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Exploring bacterial functionality in mangrove sediments and its capability to overcome anthropogenic activity

Simone Raposo Cotta[a], Luana Lira Cadete[a], Jan Dirk van Elsas[b], Fernando Dini Andreote[a], Armando Cavalcante Franco Dias[a,*]

[a] Department of Soil Science, ESALQ/USP, University of São Paulo, Piracicaba, Brazil
[b] Microbial Ecology Group, Institute for Evolutionary Life Sciences, University of Groningen, AG, Groningen, the Netherlands

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ABSTRACT

Mangrove forests are highly productive yet vulnerable ecosystems that act as important carbon sinks ("blue carbon"). The objective of this work was to analyze the impact of anthropogenic activities on microbiome structure and functioning. The metagenomic analysis revealed that the taxonomic compositions were grossly similar across all mangrove microbiomes. Remarkably, these microbiomes, along the gradient of anthropogenic impact, showed fluctuations in the relative abundances of bacterial taxa predicted to be involved in sulfur cycling processes. Functions involved in sulfur metabolism, such as APS pathways (associated with sulfate reduction and sulfur oxidation processes) were prevalent across the microbiomes, being *sox* and *dsr*AB genes highly expressed on anthropogenically-impacted areas. Apparently, the oil-impacted microbiomes were more affected in taxonomic than in functional terms, as high functional redundancies were noted across them. The microbial gene diversity found was typical for a functional system, even following the previous disturbance.

1. Introduction

Mangroves are listed among the environments of which the 'health' is threatened due to their exposure to anthropogenic contamination (Barbier et al., 2011; Barbier, 2016). The resulting loss of biodiversity and ecosystem functions in mangroves may contribute to decreases of coastal protection against flooding and storm events, reducing the resilience of the ecosystem to disturbances (Barbier, 2016; Lovelock et al., 2017; Micheli and Halpern, 2005). It is imperative to assess the impact of the biodiversity in mangroves to support strategies for the recovery of affected areas (Alongi, 2014; Holguin et al., 2006).

Given the fact that key life support processes occur in microbiomes, such functions need to be preserved in mangrove microbiomes. Thus, understanding the response of the latter to disturbances is essential to properly assess the stability and functioning of these ecosystems (Griffiths and Philippot, 2012). Mangroves often have high productivity, which is associated with a high organic matter content in a predominantly anaerobic environment (Behera et al., 2014; Dutta et al., 2017). The turnover of this organic matter is often associated with sulfate reduction, and hence a functioning sulfur cycle is a major determinant of processes in mangrove sediments (Varon-Lopez et al., 2014). Sulfate reduction is usually considered to constitute the most important respiration process in mangrove sediments, accounting for > 75% of total mineralization. Besides that, the magnitude and fate of organic matter in mangroves is highly variable, as it is regulated by the local redox states (Friesen et al., 2018).

Adaptive power is key to life in basically all ecosystems, in particular for microorganisms. One key phenomenon that spurs the adaptability of bacteria is horizontal gene transfer (HGT) (Aminov, 2011). Genetic elements such as plasmids, conjugative transposons and phages are frequently associated with the dissemination of genes conferring adaptive traits, e.g. xenobiotic degradation genes (Sreeshan et al., 2014; Rajan and Sobecky, 2017). The selective pressure exercised by oil and sewage contamination is thought to stimulate the occurrence of successful HGT events, as microorganisms may need to rapidly adapt to the new stressful condition (Johnsen and Kroer, 2007; Top and Springael, 2003). For instance, genes that encode the degradation of both natural and xenobiotic organic compounds are frequently located on plasmids, transposons, or other mobile and/or integrative elements (Top et al., 2002).

Several mangrove areas along the coastline of the state of São Paulo in Brazil constitute a gradient of environmental contamination. For instance, one area was impacted by an oil spill in the 1980's and another area by sewage disposal. The impact on the system in each area

* Corresponding author at: Laboratory of Soil Microbiology, Department of Soil Science, "Luiz de Queiroz" College of Agriculture, University of São Paulo, Brazil.
E-mail address: dias147@gmail.com (A.C.F. Dias).

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can be seen through changes in the vegetation, as described in recent studies (Andreote et al., 2012; Dias et al., 2012; Cabral et al., 2016). Next to the impacted areas, a pristine (control) mangrove site was found inside the preserved area of the Cardoso Island.

Here, we performed a comparative analysis of the microbiomes of the aforementioned (impacted versus control) mangrove sites (mainly focusing on the bacterial communities), using metatranscriptomics in comparison to metagenomics. Evaluations of the impacts on microbiomes are essential to ascertain the stability of functioning of these ecosystems (Griffiths and Philippot, 2012). Our objectives were: *i*) to describe the microbiomes of the selected mangrove sediments according to taxonomy and functionality; *ii*) to evaluate the impact of anthropogenic activities on the diversity and richness of taxa and functions; *iii*) to select key cycling processes and evaluate the impact of anthropogenic influences on these, and *iv*) to evaluate the potential of the microbiomes to adapt to new conditions based on HGT mechanisms. We hypothesized that anthropogenic activities shift the diversity of microbial taxa and the functionality of microbiomes, impacting the maintenance of ecosystem services provided by mangrove forests.

2. Materials and methods

2.1. Mangrove areas and samples collection

This approach yielded a total of 12 samples: four mangrove areas × three replicates. The mangrove areas consisted of areas BrMgv01 and BrMgv02, characterized as oil-contaminated (resulted from an oil-spill occurred in 1983), with BrMgv01 receiving less oil than BrMgv02; BrMgv03 (Bertioga city-sewage contamination) and BrMgv04 (pristine). BrMgv01, BrMgv02 and BrMgv03 are located at Bertioga and BrMgv04 adjacent to Cananéia City (for more details see Andreote et al., 2012; Cabral et al., 2016; Dias et al., 2010, 2011, 2012). Samples were collected in each mangrove with replicates separated by at least 30 m from each other. Sediment cores were obtained with a 30 cm sampler, 7 cm in diameter, resulting in sediment cores of approximately 1 kg. The physical and chemical characteristics of the sediments are presented in Supplementary Table S2. Information about hydrocarbon composition and concentration can be found in Table S3.

2.2. DNA and RNA extractions

Total DNA was extracted from 0.5 g of each sediment sample using the PowerSoil DNA Isolation kit (Mobio Labs, Inc. Solana Beach, USA) according to the manufacturer's protocol. RNA extraction was performed immediately after sample collection. 2.0 g of sampled mangrove sediment was used in the total RNA Isolation PowerSoil® kit (Mobio Labs, Inc. Solana Beach, USA) according to the manufacturer's protocol. The amounts of DNA and RNA were measured in 1% (w/v) agarose gels electrophoresis and the final concentrations quantified with NanoDrop (Thermo Fischer Scientific, Waltham, MA, USA). The total removal of DNA was checked using Agilent 2100 Bioanalyzer DNA electropherogram (Agilent, Santa Clara, CA, USA). Low or absent concentration of DNA was considered as a result of efficient removal of total DNA.

2.3. Preparation of RNA for sequencing

Aliquots of 15 μl of total RNA (20 ng μl^{-1}) were used for the removal of rRNA, promoted by the application of the Ribo-Zero rRNA Removal Kit™ (Target-bacteria Epicentre®, Illumina). This fraction was named as mRNA in further analyses. Either total or purified RNA were used to prepare cDNA with the ScriptSeq™ mRNA-Seq Library Preparation Kit (Epicentre). This approach was similar to the ones used to perform metatranscriptomic analysis in soils in previous studies (Cabral et al., 2016; Cabral et al., 2018; Carvalhais et al., 2012).

2.4. Sequencing of DNA and RNA from mangrove sediments

A total of 36 samples was shotgun-sequenced (12 samples × 3 fractions (DNA, mRNA and total RNA)). Libraries were sequenced on the Illumina® HiSeq 2000 platform, with sequencing procedures performed at the Laboratory for Functional Genomics Applied to Agriculture (http://www.esalq.usp.br/genomicafuncional/) located at "Luiz de Queiroz" College of Agriculture (University of São Paulo, Piracicaba, SP, Brazil).

2.5. Sequence analysis

Raw sequences were initially processed at CLCbio® software version 6.5.1 (CLC Bio, Denmark), where datasets were de-multiplexed (according to their tags) and low-quality sequences were filtered (score limit of 0.05; maximum 1 ambiguous nucleotide allowed; minimum sequence length of 100 nt). Resulting sequences were then uploaded to the MG-RAST (http://metagenomics.anl.gov) bioinformatics pipeline for analysis (Meyer et al., 2008). These datasets are publicly available at MG-RAST. Sequences derived from DNA are available under the codes 4533988.3, 4533989.3, 4533990.3, 4533991.3, 4533992.3, 4533993.3, 4534574.3, 4534575.3, 4534576.3, 4534058.3, 4534060.3, 4534815.3. Datasets generated from total RNA are available under the codes 4534057.3, 4534059.3, 4538651.3, 4534489.3, 4534385.3, 4534386.3, 4538683.3, 4538684.3, 4534934.3, 4534657.3, 4534658.3 and 4534659.3. Sequences obtained from mRNA-enriched samples are codified as 4532366.3, 4532600.3, 4532601.3, 4533823.3, 4550847.3, 4534061.3, 4533695.3, 4534490.3, 4534820.3, 4534002.3, 4533831.3 and 4534003.3.

2.6. Comparison of taxonomic and functional patterns of microbiomes

The taxonomic profiling of the microbiomes based on ribosomal RNA sequences (from DNA and total RNA) was performed using the databases available in MG-RAST (Annotation source: M5NR; Max e-value cutoff: $1e^{-5}$, min % Identity cutoff: 60%). The functional annotation of sequences was performed based using MG-RAST/SEED subsystem hierarchical classification with an *E*-value cutoff $< 10^{-5}$ (Overbeek et al., 2005). The hierarchical categories Phages, Prophages, Transposable elements, Plasmids and Sulfur Metabolism (Level 1 of the SEED Subsystems annotation) were specifically evaluated. In order to gain detailed information about sulfur metabolism, the relevant sequences (Level 3: inorganic sulfur assimilation, sulfate reduction-associated complex and sulfur oxidation) were uploaded to the virtual Workbench in MG-RAST for proper taxonomic affiliation, achieved by the subjection to Best Hit Classification using the M5NR database.

Taxonomic or functional patterns were compared among the mangrove microbiomes based on Principal Correspondence Analysis (PCoA). The microbiome diversity and richness values (Shannon and Chao-1 index, respectively) were determined using PAST software package version 2.10 (Hammer et al., 2001). Every dataset used for the comparisons among DNA, RNA or mRNA data from distinct mangroves were normalized in the unit reads per million. Statistical analysis of the sequencing data was performed using STAMP and PAST software (Parks and Beiko, 2010).

3. Results

3.1. Characteristics of datasets from metagenomics and metatranscriptomics approaches

After trimming and removal of low-quality sequences, 4619 to 9430 million reads were recovered for total DNA samples; 9404 to 13,080 million reads for total RNA samples and 13,225 to 15,926 million reads to mRNA samples. Regarding the DNA dataset, the replicates of BrMgv01 (4973 to 9230 million reads) and BrMgv04 (6809 to 8201

Table 1
Sequencing information.

	MG-RAST ID	Sample	N. of reads before QC*	N. of reads after QC*	Mean sequence length	% of predicted rDNA*	Total bp*
Total DNA	4533988.3	MgvD1a	5.188.484	4.973.276	85 ± 14	1.007.519	444.933.880
	4533989.3	MgvD1b	7.456.064	7.423.367	86 ± 14	1.468.443	643.394.015
	4533990.3	MgvD1c	9.298.728	9.230.765	86 ± 14	1.799.403	806.673.737
	4533991.3	MgvD2a	4.647.188	4.619.735	84 ± 15	966.547	394.943.871
	4533992.3	MgvD2b	8.046.086	7.798.081	86 ± 14	1.545.678	695.080.917
	4533993.3	MgvD2c	6.397.854	6.106.683	86 ± 14	1.218.952	552.412.030
	4534574.3	MgvD3a	7.371.498	7.127.865	86 ± 14	1.422.015	638.113.083
	4534575.3	MgvD3b	7.395.306	7.197.365	86 ± 14	1.370.288	637.211.942
	4534576.3	MgvD3c	9.636.928	9.430.338	86 ± 14	1.764.698	836.530.711
	4534058.3	MgvD4a	7.367.110	7.052.357	86 ± 14	1.346.352	635.206.262
	4534060.3	MgvD4b	8.395.634	8.201.089	86 ± 14	1.556.360	726.481.661
	4534815.3	MgvD4c	7.019.218	6.809.639	86 ± 14	1.336.367	606.713.612
Total RNA	4534057.3	MgvR1a	27.270.176	11.309.770	98 ± 5	12.229.555	1.113.642.205
	4534059.3	MgvR1b	30.521.812	13.051.720	98 ± 5	14.624.329	1.285.531.606
	4538651.3	MgvR1c	25.954.364	9.975.522	98 ± 5	10.544.893	982.411.233
	4534489.3	MgvR2a	25.329.292	10.697.654	98 ± 5	11.163.970	1.058.727.822
	4534385.3	MgvR2b	23.964.042	10.214.457	98 ± 5	10.495.595	1.010.816.933
	4534386.3	MgvR2c	25.258.256	11.185.476	98 ± 5	11.559.863	1.107.107.289
	4538683.3	MgvR3a	24.431.564	11.577.911	98 ± 5	12.227.518	1.145.903.971
	4538684.3	MgvR3b	23.782.274	13.080.989	98 ± 5	10.942.380	1.292.087.592
	4534934.3	MgvR3c	26.775.362	13.040.848	98 ± 5	13.703.727	1.290.679.161
	4534657.3	MgvR4a	24.800.702	10.506.481	98 ± 5	10.990.888	1.038.981.281
	4534658.3	MgvR4b	22.230.590	10.130.485	98 ± 5	10.131.576	1.002.192.185
	4534659.3	MgvR4c	22.787.310	9.404.278	98 ± 5	9.732.570	930.680.131
mRNA	4532366.3	MgvRC1a	24.637.540	15.522.810	99 ± 1	4.136.138	548.246.211
	4532600.3	MgvRC1b	27.216.082	18.730.386	98 ± 5	5.504.905	851.261.989
	4532601.3	MgvRC1c	22.134.272	13.517.823	98 ± 5	6.562.695	1.338.009.604
	4533823.3	MgvRC2a	27.020.008	13.563.725	98 ± 5	2.329.098	341.873.216
	4550847.3	MgvRC2b	24.550.860	13.431.264	98 ± 5	6.703.447	1.328.582.129
	4534061.3	MgvRC2c	24.559.378	13.225.022	98 ± 5	2.527.732	1.308.038.558
	4533695.3	MgvRC3a	24.082.767	14.713.089	99 ± 4	8.456.768	1.456.607.520
	4534490.3	MgvRC3b	17.975.953	16.591.809	98 ± 5	3.719.541	1.636.039.772
	4534820.3	MgvRC3c	24.343.772	14.848.251	99 ± 4	8.540.230	1.470.034.367
	4534002.3	MgvRC4a	26.081.434	15.926.125	98 ± 5	6.786.881	1.575.194.424
	4533831.3	MgvRC4b	24.078.358	14.991.925	98 ± 5	2.428.376	1.482.586.859
	4534003.3	MgvRC4c	24.258.790	14.753.201	98 ± 5	4.635.247	1.460.416.313

* Quality Control.

million reads) were more variable than the replicates from the other sites (BrMgv02: 4647 to 8046 million reads; BrMgv03: 7371 to 9636 million reads). With respect to the total RNA dataset, the replicates were consistent across all evaluated sites, with slight fluctuations only in the BrMgv01 site (9975 to 13,051 million reads). However, the mRNA dataset had a higher variation between the replicates, with the exception of BrMgv02 (Table 1). The percentages of reads matching ribosomal RNA (rRNA) were, on average, 1.5%, 62.28% and 20.94% in the databases from DNA, rRNA and mRNA, respectively (Table 1). These data show the efficiency of the methodology applied to enrich mRNA from the total RNA extracted from the samples.

3.2. Comparison of taxonomic and functional patterns

3.2.1. Microbiome comparison by DNA based analyses

Principal coordinates analysis (PCoA) (explaining 55.5% of the variance) showed that, on the basis of the 16S rRNA gene sequences, the taxonomic compositions of the microbiomes of the four mangroves were distinct (Suppl. Fig. 1), with the replicates being highly consistent for sites BrMgv01, BrMgv03 and BrMgv04 and differing slightly for BrMgv02. Remarkably, the latter (oil-contaminated) site clustered away from all other sites. The BrMgv03 (anthropogenically-impacted) and BrMgv04 (pristine) replicates also clustered in separate, yet these were more similar to each other (Suppl. Fig. 1a).

The PCoA based on the (metagenome-derived) functional profiles explained 61.4% of the variance. A higher variation was found between the replicates from BrMgv01 samples. However, a pronounced separation of sites was noted. The BrMgv02 derived profiles were, again, most distinct from those from the other sites (Suppl. Fig. 1b). This was followed by samples from BrMgv04, and finally by those from BrMgv01 and BrMgv03.

The lowest values of (phylogenetic) diversity were found in the BrMgv02 site (Suppl. Table S4), i.e. 2.85 ± 0.05 (Shannon's diversity index). The other mangrove sites showed values of 3.08 ± 0.03, 3.00 ± 0.02 and 2.90 ± 0.02, for BrMgv01, BrMgv03 and BrMgv04, respectively ($p < 0.05$). Richness values (calculated using Chao-1 index) were similar between mangrove sites, with slight declines in BrMgv01 ($p > 0.05$) (Suppl. Table S4). The diversity of functions across all evaluated sites was stable, not differing between the samples (Suppl. Table S4).

3.2.2. Taxonomic patterns derived from total RNA sequences

A range of 94 to 97% of the sequences derived from the total RNA samples consisted of rRNA sequences (Table 1). Hence, we used this information to derive meta-taxonomic information from the samples and verify which microbial group was more “active” (high amount of 16S rRNA transcripts). Using PCoA, all samples distributed quite randomly at 58.8% of the variance explained. Hence, any differences in the taxonomic patterns of the respective microbiomes were blurred at this level, probably due the high variability observed between replicates of each evaluated site (Suppl. Fig. 1c). As observed for the DNA-based sequences, BrMgv02 site showed the lowest Shannon diversity index (2.59 ± 0.01), as compared to the other evaluated sites (Suppl. Table S4) ($p < 0.05$). The richness values were stable between the mangrove sites, only showing slight variations (Suppl. Table S4).

3.2.3. Functional patterns derived from mRNA sequences

We then used a clustering approach to discern any differences in the

functional profiles across the mangrove microbiomes. Clearly, using this approach, a PCoA explaining 95.2% of the variance was generated. The replicates of BrMgv01, BrMgv02 and BrMgv04 were consistent. Here, the BrMgv01 mangrove samples clustered clearly away from all other mangrove samples. BrMgv03 site showed a high variability across the replicates (Suppl. Fig. 1d). BrMgv01 showed the lowest diversity of functions realized by the mangrove sediment microbiomes (5.73 ± 0.06) ($p < 0.05$) (Suppl. Table S4).

3.3. Comparison of occurrence of taxonomical groups in the DNA- and RNA-derived sequence data sets

In all samples, the most representative taxa (> 3% relative abundance) composing the bacterial communities revealed by the DNA- and RNA-based approaches were, in order of descending prevalence, Proteobacteria (mainly Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria and Alphaproteobacteria), Bacteroidetes, Chloroflexi and Firmicutes (Clostridia and Bacilli) (Table S1). In detail, on the basis of the DNA-derived sequences, this distribution was roughly constant between the mangrove samples, with a slight difference for Gammaproteobacteria (more prevalent in the oil-impacted BrMgv02 samples than in the remainder).

Remarkably, an analysis of the groups with prevalence below 3% (DNA-derived data) revealed Dehalococcoidetes to be more prevalent in BrMgv03 (3.60%) than in all other samples (BrMgv01: 2.30%; BrMgv02: 0.60%; BrMgv04: 1.40%; Table S1).

On the basis of the RNA-derived data, the less abundant taxa (< 3%) Acidobacteria, Chlorobia, Chloroflexi, Dehalococcoidetes and Deinococci were significantly more abundant in the BrMgv04 than in all other samples (Table S1).

3.4. Functional patterns in the mangrove microbiomes

A comparison of the frequency of each annotated function in the datasets derived from DNA and mRNA allowed us to differentiate between traits that were highly or poorly expressed. Per mangrove system, the correlation between the two functional patterns based on the relative abundances of the reads was surprisingly high, with values of R^2 varying from 0.508 (BrMgv01) to 0.782 (BrMgv03) (Fig. 1).

The most representative functions determined at the first (highest) hierarchical level of the SEED database (for mRNA sequences) were similar across all mangrove areas. These consisted of traits involved in protein metabolism, carbohydrate metabolism, stress responses, amino acid and derivative transformations, respiration, and sulfur metabolism ($p > 0.01$) (Fig. 1). For protein metabolism, the prevalent function was protein degradation, corresponding an average of 20% of the transcripts and for carbohydrate metabolism, CO_2 fixation (16%) and central carbohydrates metabolisms (50%) (data not shown). Transcripts associated with carbohydrate metabolism were more prevalent in BrMgv03 as compared to BrMgv01, BrMgv02 and BrMgv04 ($p = 0.05$) (Fig. 1c).

In contrast, the functions prevalently found in the DNA-derived data were associated with amino acid and derivative transformations and phages, prophages, transposable elements and plasmids (Fig. 1). Significant differences in functions that are directly associated with anthropogenic activity (e.g. xenobiotic degradation and polycyclic aromatic compound use) were not detected between the sampled mangrove sites.

3.5. Traits associated with sulfur transformations

Reads for genes of all steps of the major sulfur transformations (reductive and oxidative pathways) were detected across all mangrove areas. A high number of mRNA based reads was found, as compared to DNA based ones. Considering the mRNA sequences, the abundances of the reads for sulfur metabolism was similar across the microbiomes, and hence these were not strongly influenced by prior oil or sewage/sludge spills (Fig. 2d–f). Besides, the replicates of the mRNA dataset were more variable than of the DNA dataset (Fig. 2).

In a general view, the reads representing the APS pathway for sulfite oxidation to sulfate (*apr*AB genes) were prevalent, and the numbers greatly exceeded those of other genes (in both DNA and mRNA based datasets). Besides, in the DNA based dataset, this function was more dominant in the BrMgv02 microbiome in relation to the other ones (Fig. 2b). However, this pattern was not reproducible on the basis of the mRNA dataset. Remarkably the latter dataset indicated a gene expression reduction for *apr*AB genes in the BrMgv03 microbiome (Fig. 2e).

In detail, in the oil-contaminated areas BrMgv01 and BrMgv02, the mRNAs of both sulfur oxidative (*sox* complex) and sulfate reductive processes (*dsr*AB genes) were only slightly raised in comparison to the relative abundances in the other microbiomes (Fig. 2d and f). In the BrMgv03 mangrove (sewage- and sludge-contaminated), the reads for the *apr*AB genes were slightly lower than those in the other areas (Fig. 2e). In contrast, the DNA-derived data revealed a different pattern, that is, the prevalence of all traits (*apr*AB, *sox* complex and *dsr*AB) was raised in the oil-contaminated area BrMgv02 (Fig. 2a–c).

When using the genes of the *sox* complex, *apr*AB and *dsr*AB, as proxies to verify taxonomic distinctions across the organisms involved in sulfur cycling among the mangrove microbiomes, the RNA-derived data revealed more differences than the DNA-based ones (Fig. 3). The DNA-derived data revealed that the BrMgv04 microbiome had lower diversity (1.72 ± 0.04) than the BrMgv02 one (2.16 ± 0.03), while on the basis of the RNA-based sequences, BrMgv03 showed higher diversity (1.89 ± 0.26) ($p < 0.05$) (Suppl. Table S5). The richness values followed the patterns observed for the diversity ranking (Suppl. Table S5).

The taxonomic affiliation of the sequences annotated as *apr*AB revealed similar patterns among the four mangrove areas for the DNA-based analyses, but differentiated according to the RNA-based sequences. In the latter case, a prevalence of Betaproteobacterial and Deltaproteobacterial *apr*AB genes was found in BrMgv01, whereas in the other areas these classes occurred in lower abundances than Clostridia (Fig. 3c and d).

With respect to the *dsrAB* sequences, the DNA-derived patterns were similar between the mangrove areas, showing slight variations on relative abundance, both between the replicates and between the samples. The lowest diversity was observed in the BrMgv02 (1.55 ± 0.10) mangrove microbiome for DNA-derived sequences ($p < 0.05$). For RNA-database, the BrMgv04 microbiome showed higher diversity (1.87 ± 0.39) ($p > 0.05$) (Suppl. Table S5). Overall, the largest number of *dsr*AB reads was affiliated to similar sequences found in Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria and Clostridia on DNA-database. However, the RNA-based analyses revealed clear distinctions between the sites, with the absence of Clostridia-derived sequences in BrMgv01 and a high prevalence of Deltaproteobacterial ones in BrMgv04 (Fig. 3e and f).

Finally, a similar pattern occurred with respect to *sox* genes, involved in sulfur oxidation. The DNA-based analyses showed a similar composition of this functional community across all mangrove sites, with a prevalence of sequences affiliated to those of the Betaproteobacteria and a slight prevalence of Chlorobia on BrMgv02 (Fig. 3a). The metatranscriptomics approach, on the other hand, suggested distinctions among the communities from each area, mainly caused by variations in the frequencies of Betaproteobacteria (more frequent in BrMgv01 and BrMgv03) and Epsilonproteobacteria (found in higher frequencies in BrMgv02 and BrMgv04). The class Chlorobia, prevalent on almost all evaluated sites for DNA database was absent on BrMgv01 site for RNA dataset (Fig. 3b). The diversity of BrMgv04 site (2.30 ± 0.08^{bc}) was higher than that of BrMgv02 (1.78 ± 0.09^{ba}) on the basis of the DNA-dataset. Based on RNA-derived data, BrMgv01 showed the lowest diversity (0.98 ± 0.24). BrMgv02 showed higher richness (25 ± 4) on RNA-derived data when compared to the other sites ($p < 0.05$) (Suppl. Table S5).

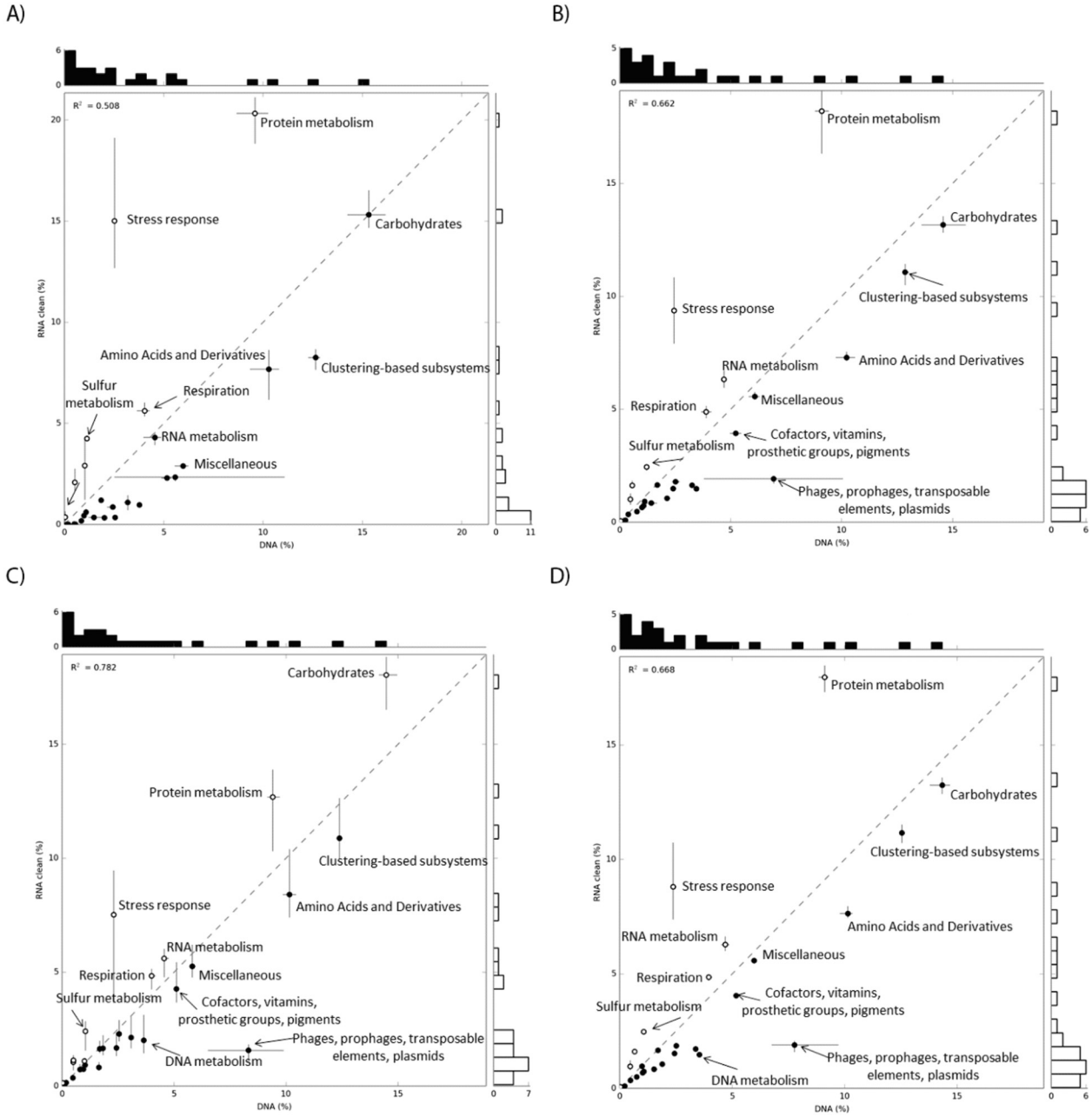

Fig. 1. Comparison of functional profiles for metagenome (DNA) and metatranscriptomics (mRNA) of all mangrove areas. The letters represent the mangrove areas; A) BrMgv01; B) BrMgv02; C) BrMgv03 and D) BrMgv04. Dots on either side of the dashed trend line are enriched in either DNA or RNA.

3.6. Tracking sequences of phages, prophages, transposable elements and plasmids

Reads representative of phages (including prophages), plasmids and related functions were prevalently found across all mangrove areas. The relative abundances of these elements were around 12% (range 5.98 to 15.16%) and 3.35% (range 1.81 to 3.95%) of the total reads from the metagenomes and metatranscriptomes, respectively (data not shown). Among these reads, phages/prophages were the most prevalent, amounting to up to 90% of them, being consistent across all replicates of the DNA-dataset. The putative pathogenicity island was absent on BrMgv04 sample. For RNA-dataset, phages/prophages were prevalent on BrMgv02 (84.70%) and putative (pathogenicity) islands were prevalent on BrMgv01 (83.97%). For the other sites, phages/prophages prevalence ranged 15.92 to 39.95% and putative pathogenicity island prevalence ranged 11.15 to 58.02%. The frequency of Gene Transfer Agents was very low in all areas (Fig. 4a). The metatranscriptomics approach, however, suggested the existence of distinctions among the communities from each area, mainly caused by variations in the frequencies of Pathogenicity Island, more frequent on BrMgv01 and Phages and Prophages, prevalent on BrMgv02 (Fig. 4b).

4. Discussion

The pressure of anthropogenic activities on natural biomes around the world is increasing exponentially, giving rise to a new geological era, the Anthropocene (Worm and Paine, 2016). Mangroves that are affected by human contamination may be impacted in microbial ecosystem functions, such as carbon and sulfur cycling processes (Sreeshan et al., 2014). Alterations of the diversity and structure of sediment microbiomes can disrupt the ecological functions that depend on specific species assemblages (Micheli and Halpern, 2005).

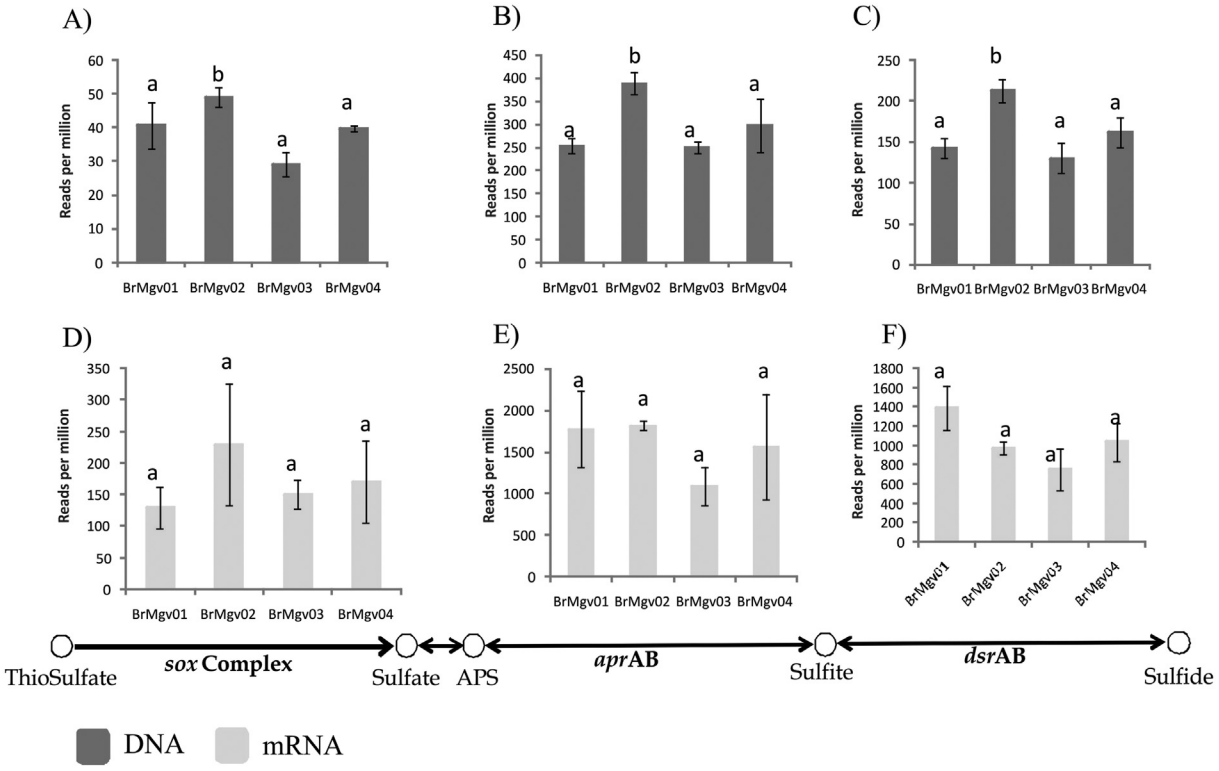

Fig. 2. Sulfur metabolism profile. Letters A, B, D and E letters represent sulfur oxidation and letters C and F represented sulfate reduction. A, B and C correspond to metagenome dataset and D, E, F correspond to metatranscriptomic dataset. Different lower letters correspond to statistical differences between mangroves sites evaluated by Tukey test ($p < 0,05$).

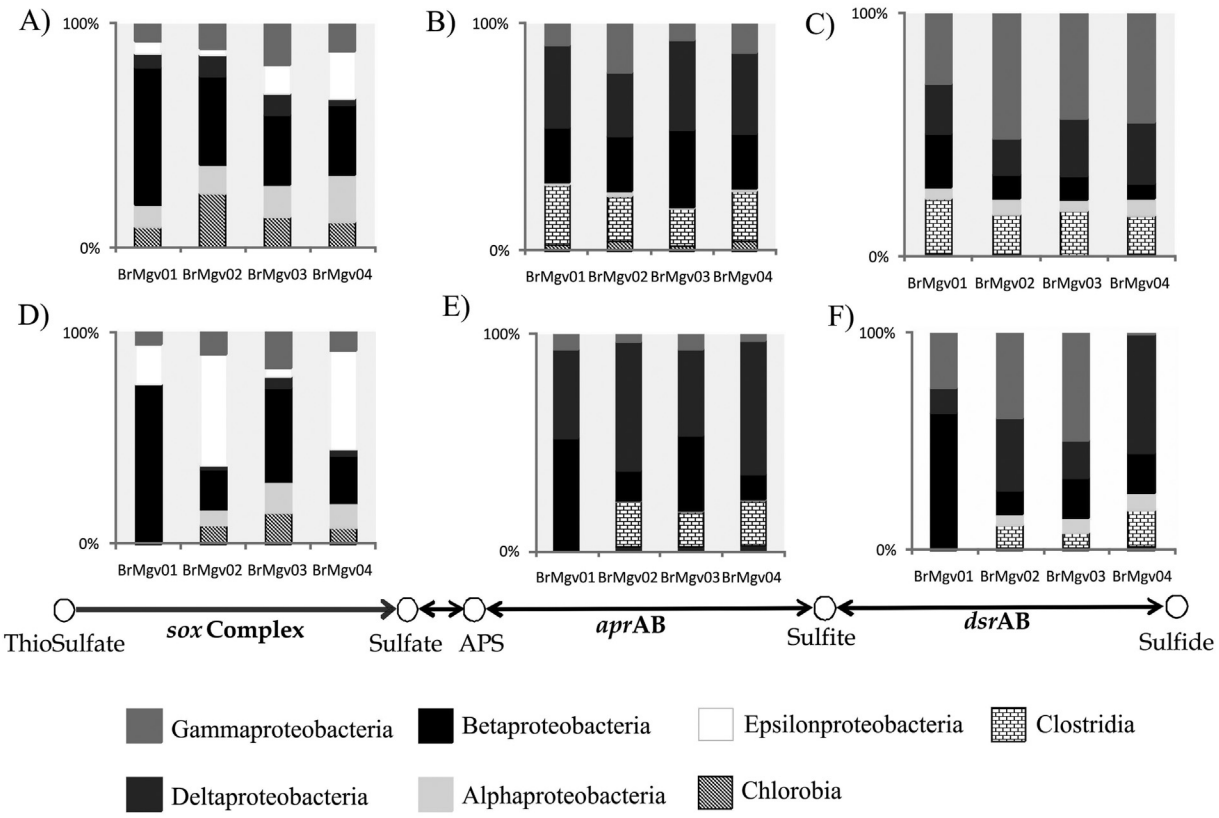

Fig. 3. SSU RNA sequences associated with sulfur oxidation (A, B, D and E) and sulfate reduction (C and F) from the dataset was classified by BlastN against the M5RNA and M5NR database available on MG-RAST. A, B and C correspond to metagenome dataset and D, E and F correspond to metatranscriptomic dataset.

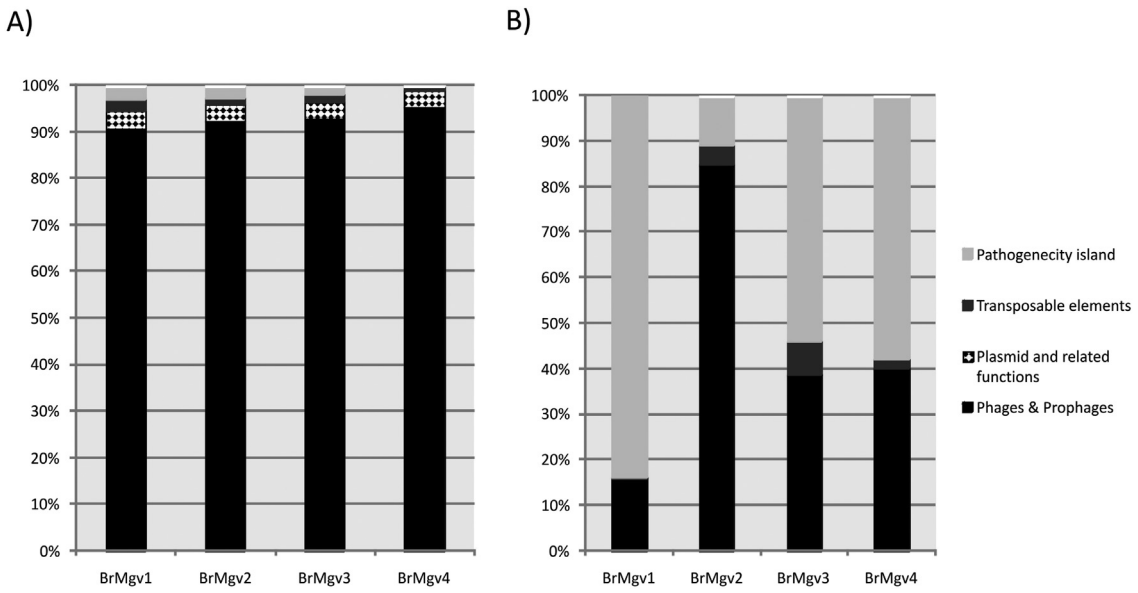

Fig. 4. Relative abundance of reads associated with phage, prophages, transposable elements and plasmids annotated using SEED database on metagenome (A) and metatranscriptome (B) dataset.

Although at a high taxonomic level the taxonomic compositions of the mangrove sediment microbiomes were rather constant for abundant bacterial groups, BrMgv02 presented the lowest diversity (based on DNA and RNA-derived data) as compared to the other sites. Considering the diversity of functions, all sites were very similar (DNA and mRNA-based data). Apparently, mangrove sediment taxa are rather susceptible to local environmental influences such as caused by oil or sewage, while the functions carried in microbiomes are more stable, changing with less intensity.

The impacted mangrove areas showed a microbiome structure that differed from the pristine areas, especially the oil-contaminated mangroves, although at high taxonomic ranks these differences dwindled away. The observed prevalence of Proteobacteria (Gammaproteobacteria, Deltaproteobacteria, Betaproteobacteria and Alphaproteobacteria), Bacteroidetes, Chloroflexi and Firmicutes (Clostridia and Bacilli) is in accordance with the literature, and these groups have been suggested to be members of the core microbiome of mangrove sediments in subtropical areas (Andreote et al., 2012; Dias et al., 2012). Interestingly, the class Epsilonproteobacteria appeared to be affected by the factors reigning in the anthropogenically affected mangroves (Fig. 3). This effect was remarkable, but it is too early to imply effects of oil or sewage as the true drivers. The class comprises chemolitotrophic organisms that obtain energy for metabolism through the oxidation of reduced sulfur compounds in an anaerobic environment (Mendes and Tsai, 2014; Takai et al., 2005). The sulfur oxidation realized by these organisms was relative stable based on the RNA-derived data (in particular, when BrMgv02 is compared with BrMgv04) and for that, these microbial groups may not be detectable or “active” in the sample at the applied sequencing depth (Yu and Zhang, 2012).

Bacteria are responsible for most of the carbon flux in tropical mangrove sediments under the local conditions of low availability of oxygen. In surface layers, they generate most of the energy and nutrients, supporting primary productivity (carbon dioxide fixation by cyanobacteria and other bacteria), whereas in lower layers they are key in the anaerobic degradation (supported by nitrate, iron, manganese or sulfate reduction) of organic matter and C cycling (Bouillon et al., 2003; Holguin et al., 2001; Rigonato et al., 2018). Besides, high productivity of mangroves may be associated with litter decomposition (Moitinho et al., 2018). It has been posited that systems with similar levels of productivity tend to show a similar microbial community composition (Horner-Devine et al., 2003), however this tenet has been questioned (Geyer et al., 2017). Here, we investigated the propensity of four selected mangrove microbiomes – with different level of environmental insult - to serve as carriers of the key cycling functions of the system.

In all mangroves, we found evidence for high productivity, as indicated by the high abundance of transcripts associated with energy flux and central carbon metabolism [≈16% of mRNA sequences annotated as CO_2 fixation (photosynthesis); ≈20% as Protein Degradation and ≈50% of mRNA as Central Carbohydrates Metabolism (degradation of organic matter)]. This high mangrove productivity is clearly a key facet that structures the microbiomes, and apparently this process worked to a similar extent in terms of shaping taxonomically similar communities.

The high prevalence of transcripts annotated as Central Carbohydrates and Protein Metabolism (14.90% of total mRNA sequences were annotated as Protein Degradation) suggests that involvement in organic matter breakdown is the main feature that supports the high mangrove productivity. Mangrove leaves form a large pool of carbon, nitrogen and other nutrients, that is a driver of detrital food web inside mangrove forests (Nordhaus et al., 2017). One limiting factor for litter decomposition on mangroves could be the availability of oxygen. But the mostly anoxic or sub-oxic conditions observed in the non-surface layers of the mangrove systems indeed are propitious for sulfate reduction (some nitrate reduction) supported organic matter degradation (Fernandes et al., 2012; Horner-Devine et al., 2003; Varon-Lopez et al., 2014).

In our samples, the bacteria associated with sulfur cycling processes were mainly members of the Proteobacteria, in particular Deltaproteobacteria, Gammaproteobacteria and Betaproteobacteria, corroborating previous studies (Taketani et al., 2010; Acosta-González and Silvia, 2016; Tiralerdpanich et al., 2018). In previous reviews that described microbial communities associated with sulfur cycle on mangroves sites around the world, bacterial taxa next to Alphaproteobacteria, Epsilonproteobacteria and Chlorobia, had already been identified (dos Santos et al., 2011; Behera et al., 2014).

Here, the RNA-based analyses enabled a differential representation of these groups per mangrove. While Deltaproteobacteria were prevalent under pristine conditions, Gammaproteobacteria was the most active group in sulfate reduction in anthropogenic-impacted areas. Besides, it was possible to note an absence of Chlorobia and Clostridia groups, associated with *sox* complex and *apr*AB and *dsr*AB genes respectively, in the BrMgv01 microbiomes. The absence of these groups

was not considered as a technical artifact due the detection of these groups on metatranscriptomic studies on another mangroves sites (Rampadarath et al., 2018). Thus, considering that loss of function was unlikely in this microbiome, this result could indicate that other microbial groups are performing these functions. The functional redundancy existing in virtually all microbiomes is thought to promote the functional resilience/resistance of the system upon perturbances (Shade et al., 2012).

The higher prevalence of transcripts associated with carbohydrate transformations in the anthropogenically-impacted mangrove BrMgv03 corroborates data from a previous study where the main source of contamination was sewage (Andreote et al., 2012; Cabral et al., 2016). This finding (associated with gene expression pattern) indicates a persistent microbiome response to this contamination. However, the role of the change in vegetation resulting from anthropogenic activities needs to be addressed as well (Andreote et al., 2012), as it alters local conditions and consequently provides an alternative scenario for gene expression by the sediment microbiome. Changes in mangrove vegetation promote modification of exudate and aeration patterns, besides hydrocarbons derived from higher plants, which could affect microbial functionality (Zheng et al., 2017). This idea is reinforced by the high prevalence of transcripts associated with organic acid metabolism observed in BrMgv03 when compared to the other sites, as this function is directly related to changes on root exudates (Song et al., 2012).

An as-yet-poorly explored process driving microbial adaptation in mangroves is HGT. The horizontal acquisition of genetic information permits bacteria to explore new (niche) environments (Van de Guchte, 2017). Dioxygenase genes associated with plasmids of the PromA group were previously detected at the oil-contaminated area used at this work (BrMgv02) (Dias et al., 2018). Here, we found genes associated with phages, prophages, transposable elements and plasmids via the analysis of DNA-derived sequences, indicating a potential for adaptation to a fluctuation on environmental conditions. This finding of a huge prevalence of mobile genetic elements corroborates a recent study performed in a Saudi Arabia mangrove (Alzubaidy et al., 2015).

Notwithstanding the recorded past and present impacts, significant differences in functions that are directly associated with anthropogenic activity (e.g. xenobiotic degradation and polycyclic aromatic compound utilization) were not detected between the sampled mangrove sites. These metabolic pathways were presented at these sites (Cabral et al., 2018). Recently, genes encoding aromatic hydroxylating dioxygenase genes were detected in the same oil-impacted areas used at this study (BrMgv02) using a clone library and metagenomic/metatranscriptomic methodology (Sousa et al., 2017; Cabral et al., 2018).

5. Conclusions

Ecosystem recovery from anthropogenic disturbance, either without human intervention or assisted by ecological restoration, is increasingly occurring worldwide (Moreno-Mateos et al., 2017). Microbial diversity reinstates the dynamic balance in degraded ecosystems establishing a functional equilibrium, which may facilitate the maintenance of sustainability (Singh, 2015). In the present work, we describe the taxonomic and functional diversity of the microbial communities present in mangrove sites that were differentially impacted by human activity. Our findings reveal that, despite similar taxonomic compositions, these communities are distinctly organized. No evidence of loss or impairment of system functionality was observed. In addition, the microbiota in the sediment is possibly capable of modifying and adapting to new conditions by HGT mechanisms.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2019.03.001.

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