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# Microbial diversity and anaerobic hydrocarbon degradation potential in an oil-contaminated mangrove sediment

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## Abstract

**Background:** Mangrove forests are coastal wetlands that provide vital ecosystem services and serve as barriers against natural disasters like tsunamis, hurricanes and tropical storms. Mangroves harbour a large diversity of organisms, including microorganisms with important roles in nutrient cycling and availability. Due to tidal influence, mangroves are sites where crude oil from spills farther away can accumulate. The relationship between mangrove bacterial diversity and oil degradation in mangrove sediments remains poorly understood.

**Results:** Mangrove sediment was sampled from 0–5, 15–20 and 35–40 cm depth intervals from the Suruí River mangrove (Rio de Janeiro, Brazil), which has a history of oil contamination. DGGE fingerprinting for *bamA*, *dsr* and 16S rRNA encoding fragment genes, and qPCR analysis using *dsr* and 16S rRNA gene fragment revealed differences with sediment depth.

**Conclusions:** Analysis of bacterial 16S rRNA gene diversity revealed changes with depth. DGGE for *bamA* and *dsr* genes shows that the anaerobic hydrocarbon-degrading community profile also changed between 5 and 15 cm depth, and is similar in the two deeper sediments, indicating that below 15 cm the anaerobic hydrocarbon-degrading community appears to be well established and homogeneous in this mangrove sediment. qPCR analysis revealed differences with sediment depth, with general bacterial abundance in the top layer (0–5 cm) being greater than in both deeper sediment layers (15–20 and 35–40 cm), which were similar to each other.

**Keywords:** Mangrove, Bacterial diversity, Anoxic sediment, Sulphate, Petroleum, Hydrocarbons

## Background

The Deepwater Horizon oil spill of 2010 in Gulf of Mexico serves as a reminder of the potential adverse impacts of petroleum compounds to the environment [1,2]. Petroleum is a complex mixture of saturated and aromatic hydrocarbons, polar compounds, resins and asphaltenes. Saturates are proportionally the most significant fraction by mass while the most toxic and persistent compounds are the polar and aromatic hydrocarbons [3]. Such compounds can be responsible for massive wildlife death soon after oil spills and, as well as over the medium and long-term [1]. Unfortunately, accidents resulting in oil spills

happen routinely, and due to tidal activity spilled oil is commonly transported to coastal regions. In temperate to tropical latitudes mangrove forests are one of the ecosystems that are most detrimentally affected [4].

Mangroves are vital ecosystems for coastal protection. Their features make them a unique environment, with high biological diversity and activity. Salinity and organic matter availability vary in different parts of mangrove forests [5]. Beneath a thin aerobic surface layer, mangrove sediments are predominantly anaerobic, i.e., anaerobic biochemical processes are catalyzed by sediment microbial communities [6]. In previous studies about microbial populations, it was shown that *Alphaproteobacteria* dominated the bacterial community in a non-disturbed Brazilian mangrove sediment [5] and that after crude oil exposure, bacterial groups such

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as *Anaerolinea* decrease in population abundance whereas *Deltaproteobacteria* increase [7]. The anoxic nature of mangrove sediment is a key feature that allows oil accumulation in such ecosystems [8]. For example, after an oil spill it is possible to detect higher amounts of oil in deeper sediment than at the surface, showing that oil tends to percolate through the sediment down to deeper layers [9,10].

Several microorganisms are capable of degrading aliphatic and aromatic hydrocarbons under anoxic conditions [11]. Boopathy [12] studied diesel degradation in estuarine sediment microcosms in the presence of different terminal electron acceptors. In the presence of nitrate, sulphate and carbonate, 99% of the crude oil was removed within 510 days, whereas stimulating only sulphate reduction, methanogenesis, or nitrate reduction resulted in 62, 43, and 40% oil removal, respectively. Boopathy and colleagues observed the same interesting results on anaerobic oil hydrocarbon degradation in follow-up studies, showing that sulphate-reducing condition is the most efficient redox condition in experiments using individual electron acceptors [13,14].

Petroleum hydrocarbon degradation pathways are distinct. It is believed that n-alkane-utilizing strains do not grow with aromatic hydrocarbons, and vice versa [15]. There are two elucidated mechanisms for anaerobic alkane degradation. One involves fumarate addition to the alkane subterminal carbon to produce alkylsuccinate compounds, and in the other process the alkane is carboxylated [16]. The enzymes responsible for fumarate addition in anaerobic alkane metabolism are alkylsuccinate synthases, AssA1 and AssA2, encoded by *assA1* and *assA2* genes, respectively [17,18].

Aromatic hydrocarbons are converted to a few central intermediates before being further metabolized. The most common central intermediate of the anaerobic aromatic hydrocarbon transformation is benzoyl-CoA [19], which is then converted to dienoyl-CoA. The next set of reactions ends with a 6-OCH-hydrolase enzyme opening the aromatic ring of the compound. This enzyme is encoded by *bamA* which is considered as a good genetic marker for studying anaerobic aromatic hydrocarbon degradation, since it contains highly conserved regions [20]. However, *bamA* is involved in anaerobic aromatic hydrocarbon degradation in general, and not exclusively degradation of petroleum-derived compounds. Another good target for the detection of anaerobic aromatic hydrocarbon-degrading microorganisms is the enzyme benzylsuccinate synthase (Bss), which is involved in the anaerobic degradation of toluene and xylene, via fumarate addition to the methyl group, transforming these compounds into benzylsuccinates. Bss has been identified in all anaerobic toluene-degrading microorganisms studied to date, and is composed by three subunits, of

which,  $\alpha$  subunit, encoded by *bssA* gene is the target for molecular studies. This gene is highly conserved and has been employed as a molecular marker for the characterization of environmental samples [20-22].

Despite the importance of crude oil pollution in coastal environments, little attention has been paid to bacterial diversity and anaerobic degradation potential of crude oil hydrocarbons in mangrove sediments. Therefore, the aims of this study were: to compare microbial community profiles in sediments from different depths; to quantify total bacteria and sulphate-reducing bacteria (SRB) as a function of depth; and to screen for the presence of key genes involved in anaerobic hydrocarbon degradation in mangrove sediment.

## Results

### Sediment porewater sulphate concentration

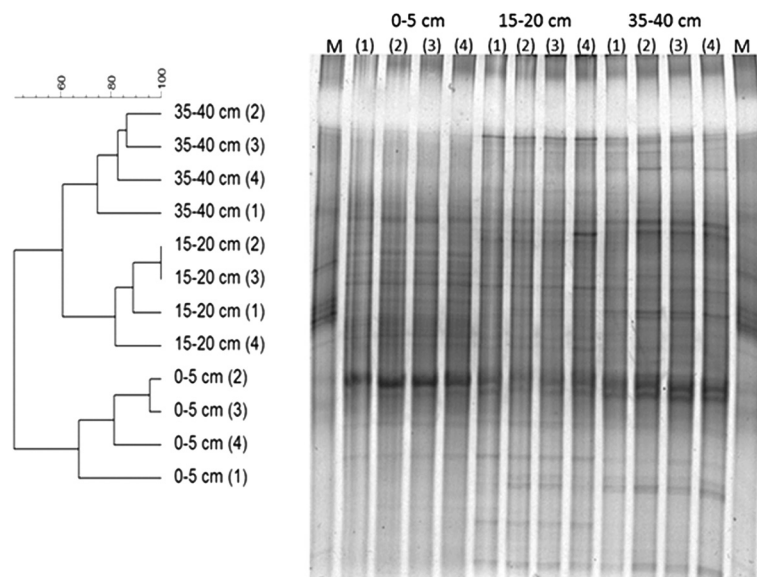
In the current study, sulphate was measured at each studied depth, and in the surface sediment (0–5 cm layer), its concentration was 14.9 mM. Sediment from the two other studied depths, 15–20 cm and 35–40 cm, had a sulphate concentration of 3.6 mM. This suggests an active sulphate reduction zone in the top 15 cm of the sediment. These values reflect the influence of seawater (28 mM sulfate) in mangrove ecosystems, which is introduced by tidal activity.

### Sediment microbial community analyses: PCR-DGGE for 16S rRNA, *bamA* and *dsr* genes

To study the bacterial community profile, genomic DNA extracted from sediment samples was analysed by PCR using universal primers to amplify 16S rRNA gene fragments. Amplicons with the expected size of 430 kb were separated by denaturing gradient gel electrophoresis (DGGE) and the results showed a clear distribution of the bacterial populations within the three studied depths (Figure 1), revealing the occurrence of two main clusters: one cluster from the 0–5 cm layer, and another associated with sediment samples from both 15–20 and 35–40 cm depth.

To study the SRB community at different sediment depths PCR-DGGE was performed using primers targeting the *dsr* gene that encodes the dissimilatory bisulphite reductase enzyme that is present in all sulphate reducers [23]. This revealed the occurrence of two main clusters, one cluster derived from the 0–5 cm sediment and the other was associated with sediment samples from both 15–20 and 35–40 cm depth (Figure 2).

PCR-DGGE using primers targeting the *bamA* gene, responsible for anaerobic aromatic hydrocarbon degradation, revealed a distribution of two main clusters. Unlike the 16S rRNA gene and *dsrAB* patterns, *bamA* distributions were revealed by one distinct banding pattern common to both the 0–5 and 15–20 cm depths, and a



**Figure 1 16S rRNA dendrogram for different depths of mangrove sediment and the gel image.** Dendrogram generated based on denaturing gradient gel electrophoresis (DGGE) fingerprints of 16S rRNA gene fragments from triplicates of mangrove sediment from 3 different depths: 0–5, 15–20 and 35–40 cm, and the DGGE gel image.

different pattern in the deeper 35–40 cm sediment (Figure 3). The patterns in the shallower sediments can further be clustered specifically to the 0–5 and 15–20 cm sediment depths.

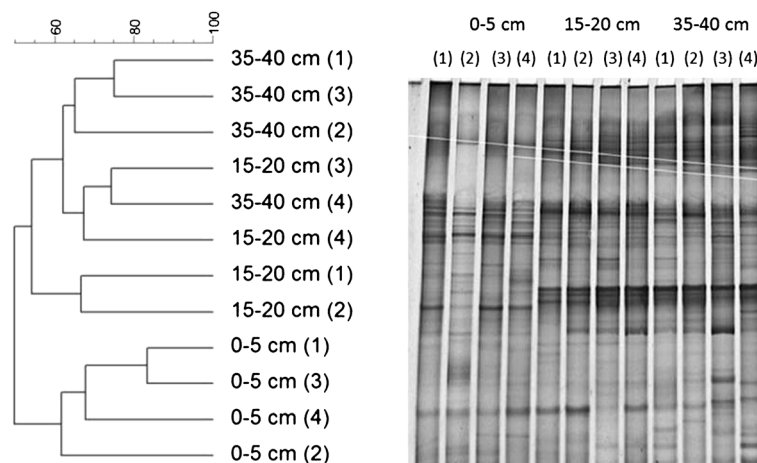
#### Molecular techniques for sediment: PCR for *assA* and *bssA*

To further verify the potential for anaerobic petroleum hydrocarbon degradation within the sediment microbial populations, end-point PCR analyses targeting *assA* and *bssA* genes were performed. Genomic DNA from all three sediment depths did not give rise to a PCR

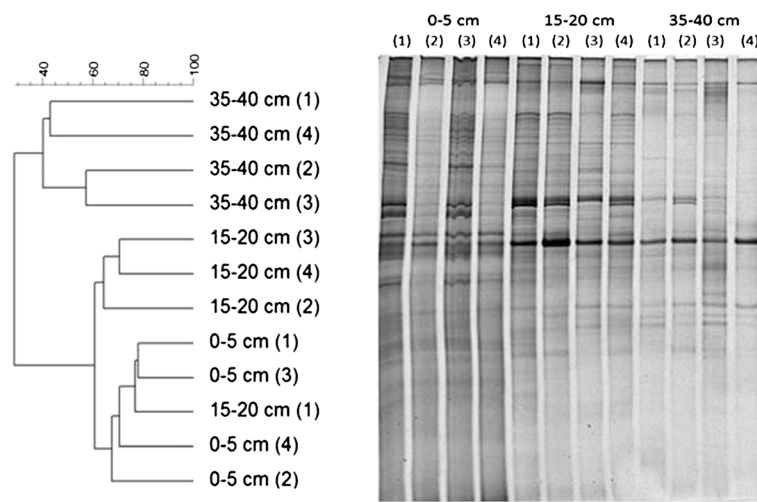
product using these primers, despite the fact that this mangrove sediment has a history of petroleum contamination.

#### Molecular techniques for sediment: q-PCR for 16S rRNA and *dsr* genes

To estimate the bacterial abundance within the three depth horizons, a quantitative (q-) PCR assay was performed for 16S rRNA genes using sediment genomic DNA samples as templates. Results presented in Figure 4a show depth variations of total bacterial 16S



**Figure 2 *dsr* gene dendrogram and gel image for different depths of mangrove sediment.** Dendrogram generated based on denaturing gradient gel electrophoresis (DGGE) fingerprints of *dsr* gene from triplicates of mangrove sediment from 3 different depths: 0–5, 15–20 and 35–40 cm, and the gel image.



**Figure 3** *bamA* gene dendrogram and gel image for different depths of mangrove sediment. Dendrogram generated based on denaturing gradient gel electrophoresis (DGGE) fingerprints of *bamA* gene from triplicates of mangrove sediment from 3 different depths: 0–5, 15–20 and 35–40 cm, and the gel image.

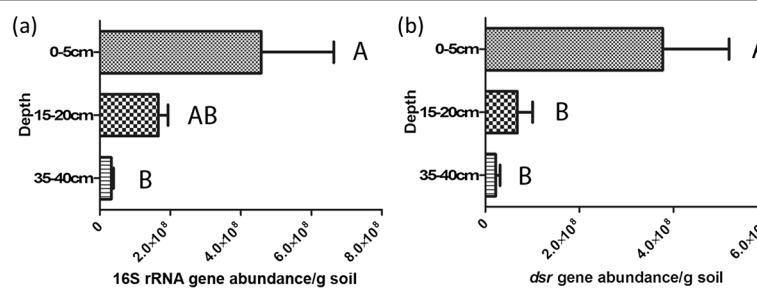
rRNA genes. In the top sediment, q-PCR detected  $4.6 \times 10^8$  genes/g of sediment, in the middle layer,  $1.78 \times 10^8$  genes/g of sediment, and in the deep sediment, the abundance was  $3.2 \times 10^7$  genes/g of sediment. One-way ANOVA indicated that the only significant difference was detected between the 0–5 and the 35–40 cm layers.

In order to estimate the abundance of SRB in the sediment samples, q-PCR was performed for *dsr*. The results were used to compare SRB abundance as a function of sediment depth and are shown in Figure 4b. In the top sediment, q-PCR detected  $3.6 \times 10^8$  genes/g of sediment, in the middle layer  $6.6 \times 10^7$  genes/g of sediment were detected, and in the deeper layer the abundance was  $2.1 \times 10^7$  genes/g of sediment. As such, SRB abundance decreases with depth, with one-way ANOVA confirming that the abundance in the surface sediment is significantly different from the abundance in the two deeper layers.

## Discussion

Pore-water sulphate concentration decreases from 14.9 to 3.6 mM in the top centimeters and remains low in the deeper sediment, indicating a near-surface sulphate reduction zone, as observed elsewhere [24–29]. Sulphate concentration in seawater and marine sediments is around 28 mM [11]. Mangroves are brackish ecosystems, due to tidal activity, and have a higher sulphate concentration than freshwater sediments.

In accordance with the sulphate profile, q-PCR showed a significantly larger population of *dsr*-containing microorganisms in the 0–5 cm layer relative to the deeper sediments. This is consistent with the sulphate-reduction zone being located in the shallower sediment interval and suggests that SRB populations are active there. High microbial abundance in the shallow sulphate-containing sediment was also reported in previous studies [28], where it was associated with intense sulphate reducing activity likely owing to organic matter availability.



**Figure 4** Bacterial abundance at different depths of mangrove sediment. Abundance of bacterial populations on mangrove sediments of three different depths tested with q-PCR using oligonucleotide primers for 16S rRNA gene encoding fragment (a) and oligonucleotide primers for *dsr* gene (b). Bars with the same letter are not significantly different (one-way ANOVA).

DGGE was used to assess the sediment bacterial community, using as targets the genes encoding 16S rRNA, BamA and DsrAB. DGGE analysis of 16S rRNA gene diversity revealed depth-dependent differences. A distinct bacterial community composition was identified below 5 cm (i.e., below the sulphate-reduction zone) and is similar in the two deeper sediments, possibly due to lower organic matter availability.

Positive PCR amplification of *bamA* indicates the potential for anaerobic aromatic hydrocarbon-degrading microorganisms at all sediment depths. BamA is involved in the degradation of aromatic hydrocarbons in general, not only petroleum-derived aromatics. BamA-encoding microorganisms are found in the environment independently of contamination [20,30]. Plant matter is a major source of aromatic hydrocarbons [31], which may explain the prevalence of BamA-encoding microorganisms throughout the sediment. Alternatively spilled crude oil percolates deep into the sediment, and the close contact with aromatic compounds in more recalcitrant crude oil fractions might enrich *bamA* containing microorganisms. The apparent absence of Bss-encoding bacteria might be explained because the *bssA* variants targeted by our PCR primers may be mainly involved in anaerobic degradation volatile aromatic compounds (e.g., toluene and o-xylene [22]) which evaporate soon after the oil is spilled. Alternatively, other metabolic pathways and functional genes could be involved in the degradation of oil-derived aromatics in this mangrove sediment. The DGGE analysis of *bamA* diversity showed that the population structure of aromatic hydrocarbon degrading bacteria also changes with depth, being more similar in the top 20 cm, where the influence of plant detritus is greatest. In deeper sediment, 35–40 cm, the DGGE pattern contains fewer bands than the other two analyzed depths.

Küntze and colleagues [20] recommended the combination of PCR for *bamA*, which gives an overview of the anaerobic aromatic hydrocarbons degrading microorganisms present in the studied material, with PCR for *bssA*, which is specific for toluene and xylene degradation – although this gene also seems to be involved in the degradation of some long-chain aromatic hydrocarbons (L. Andrade, unpublished data). In the current study, sediment samples from the three depths tested negative for *bssA* (data not shown). Samples were also similarly screened with PCR primers targeting *assA*, involved in anaerobic alkane degradation, and results were also negative. Our failure to amplify *bssA* and *assA* do not necessarily mean that anaerobic aromatic hydrocarbon-degrading microorganisms are absent from the Surui mangrove

sediment; they may be present at abundances too low to be detected with the PCR protocol used. Alternatively, anaerobic hydrocarbon degraders possessing *ass/bss* sequence variants lacking homology to our PCR primers [18] or that employ degradation pathways altogether different to the ones tested here (e.g., carboxylation reactions [32] or the two-step oxidation of methylene observed in the degradation of ethylbenzene by a nitrate-reducing strain [33]) for catabolism of anaerobic hydrocarbons.

PCR-DGGE analyses for *dsr* showed that the bacterial community profile in the top 5 cm differs from the two deeper sediment intervals, which was also observed in DGGE analysis of 16S rRNA genes. Nevertheless, the similarities in banding pattern are large concerning sediments of the two deeper layers, while both change a little when comparing to superficial sediment. Similar diversity among dissimilatory sulfite reductase sequences in deeper sediment layers was also observed by Fan and colleagues [34] who analysed *dsrAB* from the surface to 50 cm depth. They suggest that different surficial and deeper sediment SRB community structure is related to tidal variation, which makes sediment temporarily oxic, hypoxic or anoxic. Moreover, tidal inundation also transports sulphate from the sea to the coastal sediment, which shows a high sulphate concentration in the first centimetres of sediment, but diluted in the freshwater presents a low concentration downward. Taketani and colleagues [35] also studied SRB community structure using DGGE and showed that SRB diversity decreases with depth in mangrove sediment, as well as revealing a drop in the relative abundance of SRB, in agreement with the qPCR results presented here (Figure 4). However they noted little variation in diversity in the first 30 cm of that sediment [35]. In anaerobic sediments, SRB play an important role in nutrient cycling and organic matter remineralization [6], and they can be especially important in oil-polluted locations where certain SRB are capable of anaerobic hydrocarbon degradation [11]. Taketani and colleagues confirmed the importance of SRB populations in mangrove sediments, particularly after an oil-contamination event. In a study using mesocosms with pristine and polluted mangrove sediments, they reported an increase in SRB abundance in pristine sediment after oil input, and observed that a mangrove with history of oil contamination is better prepared to respond to such an adverse situation than a non-contaminated one [7].

General bacterial abundance determined by 16S rRNA-targeted qPCR was highest in the 0–5 cm layer sediment, and decreased with depth (Figure 4). The same phenomenon occurs for sulphate-reducing bacteria, in

agreement with sulphate concentrations measured in the sediment depths investigated. Comparing q-PCR results for *dsr* and 16S rRNA gene fragment genes suggests that a large fraction of the bacteria present may be sulphate-reducers. It is remarkable that in the top sediment, *dsr* genes represent almost 80% of the number of genes for general bacteria (16S rRNA gene encoding fragment gene). For the deeper sediments these values are almost 40% (15–20 cm) and almost 65% (35–40 cm). It is well known that microorganisms contain more than one copy of 16S rRNA gene. This also might happen for *dsr* gene [36]. Moreover, the primers for 16S rRNA gene encoding fragment gene used in the present study target bacteria, while in their study, Geets and colleagues [36] also detected archaeal *dsr* with the same primer pair that was used here. In principle *dsr* detected in these mangrove sediments by q-PCR could have archaeal species, and as such, the values we report could overestimate the number of sulphate-reducing bacteria.

This is one of the few studies on anaerobic bacterial diversity in mangrove sediments at different sediment depths. Results presented in this study shows that the

bacterial diversity and abundance change with depth. This might explain why petroleum and other xenobiotic compounds that percolate to the deep anoxic sediment layers may remain undegraded for years.

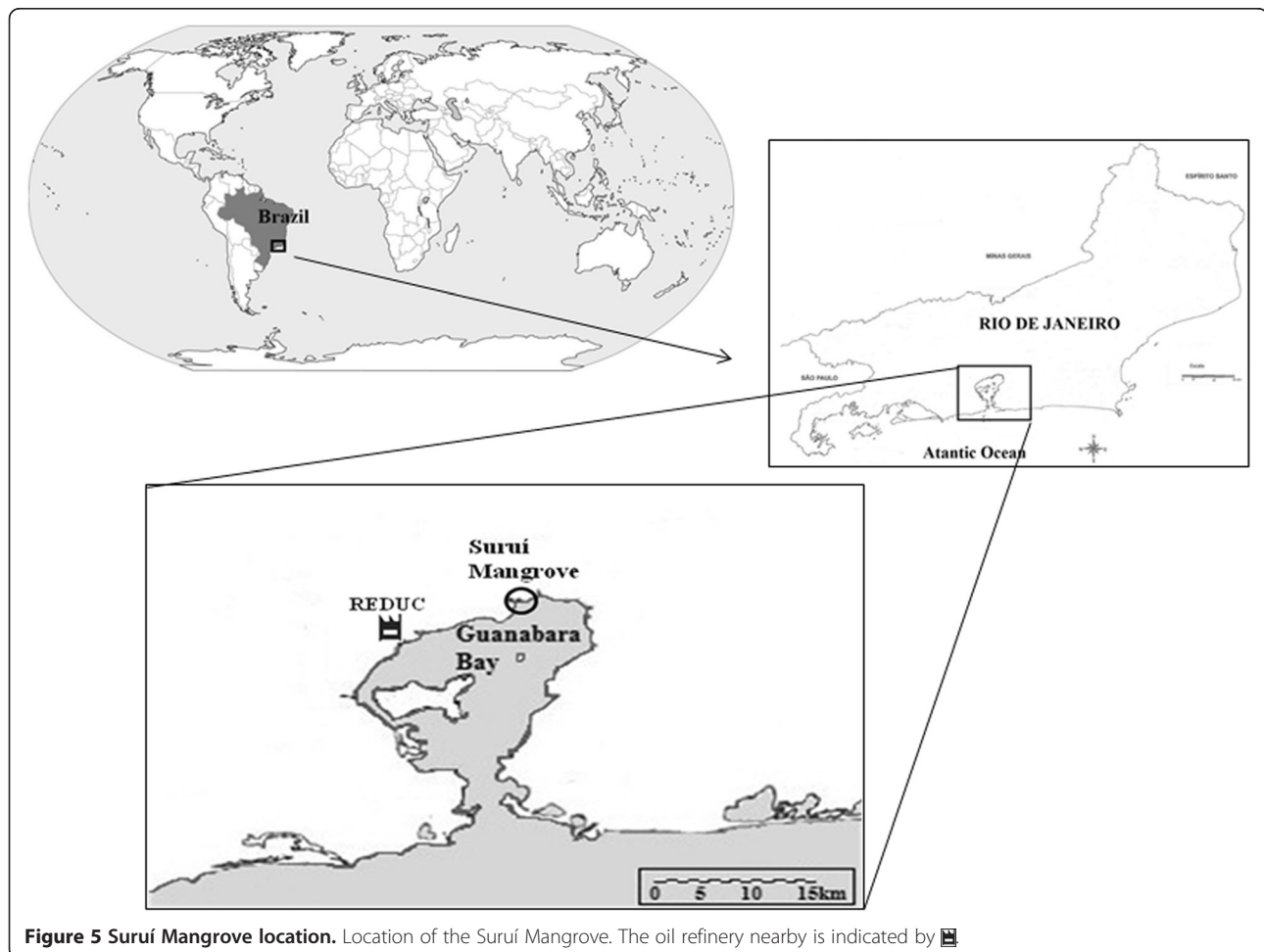
## Conclusions

Sulphate decreases dramatically in the first centimetres of the mangrove sediment, and overall bacterial diversity and abundance from the surficial interval (0–5 cm) differs from deeper layers (15–20 and 35–40 cm), which are very similar to each other. Genes involved in anaerobic alkane and aromatic petroleum hydrocarbon degradation were not detected by PCR, perhaps because gene targets for the PCR primers chosen may not have matched to *in situ* genetic diversity.

## Methods

### Sediment sampling

The sampling site was the Suruí mangrove in Guanabara Bay, situated in Magé, state of Rio de Janeiro, Brazil (Figure 5). In the year 2000, there was an oil spill in Guanabara Bay, impacting the Suruí mangrove. More



**Figure 5 Suruí Mangrove location.** Location of the Suruí Mangrove. The oil refinery nearby is indicated by 

than 1 million liters of oil leaked from a broken pipeline of an oil refinery nearby, and the most affected region was the northern part of the bay [37].

Samples were collected at one point of the mangrove (S 22°41'50", W 043°07'00"), during the low tide period. Four aluminum tubes 60 cm in length were used to obtain sediment cores down to 40 cm depth, with less than 1 m of distance of each other sampling point. After sampling, tubes were wrapped in plastic material to limit oxygen exposure, and transported immediately to the laboratory for further processing steps.

In the laboratory, each core was sectioned to obtain samples of the following intervals: 0–5, 15–20 and 35–40 cm deep. Sediment samples of the four replicate cores for each interval were each divided into two parts: a portion reserved for total genomic DNA extraction and molecular based studies, and another one reserved for porewater sulphate analysis.

#### Sediment porewater sulphate concentration

Sulphate was analysed by chromatography through Metrohm ion chromatograph with conductivity detection, isolated in a 100 × 4.0 mm polyvinyl ethanol column, using sodium carbonate and sodium bicarbonate as eluent.

#### Molecular techniques for sediment: PCR-DGGE for 16S rRNA, *bamA* and *dsr* genes

Total genomic DNA was extracted from bulk sediment of each replicate using FastDNA<sup>®</sup> SPIN kit, accordingly to manufacturer recommendations.

PCR reactions for further DGGE analysis were performed using U968f-GC1 and L1401, universal primers for the 16S rRNA gene, as previously described by Heuer and Smalla [38]. Before DGGE analysis, PCR products were confirmed to have been amplified by electrophoresis in a 1.2% agarose gel run at 80 V in Tris-Borate-EDTA buffer, and further staining step for 15 min immerse in a solution containing 0.5 g/ml ethidium bromide and revealed under short-wavelength ultraviolet light.

PCR products were submitted to DGGE analysis [39] using a DCode System (universal mutation detection system, BioRad, Richmond, USA), using a 6% acrylamide gel within a denaturing gradient of 40% to 70% of a mixture of urea and formamide. Electrophoresis was performed in 1x Tris-acetate-EDTA buffer at 60°C and at 75 V for 16 h. For the staining step, Sybr Gold (Invitrogen) was used, and the gel was visualised using a Storm 860 Imaging System (GE Healthcare). DGGE images were analysed using BioNumerics software (Applied Maths, Belgium) and similarities between lanes were calculated using the band-based Jaccard correlation coefficients, and cluster analysis was performed by the unweighted pair group method with average linkages (UPGMA). PCR-DGGE was also performed for *bamA* to compare the profile of diversity of anaerobic hydrocarbon-degrading bacteria at the three studied depths. PCR mixture and conditions for the *bamA* reactions were as previously described by Kuntze and colleagues [20]. Primers SP9 and ASP1 were used and PCR products run on a 9% acrylamide gel within a denaturing gradient of 50% to 70% of urea and formamide. To primer SP9 a GC clamp (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G-3') was attached to stabilize transition forms of the DNA molecule, improving band pattern results [40].

To compare the diversity of SRB at different depths, a PCR-DGGE was executed using two pairs of primers for *dsr* gene (Table 1). Formerly, a PCR reaction was carried out using the Primer Set 1. The resulting amplicons of this reaction became templates for a second PCR reaction using Primer Set 2.

Reaction with Primer Set 1 consisted of a 25 µl mixture, containing 1× 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40 (Fermentas), 1.75 mM MgCl<sub>2</sub>, 50 mM of each dNTP, 200 nM of each oligonucleotide primer (Set 1), 2.5 U of Taq DNA polymerase (Fermentas), 0.5 µl of bovine serum albumin (BSA) 1% (V/V), and 1 µl of DNA. Amplification conditions comprised an initial denaturation step of 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 90 s, and a final extension step of 72°C for 10 min. PCR with Primer Set 2 consisted of a 50 µl

**Table 1 Primers for sulphate-reducing bacteria detection**

	Primer Set	Forward (F) and Reverse (R) Oligonucleotide Primer Sequences	Reference
Primer Set 1	DSR1F	F: 5'-ACS CAC TGG AAG CAC GGC GG-3'	[23]
	DSR4R	R: 5'-GTG TAG CAG TTA CCG CA-3'	[36]
Primer Set 2	DSRp2060F-GC	F: 5'-CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CCA ACA TCG TYC AYA CCC AGG G-3'	[36]
	DSR4R	R: 5'-GTG TAG CAG TTA CCG CA-3'	[36]

Oligonucleotide primers used in PCR reactions for assessment of the sulphate-reducing bacterial communities and comparison between the 3 studied depths.

mixture, containing 1x 100 mM Tris-HCl (pH 8.8 at 25°C), 500 mM KCl, 0.8% (v/v) Nonidet P40 (Fermentas), 1.75 mM MgCl<sub>2</sub>, 50 mM of each dNTP, 200 mM of each oligonucleotide primer (Set 2), 2.5 U of Taq DNA polymerase (Fermentas), 0.5 µl of bovine serum albumin (BSA) 1% (v/v), and 2 µl of amplicon from the previous reaction. Amplification conditions comprehended an initial denaturation step of 95°C for 5 min, followed by 20 cycles of 95°C for 40 s, 65 down to 55°C (-0.5°C at each cycle) for 1 min and 72°C for 1 min, 20 cycles of 94°C for 40 s, 55°C for 40 s and 72°C for 1 min, and a final extension step of 72°C for 5 min. Amplification success was confirmed with electrophoresis on agarose gel 1.2% (m/v) in TBE buffer 0.5x at 90 V for 90 min. Gel was stained in a solution of GelRedT™ 1x (Biotium, CA, USA). PCR products of the second reaction were separated based on GC composition by DGGE analysis, using 9% acrylamide gel within a denaturing gradient of 45% to 65% of urea and formamide.

#### Molecular techniques for bulk sediment: PCR for *assA* and *bssA*

To assess the presence of potential anaerobic hydrocarbon degraders at the mangrove, bulk sediment of the three studied depths were submitted to PCR targeting the genes responsible for anaerobic alkane degradation, and anaerobic toluene and xylene degradation. For these the oligonucleotide primers used were *assA* 2 F/R (Aitken *et al.*, unpublished observations) and *bssA* [22] (Table 2). PCR mixture for *assA* 2 F/R consisted of a 50 µl mixture, containing 5 µl of 10x buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM dNTP, 0.2 µM of each primer, and 1 U of Taq DNA polymerase (Promega), and 1 µl of the template DNA. Amplification conditions included an initial denaturation step of 95°C for 5 min, followed by 35 cycles of 94°C for 1 min, 56°C for 1 min and 72°C for 1 min, and a final extension step of 72°C for 5 min. PCR mixture and conditions for *bssA* followed what was previously described elsewhere [23].

#### Molecular techniques for bulk sediment: q-PCR for 16S rRNA and *dsr* genes

Quantitative PCR (q-PCR) assays were carried out using ABIPrism 7500 (Applied Biosystems) detection system, to quantify abundance of the gene encoding the 16S rRNA, following manufacturer's recommendations.

Amplification consisted of a 25 µl reaction containing 12.5 µl of GoTaq® q-PCR Master Mix 2x (Promega), 40 mM Tris-HCl (pH 8.4), 100 mM KCl, 6 mM MgCl<sub>2</sub>, 400 µM dATP, 400 µM dCTP, 400 µM dGTP, 800 µM dUTP, 40 U/ml UDG (Invitrogen), 200 nM of each primer, 0.5 µl ROX Reference Dye 50 mM (Invitrogen), 0.5 µl BSA (1 mg/ml), 5.5 µl H<sub>2</sub>O and 2 ng DNA. Oligonucleotide primers used were 357 F (5'-CTA CGG GRS GCA G-3') and 529R (5'-CGC GGC TGC TGG CAG-3'), modified from Muyzer and colleagues [39]. The assays were performed in triplicates. A standard DNA sample was previously used to make a standard curve, and H<sub>2</sub>O was used as the negative control. PCR conditions consisted of an initial denaturation step of 94°C for 3 min, followed by 30-40 cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 45 s.

A q-PCR was also used to quantify SRB population, with ABIPrism 7500 (Applied Biosystems) detection system, to quantify abundance of the gene *dsr*. Amplification step was carried out with a 25 µl mixture containing 12.5 µl of GoTaq® q-PCR Master Mix 2x (Promega), 0.5 µl of each primer 10 µM, 0.5 µl BSA (1 mg/ml), 4.5 µl H<sub>2</sub>O and 2 ng DNA [41]. Oligonucleotide primers used were DSR1F (5'-ACS CAC TGG AAG CAC GGC GG-3') and DSR-R (5'-GTG GMR CCG TGC AKR TTG G-3') [23]. PCR conditions consisted of an initial denaturation step of 95°C for 5 min, 35 cycles of 95°C for 1 min, 57°C for 1 min and 72°C for 45 s. All samples were used in triplicates and H<sub>2</sub>O was used as the negative control.

To both reactions (16S rRNA and *dsr* gene) efficiencies and melting curves were determined and analysed using ABIPrism 7500 Detection System (Applied Biosystems).

**Table 2 Primers for anaerobic hydrocarbon degradation genes detection**

Primer Set	Forward (F) and Reverse (R) Oligonucleotide Primer Sequences	Expected amplicon size (bp)	Reference
SP9/ASP1 ( <i>bamA</i> )	F: 5'-CAG TAC AAY TCC TAC ACV ACB G-3' R: 5'-C MAT GCC GAT YTC CTG RC-3'	~300	[20]
<i>assA</i> 2F/R ( <i>assA</i> )	F: 5'-YAT GWA CTG GCA CGG MCA-3' R: 5'-GCR TTT TCM ACC CAK GTA-3'	440	Aitken <i>et al.</i> , unpublished observations
7772 F/8546R ( <i>bssA</i> )	F: 5'-GAC ATG ACC GAC GCS ATY CT-3' R: 5'-TCG TCG TCR TTG CCC CAY TT-3'	~794	[22]

Oligonucleotide primers used in PCR reactions for anaerobic hydrocarbon degradation detection.



## Abbreviations

BSA: Bovine serum albumin; DGGE: Denaturing gradient gel electrophoresis; PCR: Polymerase chain reaction; PRAS: Pre-reduced anaerobic sterilized; q-PCR: Quantitative polymerase chain reaction; SRB: Sulphate reducing bacteria; UPGMA: Unweighted pair group method with average.

## Authors' contributions

LLA conceived of the study, and participated in its design and coordination and wrote the manuscript. DCAL carried out some of the molecular genetic studies. EMF helped with sampling and processing steps. LQF and GRP helped with anaerobic manipulation of samples and design of the experiments. MJM participated in the data interpretation. CH participated in the data interpretation and writing. RSP helped in the experiment design, data interpretation and wrote the manuscript. RMCPD and ASR were the major responsible by the experiment design, and helped in data interpretation and wrote the manuscript. All authors read and approved the final manuscript.

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