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CHEMICAL AND MICROBIOLOGICAL CHARACTERIZATION OF MANGROVE SEDIMENTS AFTER A LARGE OIL-SPILL IN GUANABARA BAY - RJ - BRAZIL

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SHORT COMMUNICATION

ABSTRACT

Seventeen months after a 1,3 million L oil spill into Guanabara Bay, analyses of mangrove sediments showed that the three sites closest to the spill remain highly polluted (>10 μg-g^{-1} polyaromatic hydrocarbons). A fourth site was less polluted, from which most hydrocarbon degrading bacteria were isolated.

Key words: mangrove, PAH, bioprospecting, bioremediation, oil spill, polyaromatic hydrocarbons

Mangroves are biologically important and productive ecosystems along subtropical and tropical coastlines, lagoons and estuaries. Undisturbed mangroves provide habitats for a wide variety of plants, animals and microorganisms. They have an important role in shore protection and are frequently associated with commercial fishing (11).They are at risk from acute/point pollution caused by freak accidents and from chronic pollution when associated with ports and petrochemical industries where repeated spills are common. In January 2000, more than 1,3 million L of heavy oil leaked from a refinery pipeline with visible pollution seen along 5 km of the Guanabara Bay coastline in Rio de Janeiro, oil covered sediments at points S1-S4 (Fig. 1) (9,17). This was not the first spill from the Duque de Caxias refinery but the largest in recent history. Although some of the effects of oil spills on mangrove ecosystems have been reported (3,8,10,11,12,16,20), little is known about chronic spill sites and the oil-degrading bacteria in such mangrove systems. Here we report on the physicochemical and microbiological state of four polluted Guanabara mangrove sites 17 months after being covered by oil and we compare that data with a nearby pristine mangrove. The chemical data is presented in the context of legal standards, the microbial data in colony forming unit (CFU) counts for heterotrophic bacteria (HB) and most probable number (MPN) for oil degrading bacteria. Oil degrading bacteria (DB) with biotechnological potential were isolated and identified from the rhizospheres of two mangrove plants: *Rhizophora mangle* and *Laguncularia racemosa*.

Bacterial counts and polyaromatic hydrocarbons (PAH) analyses were investigated from water, sediment and rhizosphere samples from the mangrove ecosystems in Guanabara Bay and Sepetiba Bay, Rio de Janeiro, Brazil. In Guanabara Bay, four contaminated sites were chosen. Sites S1 (22º 44' 06S; 043º 14' 21W), S2 (22º 44' 13S; 043º 14' 12W) and S3 (22º 58' 29S; 043º 13' 33W) are a transect extending from the spill site (S1) at the edge of the refinery mangrove along to the inner edge of Guanabara Bay (S3) on land belonging to the refinery. Site 4 (22º 41' 46S; 043º 06' 58W) is a more distant site that was also covered by oil as a result of the spill (Fig. 1). Site 5 samples (not shown in Fig 1) were collected from the mangrove at Marambaia Restinga, Sepetiba Bay, Rio de Janeiro, (23º 02' 30S; 043º 35' 44W), and represent a pristine mangrove and a reference to which analyses were compared.

Water collection for microbial sampling was done using 500 mL sterilized flasks. Sediment samples (0-5 cm depth) were

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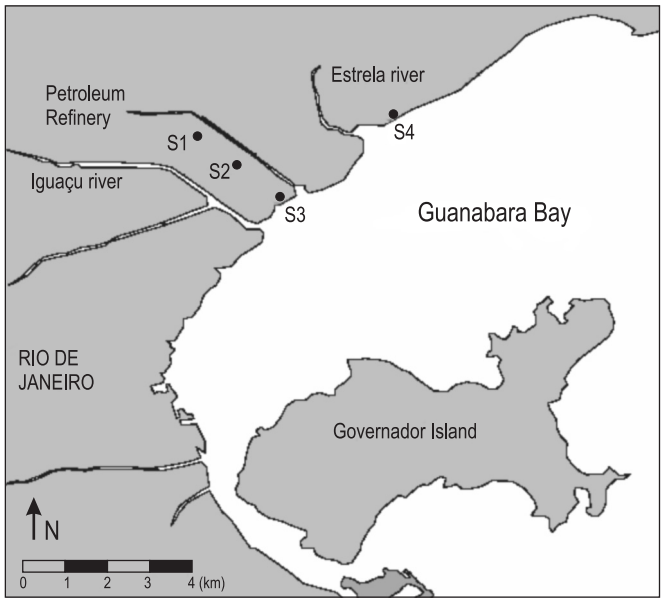

Figure 1. Map of the study area and the location of mangrove sampling sites S1, S2, S3 and S4 at Guanabara Bay.

collected using a sterile stainless steel spatula. Three replicate samples were randomly collected from each site to make a composite sample and this was used for all subsequent analyses. Two mangrove tree species, *R. mangle* and *L. racemosa*, were selected for this study. To obtain sufficient rhizosphere sediment for microbial counts, 5 samples of sediment that adhered to the roots of five mangrove plants of similar size (~80 cm) were pooled to form one composite sample. This process was repeated six times to provide six different samples for analyses. All samples were transported on ice to the laboratory where they were stored at 4ºC until be analyzed. Temperature, pH, salinity, oxygen, biochemical oxygen demand (BOD), phosphorous and nitrogen were analyzed from water samples using standard methods (2). Extraction of n-alkanes and PAHs from sediment samples followed the USEPA (21) standard protocol 8260 and 827ºC. The concentrations of n-alkanes and PAH compounds were analyzed from six replicate samples from each site using a gas chromatograph Varian (GC/MS) at the Innolab Laboratory, Harburg, Germany. Heterotrophic bacteria (HB) colony forming unit (CFU) counts from water, sediment and rhizosphere samples were carried out using the spread-plate technique on Marine Agar (Difco). Rhizosphere samples, 5 g of sediment, were suspended in 45 mL of 0.1% sodium pyrophosphate (Merck) containing 0.1% Tween 80 in Erlenmeyer flasks with glass beads. After 30 min shaking at 200 rpm, serial dilutions were made with sterile saline solution (0.85%). Plates were prepared in triplicate and incubated at 29ºC for 48 hours. Hydrocarbon-degrading bacteria (DB) were estimated by serial dilution in tubes with 10 mL of mineral salts medium (MM) using the MPN method (2). The MM was prepared as follows: 0.05% K_2HPO_4; 0.05% KH_2PO_4; 0.02% $MgSO_4 .7\ H_2O$; 0.01% $CaCl_2 .2\ H_2O$; 0.01% NaCl; 0.0002% $FeCl_3 .6\ H_20$; 0.05% NH_4NO_3; pH 7.0. Arabian Light crude oil was added, as the sole carbon source, to the sterilized media to a final concentration of 0.5% (vol/vol). The media was then inoculated and incubated for 7 days at 29ºC. After incubation, 50 mL aliquots from growth-positive tubes of rhizosphere samples were inoculated in MM plus crude oil and incubated for further 7 days at 29ºC. Sub-samples (1 mL) from the MPN tubes were taken and plated onto Tryptic Soy Agar (Difco). Isolates were described as petroleum degraders after growth in media with crude oil as the sole carbon source. Those bacteria that degraded the C15, C18 fractions and the Arabian Light crude oil at 0.5% (vol/vol) within 48 hours, resulting in an emulsion, were identified by 16S rDNA sequencing and stored for future biotechnological purposes. DNA was extracted from isolates using MoBio Ultra Clean DNA Isolation kit (Mo Bio Inc. Solana Beach – CA, USA) and 16S rDNA Polymerase Chain Reaction (PCR) amplified using primers U968*f* (5' AAC GCG AAG AAC CTT AC 3') and L1401*r* (5' GCG TGT GTA CAA GAC CC 3') (Interactiva, Germany) and protocols of Nubel *et al.* (15). Partial 16S rDNA sequences (250-400 base pairs) were obtained by automatic sequencing using an ABI 310 sequencer and the PRISM Big Dye™ Termination Cycle Sequencing kit. Sequences were compared with those on public databases using BLAST in order to identify the most efficient oil degrading bacteria (1).

Data in Table 1 A, B, C demonstrate that concentrations of n-alkanes, total PAHs and individual PAH in surface sediments varied considerably among the Guanabara mangrove sites (S1-S4) and between Guanabara and Marambaia site (S5). The amounts of PAHs accumulated in Sites S1 to S3 sediments were much higher than S4. N-alkanes and PAH values were below detectable levels at the pristine reference site (S5). Data in Table 1C showed increased BOD with distance from the oil spill (S1 to S4) but did not reach the characteristically high BOD levels typical of mangrove sediments (S5). At Sites S1, S2 and S3 the ammonia levels were four to five times higher than the Brazilian National Environment Agency (IBAMA) guidelines (6). CFU counts of heterotrophic bacteria (HB) in the water samples were similar for all five sites (10^5 CFU mL^{-1}). No impact on HB counts in water was apparent 17 months after the spill (Table 2). The counts for degrading bacteria (DB) were higher in water from the four polluted sites than at the pristine site S5. The sediment samples showed one order of magnitude increase in HB compared with the water samples at sites S1-S3 and a two order of magnitude increase for sites S4 and S5 (ranging from 10^4 - 10^7 MPN/100 mL^{-1}). Counts of DB from sediments at S1 to S5 were an order of magnitude higher than their equivalents from water (Table 2). Counts of rhizosphere HB and DB remained relatively constant for *R. mangle* and *L. racemosa* during the study period. No rhizosphere effect on counts could be seen. The trend of a lower MPN for DB from rhizosphere sediments at the pristine site

Table 1. Chemical characterization of mangrove sediments of Guanabara Bay (S1-S4) and Marambaia *Restinga* Mangrove (S5).

A	SAMPLING SITES					Guidelines[a]	
PAH compounds	S1*	S2*	S3*	S4*	S5*	ER-L	ER-M
Naphthalene	883	1416	1066	ND	ND	160	2100
Acenaphthalene	ND	ND	ND	ND	ND	44	640
Acenaphtene	166	166	ND	ND	ND	16	500
Fluorene	150	233	266	ND	ND	19	540
Phenanthrene	483	616	416	ND	ND	240	1500
Antracene	366	333	66	ND	ND	85	1100
Fluoranthene	183	333	83	100	ND	600	5100
Pyrene	3533	6150	2000	200	ND	665	2600
Benz(a)anthranecene	2533	5416	1650	300	ND	261	NA
Chrysene	4966	7050	3016	100	ND	384	2800
Benzo(b)fluoranthene	750	1416	433	266	ND	NA	NA
Benzo(k)fluoranthene	183	183	ND	66	ND	NA	NA
Benzo(a)pyrene	1950	3333	1500	200	ND	430	1600
Indeno(123-cd)pyrene	ND	ND	50	ND	ND	-	NA
Dibenzo(ah)anthracene	ND	ND	ND	ND	ND	63	260
Benzo(ghi)perylene	700	1633	980	ND	ND	NA	NA
∑ PAHs	16846	28278	11526	1232	-	4022	44792

[a] Adapted from Tam *et al.* (20); ER-L, Effects Range Low; ER-M, Effects Range Median; (ng g^{-1}). *Mean values of six replicates; S1, S2 and S3: Refinery sampling sites; S4: Sampling site close to the Refinery; S5: Pristine site; NA - not available; ND - not detected.

B	SAMPLING SITES				
n-Alkanes	S1	S2	S3	S4	S5
nC10	ND	ND	ND	ND	ND
nC11	6.3	3.6	10.8	ND	ND
nC12	12.6	21.3	15.1	ND	ND
nC13 – nC38	206	300	281.8	44	ND
∑ n-Alkanes	225.7	324.9	307.7	44	-

*Mean values of six replicates; S1, S2 and S3: Refinery sampling sites; S4: Sampling site close to the Refinery; S5: Pristine site; ND - not detected.

C	SAMPLING SITES					CONAMA*
General parameters	S1	S2	S3	S4	S5	**Guidelines**
BOD (mg L^{-1})	3.6 ± 1.2	5.2 ± 1.7	6.0 ± 2.3	7.4 ± 3.9	11.4 ± 9.7	d≤5
Dissolved oxygen (mg L^{-1})	5.2 ± 2.4	3.7 ± 1.5	5.1 ± 3.5	7.1 ± 4.5	1.6 ± 0.1	e≥5
N-NH_3 (mg L^{-1})	1.7 ± 0,5	1.7 ± 0.7	2.1 ± 0.4	0.17 ± 0.2	0.3 ± 0.1	0.4
N-NO_3^- (mg L^{-1})	0.17 ± 0.1	0.16 ± 0.1	0.11 ± 0.1	0.02 ± 0.01	0.02 ± 0.01	-
N-NO_2^- (mg L^{-1})	0.06 ± 0.03	0.04 ± 0.02	0.05 ± 0.04	0.003±0.003	0.001±0.001	-
pH	7.1 ± 0.6	7.4 ± 0.1	7.5 ± 0.2	8.3 ± 0.3	7.6 ± 0.3	6.5 - 8.5
Phosphorous (mg L^{-1})	0.7 ± 0.6	1.2 ± 0.5	1.2 ± 0.5	0.3 ± 0.3	0.12 ± 0.08	-
Salinity (mg L^{-1})	19 ± 3.1	20 ± 2.6	19 ± 2.3	22 ± 5.0	29.6 ± 1.9	-
Temperature (ºC)	25 ± 0.7	25 ± 0.7	25 ± 0.7	27	26.5 ±0.5	-

Mean and Standard Deviation (n = 6) for S1, S2 and S3: Refinery sampling sites; S4: Sampling site close to the Refinery; S5: pristine site; *Brazilian Guidelines based on CONAMA Resolution Nº 20/86 (www.ibama.gov.br) - not defined.

Table 2. Microbial counts from Guanabara Bay (S1-S4) and Marambaia *Restinga* Mangrove (S5).

Sampling Sites	BACTERIAL COUNTS							
	Water		Sediment		*Rhizophora mangle* Rhizosphere		*Laguncularia racemosa* Rhizosphere	
	HB	DB	HB	DB	HB	DB	HB	DB
S1	4.9 ± 0.2	2.5 ± 0.6	5.8 ± 0.7	4.4 ± 0.2				
S2	4.9 ± 0.3	2.6 ± 0.6	5.9 ± 0.6	4.4 ± 0.3	5.4 ± 0.8	3.6 ± 0.5	5.0 ± 0.8	3.5 ± 0.3
S3	5.0 ± 0.5	2.4 ± 0.4	6.2 ± 0.9	3.8 ± 0.6				
S4	4.7 ± 0.8	2.7 ± 0.4	7.1 ± 0.8	4.0 ± 0.4	6.8 ± 0.5	3.8 ± 0.4	7.0 ± 0.4	3.8 ± 0.3
S5	4.9 ± 0.5	1.8 ± 0.2	7.3 ± 0.5	3.3 ± 0.4	7.2 ± 0.2	2.5 ± 0.2	7.3 ± 0.2	2.4 ± 0.1

S1, S2 and S3: Refinery sampling sites; S4: Sampling site close to the Refinery; S5: Pristine site; Water Samples HB: Heterotrophic Bacteria (Log CFU/mL); DB: Petroleum-Degrading Bacteria (Log MPN/100 mL) Sediment samples: HB: Heterotrophic Bacteria (Log CFU/g fresh wt.) DB: Petroleum-Degrading Bacteria (Log MPN/g fresh wt.); Rhizosphere samples: HB: Heterotrophic Bacteria (Log CFU/g fresh wt.) DB: Petroleum-Degrading Bacteria (Log MPN/g fresh wt.) Values are mean ± 1 standard deviation (n = 6).

compared with rhizosphere sediments from polluted sites was again observed. Of the 400 degrading bacterial strains initially isolated from rhizosphere soils, 78 repeatedly grew under laboratory conditions and were tested for oil degradation potential. From the 78, eleven strains degraded oil within 48 hours and were selected for identification by partial 16S rDNA sequencing. Ten of the eleven strains sequenced were identified as *Bacillus* and the remaining strain as *Acinetobacter*. Three *Bacillus* strains (MC6, MC7, MC11) shared less than 95% 16S rDNA partial sequence similarity (~500 base pairs) compared with described type strains. These strains require further investigation to determine whether they merit new species status. The remaining strains had partial sequences (~500 base pairs) which were >97% similar to well described and known type strains.

Total PAH levels in surface sediments up to 20 $\mu g\text{-}g^{-1}$ at S3 area were detected ten days after the spill in 2000 by Gabardo *et al.* (9). They importantly raised the issue that previous spills into Guanabara Bay from the same refinery made it difficult to determine whether their values recorded ten days after the large spill could be solely attributed to that particular spill. Seventeen months later, evidence of oil could still be seen on the trunks and roots of mangrove vegetation and sediments at all four Guanabara sites (S1-S4). Mean total PAHs for S1 and S2 were found to be 16.8 and 28.3 $\mu g\text{-}g^{-1}$ respectively, which are similar to values reported by Gabardo *et al.* (9). Site 3 was lower at 11.5 $\mu g\text{-}g^{-1}$ and S4 much lower at 1.23 $\mu g\text{-}g^{-1}$ and as expected PAHs were not detected at S5. Placing these results in a global context, it is noted that the PAH levels in Guanabara Bay were between two and six times higher than the highest levels (6.19 $\mu g\text{-}g^{-1}$) found from 20 sampling sites in four mangroves in Hong Kong (20). Moreover, Tam *et al.* (20) compared their data with further fourteen sites in Hong Kong, China, the Caribbean and Puerto Rico where the highest PAH level reported was 2.2 $\mu g\text{-}g^{-1}$. Clearly the levels of PAHs reported here are very high by global comparison. In another study on PAHs (7) the important role of mangrove sediments as traps, or concentrating sites, for oil pollution was stressed. Medeiros *et al.* (17) reporting on an estuarine lagoon in Southern Brazil found mean total PAHs at 11.8 $\mu g\text{-}g^{-1}$ from sediments next to a petroleum distribution plant and 4.4 $\mu g\text{-}g^{-1}$ from refinery sediments. These sites were 100 and 40 times more contaminated than a nearby lagoon and were described as sites of chronic/repeated pollution. From this preliminary study, we suggest that sites S1-S3, which continued to be highly PAH contaminated 17 months after the spill, are sites of repeated pollution (18,24). High levels of ammonium, a labile compound (Table 1C), are indicative of chronic industrial and sewage pollution, and were also found at sites S1-S3. This type of pollution has been independently reported by Paranhos *et al.* (21) in vicinity of S1-S3. The much lower PAH levels at S4 (1.23 $\mu g\text{-}g^{-1}$) might indicate that this site is not repeatedly polluted and natural bioremediation is reducing PAH levels (15), however, to test this hypothesis new experiments are needed. Seventeen months after the spill, CFU counts did not reveal a disturbance in the numbers of heterotrophic bacteria in the water samples; however, they were lower in the polluted sediments than at the pristine site. Conversely, MPN numbers of degrading bacteria were higher from sites S1-S4 than S5. These findings are reasonable and are explained as an overall decrease in total HB bacteria as a result of the pollution and an enrichment of degrading bacteria in response to the enrichment of hydrocarbons spilled into the environment. A rhizosphere effect was not observed for *R. mangle* and *L. racemosa* which could reflect the difficulties in representative sampling from firstly the rhizosphere and secondly from tidal sediments (15). Seven of the best eleven petroleum-degrading strains were isolated from the rhizosphere of plants at S4, an interesting Site. The Site of intermediary pollution and possibly

in recovery warrants further investigation to establish if there is a link between PAH concentration and biodegradation rates; similarly, is there an important difference between point versus chronic pollution sites for bioprospecting? Strains from the genera isolated in this study have frequently been reported as DB (6). Ten of the eleven most efficient petroleum degraders belonged to genus *Bacillus*, already recognized as a bacterial group of considerable practical importance for biotechnological applications. Several *Bacillus* strains from soils and mangrove sediments have already been reported as hydrocarbon degraders and emulsifier producers (10,13,23). Our results support those of Macrae *et al*. on soil (16), who found bacilli as dominant rhizosphere organisms and suggested that they should be targeted to provide microbial solutions which ameliorate polluted environments. Clearly, the desire to remediate pollution exists and our preliminary findings support the notion that it is in polluted sites that we are likely to find the microbes best adapted to carry out bioremediation.

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RESUMO

Caracterização química e microbiológica de sedimentos de manguezal após um grande derramamento de óleo na Baia de Guanabara, RJ, Brasil

Dezessete meses após um derramamento de 1,3 milhões de litros de óleo na Baía de Guanabara, análises de sedimento do manguezal mostraram que os três pontos de amostragem mais próximos do local do acidente permanecem altamente poluídos (>10 $\mu g\text{-}g^{-1}$ hidrocarbonetos poliaromáticos). Do quarto ponto de amostragem, o menos poluído, foi isolada a maioria das bactérias degradadoras de hidrocarbonetos.

Palavras-chave: manguezal, HPA, bioprospecção, biorremediação, derramamento de óleo, hidrocarbonetos poliaromáticos

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