in mangroves is commonly caused by industrial and port activities that promote the accumulation of different pollutants in the sediment, which has a significant impact, especially on the microbial community (Leahy and Colwell, 1990; Atlas, 1995; Kasai et al., 2001; Evans et al., 2004; Harayama et al., 2004; Bordenave et al., 2007). Despite the recognized petrochemical potential from Camamu Bay, there are no reports of contamination in the mangrove.

Ammonia oxidation is a key step in the nitrogen cycle, where the enzyme ammonia monooxygenase (AMO) is responsible for the oxidation of ammonia to hydroxylamine, which in turn is oxidized to nitrite. This step is carried out by ammonia-oxidizing bacteria (AOB), grouped in the division of the proteobacteria. The AOB are ubiquitous in all environments where ammonia is available, including in places of intense mineralization of organic matter (Koops et al., 2006). In polluted environments, AMO enzyme activity seems to be related to the beginning of the process of pollutant removal, demonstrating its importance in the understanding and distribution of AOB in degradation processes (Arp et al., 2001). Classified as chemoautotrophs, sometimes as heterotrophs, AOB are difficult to isolate as they grow slowly and are often nonculturable (Bock et al., 1991; Arp et al., 2001).

The great biotechnological interest in AOB, as well as their ecological significance, have fueled the development of culture-independent detection methods that aim to overcome procedures of isolation (Prosser, 1989; Rotthauwe et al., 1997; Stephen et al., 1998). In the case of key enzymes involved in a very specific metabolism such as AMO, the use of functional genes is more interesting and has been widely considered in relation to 16s rRNA studies, due to the low numerical dominance of these bacteria in environmental samples (Rotthauwe et al., 1997; Stephen et al., 1998). In all known AOB, functional genes that encode the enzyme AMO are organized in an operon called amoCAB, and thus, only a portion of the amoA gene needs to be used as a molecular marker for studying the diversity of AOB (Sayavedra-Soto et al., 1998). The use of the DGGE (denaturing gradient gel electrophoresis) technique has been an important tool for the study of impact on these communities, favoring direct assessment and evaluation in environmental samples by using primers of the amoA gene (Nicolaisen and

Ramsing, 2002; Zhang et al., 2009). DGGE allows a comparative assessment of gene diversity amplified by PCR in a large number of samples in only one gel showing changes in the community according to the conditions (Muyzer et al., 1993; Nicolaisen and Ramsing, 2002).

The impact of crude oil on the AOB community in microcosm experiments containing mangrove sediment from Camamu Bay was first investigated through the application of DGGE using functional primers of amoA. Multivariate analysis of DGGE profiles provided information on the quantitative and qualitative diversity of AOB in the presence of different concentrations of oil. CO_2 evolution in crude oil-contaminated sediment confirmed the mineralization of organic matter, suggesting processes of constant biodegradation by this important bacterial community.

MATERIAL AND METHODS

Sediment sampling

Mangrove sediment was collected from Camamu Bay located in southern Bahia (Brazil). To avoid rhizospheric effects on bacterial populations, the sediments were obtained outside the mangrove forest during lowest tide. Samples were collected at five sites within the bay area (site 1 = 13° 93' 94.8" S, 39° 06' 89.4" W; site 2 = 13° 93' 53.1" S, 39° 5' 7.4" W; site 3 = 13° 92' 31.5" S, 39° 5' 32" W; site 4 = 13° 90' 71.5" S, 39° 4' 96.7" W; site 5 = 13° 89' 46.7" S, 39° 3' 88" W) (Figure 1). For each site, five sub-samples were randomly collected in the first 10 cm of sediment, separated by at least 1 m. Samples were placed in sterile plastic bags on wet ice and immediately transported to the laboratory. A mixed sample was dried at room temperature and sieved through a 2-mm mesh.

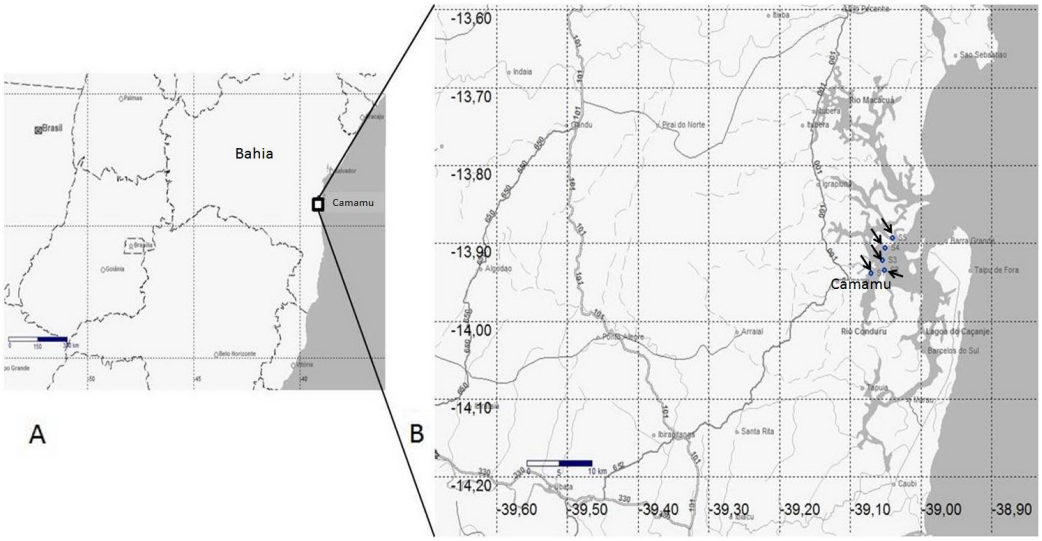

Figure 1. A. Camamu Costa do Dendê, Bahia, Brazil. **B.** The tip of the arrows show the location of the mangroves in the bay where the collections were made from the sediment. Location 1 = 13° 93' 94.8" S, 39° 6' 89.4" W; location 2 = 13° 93' 53.1" S, 39° 5' 07.4" W; location 3 = 13° 92' 31.5" S, 39° 5' 32" W; location 4 = 13° 90' 71.5" S, 39° 4' 96.7" W; location 5 = 13° 89' 46,7" S, 39° 03' 88" W. 10 km in B.

The physico-chemical soil analysis was carried out by Laboratório de Solos e Nutrição de Plantas of Embrapa and is summarized in Table 1.

Table 1. Chemical and physical characteristics of mixed samples from Camamu Bay mangrove.

pH of water	P (mg/dm³)	K	Ca	Mg	Ca + Mg	Al	Na	H + Al	S	CCC[a]	V (%)	Organic matter (g/kg)
4.1	15	(Cmol/dm³)[b]										62.22
		1.05	16.3	5.1	21.4	0.7	20.87	7.59	43.32	50.91	85	
Granulometry (g/kg) with NaOH dispersion												
		Sand silt clay				Silt	Clay	Textural classification				
		725				154	121	Sandy soil				

Sediment analysis done by Embrapa dos Solos (Cruz das Almas, Bahia, Brazil). [a]Cation exchange capacity. [b]Base saturation.

Basal respiration of microcosms

The study was designed to mimic an oil spill approaching the mangroves from seaward. The oil used was a mixture of crude oil from Reconcavo Bahia provided by Landulpho Alves Refinery, São Francisco do Conde, BA, Brazil. Its characteristics were of a degree API of 36.5 and 0.06% sulfur content. Microbial respiratory activities were evaluated based on cumulative CO_2 evolution, modified from Bartha and Pramer (1965). Microcosms were set up in triplicate in tightly closed glass flasks. To determine metabolic activity in each microcosm, respirometric analyses by monitoring CO_2 emission were periodically performed. Each 2-L glass flask contained 250 g mangrove sediment to which crude oil was added. Final oil concentrations were as follows: 0.1, 0.5, 1, 2, and 5% (w/v). Ten milliliters of NaOH (0.1 M) was added to plastic tubes inserted into each microcosm. Autoclaved sediment with 1% oil and non-contaminated sediment were used as control to visualize volatility of oil compounds and microbial communities without oil interference, respectively. The microcosms were incubated for a period of 22 days at room temperature, and every two days the basal respiration was measured by titration. NaOH (0.1 M) present in the microcosms was removed on reading days to be titrated with 0.1 M HCl. Three drops of phenolphthalein were used as pH indicator. The respiration mean of the blanks was subtracted from the treated microcosms, and the difference in CO_2 production between the blanks and the treated microcosms was used as the amount of CO_2 produced. Analysis of released CO_2 was done in triplicate, and data were subjected to statistical analyses, which were performed with GraphPad Prism v.5. One-way analysis of variance (ANOVA) was used to compare basal respiration data. The Tukey multiple comparison test was used to determine the significance of differences between microcosms.

Soil DNA extraction, PCR and DGGE analysis

DNAs from uncontaminated mangrove, landfarm soil and oil-contaminated microcosms were extracted using the PowerSoil™ DNA Isolation kit (MoBio, UK) according to manufacturer instructions. DNAs from microcosms contaminated with 0.1, 0.5, 1, 2, and 5% oil were extracted after 22 days of contamination. The sample from landfarm was used for comparison (Maciel et al., 2009). For DGGE analysis, the samples were labeled MG, 0.1, 0.5, 1, 2, 5% and Land for mangrove, crude oil-contaminated mangrove and landfarm, respectively. A set of primers was used to amplify specific regions: AmoA1F (5'-GGGGTTTC

TACTGGTGGT-3') and AmoA2R (5'-CCCCTCKGSAAAGCCTTCTTC-3') for the group of ammonia-oxidizing bacteria (Rotthauwe et al., 1997). Forward primers contained a 40-bp GC-clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') attached to the 5' end (Muyzer et al., 1993). PCR was performed using a mix with 1.25 U Taq polymerase, 1X Taq buffer, 0.2 mM sense and antisense primers, 200 mM deoxynucleotides, 3.0 mM $MgCl_2$, 10 ng metagenomic DNA and sterile Milli-Q water, in a final volume of 50 µL. The amplified rDNA sequences were analyzed on an 8% polyacrylamide gel (w/v; 37.5:1 acrylamide:bis-acrylamide), consisting of a denaturing gradient of 40-70%. The gels were run in 0.5X TAE buffer (20 mM Tris acetate, pH 7.4, 10 mM sodium acetate, 0.5 mM EDTA) at a constant voltage of 60 V for 18 h at 60°C. The bands were visualized by staining with silver nitrate according to Heuer et al. (1997). Each band was considered to be an operational taxonomic unit (OTU). All procedures were done in triplicate.

Multivariate analysis

Three methods of multivariate analysis were applied: 1) cluster analysis; 2) cluster analysis preceded by principal component analysis (PCA); 3) Venn diagram, for comparison of the results. The first method of cluster analysis was based on a binary matrix, representing the presence/absence of bands in each treatment. The similarity matrix was obtained by calculating Jaccard's coefficient. In the second method, PCA was applied to reduce the dimension of the original variables. In the third method, Venn diagrams were constructed manually, taking into account the intersections of the DGGE bands obtained.

RESULTS

Basal respiration

Analysis of microbial activity during a period of 22 days showed no significant increase ($P < 0.05$) in the amount of CO_2 produced in all samples (Figure 2A). The average total amount of CO_2 produced showed no significant differences between contaminated and non-contaminated samples except for the sample treated with 5% crude oil (Figure 2A and B). However, during the first 8 days, high oil-contaminated sediments showed decreased levels of CO_2 evolution (Figure 2A).

DNA, PCR and DGGE analyses

In this study, the DNA extracted in triplicate from all samples showed high yield and quality, allowing further molecular analysis. The PCR products amplified with primers AmoA1F and AmoA2R generated fragments of approximately 491 bp. The PCR-DGGE revealed that AOB communities were present in all samples analyzed. Based on DGGE analysis, uncontaminated sediment, oil-contaminated sediment and landfarm soil showed different band patterns with variable composition, suggesting a great diversity within the AOB group (Figure 3A and B). Samples from Land and of 5% oil-contaminated sediment showed 9 and 8 bands, respectively, the lowest number of bands on the gel (Figure 3B), whereas the 2% oil-contaminated sample with 22 bands revealed the highest complexity.

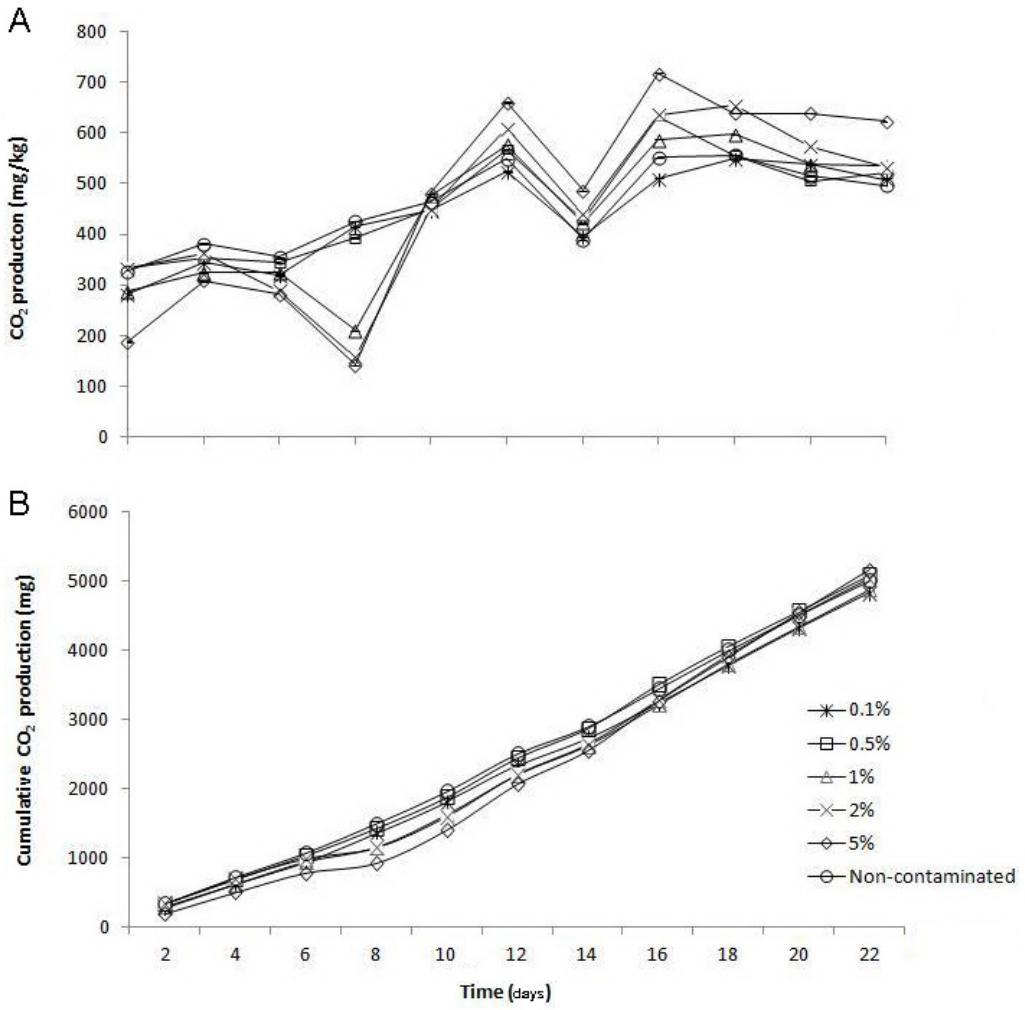

Figure 2. CO_2 production (**A**) and cumulative CO_2 production (**B**) for sediment non-contaminated and contaminated with 0.1, 0.5, 1, 2, and 5% crude oil.

The band profiles were clustered according to similarity using Jaccard's similarity coefficient (Figure 4). The dendrogram revealed common and specific bands in each sample group. The similarity between the DGGE profiles of the community in sediment exposed to 0.5, 1 and 2% oil contamination was higher than of communities exposed to 0.1 and 5% oil contamination (Figure 4 and Figure 5C). DGGE profiles in 0.1, 1 and 2% oil-contaminated samples suggested AOB communities with higher similarity than those generated for the uncontaminated and 5% oil-contaminated MG sediment and for Land as the latter samples showed the lowest similarity in band profiles (Figures 4 and 5A).

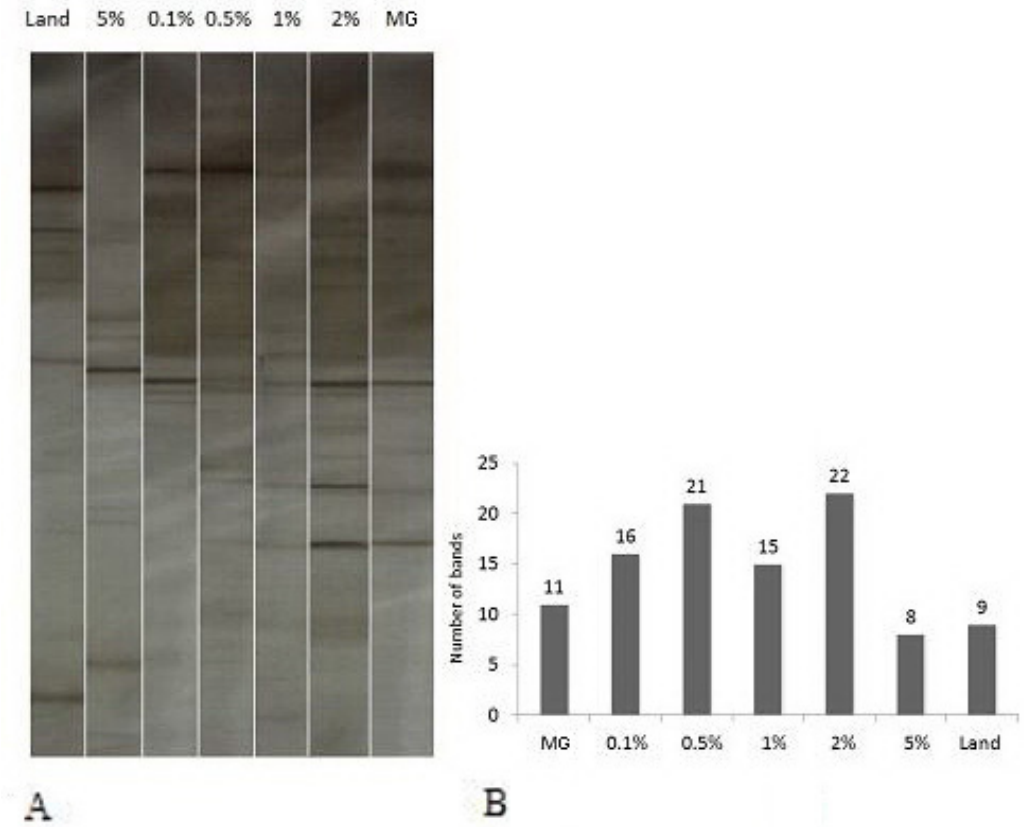

Figure 3. A. DGGE of DNA amplified for AOB using amoA1F (containing a 40-bp GC-clamp) and amoA2R primers. The gel conditions were 8% (w/v) polyacrylamide (37.5:1 acrylamide:bis-acrylamide), consisting of a denaturing gradient of 40-70%, at a constant voltage of 60 V for 18 h at 60°C. Samples were labeled MG, representing the non-impacted mangrove, 0.1, 0.5, 1, 2, and 5%, representing the profile after 22 days of microcosm contamination with oil at 0.1, 0.5, 1, 2, and 5%, respectively, and Land, representing the DGGE profile for the AOB group of a sample from landfarm soil. **B.** Number of bands by treatment.

The first PCA analysis allowed a distribution of the sample profiles along axes of ordering based on the presence and absence of DGGE-detected bands (Figure 5A). When the PCA was generated without the sample Land (Figure 5B), the distance between the samples MG and 5% could be demonstrated. When the PCA analysis suppressed the samples MG and Land, the sample 5% is distant from the others (Figure 5C).

The Venn diagram generated from the DGGE band profiles of all samples, forming five groups labeled MG, Land, 5%, 0.1% and G1 (comprising 0.5, 1 and 2%), showed the absence of common OTUs among the samples, but the sharing of 8 OTUs between G1 and MG (Figure 6). The Venn diagram considering only the samples contaminated with oil showed the sharing of a single band (Figure 7). However, excluding the sample 5%, there were 7 common bands between the other samples, confirming a higher similarity of these samples with a Jaccard similarity index of 50% (Figure 4 and Figure 8) and 23 bands present in oil-contaminated samples and absent in MG and Land (Figure 6).

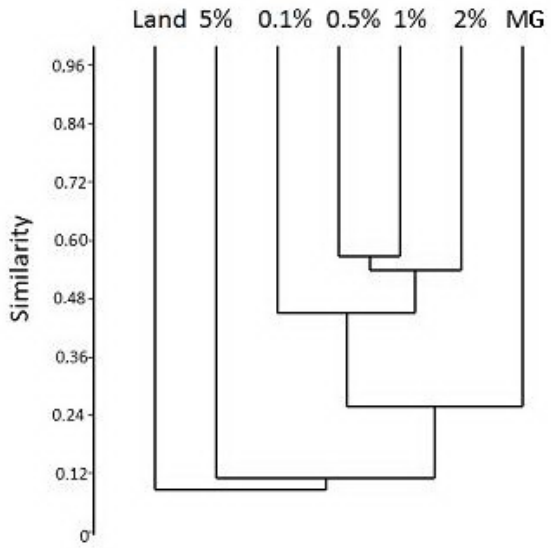

Figure 4. Dendrogram based on Jaccard's coefficient generated using cluster analysis from the PAST program after matrix generation from DGGE of amoA. The point labeled MG represents the cluster analyses for non-impacted mangrove, and 0.1, 0.5, 1, 2, and 5% representing the profile after 22 days of microcosm contamination with oil at 0.1, 0.5, 1, 2, and 5%, respectively; Land represents the AOB group of a sample from landfarm soil.

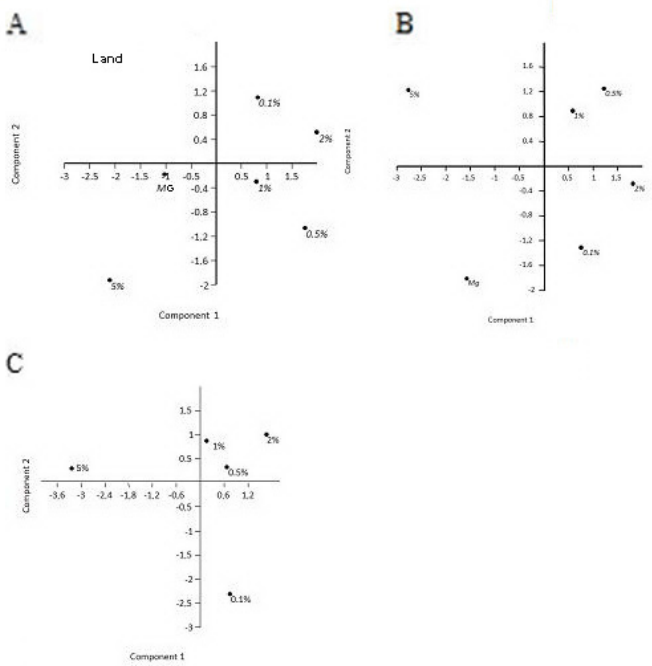

Figure 5. A. Principal component analysis (PCA) generated from DGGE matrix profile using all samples. **B.** PCA considering only the MG and contaminated samples. **C.** PCA considering only the contaminated samples. The point labeled MG represents the non-impacted mangrove, and 0.1, 0.5, 1, 2, and 5% represent contamination with oil at 0.1, 0.5, 1, 2, and 5%, respectively; Land is a sample from landfarm soil.

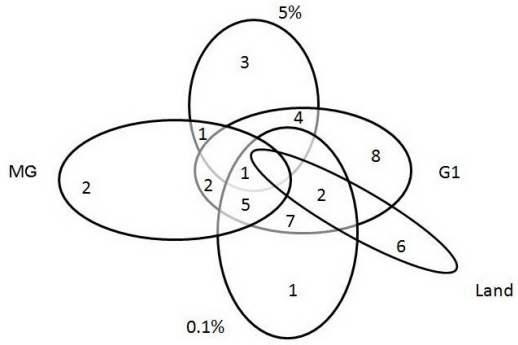

Figure 6. Venn diagram showing the richness of bands detected by DGGE profiles, considering all samples. To develop the riches of this diagram, bands of treatments 0.5, 2 and 1% were grouped together and labeled G1. Groups denominated MG and Land correspond to samples from non-contaminated mangrove and from landfarm, respectively.

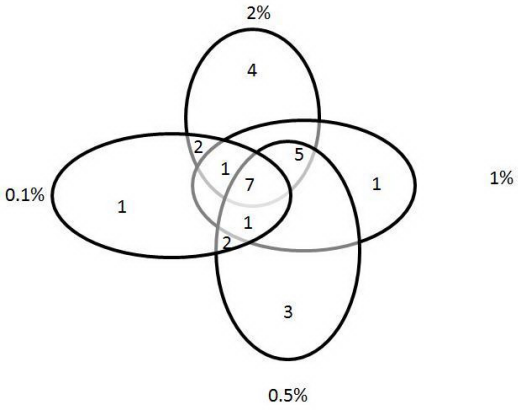

Figure 7. Venn diagram showing the richness of bands detected by DGGE profiles, considering only the oil contamination of 0.1, 0.5, 1, and 2%.

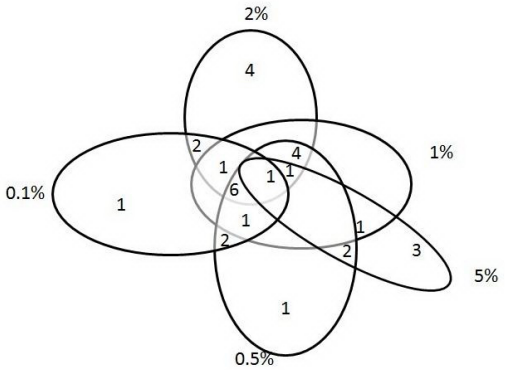

Figure 8. Venn diagram showing the richness of bands detected by DGGE profiles, considering only the oil-contaminated samples, where individual groups were considered for analysis of similarity between them.

DISCUSSION

Ammonia oxidation is an important step in the global nitrogen cycle, where the enzyme AMO, produced by AOB, is responsible for the conversion of ammonia to nitrite (Hollocher et al., 1981; Prosser, 1989; Bothe et al., 2000). Monitoring of this community in petroleum degradation processes has become of great importance as it gives us more information on bacterial diversity and metabolic activity. As reported by Arp et al. (2001), the AMO enzyme may be used as a tool to initiate, along with other enzymes, the metabolism of pollutants such as alkanes, alkenes, or aromatics from soils, waters, and sediments. In this study, we investigated in microcosm experiments the impact of crude oil contamination on the AOB community of mangrove sediment.

The physical and chemical characteristics of the non-contaminated MG sediment demonstrated that it is rich in organic matter (Table 1). The sample from oily sludge landfarm soil, used for comparison, showed organic matter above 60 g/kg (Maciel et al., 2009). According to the literature, AOB communities are found in samples rich in organic matter, and although they are usually classified as chemoautotrophs, heterotrophic growth has also been described for several species (Arp et al., 2001). This reveals its importance in processes of degradation of organic matter in MG. In agreement with the literature, the occurrence of AOB is rare at pH values below 7 (Burton and Prosser, 2001; Gieseke et al., 2006). However, other studies show the presence of AOB at acidic pH (Nicol et al., 2008), which supports our detection of the amoA gene in MG at pH 4.1 (Table 1).

The experiments showing CO_2 production in the oil-contaminated MG microcosms demonstrated the presence of a bacterial community with potential for mineralization of organic compounds, a fact corroborated by other studies (Islam and Weil, 2000; Karlen et al., 2001). Among the oil-contaminated samples, those with the highest oil concentrations had the lowest CO_2 production within the first 8 days, suggesting difficulty in initiating mineralization at high levels of contamination by non-adapted microorganisms. The increased metabolic activity after 8 days in these microcosms, however, suggests a successful adaptation of the microbes for the enzymatic degradation of organic matter.

The success of DGGE analysis obtained here is directly related to the quality of the extracted DNA, a crucial step in detecting nonculturable bacteria. The attempts of extraction with other DNA extraction methods described by Maciel et al. (2009) and Amorim et al. (2008) were not successful. We attribute this to our specific sample conditions, i.e., to the presence of humic acids and, especially, to the presence of oil contamination in our samples. These obstacles were overcome by the use of the Power Soil kit (MOBIO), and the DNA extracted from MG confirmed the presence of AOB after amplification with the primer set for amoA. The use of functional markers, i.e., genes of key enzymes involved in specific metabolism such as AMO, has been greatly encouraged for studies of specific communities as AOB (Rotthauwe et al., 1997). Thus, the use of specific 16S rRNA gene primers can be avoided, as this would also allow the amplification of other β-proteobacteria (McCaig et al., 1994; Rotthauwe et al., 1997; Junier et al., 2009).

The DGGE analysis revealed not only the richness and similarity of bands but also the impact generated by oil on the AOB community. Notably, the landfarm sample and the sample contaminated with 5% oil showed less diversity/similarity than the other samples. This can be explained by a negative pressure on community members of AOB for the sample

contaminated with 5% oil with an increase of 150% compared to the 2% oil-contaminated sample, where a higher bacterial diversity was found. In relation to the landfarm sample, where microorganisms would be theoretically habituated to the adverse conditions produced by the compounds present in oil wastes, this diversity could be explained by the selective pressure exerted by oil, and the OTUs represent the microorganisms already accustomed to this environment. Land did not show similarity with the other samples because of its different origin and characteristics.

In one universe of 42 OTUs revealed by the Venn diagram (Figure 6), 36 were among the mangrove samples. The contaminated mangrove samples, disregarding the 5% sample, shared only 7 OTUs. Considering that all samples must be assessed in relation to the uncontaminated mangrove sample, 8 OTUs showed similarity or approximately 72% of OTUs remained or did not change with the presence of oil. Apparently, this contaminant seemed to favor even more the diversity of AOB as can be seen with 2% contamination, and there are reports showing that numerical dominance may not indicate activity (Jia and Conrad, 2009). Interestingly, Vanelli et al. (1990) and Vanelli and Hooper (1992) affirm the importance of AOB not only because of their being part of the global nitrogen cycle, but also due to their capability of degrading a wide range of hydrocarbons and halogenated hydrocarbons.

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