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Abstract

Mangrove sediment harbors a unique microbiome and is a hospitable environment for the growth of a diverse group of bacteria capable of oil biodegradation. Our goal was to understand bacterial community dynamics from mangrove sediments under heavy-oil contamination stress, and to look for common patterns that may be associated with oil biodegradation is such environments. We tested the hypothesis of a two-phase pattern of petroleum biodegradation, already reported in the literature, where key events in the degradation process take place in the first three weeks after the contamination. Two sample sites with different oil pollution history were compared through T-RFLP analyses and using a pragmatic approach based on the Microbial Resource Management Framework. Our data corroborated the already reported two-phase pattern of oil biodegradation, although the original proposed explanation is questioned, opening up the possibility to consider other plausible hypothesis of microbial interactions as the main drivers of this pattern.

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Introduction

Microorganisms are the most abundant and diverse organisms on Earth (Whitman et al. 1998). Yet, little is known about the various patterns of microbial distribution across different environments. Microbial communities in contaminated ecosystems tend to be dominated by the organisms that can degrade or tolerate the contaminant. Since contamination is a strong selection force, these communities are typically less diverse than those in non-stressed ecosystems. Several studies on oil contamination reported a drastic short-term reduction in the diversity of the bacterial communities, which could be accounted for by oil toxicity and strong selection for particular hidrocarbonoclastic bacteria, such as *Alcanivorax spp* and *Cycloclasticus spp* (Hazen et al. 2010; Kostka et al. 2011; Jurelevicius et al. 2013; Kimes et al. 2013; Sutton et al. 2013). Loss of biodiversity has implications for ecosystem functioning as well as the delivery of ecosystem services (Cardinale et al. 2012), and are of comparable magnitude to the effects of many other global environmental changes (Hooper et al. 2012). It is estimated that worldwide mangrove forests provide at least US $ 1.6 billion each year in ecosystem services (Polidoro et al. 2010).

Several studies suggest that mangrove is a hospitable environment for the growth of a diverse group of bacteria capable of oil biodegradation (Ramsay et al. 2000; Brito et al. 2006; Gomes et al. 2008; Tian et al. 2008; Liu et al. 2011; Santos et al. 2011; Jurelevicius et al. 2013). Mangroves are intertidal ecosystems along the coastlines of tropical and subtropical regions, with unique features such as high primary productivity, abundant detritus, rich organic carbon content and anoxic/reduced conditions (Ghizelini et al. 2012). In tropical mangroves, bacteria and fungi constitute 91% of the total microbial biomass, whereas algae and protozoa represent only 7% and 2%, respectively (Alongi 1988). It has been proposed that the microbial structure and function of mangroves are directly responsible for this ecosystem functioning (Holguin et al. 2001). Mangrove sediments harbor a unique microbiome and metabolic reconstructions suggest that ecological processes may be modulated by the prevailing conditions found in mangrove (Andreote et al. 2012).

Diversity is a function of two components: (i) species richness and (ii) species evenness or equitability. These two concepts are difficult to assess, especially when considering microbial diversity of complex ecosystems. We approached this problem by using a pragmatic approach aiming at understanding bacterial community dynamics from mangrove sediments under heavy-

oil contamination stress, and at looking for common patterns that may be associated with oil biodegradation is such environments (that is, a process that may govern community dynamics under such conditions).

Materials and Methods

Sampling sites and sample collection

Four sampling sites were chosen with respect to their different hydrocarbon pollution history. Sampling sites GBA (R22º41'14.5"S; 43º05'6.83"O) and GBB (22º41'1.55"S 43º05'9.21"O) were located in the Guanabara Bay, in the city of Rio de Janeiro, Brazil, and sampling sites GR (21º36'27.85"S 41º03'05.74"O) and GV (21º35'9.11"S 41º03'39.70"O) were located in Gargaú, in the city of São Francisco do Itapaboana, in the northern part of the state of Rio de Janeiro, Brazil (Fig 1). Physicochemical parameters for the four sampling sites are shown in Table 1.

Figure 1: Location of the sampling sites considered in this study.

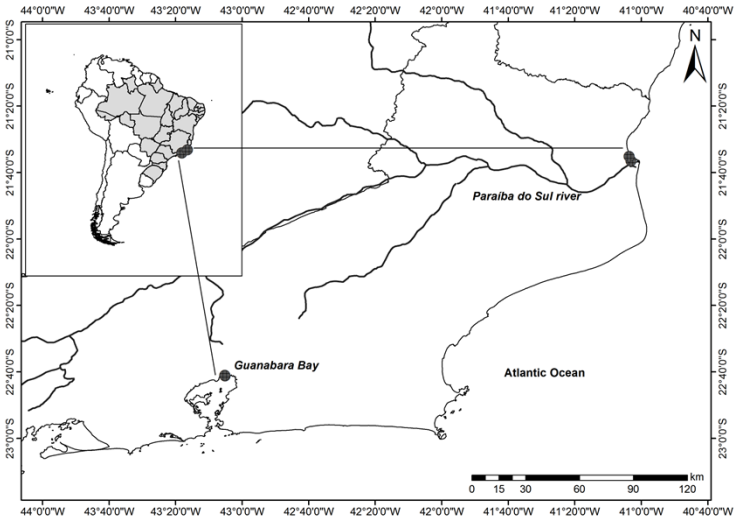


Table 1: Physicochemical parameters of the four sampling sites considered in this study.

		GBA	GBB	GV	GR
pH		7,7	7,6	6,8	6,1
Salinity		24	24	4	3
Granulometry (%)	Sand	30	76	14	12
	Clay	13	6	18	20
	Silt	57	18	68	68
C_{org} (%)		5,72	0,75	5,86	7,56
N (%)		0,24	0,04	0,39	0,43

Guanabara Bay is notorious for its chronically polluted conditions, with a history of oil spill accidents (Ghizelini et al. 2012). The mangrove in Gargaú is located in the estuary of Rio Paraíba do Sul, the biggest estuary in the northern region of the state of Rio de Janeiro. The degradation of this mangrove is related primarily to selective logging and deforestation for the implantation of pastures for cattle ranching, raw sewage, urban runoff, industrial waste release, and construction of roads and landfills (Bernini et al. 2010). There is no record of oil spill in this area.

For each site, three composite samples consisting of five sediment cores each (c. 10 cm of top sediment with 8 cm diameter) were randomly collected. The samples were at least 10 m apart from each other and within each sample the cores were at least 1m distant from each other. Sampling was done during the low tide and transported to the laboratory in a insulated container with ice. Upon arrival at the laboratory, the composite samples were thoroughly homogenized to one representative sample per locality and immediately processed.

Molecular analyses

Total genomic DNA was extracted from the sediments at each sampling site using the UltraClean Soil DNA Isolation Kit (MoBio), following the manufacturer's instructions. The extractions were performed immediately after the sample collection at the following intervals: 7, 14, 21, 28, 60, 90, 120, and 150 days. Primers 27F (5' AGAGTTTGATCCTGGCTCAG) labeled at the 5' end with 6-carboxyfluorescein (6-FAM), and 1525R (5' AAGGAGGTGWTCCARCC) were used to amplify approximately 1500 bp of the 16S rRNA gene. The PCR reaction (20 µl)

contained 10 ng of template DNA, 5 pmol of each primers, 10µL do kit *HotStarTaq® Master Mix Kit* (Qiagen), 5µL de water of kit. The amplification conditions were 1 cycle of 94^0C for 5 min, followed by 35 cycles of 94^0C for 30 sec, 52º C for 30sec, with a final extension of 72^0C for 1 min and 30 sec. Amplicons (20ng) were digested using MnlI following the manufacture's instructions. The digested DNA was ethanol- precipitated and resuspended with 14,8 µL de Hi-Di formamida mixed with 0,2 µL de *standard Gene Scan 600 Liz* (Applied Biosystems). After this, the sample was separated by capillary electrophoresis in an ABI 3500 Genetic analyzer and analyzed with Genemapper version 4.1 (Applied Biosystems), using a baseline detection value of 5 fluorescence units. All T-RFs over this baseline value and with lengths from 50 to 600 bp were rounded to the nearest integer. Peak filtration and binning were performed with R software using the IBEST script suite (Abdo et al. 2006). True peaks (operational taxonomic units, OTUs) were distinguished from background noise, based on a three-fold standard deviation (IBEST default). Each peak corresponded to one OTU.

Data Analysis

The T-RFLP data consisted of four data sets: GBA, GBB, GR and GV. These datasets were analyzed considering two periods: the first month, when the bacterial communities were monitored weekly (time points 0, 7, 14 21 and 28 days); and the four consecutive months, when the bacterial communities were monitored monthly (time points 60, 90, 120, 150). This strategy was based in the two-phase pattern of petroleum degradation, where key events in the degradation process take place in the first three weeks after the contamination (Kaplan & Kitts, 2004). The relative abundances of the binned MnlI fragments were used to monitor changes in the bacterial community along the oil biodegradation process. A pragmatic procedure, proposed by Marzorati et al. (2008) and reviewed by Read et al. (2011), was used to describe the bacterial community structure and dynamics of each dataset. Briefly, the range-weighted richness index (Rr) was estimated as the total number of peaks in the electropherogram. The dynamics of the community (Dy) was estimated by calculating the rate of change parameter (Δt) through moving-window analyses (MWA). First, a matrix of similarity was calculated based on the Pearson correlation coefficient. The percent change (percent change = 100 – percent similarity) was then calculated. The percent change value matrix was used to perform MWA by plotting the values between day x and day x – 7 days for the first month, and day x and day x – 30 days for the

following months. The rate of change (Δt) was calculated as the average and standard deviation of the respective percent change values. In addition, moving-endpoint analyses (MEA) was performed, comparing the community profiles from different time points with the profile from the first sampling point as a reference fingerprint. The community organization (Co) was calculated as the percentage of the Gini coefficient (Wittebolle et al. 2009). To evaluate the most similar communities across time, a similarity matrix based on the Jaccard coefficient was computed considering the four datasets together and hierarchically clustered with the Ward's linkage method. All calculations were computed with the MASS and Vegan packages in the R statistical environment (version 3.0.1) (R Core Team 2013; Oksanen et al. 2013). Co was computed as described in Buckley & Damgaard (2012).

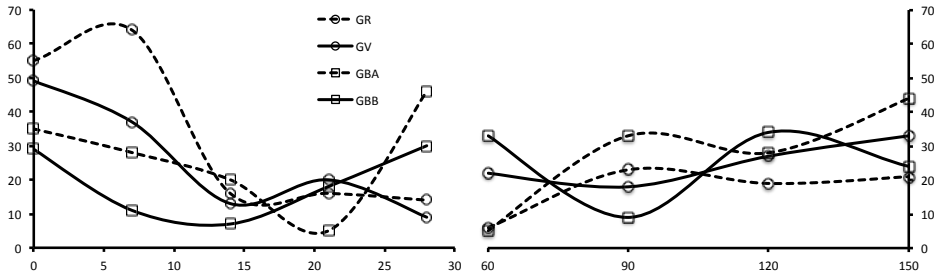
Results

Bacterial community structure and dynamics

Range-weighted richness (Rr)

This parameter translates the approximate carrying capacity for microbial diversity and ranged from 64 to 5 OTUs in the first time-interval and 46 to 5 OTUs in the second time-interval (Fig 2). GR and GV had more OTUs than GBA and GBB at the beginning of the experiment. Upon petroleum exposure, all sites showed a dramatic decrease in OTUs, except GR which first showed an increase until day 7, followed by an sharp decrease by day 14. In general, all sites lost OTUs by day 14; thereafter, there was a tendency of sudden increase in OTUs for the Guanabara sites, whereas the Gargaú sites stayed more stable. At GBA, this turning point appeared to be later at day 21. In the second time-interval, there is a fluctuation in the number of OTUs along the biodegradation process, and no common pattern is found among the four datasets. By the end of the experiment, GBA was the richest community and the only community richer than when compared to the beginning of the experiment, and GR was the least richest community.

Figure 2: Range-weighted richness (Rr) at the four sampling sites during the first and second time- intervals (x axis= number of days; y axis= number of OTUs).



Community Dynamics (Dy)

Marked changes on bacterial community composition occur within the first week of heavy-oil exposure, reaching almost 100% in GR and GBB (Fig 3). This trend was alleviated in the following weeks, and GR, GBA and GBB reached a minimum at day 21. For these communities, day 21 was a turning-point in community composition; for GV this turning-point occurred one week later, at day 28. During the second time-interval, the percent change values did not appear to have a common pattern among the communities (Fig 3). These values fluctuates around 50% and 90% , but there was a drastic decrease from day 90 to day 120 for GV and GBA. By the end of the experiment, the least and most susceptible communities were GBA and GR, respectively. In general, the communities experienced more changes during the first time-interval than during the second time-interval (Table 2). This is to say, for example, that GBB changed on average 75% during the first time-interval, whereas GV changed on average only 33% during the same period. On the other hand, when the first time-point is taken as the reference for estimating the community change percent, it can be observed that the communities in each time-point of both time-intervals are completely different from the first time-point in GBA and GBB (Fig 4). On average, GBA and GBB changed 92% and 99% and 97 and 96% in the first and second-time intervals respectively (Table 2). Interestingly, GBA differed from GBB only during the first week, when it had a lower percent change value then GBB (Fig 4). On the other hand, GR and GV changed on average less than GBA and GBB. GR and GV appeared to have a common pattern of community change with a striking difference from day 60 to day 90 (Fig 4). By the end of the experiment, GR and GV was approximately 70% different from their respective first time-point.

Figure 3: The dynamics of the community (Dy) at the four sampling sites, estimated by calculating the rate of change parameter (Δt) through moving-window analyses (MWA) (x axis= number of days; y axis= % change).

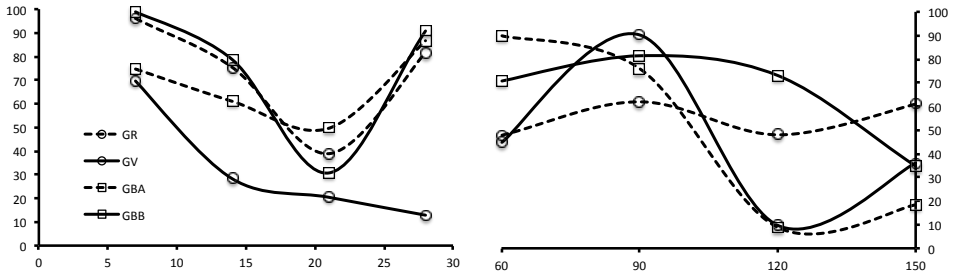


Figure 4: The dynamics of the community (Dy) at the four sampling sites, estimated by calculating the rate of change parameter (Δt) through moving-endpoint analyses (MEA) (x axis= number of days; y axis= % change).

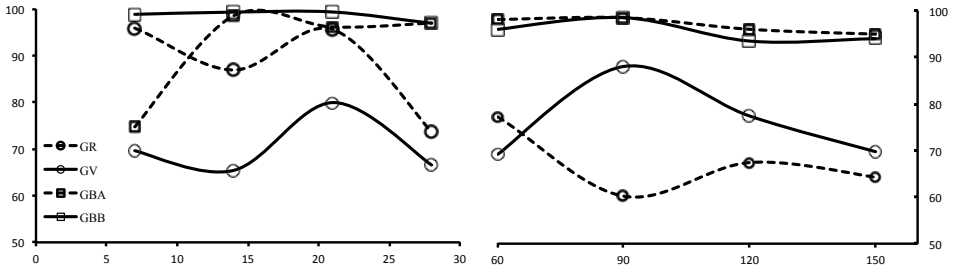


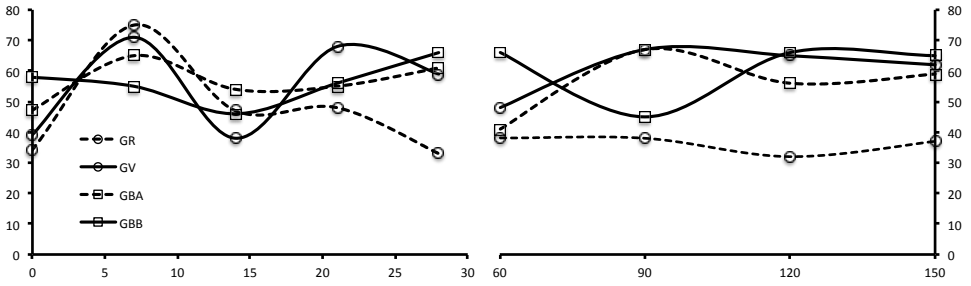
Table 2: Average change in the community dynamics at the four sampling sites, according to moving-window analysis at the first-time interval (MWA1) and the second-time interval (MWA2), as well as according to moving-endpoint analysis at the first-time interval (MEA1) and the second-time interval (MEA2).

	MWA 1	MWA 2	MEA 1	MEA 2
GR	73 ± 24.31	55 ± 7.80	88 ± 10.47	67 ± 7.29
GV	33 ± 25.29	45 ± 33.52	70 ± 6.61	76 ± 8.80
GBA	68 ± 16.12	48 ± 40.53	92 ± 11.29	97 ± 1.45
GBB	75 ± 30.48	65 ± 20.68	99 ± 1.14	96 ± 2.12

Community organization (Co)

This parameter reflects the evenness of the community. Low Co values (0 - 40) are typical for a highly even community, while uneven communities have high Co values (70 -100). The initial communities of GR and GV had a low organization, whereas GBA and GBB had a medium organization (Fig 5). During the first week, the organization values of GR, GV and GBA increased, indicating a very uneven community, especially for GR. After the third week, the organization of GV, GBA and GBB fluctuates around medium organization values, whereas GR stayed more stable, with a lower organization. By the end of the experiment, GV, GBA and GBB had a much uneven community than GR (Fig 5).

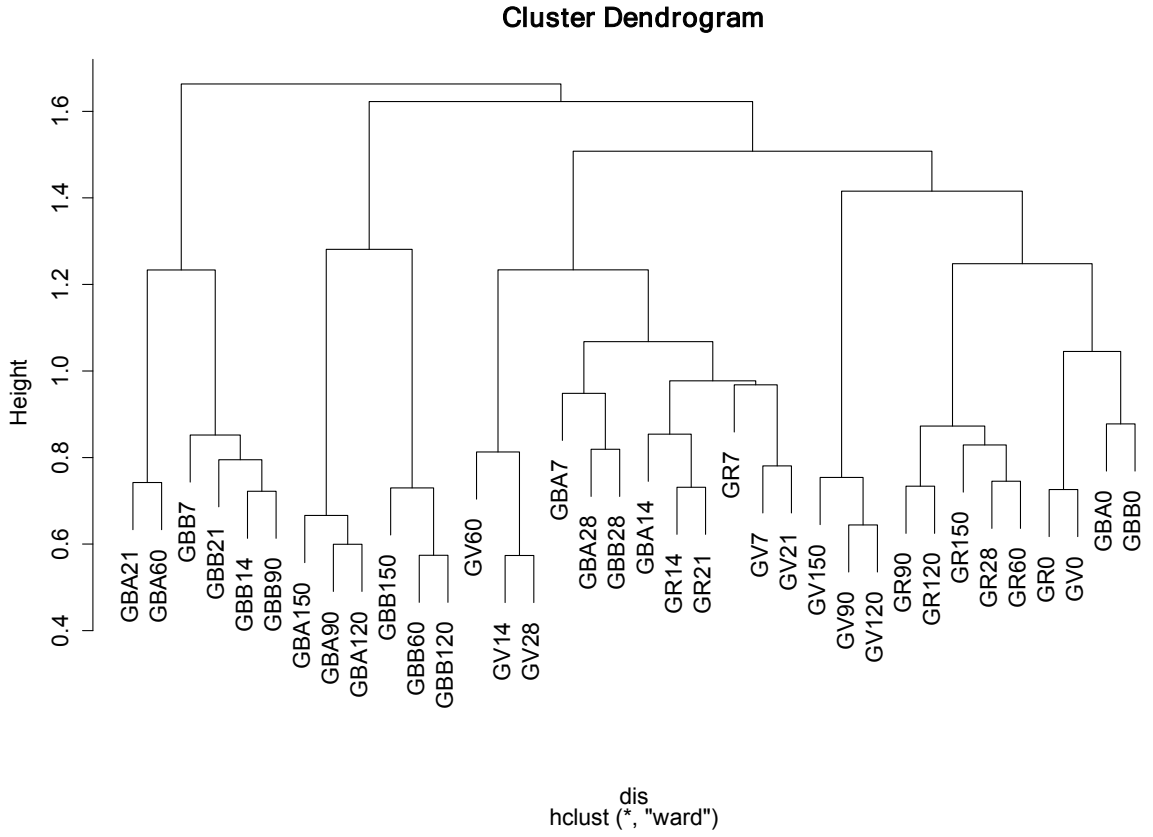
Figure 5: The community organization (Co) calculated as the percentage of the Gini coefficient (y axis) (x axis= number of days).



Hierarchical clustering

In order to compare the most similar communities, the four datasets were evaluated based on the Jaccard coefficient. Because of the complex dynamics, this coefficient was chosen, as it only takes into account the richness of the communities, whereas the Pearson coefficient is also influenced by their abundance. Six main clusters were observed (Fig 6), which were divided into initial, intermediate and final communities. The initial communities (GR0, GV0, GBA0, GBB0) were grouped together with the second phase communities from GR (GR28-GR150) in cluster 6. The final communities from GV, GBA and GBB had each a distinct cluster (clusters 5, 2 and 3, respectively), and the final communities from GV were more similar to the initial communities. The intermediate clusters 1 and 4 grouped samples mainly by time points. In these two clusters, there are samples from days 7, 14, 21, 28 and 60. The most distinct cluster (cluster 1) had samples from GBA and GBB and these samples also clustered together by temporal shifts.

Figure 6: Dendrogram of the four datasets (GBA, GBB, GR and GV for each time-interval considered - 0, 7, 14, 21, 28, 60, 90, 120, and 150 days), based on the Jaccard coefficient and using the Ward's linkage method.



Discussion

The focus of this work was to assess the ecological aspects of mangrove bacterial communities under heavy-oil exposure, using the three levels of analysis (Rr, Dy and Co). We visually followed the disappearance of the oil associated with observed changes in the bacterial community along the experiment. Our results were obtained from T-RFLP fingerprinting. This technique is a high-throughput, culture-independent method for community profiling originally developed for characterizing highly diverse bacterial communities (Liu et al., 1997). It is a reproducible and robust method that results in high-quality community fingerprints (Osborn et al. 2000). Although there are also important limitations, (Schütte et al. 2008), T-RFLP results are generally consistent with the results from clone libraries (Dunbar et al. 2000; Hackl et al. 2004)

and next-generation sequencing (NGS) technologies (Bokulich et al. 2012; Camarinha-Silva et al. 2012; Pilloni et al. 2012).

We studied the responses of two very different mangroves, especially with respect to both salinity and oil pollution. Guanabara Bay has a much higher salinity value than Gargaú and has been exposed to multiple oil spill accidents, whereas in Gargaú there has never been an oil spill. Taking these two factors together, our results indicate that oil exposure has a long-term effect in bacterial community structure, and that the bacterial assemblages are different as overall samples from Guanabara did not cluster together with samples from Gargaú. Oil contamination and the associated environmental changes have been shown as the predominant factor shaping the function composition and structure of microbial communities (Lu et al. 2012).

Based on the hierarchical clustering, three major temporal changes can be observed in the communities along the biodegradation process: (1) initial communities at day 0; (2) intermediate communities from day 7 to day 60; and (3) final communities from day 90 to day 150. The initial communities clustered together with GR, from day 28 to 150. These are the communities with low organization, indicating that they have many rare OTUs in common. These communities clustered together with the final community of GV. During this period, GV increased its richness, slightly increased its evenness and had an abrupt low dynamics from day 90 to day 120. When taking the first day as a reference point (MEA), GV showed a tendency of decreasing in percent change from day 90 on. Altogether, these data might be an indication that GV is reestablishing its initial community. On the other hand, the final communities in Guanabara Bay have their unique cluster, with two subclusters from each sampling site, indicating final communities different from the initial communities, and different from each other. Strikingly, in the GBB sub-cluster, day 60 appeared where day 90 is expected to be. The intermediate communities were separated by three clusters and in one of these clusters is the only instance where samples from different sampling sites clustered together. These three clusters are apparently grouped by temporal shifts related to different phases of the oil degradation process. The composition of the developing bacterial community varies along the biodegradation steps, as well as among the four datasets. As showed in the hierarchical cluster, most samples did not cluster by geography, but by temporal shifts in the community. A visual inspection of the data clearly indicated different assemblages of samples from the four datasets in each time point. These different assemblages may be the result of microbial endemism, which has been shown

over a range of environments (Nemergut et al. 2011). However, when considering abundance of OTUs, these different bacterial assemblages have some common most abundant OTUs, particularly OTU 249 and OTU 274. Abundant organisms were more likely to be widely distributed in soil assemblages (Nemergut et al. 2011).

Oil contamination had a significant effect on the composition of the communities, especially at the beginning of the experiment. Within seven days of exposure, GR and GBB communities changed almost 100% compared to their initial communities (Fig 3a). GV and GBA also showed a change in their respective communities during this time, although to a lesser extent. These high percent values indicate highly dynamic communities (i.e. open communities). Nevertheless, during this same period, there was a decline in richness for all sites, except GR, indicating a loss of OTUs. Also, there was a shift in community organization during this time, especially for GR and GV which went from low to high organization, indicating the dominance of few OTUs. Altogether, these data suggest a strong selection for hydrocarbon-degrading bacteria.

The dynamics of the communities along the experiment support the two-phase pattern of oil biodegradation (Kaplan and Kitts, 2004), with the breakpoint at 21 days for GR, GBA and GBB and at 28 days for GV. This two-phase pattern is characterized in the literature by a first phase of fast petroleum degradation with high abundance of few species, followed by a second phase of slower petroleum degradation with high richness of low abundant species. Our data suggest an overall tendency in richness decrease by day 21, although more marked by day 14. From day 21 on, these communities fluctuates between low (< 10) and high (>30) richness values. Moreover, the organization of the communities from day 21 on seems to reflect a marked different response between GR and the other communities.

This two-phase pattern of oil degradation has been related to the bioavailability of free total petroleum hydrocarbons (TPH) in the first phase and with a slower desorption rate of soil-sequestered TPH in the second phase (Kaplan & Kitts, 2004). Nevertheless, it has been shown that the contamination levels did not affect this two-phase pattern (Admon et al. 2001). More recently, Sutton and colleagues (2013) showed that the presence of diesel contamination, rather than its concentration, dictated changes in community diversity, regardless of the different soil matrix type considered. GBB also showed this pattern, even though this sampling site was sandy and because of this leaching is expected to happen. It has been shown that soil structure is not static in space or time and that microbes alter this structure (Crawford et al. 2012). The

biophysical properties of soil are the product of both microbial genotypic and micro-environmental diversities (O'Donnell et al. 2007; Ruamps et al. 2011). This soil-microbe system is self-organizing as a consequence of the feedback between microbial activity and particle aggregation. Therefore, another possible interpretation of the two-phase oil biodegradation pattern could be interspecies interactions that ought to happen in order to degrade complex petroleum derivatives.

Heavy oil consists of a variety of chemically distinct hydrocarbons (Head et al. 2006), which requires a diverse range of microorganisms for its degradation (McGenity et al. 2012). Complementary effects through positive species interactions have been reported as a mechanism driving community dynamics in an experimental polyculture of crude oil degrading bacteria (Venail & Vives, 2013). In that experiment, the assemblages of mixed species with complementary enzymatic metabolism was suggested as having the complete machinery to better exploit the complex mixtures in crude oil. Interestingly, indigenous communities performed better than foreign ones, suggesting that in addition to adaptation to abiotic conditions, adaptation to the biotic environment of co-occurring species is also important for bacterial community dynamics.

Bacterial species interactions have also been experimentally demonstrated to drive the evolution of alternative resource use not observed in single-species communities (Lawrence et al. 2012). In this experiment, competition among the species resulted in character displacement and the evolution of some species to use the waste generated by other species. From a systems-biology perspective, it has also been suggested that a metabolic network is responsible for the biodegradation potential of a microbial community (Pazos et al. 2003; Lorenzo 2008; Pah et al. 2013).

Gargaú was taken as the mangrove with no history of oil accidents and both sampling sites from this location had more OTUs and a lower community organization than the two sampling sites from Guanabara Bay. At the end of this experiment, oil exposure reduced the diversity in all sampling sites, except GBA. Perhaps, more importantly, community organization from day 21 on differentiated GR from the other communities as the only community with low organization (i.e., high eveness). Initial community evenness has been related to mantaining functional stability and resilience of an ecosystem (Wittebolle et al. 2009). Surprisingly, GV did not follow this pattern, even though it also had a low community evenness. Probably, the daily presence of oil

353 contaminants from the fishing boats in the area might have an impact on the composition of this
354 community.
355 Interspecies interactions can affect evolution and influence the ecosystem. Oil exposure had a
356 drastic impact on the dynamic of the communities, when taking the initial community as the
357 reference point (MEA values). During the oil incubation period, both sampling sites from
358 Guanabara Bay showed change values close to 100% from the initial community, with the
359 exception of GBA at day 7, suggesting that the initial community of GBA was already impacted
360 by the presence of oil components. GR and GV also showed large changes in community
361 composition related to the initial community, but to a lesser extent. At the end of the incubation
362 period, GR and GV were approximately 30% similar to the initial community. We propose to
363 designate such persisting community as the core bacterial community (CBC) in mangrove
364 sediments under oil contamination, supporting the idea of community stability and resilience in
365 samples from Gargaú, in contrast to Guanabara Bay, which is chronically polluted. Time to oil
366 exposure is surely another important parameter to consider when evaluating community dynamic
367 responses, as this may cause recurrent selection of hydrocarbonoclastic bacteria and, therefore,
368 reduction in richness and increase in community organization, probably affecting the core
369 microbiota and community stability and resilience thereof. Interestingly, GV had the smallest
370 MEA values, except from day 60 on. This community increased in richness from day 90 on and,
371 at the same time, decreased in MEA values, suggesting an approximation to the initial
372 community. This is also showed in the hierarchical cluster, where the final communities of GV
373 clustered together with the initial communities and the communities from GR (GR 28 -150),
374 indicating again the tendency of reestablishing the initial community.
375 In conclusion, the effect of oil exposure on the composition of the developing bacterial
376 community is variable, time- and environmental-dependent. Our data corroborated the already
377 reported two-phase pattern of oil biodegradation, although the original proposed explanation is
378 questioned, opening up the possibility to consider other plausible hypothesis of microbial
379 interactions as the main drivers of this pattern. The decreased richness associated with the high
380 community organization at the beginning of the experiment indicates a strong selection for
381 hidrocarbonoclastic bacteria soon after oil exposure. Different hidrocarbonoclastic bacteria may
382 be selected at each sampling site at the beginning because of bacterial endemism, and this may
383 reflect the different bacterial assemblages throughout the experiment. Species interactions along

the experiment may explain the common two-phase pattern of community dynamics. Chronically polluted sites may be losing other functional groups as a result of recurrent selection for hidrocarbonoclastic bacteria, which affects ecosystem functioning. The cooperative behavior of microbes to self-construct a functioning community is central to their success (McGenity at al. 2012), and community evenness is critical for the maintenance of functional stability and resilience of an ecosystem (Wittebolle et al. 2009). Although our data do not come from functional genes and caution need to be taken when interpreting community organization in relation to function organization (Read et al. 2011), there is clearly a different response when comparing a community without any oil contamination history and other communities with different levels of oil exposures.

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