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# Greenhouse gas emission potential of tropical coastal sediments in densely urbanized area inferred from metabarcoding microbial community data

Vanessa Almeida Moreira<sup>a,b</sup>, Cristiana Cravo-Laureau<sup>b</sup>, Angelo Cezar Borges de Carvalho<sup>a,b</sup>, Alice Baldy<sup>b</sup>, Edison Dausacker Bidone<sup>a</sup>, Elisamara Sabadini-Santos<sup>a</sup>, Robert Duran<sup>b,\*</sup>

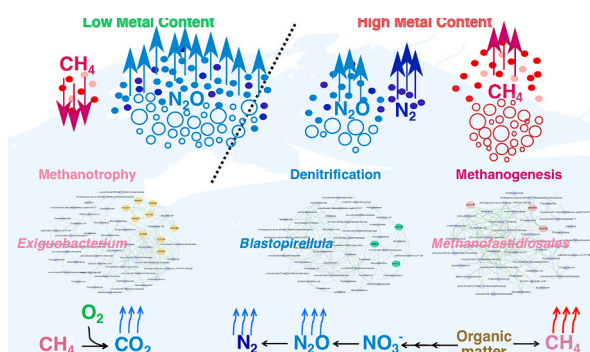
<sup>a</sup> Programa de Pós-Graduação em Geociências (Geoquímica), Instituto de Química, Universidade Federal Fluminense, Niterói, RJ 24020-150, Brazil

<sup>b</sup> Université de Pau et des Pays de l'Adour, E2S UPPA, CNRS, IPREM, Pau, France

## HIGHLIGHTS

- Metal content shapes microbial functions in Sepetiba Bay sediment.
- TOC and metals drive the energetic metabolisms.
- Potential of CH<sub>4</sub> emission thrives in high; that of depletion in low-polluted zones.
- Nitrification potential, N<sub>2</sub>O emission, prevails in oxic low polluted area.
- SB greenhouse gas emission potential alerts for mitigation strategy development.

## GRAPHICAL ABSTRACT



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## ABSTRACT

Although benthic microbial community offers crucial insights into ecosystem services, they are underestimated for coastal sediment monitoring. Sepetiba Bay (SB) in Rio de Janeiro, Brazil, holds long-term metal pollution. Currently, SB pollution is majorly driven by domestic effluents discharge. Here, functional prediction analysis inferred from 16S rRNA gene metabarcoding data reveals the energy metabolism profiles of benthic microbial assemblages along the metal pollution gradient. Methanogenesis, denitrification, and N<sub>2</sub> fixation emerge as dominant pathways in the eutrophic/polluted internal sector (Spearman;  $p < 0.05$ ). These metabolisms act in the natural attenuation of sedimentary pollutants. The methane (CH<sub>4</sub>) emission (*mcr* genes) potential was found more abundant in the internal sector, while the external sector exhibited higher CH<sub>4</sub> consumption (*pmo* + *mmo* genes) potential. *Methanofastidiosales* and *Exiguobacterium*, possibly involved in CH<sub>4</sub> emission and associated with CH<sub>4</sub> consumers respectively, are the main taxa detected in SB. Furthermore, SB exhibits higher nitrous oxide (N<sub>2</sub>O) emission potential since the *norB/C* gene proportions surpass *nosZ* up to 4 times. *Blastopirellula* was identified as the main responsible for N<sub>2</sub>O emissions. This study reveals fundamental contributions of the prokaryotic community to functions involved in greenhouse gas emissions, unveiling their possible use as sentinels for ecosystem monitoring.

\* Corresponding author.

E-mail address: [robert.duran@univ-pau.fr](mailto:robert.duran@univ-pau.fr) (R. Duran).

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## 1. Introduction

Coastal areas, which account for 20 % of the world's land surface (Shi and Singh, 2003) are home of approximately 40 % of the world's population. They thus concentrate major human activities (World Resources Institute, 2000) that generate various chemical pollutions threatening the environment (Carvalho et al., 2021; Moreira et al., 2021, 2022; Ramsar Convention Secretariat, 2016), ultimately impacting the Earth system (Persson et al., 2022; Steffen et al., 2015). Therefore, they require special attention in order to mitigate the effect of pollutants.

Governmental regulations for monitoring coastal zones focus primarily on quantifying eutrophication and pollutant contents (CONAMA, 2012; WFD, 2000). Curiously, they pay little attention to changes in the structure and functioning of benthic microbial communities, which can nevertheless signal the harmful effects of pollutants (Bourhane et al., 2022; Moreira et al., 2023; Seidel et al., 2021). Indeed, benthic microbial communities, which plays a crucial role in biogeochemical cycles, are at the basis of the trophic web (Caruso et al., 2015). Thus, disruptions in benthic microbial communities are transmitted along the food web impacting higher trophic levels through the so called bottom-up effect (Begon et al., 2005). Additionally, they respond rapidly to environmental changes thanks to their metabolic versatility and their exceptional adaptation capacity by the acquisition of genes conferring tolerance/resistance to diverse pollutants such as metals and antibiotics via lateral gene transfer (Dick, 2018; Flemming and Wingender, 2010; Harrison et al., 2007; Moreira et al., 2022). For example, anaerobic microbial taxa have been found predominant in chronically metal-polluted coastal sediments indicating the lack of oxygen (Li et al., 2019; Moreira et al., 2023). As consequence of oxygen depletion, fisheries resources can be affected (Cheung et al., 2018) and greenhouse gas emissions increased (Ayangbenro et al., 2018; Coclet et al., 2021; Tamburini et al., 2020; Wang et al., 2022a, 2022b, 2022c). Therefore, microbial communities have a real potential to serve as indicators for reporting environmental quality (Bourhane et al., 2022; Moreira et al., 2022, 2023). Interestingly, the modification of microbial communities functioning in response to the presence of pollutants can be assessed by targeting the expression of functional genes. In this way the expression of *nor* (denitrification) and *mcr* (methanogenesis) genes has been reported to increase in polluted sediments (Falk et al., 2019; Li et al., 2019), indicating that pollution leads to increase the greenhouse gases emission, specifically nitrous oxide (N<sub>2</sub>O) and methane (CH<sub>4</sub>). Thus, accessing microbial functions will provide valuable information on not only the direct impact of pollution to microbial community but also the consequences at global scale.

Sepetiba Bay (SB; Rio de Janeiro - Brazil) stands as an illustrative case of a coastal region impacted by over half a century by chronic metal pollution due to historical mining activities (Carvalho et al., 2021, 2022; Lacerda and Molisani, 2006; Moreira et al., 2022). Currently, metallic contaminants enter the bay through diverse activities including harbor, industrial, tourism, and domestic waste discharge. The latter is a predominant source, flowing into the bay's main tributaries covering a population of over 8 million inhabitants (IBGE, 2011; Moreira et al., 2021). Prior studies in SB revealed the prevalence of anaerobic prokaryotic taxa (Thermodesulfobionria, Methanomicrobia, Anaerolineae, Gammaproteobacteria, and Thermoplasmata classes) in the sites receiving the most important loads of pollutants from the surroundings, which exhibited strong correlation ( $p < 0.05$ ) with metallic contamination (Moreira et al., 2022, 2023). The presence of such prokaryotic taxa with anaerobic metabolisms suggests greenhouse gas emissions (Moreira et al., 2023). Whith climate change forecasts predicting an intensification of tropical rainfall (Rabalais et al., 2009; Seidel et al., 2021), the current SB eutrophication and pollution (Carvalho et al., 2022; Moreira et al., 2021) is likely to increase. Thus, it is of paramount importance to early detect the disturbance in order to implement mitigation strategies. Particularly, it is essential to understand the ecological role played by the microbial community and how biogeochemical

processes are affected by pollutants (Coclet et al., 2021; Hur and Park, 2019; Ortiz-Estrada et al., 2018; Su et al., 2020). Predictive tools, based on 16S rRNA gene metabarcoding, have provided valuable insights into prokaryotic functions in coastal sediments (Bourhane et al., 2022, 2023; Moreira et al., 2022; Raiyani and Singh, 2023; Rajeev et al., 2021), and assessed the greenhouse gas emission potentials (Li et al., 2020a; Wemheuer et al., 2020). They can provide reliable data for bio-monitoring consistent with enzymatic activity (Su et al., 2018; Wang et al., 2022b; Wang et al., 2023) in order to propose countermeasures for the mitigation of pollutant effects and limit greenhouse gas emissions. We hypothesize that understanding the benthic microbial community functions stake in response to pollutant at SB would provide new information on the factors responsible for greenhouse gas emissions. To this aim, the main energy metabolisms prevailing at the SB polluted sites were predicted in silico from 16S rRNA metabarcoding data in order to determine the microbial taxa potentially associated with greenhouse gas emissions.

## 2. Materials and methods

### 2.1. Study area

Sepetiba Bay (Rio de Janeiro, Brazil – Fig. 1) is one of the most impacted bays in the region in terms of human activities (Barroso et al., 2022; Carvalho et al., 2022; Moreira et al., 2021). Spanning an area of approximately 419–447 km<sup>2</sup>, which varies with the tidal cycle, the bay exhibits an average depth of 6 m, a volumetric capacity of approximately  $2.56 \times 10^9$  m<sup>3</sup>, and a water renewal time of approximately 6 days (Barcellos et al., 1997; Molisani et al., 2004, 2006).

Since the 1950s, Sepetiba Bay (SB) has been subjected to persistent metal contamination, primarily resulting from the extraction of zinc (Zn) and cadmium (Cd) from calamine ore. This operation ceased in the 1990s, leaving an environmental liability that impacts the ecosystem until nowadays (Kütter et al., 2021).

In addition to the long-term contamination, the current state of the bay is heavily influenced by industrial, tourism, and harbor activities. Besides, the urban expansion that surrounds the bay contributes to eutrophication and pollutant loads from the release of untreated sewage (Barroso et al., 2022) and effluents discharged into the tributary rivers, particularly impacting the stations situated in the internal sector. Conversely, the stations in the external sector experience comparatively moderate pollution levels due to reduced population density and the mitigating influence of ocean currents (Carvalho et al., 2022; Moreira et al., 2021).

### 2.2. Sampling

A total of 26 sediment samples (Fig. 1) were collected from Sepetiba Bay using a stainless steel Van Veen sampler. The sample stations were located along the coastline, striving to catch the influence of continental drainage and the contaminant gradient. Internal (eastern portion – SB01 to SB12) and external (western portion – SB13 to SB26) sectors are characterized by increased and reduced anthropogenic pressure, respectively (Moreira et al., 2021).

At each station, a single sample was collected using a pre-decontaminated (10 % HCl) plastic spatula. For molecular analysis, sediment aliquots were stored in sterile Whirl-Pak® bags and kept cool until arrival at the laboratory, where they were frozen at -20 °C until DNA extraction. Sediment aliquots for Total Organic Carbon (TOC), fine-grain (silt + clay content), and metals quantification were stored in Ziplock bags and kept cool until arrival at the laboratory.

### 2.3. Environmental variables quantification

#### 2.3.1. Fine-grain and total organic carbon (TOC) content

For a more detailed description of the pre-treatment of samples and

the methodology employed for the quantification of the environmental parameters presented in this study, consult Moreira et al. (2021). Briefly, the fine-grain content was determined using a laser particle analyzer (CILAS Shimadzu 1064) after removing organic matter and carbonate through calcination (Heiri et al., 2001). The fine-grain content is reported as the % of silt and clay content ( $<63 \mu\text{m}$ ). Total organic carbon (TOC) quantification was conducted using an elemental analyzer (Flash EA1112, Thermo Electron Corporation, Milan, Italy) following the NCEA-C-1282 method (U.S. EPA, 2002). The environmental descriptors chosen and the methods adopted in this study align with the guidelines outlined in the Brazilian legislation for monitoring dredged coastal sediments (CONAMA, 2012).

### 2.3.2. Metal quantification, contamination factor (CF), and degree of contamination ( $\Sigma\text{CF}$ )

Aliquots of dry and homogenized sediment samples were subjected to microwave-assisted acid digestion (aqua regia – method US EPA 3051A; US EPA, 2007) to extract the seven priority toxic metals (Hg, Cd, As, Pb, Cr, Cu, and Zn), according to the Brazilian legislation responsible for monitoring dredged coastal sediments (CONAMA, 2012). The metal quantification was carried out using ICP-MS (Agilent Technologies - 7900). Further details regarding the detection limit, certified reference materials, and metal recovery percentages are available in Moreira et al. (2021).

For assessing the potential for anthropogenic pollution in Sepetiba Bay sediments, the contamination factors ( $\text{CF} = C/C_0$ ) were determined for each sample station based on the metal quantification results. Here,  $C$  represents the metal concentration ( $\text{mg}\cdot\text{kg}^{-1}$ ) in the sediment sample, while  $C_0$  denotes the metal background concentration ( $\text{mg}\cdot\text{kg}^{-1}$ ). The ambient background (Galuszka, 2007) was set using metal concentrations from the current oceanic disposal area for dredged sediments (Dump area - Fig. 1). Station SB26, which was formerly used as the disposal area for dredged sediments in Sepetiba Bay, was included as a control. The overall degree of contamination ( $\Sigma\text{CF}$ ) reflects the cumulative sum of the contamination factors for all seven metals quantified at a sample station.

### 2.4. DNA extraction, PCR amplification, and sequencing

For the environmental DNA extraction, aliquots of 0.25–0.40 g of wet sediment were used following the DNeasy® PowerSoil® Kit (QIAGEN) protocol. Primers 515F (Parada et al., 2016) and 928R (Wang and Qian, 2009) were employed to target the amplification of the V4-V5 region of the 16S rRNA gene. The PCR amplification was performed using the Eppendorf 5333 Mastercycler Thermal Cycler (Germany). The quality and size of the amplified DNA sequences were checked by electrophoresis visualized under UV light as previously described (Moreira et al., 2022).

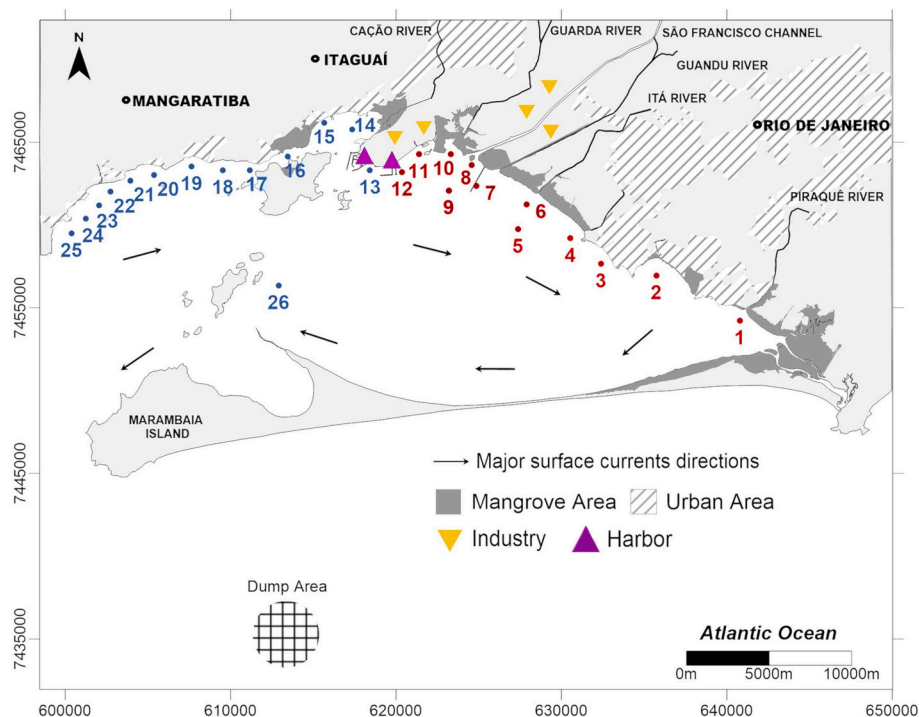
The sequencing analysis using Illumina MiSeq technology was performed by the NED team (UMR1388 GenPhySE) and the GeT core facility (Toulouse, France). The complete protocol is displayed at <https://sites.google.com/site/olivierzembwebsite/16s-sequencing>. For data processing, the QIIME 2 2019.4 pipeline (Caporaso et al., 2010) was used, including demultiplexing, denoising (dada2 package), and rarefying sequences. Taxonomy assignment at 97 % similarity was conducted by comparing representative amplicon sequence variants (ASV) to the SILVA (v. 132) database of known 16S rRNA gene sequences (Beccati et al., 2017; Quast et al., 2013).

The complete dataset was deposited in the NCBI Sequence Read Archive (SRA) database with the accession numbers SAMN20003651 to SAMN20003676, and it is associated with the BioProject number PRJNA743207.

### 2.5. Energy metabolism pathways/marker genes prediction

Tax4Fun2 (Wemheuer et al., 2020) was used to infer the energy metabolism pathways and the related marker genes from the 16S rRNA gene sequence data. The relative proportions of functional profiles were determined by analyzing protein sequences using the Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) database as a reference specifically designed for prokaryotes.

The KOs belonging to nitrogen ( $\text{N}_2$  fixation, nitrate reduction (assimilatory + dissimilatory reductions:  $\text{NO}_3 \rightarrow \text{NH}_4$ ), complete



**Fig. 1.** Location of sampling station in Sepetiba Bay (Rio de Janeiro – Brazil). The stations highlighted in red make up the internal sector (greater anthropic influence), while those highlighted in blue make up the external sector (greater oceanic influence).

nitrification ( $\text{NH}_4 \rightarrow \text{NO}_3$ ), and denitrification ( $\text{NO}_3 \rightarrow \text{N}_2$ ), methane (methanogenesis and methanotrophy), and sulfur ( $\text{SO}_4$  reduction – assimilatory + dissimilatory - and  $\text{S}_2\text{O}_3$  oxidation) energy metabolisms, as well as their main marker genes, were selected for this work, following the KEGG reference pathway database (<https://www.genome.jp/kegg/pathway.html>). The relative proportions of KOs belonging to the same module in a specific energy metabolism pathway (e.g., KOs belonging to the  $\text{N}_2$  fixation module) were summed and then standardized using the ranging for variables with an arbitrary zero approach (Milligan and Cooper, 1985).

## 2.6. Data analysis

Before statistical analysis, environmental (TOC, fine-grain, metal CFs, and  $\Sigma\text{CF}$ ), microbial community (classes and genera abundances), and predicted data (energy metabolisms modules) - were standardized using a min-max scaling approach, which transformed the variables to a common scale with arbitrary zero points (Milligan and Cooper, 1985). Analyses involving marker genes did not require standardization.

Statistical analyses, including Spearman correlation, Mann-Whitney's  $U$  test, and Permutational Analysis of Variance (perMANOVA – ‘Vegan’ package; Oksanen et al. (2019)), were conducted using the R programming language.

Principal Component Analysis (PCA) is a robust statistical approach used to explore relationships between variables within complex datasets. Using the ‘Vegan’ package (Oksanen et al., 2019), PCA was applied to investigate the correlation between environmental parameters (TOC, fine-grain content, and metal CFs) and predicted energy metabolisms in a diverse microbial community inhabiting Sepetiba Bay sediments.

The ‘pheatmap’ package (Kolde, 2019) was used to build heatmaps and cluster correlations between the predicted energy metabolisms/marker genes and the metal CFs/microbial taxa abundance. Spearman's correlation was used when the data did not have a normal distribution (Shapiro-Wilk test,  $p < 0.05$ ).

The Linear discriminant analysis effect size analysis (LefSe - Segata et al. (2011)) was performed to detect indicator energy metabolisms according to the SB sector. Briefly, the non-parametric Kruskal-Wallis sum-rank test was used to detect predicted energy metabolisms with significant differential proportions ( $p < 0.05$ ). Biological consistency was analyzed using the pairwise Wilcoxon test ( $p < 0.05$ ). An LDA threshold score of 2.0 and 1000 bootstrap interactions was applied.

The ‘VennDiagram’ package (Chenn, 2018) was used to create a Venn diagram showing the distribution of the main 39 energy metabolism marker genes according to the SB sectors.

To establish the relationship between predicted energy metabolisms/marker genes and prokaryotic abundance, co-occurrence networks were constructed using the Cytoscape (CoNet app) and visualized with the Gephi software. The analysis was performed with Spearman correlation and a 0.05  $p$ -value threshold. In the networks, nodes represented (i) energy metabolisms and prokaryotic classes (analysis restricted to 150 edges), and (ii) marker genes and prokaryotic genera (analysis restricted to 150 edges). Edges represent highly significant correlations (Spearman;  $p < 0.01$ ), where red edges indicate mutual exclusion and green edges indicate a co-occurrence distribution pattern.

## 3. Results and discussion

### 3.1. Contamination in the Sepetiba Bay sediments

The SB sediments have accumulated contaminants (mainly metals) since the '50s, due to the extraction of Cd and Zn from calamine ore. This activity ceased in the 1990s. In addition to the environmental liabilities left behind by the mining industry, the bay is currently suffering the input of pollutants and urban effluents by the main tributary rivers that contribute densely to the internal SB sector (Carvalho et al., 2021; Moreira et al., 2021). Previous report revealed that the internal SB sector

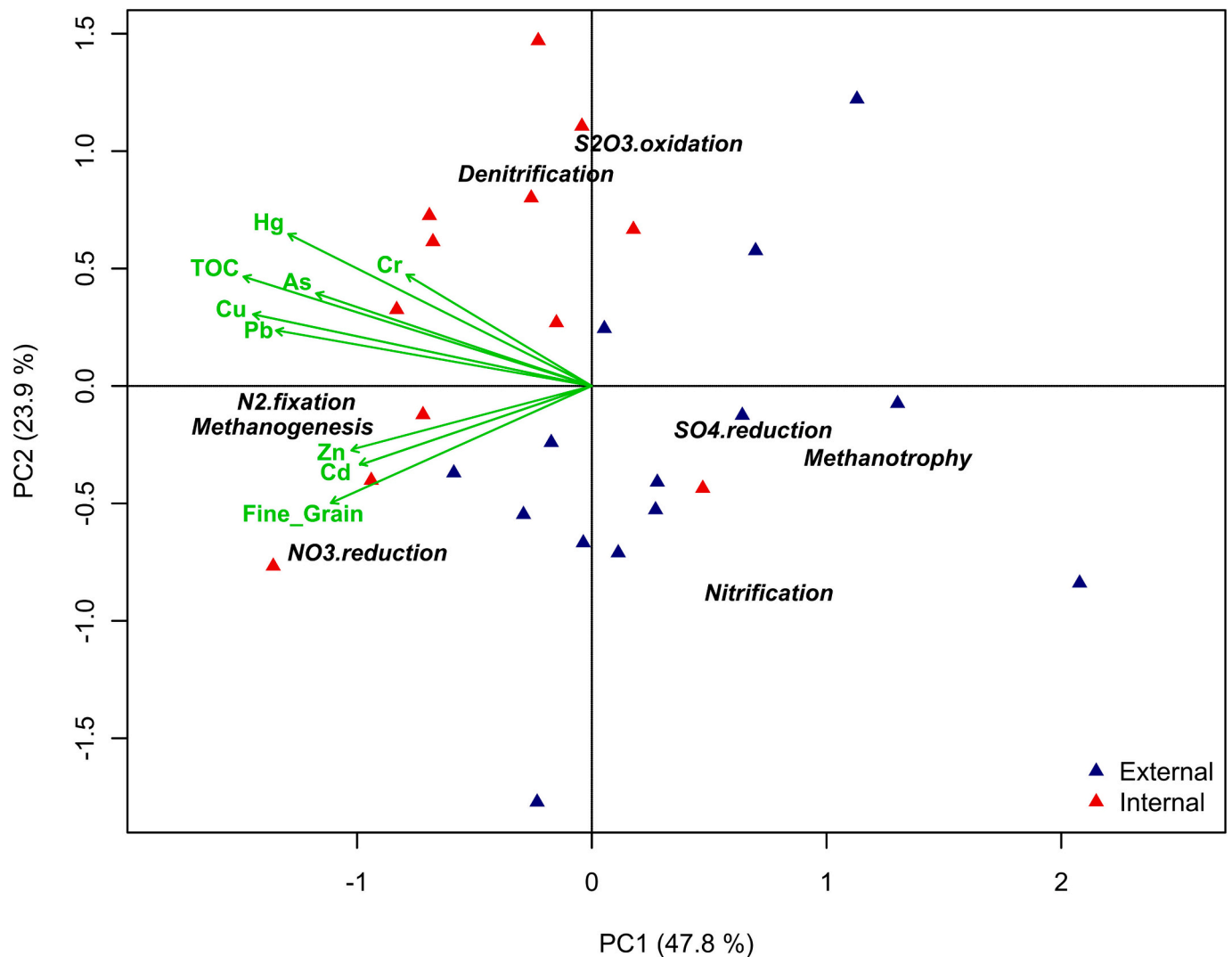
(SB01 to SB12) exhibited significant differences (Spearman;  $p < 0.05$ ) in the fine-grain and total organic carbon (TOC) contents (Moreira et al., 2021), the silt + clay content being twice to that observed in the external SB sector (SB13 to SB26), and TOC triple (Supplementary material - Table S1). In addition, the average  $\Sigma\text{CF}$  in the internal SB sector represents 11-fold that of the external SB sector (Spearman;  $p < 0.05$  – Supplementary material - Table S1). These observations align with the hydrodynamic clockwise circulation, which favours the deposition and accumulation of fine-grain sediments in the SB internal sector (Signorini, 1980), also explaining the higher TOC and contaminant contents found in the internal SB sector because they likely bind at the fine-grain sediment surface (Förstner and Schoer, 1984; Förstner and Wittmann, 1981). In contrast, the strong influence of oceanic circulation, the reduced number of tributaries, and lower urbanization likely explain that the lower metallic contamination level observed at the external SB sector (Moreira et al., 2021). According to the Brazilian legislation (CONAMA, 2012), all the stations located in the external SB sector had a  $\Sigma\text{CF}$  below L1 level (low adverse effects on biota) while nine stations located in the internal SB sector exhibit  $\Sigma\text{CF}$  over L1, and two other stations exceed the L2 (high adverse effects on biota) level (Supplementary material - Table S1). These results underscore the alarming extent of metallic contamination in the internal sector, posing potential risks to the biota.

### 3.2. Distribution of predicted energy metabolic functions in Sepetiba Bay

The Tax4Fun2 prediction, based on 16S rRNA gene sequence data in which the unused sequences was 34 %, generated 8321 predicted functional orthologues (KO). Only the KOs belonging to nitrogen (33), sulfur (21), and methane (64) energy metabolisms were considered in the present study.

The Principal Component Analysis (PCA) showed that fine-grain content, TOC, and contamination factor (CF) of Cr, Hg, Zn, Cd, As, Cu, and Pb explain 71.7 % of the distribution of the predicted energy metabolic functions in the SB sediments (Fig. 2). Nitrogen ( $\text{N}_2$ ) fixation, methanogenesis, nitrate ( $\text{NO}_3$ ) reduction, thiosulfate ( $\text{S}_2\text{O}_3$ ) oxidation, and denitrification were the main predicted energy metabolic functions detected in the internal SB sector stations, characterized by highest metallic and organic pollution (Carvalho et al., 2021; Moreira et al., 2021, 2022, 2023). Noteworthy, these energy metabolic functions exhibited significant higher relative abundance in the internal SB sector (Fig. 3A), further confirmed by their correlation (Spearman correlation,  $p < 0.05$ ) with metal contents (Fig. 3B). Additionally, taxa affiliated with the Thermoplasmata, Thermococci, Thermodesulfobionia, and Dehalococcoidia classes, anaerobic taxa resistant to metallic pollution typically found in reduced environments (Ma et al., 2016; Maturro et al., 2016; Nuzzo et al., 2017; Tangherlini et al., 2020; Teske, 2009), were found dominant in the sediments of the internal SB sector stations (Moreira et al., 2022, 2023), reduced environments due to high TOC and nutrient inputs. The presence of sulfide producing taxa (Thermodesulfobionia, Dehalococcoidia, and Thermococci) (Govindarajan et al., 2022; Lallar et al., 2023; Löffler et al., 2013) likely generate reduced conditions, which are potentially conducive to conditions favouring the development of greenhouse gas (GHG) emitting taxa such as Thermoplasmata, archeon methanogen (Lin et al., 2011). In the same way, the presence of metals favoured denitrification functions (Fig. 2A) consistent with previous reports (Andreote et al., 2012; Liu et al., 2016; Lu et al., 2022; Xi et al., 2021), leading to the GHG  $\text{N}_2\text{O}$  production, while nitrification functions were correlated with the external SB sediments (Figs. 2 and 3B). It is thus likely that the conditions observed in the sediments of the internal SB sector may favour GHG emissions. In contrast, the sediments of the external SB sector have the potential to consume methane and other GHGs via the prevalence of methanotrophy and nitrification metabolisms, as shown by Tax4Fun2 prediction (Figs. 2 and 3A), being thus GHG sink.

In addition, several genera belonging to diazotrophic heterotrophs



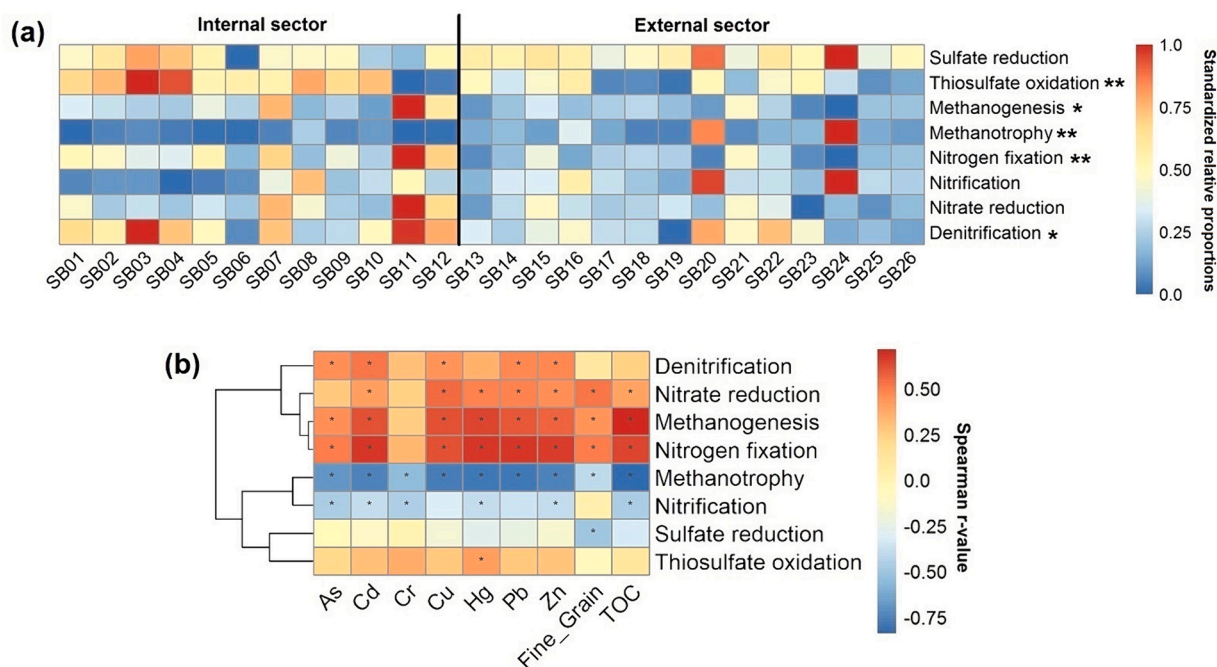
**Fig. 2.** Distribution of energy metabolism modules in SB sediments. Principal Component Analysis (PCA) shows the distribution of energy metabolism modules across Sepetiba Bay (SB) sectors and their correlations with the environmental variables - fine grain content (<63  $\mu\text{m}$ ), TOC, and metal Cfs. The red triangles represent internal sector stations, while those in blue represent external sector stations.

such as Proteobacteria, Firmicutes, and Spirochetes, shown as responsible for N<sub>2</sub> fixation in eutrophic coastal environments (Jabir et al., 2021) with capacity of pollutant degradation (Bazylnski et al., 2000; Ferreira et al., 2018; Odokuma and Inor, 2002), were defined as bio-indicators of metallic contamination in the internal SB sector stations (Moreira et al., 2023). The predicted nitrogen fixation functions showed similar correlation patterns with metal contents as methanogenesis (Figs. 2 and 3B), suggesting that both metabolisms are closely coupled. Such observation is in agreement with previous studies showing the diazotroph/methanogen association, nitrogen fixation releasing hydrogen that is then consumed by methanogens (López-García et al., 2017).

Regarding sulfate reduction, the Tax4Fun2 prediction showed that the related genes were evenly distributed in both sectors with however a slight higher abundance in the external sector stations (Fig. 3A). Noteworthy, sulfate-reducing metabolisms were predominant over those of methanogenesis in external SB stations (Figs. 2 and 3A), which was consistent with a previous study showing that methane emissions were reduced in the permanently flooded sediments close to the SB14 and SB15 stations (Marinho et al., 2012), which was also observed in flooded wetlands (Bourhane et al., 2023). In contrast, methanogenesis outcompetes sulfate reduction in the internal SB sector that may be explained by

the fact that high amounts of labile organic substrates promotes the selection of methanogens (Chen et al., 2011) as methanogens compete with sulfate-reducing taxa for the same substrate, acetate (Sela-Adler et al., 2017). Thus, it is likely that the input of organic matter (e.g. raw sewage) is one of the key factors driving methanogenesis at SB. It is also important to note that methanogenesis can be favoured by the lower salinity prevalent in the internal SB sector stations, due to continental freshwater input (Marinho et al., 2012), as well as by syntrophic association with sulfate reducers (Morris et al., 2013).

The high abundance of predicted genes involved in methanogenesis and denitrification indicated the degradation capacities of the microbial community in the internal SB sector. Indeed, it has been shown that methanogenesis and denitrification are related to natural attenuation ability of the benthic microbial community (Li et al., 2019; Zhang et al., 2015). Methanogens can degrade complex organic compounds present in sediments, including hydrocarbons (Zhou et al., 2022), generating methane as end product (Shima et al., 2002). Likewise, denitrifying bacteria, that use nitrate as electron acceptor, can degrade organic matter and oil-derived contaminants (Zhang et al., 2015). Thus, the potential degradation processes that may occur in the sediments of the internal SB sector are conducive to the emission of GHG, which is of concern at the regional and global scales. The disturbances reported in

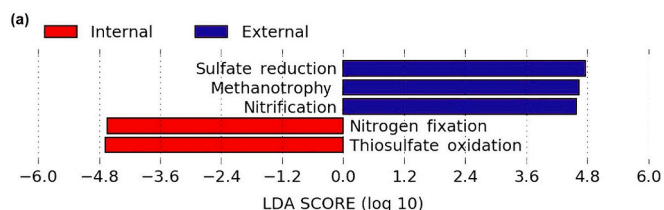


**Fig. 3.** Comparison of energy metabolism modules distribution among the different Sepetiba Bay stations. The Heatmap (a) shows the energy metabolism modules' relative proportions (%) in different Sepetiba Bay (SB) stations - significant differences between SB sectors are indicated by '\*\*' (Spearman;  $p < 0.05$ ) and extremely significant differences are indicated by '\*\*\*' (Spearman;  $p < 0.01$ ). The cluster correlation (b) shows the relationship between the energy metabolism modules and the environmental variables - fine grain content ( $<63 \mu\text{m}$ ), TOC, and metal CFs.

the internal SB sector, eutrophication and chemical pollution, are among the planetary boundaries that have been transgressed (Persson et al., 2022; Steffen et al., 2015). The excessive amount of contaminants released in coastal ecosystems participate to climate imbalances (Herbert-Read et al., 2022). Approximately 5 % of the Brazilian territory covers coastal areas (Governo do Estado da Bahia, 2022), where the main economic activities are developed (IBGE, 2011), concentrating 30 % of the Brazilian population. The disturbance of coastal ecosystems, due to pollutant input results in the installation of anaerobic microbial metabolism, generating GHG ( $\text{N}_2\text{O}$  and  $\text{CH}_4$ ) emissions (Chen et al., 2010). Alas, the situation will be aggravated as surface runoff is expected to increase as a consequence of intensification of tropical rainfall anticipated by climate change prediction (Rabalais et al., 2009; Seidel et al., 2021). It is thus urgent to implement sanitary countermeasures in order to reduce metal and organic inputs because such pollution contribute to GHG emission (Coclet et al., 2021; Li et al., 2019) with global repercussions (Steffen et al., 2015).

### 3.3. Predicted energy metabolic indicators of metallic contamination

The Linear discriminant analysis Effect Size (LEfSe), performed with the eight energy metabolism modules, identified differentially abundant



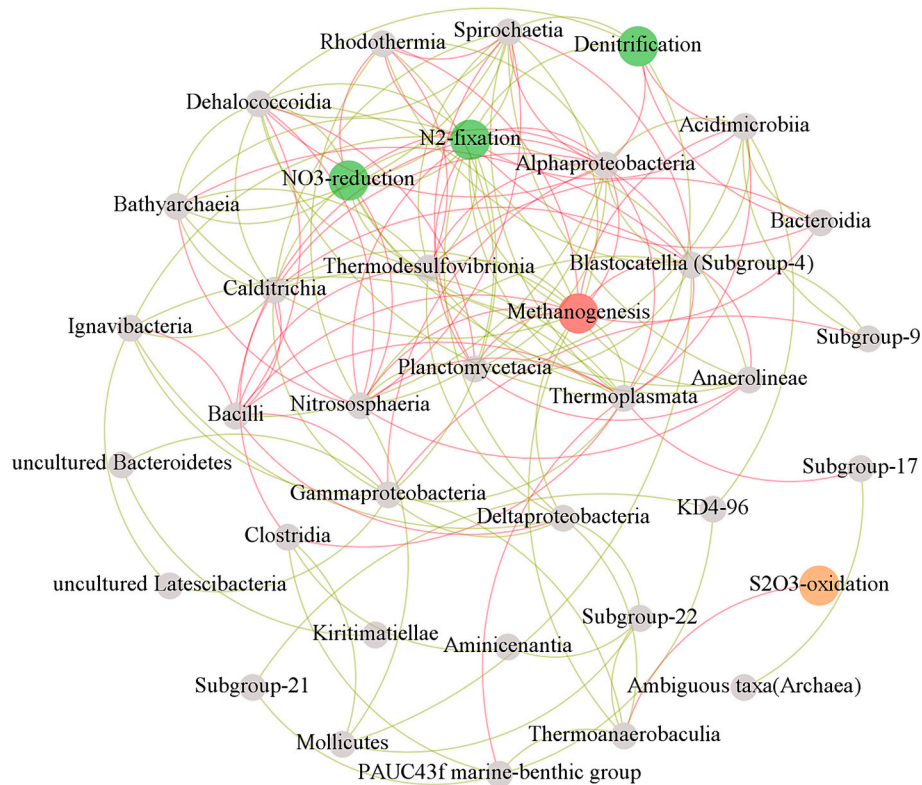
**Fig. 4.** Comparison of energy metabolism modules at Sepetiba Bay sectors. Linear Discriminant Analysis Effect Size (LEfSe) shows energy metabolism modules that have significantly different abundances between Sepetiba Bay (SB) sectors. These metabolisms are indicators of the environmental conditions of each sector.

metabolisms according to the SB sector (Fig. 4), which can be considered as functional indicators (Segata et al., 2011). For the external SB sector, methanotrophy, sulfate reduction, and nitrification metabolisms were identified as function indicators (Fig. 4). Consistently, these metabolisms were negatively correlated with metal CFs (Fig. 3B).

For the internal SB sector, thiosulfate oxidation and nitrogen fixation were found as metabolism indicators (Fig. 4). This observation was supported by the higher abundance of thiosulfate oxidation and  $\text{N}_2$  fixation predicted genes in the internal SB sector (Fig. 3A). Noteworthy, the abundance of these genes was significantly correlated with metal CFs (Spearman;  $p < 0.05$ ; Fig. 3B), suggesting that metallic pollution select such energy metabolisms. Thiosulfate oxidation, universal metabolism in the environment (Newsome and Falagán, 2021), has been found coupled with denitrification and nitrogen fixation in eutrophic environments (Zhang et al., 2024). Nitrogen fixation likely compensate for the lack of nitrogen (Li et al., 2022), favouring the development of microbial taxa involved in the metabolization of environmental pollutants (Bazylnski et al., 2000; Ferreira et al., 2018; Odokuma and Inor, 2002; Onwurah, 1999). Additionally, thiosulfate oxidation has been shown to be involved in metal transformation mechanisms, reducing their bioavailability and toxicity (Juyakumar et al., 2022; Nguyen et al., 2022; Srichandan et al., 2019).

### 3.4. Identification of microbial classes linked to the energy metabolisms selected by metallic pollution

The co-occurrence network, correlating the predicted energy metabolisms linked to metal contamination (CFs) with the microbial classes, identified the microorganisms potentially involved with the functions stake in the environment as well as the metabolism coupling (Fig. 5). The co-occurrence network, restricted to 150 edges considering the extremely significant (Spearman;  $p < 0.01$ ) interactions, showed positive correlations between methanogenesis,  $\text{N}_2$  fixation, and  $\text{NO}_3$  reduction metabolisms (Spearman;  $p < 0.01$  - Fig. 5). Several studies have reported biogeochemical cycles coupling in various environments (Bae et al., 2018; Dang et al., 2021), particularly in eutrophized coastal



**Fig. 5.** Co-occurrence network between the energy metabolism modules that prevail at SB internal sector and prokaryotic classes that inhabit SB sediments. The network was inferred from the relative proportions of the five energy metabolism modules that prevail at the SB internal sector – according to Figs. 1 and 2A – and the prokaryotic classes identified at SB sediments (Moreira et al., 2023) by applying Spearman correlations (curved lines),  $p$ -value threshold 0.05. The positive correlations are indicated in green lines and mutual exclusions in red lines. Nitrogen, methane, and sulfur energy metabolism modules are represented by green, red, and orange circles, respectively.

marine ecosystems (Howarth et al., 2011). The co-occurrence network showed the coupling between methanogenesis and  $N_2$  fixation (Fig. 5), confirming the correlation observed in the internal SB sector (Fig. 3A, B). Coupling mechanisms involving methane and nitrogen biogeochemical cycles have been described in mangrove ecosystems (Qian et al., 2023) and other aquatic environments (Bae et al., 2018). The coupling between biogeochemical cycles has been shown dependent not only on the microorganisms present but also on the availability of electron donors and acceptors, pH and other abiotic factors (Handley et al., 2013) as well as the presence of metallic pollutants (Sun et al., 2020). The network showed that the abundance of methanogenesis,  $N_2$  fixation, and  $NO_3$  reduction metabolisms were positively correlated (Spearman;  $p < 0.01$  – Fig. 5) with Anaerolineae, Bathyarchaeia, Dehalococcoidia, Spirochaetia, Deltaproteobacteria, and Thermodesulfobionia classes while Thermoplasmata and Deltaproteobacteria were correlated with the abundance methanogenic and  $N_2$  fixation metabolisms (Spearman;  $p < 0.01$ ; Fig. 5). It is likely that members associated to these taxa are involved in mechanisms coupling the energy metabolisms. It has been shown that both, Thermoplasmata and Deltaproteobacteria, have the ability of nitrogen fixation (Bae et al., 2018; Jabir et al., 2021; Kapili et al., 2020; Nobu et al., 2016; Staples et al., 2007). They are key players in biogeochemical cycling in marine sediments (Li et al., 2019), being engaged in syntrophic interactions. Deltaproteobacteria releases hydrogen subsequently used by methanogens to fuel their metabolism (Compte-Port et al., 2017; Dawson et al., 2015). Deltaproteobacteria have been shown to exhibit a compelling obligate syntrophy with members of the Firmicutes (López-García et al., 2017), phylum also dominant in the internal SB sector (Moreira et al., 2023). Thus, interconnected energetic metabolisms interactions between Archaea (methanogen) and Bacteria (sulfate-reducers) are likely favoured in the internal SB sector because of the disturbed conditions

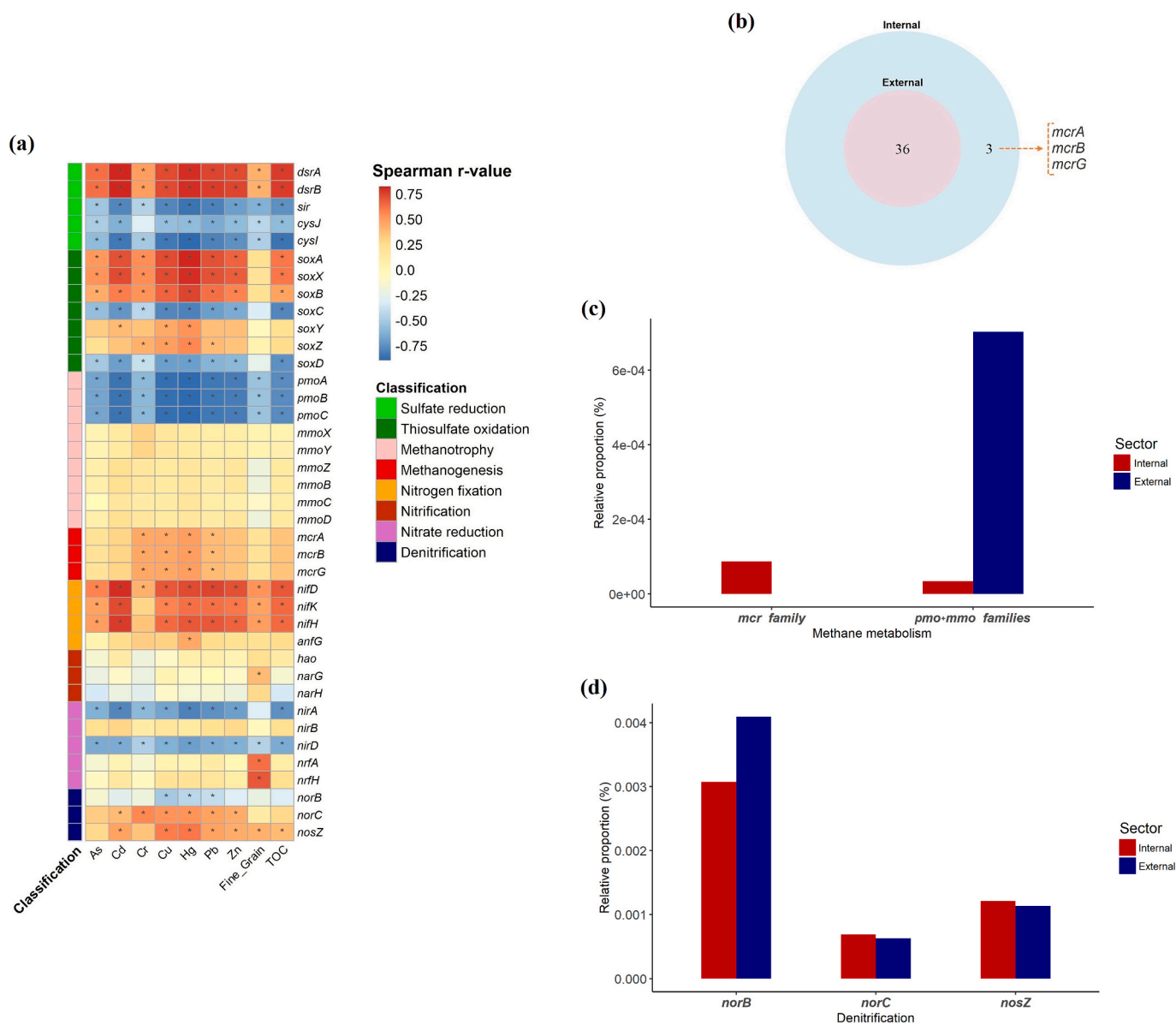
imposed by the human activities. Similar metabolic associations have been described in contaminated aquatic environments (Ahmerkamp et al., 2020; Hamann et al., 2017). However, further studies are required involving functional analysis going beyond predictive approach to decipher the mechanism coupling the different energy metabolisms in disturbed natural environments.

### 3.5. Energy metabolism marker genes under chronic metallic pollution

The predicted genes for sulfate-reduction, thiosulfate-oxidation, methanotrophy, methanogenesis,  $N_2$ -fixation, nitrification, nitrate-reduction, and denitrification metabolisms were identified via the KEGG database. Their relative abundance was correlated with environmental parameters including metal contaminant factors (CFs), TOC and fine grain contents. In total 39 specific gene markers exhibiting significant correlations with metal contamination factors were identified (Fig. 6A), which showed significant difference (perMANOVA;  $p < 0.001$ ) between the SB sectors. The methanogenesis (*mcr*) and  $N_2$ -fixation (*nif*) marker genes exhibited high correlation with metallic pollutant content (Spearman;  $p < 0.05$  – Fig. 6A), suggesting that the metal stress conditions, prevailing in the internal SB sector stations, are adequate for the development of microorganisms carrying these genes. Interestingly, the three methanogenesis marker genes (*mcrA/B/G*) were exclusively predicted for the internal SB sector (Fig. 6B), further supporting that methanogenesis is the main metabolism in the internal SB sector. Additionally, the high correlation of the *nifH* marker gene for  $N_2$ -fixation, carried by sulfate-reducers (Jabir et al., 2021; Jing et al., 2015), with metallic contaminants indicated that they play a crucial role in the bioremediation of metal-contaminated sediments as previously suggested (Li et al., 2019).

In contrast, methanotrophy marker genes (*pmo* and *mmo* family





**Fig. 6.** Specific energy metabolism marker genes selection under SB chronic metal pollution. The correlation heatmap (a) was performed with the relative proportions (%) of specific marker genes belonging to the 8 energy metabolism modules and the environmental variables considered in this study - fine grain content (<63  $\mu\text{m}$ ), TOC, and metal CFs. The heatmap shows Spearman correlations, with significant correlations ( $r < -0.39$  or  $r > 0.39$  and  $p < 0.05$ ) underscored with “\*”. The Venn diagram (b) shows the shared and exclusive marker genes between either SB sector. The bar plot (c) shows the *mcr* and *pmo* + *mmo* marker genes (methanogenesis and methanotrophy, respectively) and their relative proportions (%) across SB sectors. While, the bar plot (d) shows the *norB*, *norC*, and *nosZ* marker genes (denitrification) and their relative proportions (%) across SB sectors.

genes) were not or were negatively correlated with metal CFs (Spearman;  $p < 0.05$ ). Such observation indicated that methanotrophy was favoured at the stations of the less impacted SB sector, which was consistent with the PCA and correlation analyses showing that methanotrophy was strongly associated with sulfate-reduction metabolisms (Figs. 2 and 3B). The methane-oxidation/sulfate-reduction association has been shown as the main balance/consumption mechanism for methane emissions in coastal areas (Bhattarai et al., 2019; Weber et al., 2017).

The relative abundance of predicted *dsrA/B* genes (dissimilatory sulfate reduction) was positively correlated (Spearman correlation  $r > 0.75$ ,  $p < 0.05$ ) with metal content, while the relative abundance of predicted *sir* and *cysJ/I* genes (assimilatory sulfate reduction) was negatively correlated (Spearman correlation  $r < -0.75$ ,  $p < 0.05$ ) with metal content (Fig. 6A). Such observation indicated that sulfide production potential was detected at the SB, further supporting the

homogenous distribution of sulfate-reduction metabolism across the two SB sectors (Fig. 3A), but via assimilatory sulfate reduction in the low metal-contaminated external SB sector, while through dissimilatory sulfate reduction in the high metal-contaminated internal SB sector. Such observation suggested functional redundancy of sulfate reduction in the SB sediments. The dissimilatory sulfate reduction, sulfate used as electron acceptor, is predominant in wastewater treatment plants (Li et al., 2020b; Rodrigues et al., 2020), organic-contaminated and metal-rich ecosystems (Giloteaux et al., 2010; Stauffert et al., 2015; Wu et al., 2013), while the assimilatory sulfate reduction, incorporation to synthesize sulfur amino-acids, has been described in different environments (Zheng et al., 2017; Zhu et al., 2018).

Similarly, the relative abundance of the predicted thiosulfate oxidation related genes showed different correlations. Genes of the *sox* complex (*soxA/B/X/Y/Z*) correlated positively with metal content (Spearman  $r > 0.5$ ,  $p < 0.05$ ), while the relative abundance of the

predicted *soxC* and *soxD* genes, encoding SoxCD protein involved in the last oxidation step (Ghosh and Dam, 2009), was negatively correlated (Spearman  $r < -0.5$ ,  $p < 0.05$ ) with metal content (Fig. 6A). It is likely that microorganisms inhabiting the high metal-contaminated internal SB sector are lacking this final step. The sulfur atom probably enter another pathways or serve as sulfur storage (Ghosh and Dam, 2009). It is important to note that sulfide oxidation can also be performed through reverse dissimilatory sulfate reduction (Wu et al., 2021), which has been found correlated with metal content (Fig. 6A).

Regarding denitrification, the relative abundance of the predicted *norC* gene was positively correlated (Spearman  $r > 0.4$ ,  $p < 0.05$ ) with metal content, while that of the predicted *norB* gene was negatively correlated with metal (Spearman  $-0.3 > r > -0.5$ ,  $p < 0.05$ ) content (Fig. 6). Such observation showed once again the functional redundancy at SB. The reduction of nitric oxide (NO) to nitrous oxide (N<sub>2</sub>O) is likely performed by distinct microorganisms (Borrero-de Acuña et al., 2016), with *norC* gene-carrying microorganisms being active in the metal-contaminated internal SB sector. These microorganisms are *cnorB* denitrifiers performing the NO reduction by the cytochrome NorBC reductase (Yang et al., 2024). Although *norB* genes have been found correlated with metallic contaminants in estuarine sediments (Wang et al., 2022a, 2022b, 2022c), so far the distribution of *cnorB* denitrifiers according to the presence of pollutants has not been reported to be different to that of *qnorB* denitrifiers (quinol-oxidizing type nitric oxide reductase) carrying only a *norB* gene (Yang et al., 2024). Thus, further analysis are required in order to determine the impact of pollution on the distribution of the different type of NO reducers. Interestingly, the relative abundance of predicted *nosZ* gene was positively correlated (Spearman  $r > 0.4$ ,  $p < 0.05$ ) with metals, which was in agreement with a previous report showing the correlation with arsenic (Wang et al., 2022a, 2022b, 2022c).

The observed differences in the relative abundances of predicted genes according to the environmental conditions (Fig. 6A) rely probably on their evolution history. Evolutionary mechanisms involving mutations, duplications, and vertical and horizontal gene transfers result in orthologous genes (Adam et al., 2022; Bolhuis et al., 2010; Staples et al., 2007; Wang et al., 2021), conducting to the diversification and adaptation of organisms in a wide range of environmental conditions (Harrison et al., 2007). Therefore, the identified energy metabolism marker genes according to metal CFs can also trace the adaptation trajectory that has led to the ability to withstand different types of pollution.

Because Tax4Fun2, as any predictive analysis, has inherent limitations, particularly considering that some functional genes are species specific (Breitkreuz et al., 2021), further analyses are required to establish the functional gene inventory by shotgun sequencing or by monitoring specifically the abundance or expression of the functional genes by qPCR. Ideally, a study combining molecular approaches targeting specifically the functional genes with geochemical analysis measuring GHG fluxes would provide a comprehensive picture of the microbial processes stake in SB.

### 3.6. Potential methane and nitrous oxide emissions at Sepetiba Bay

Although predictive, our study provide useful information on the presence of potential functional genes involved in GHG emissions, laying the foundations and hypotheses for the orientation of future research. In order to draw the potential balance of GHG emissions, we considered the relative abundance of the following genes: (i) *mcrA/B* (methyl-coenzyme M reductase) catalyzing the formation of methane; (ii) *mmo/pmo* (methane monooxygenase) consuming methane by transforming it into methanol; (iii) *norB/C* (nitric oxide reductase) catalyzing the formation of nitrous oxide from nitric oxide; and (iv) *nosZ* (nitrous oxide reductase) consuming nitrous oxide by transforming it into N<sub>2</sub>. These genes showed significant different relative abundance (perMANOVA,  $p < 0.05$ ) between the SB sectors.

Regarding methane emission, only the *pmo/mmo* genes ensuring

methanotrophy were predicted in the external SB sector. Such observation indicated that the external SB sector is probably a methane sink where even if methane is produced it is likely consumed by methanotrophs. The aerobic methane oxidation is an important methane consumption in aquatic ecosystems (Hanson and Hanson, 1996) under oxic conditions, as those prevailing at the external SB sector due to the oceanic influence. In contrast, the relative abundance of the predicted *pmo/mmo* genes was two times lower than that of the predicted *mcr* (methanogenesis) genes in the internal SB sector (Fig. 6C). Even if methane consumption under anaerobic conditions can occurs through methanotrophs/sulfate-reducers association with reverse methanogenesis (Bhattarai et al., 2019; Weber et al., 2017), such observation suggested that the metal-contaminated internal SB sector acts as methane emission as shown in previous studies (Barroso et al., 2022; Marinho et al., 2012). The methane production is attributed to organic matter loads entering the internal SB sector through the many rivers that flow into SB (Carvalho et al., 2021).

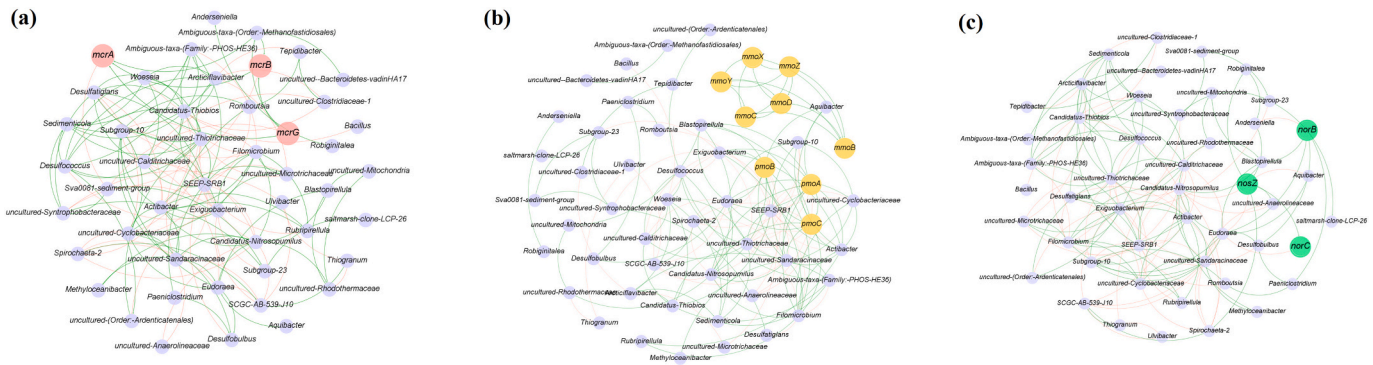
Regarding nitrous oxide (N<sub>2</sub>O) emission, the microbial community inhabiting SB sediments demonstrated potential N<sub>2</sub>O emission because the relative abundance of the predicted *norB* + *norC* genes, involved in N<sub>2</sub>O production, was higher than that observed for the predicted *nosZ* gene, involved in N<sub>2</sub>O transformation into N<sub>2</sub> (Fig. 6D). It is important to note that N<sub>2</sub>O emissions have been shown to be attributed to both denitrification and nitrification processes (Murray et al., 2015). The relative abundance of the predicted genes involved in nitrification was not correlated with metal content (Fig. 6A), being higher in the external SB sector (Figs. 2, 3, 4). Such observation indicated that nitrification is likely favoured in the external SB sector probably because the ocean currents create favourable oxic conditions (Moreira et al., 2021, 2023). Nitrous oxide is a potent GHG (Wang et al., 1976) resulting from human activities (Chen et al., 2022; Murray et al., 2015), estuaries contributing for almost 10 % of its emission although they represent <1 % of the global ocean area.

### 3.7. Potential microbial genera contributors to GHG

In order to identify the potential genera contributing to the GHG emissions, co-occurrence networks were constructed to correlate energy metabolism marker genes with the 50 most prevalent genera that colonize SB sediments. The relative abundance of *mcr*, methanogenesis marker genes, exhibited positive correlation (Spearman,  $p < 0.01$ ) with genera affiliated to the *Methanofastidiosales* order, exclusively detected in the internal sector (Fig. 7A). Members of the *Methanofastidiosales* have been detected in sediments from various aquatic ecosystems where they play a key role in methane production (Qu et al., 2024; Song et al., 2020). Thus, it is likely that *Methanofastidiosales* members are probably involved in methane emissions in the internal SB sector.

The relative abundance of *pmo* genes family, methane oxidation marker genes, was positively correlated (Spearman,  $p < 0.05$ ) with *Actibacter*, *Aquibacter*, *Blastopirellula*, *Candidatus Nitrosopumilus*, *Eudoraea*, *Exiguobacterium*, *Filomicrobium*, and *uncultured Sandaracinaceae* (Fig. 7B). *Pmo* genes were found 37 times more abundant in the low metal contaminated external SB sector (Fig. 6C), in accordance with the fact that methanotrophy has been identified as metabolism marker for the external SB sector (Fig. 4). The genera positively linked to *pmo* genes have been found in various marine ecosystems contaminated or not (López-Sánchez et al., 2024; Wu et al., 2009; Zárate et al., 2021), they are thus likely the main players involved in methane consumption (or its intermediary) in the external SB sector. Noteworthy, members of the *Exiguobacterium* genus have been found closely associated with methyloprophs, even increasing methane oxidizing activity (Veraart et al., 2018).

The relative abundance of *norB* and *norC*, nitrous oxide producing marker genes, was positively correlated with the relative abundances of *Blastopirellula*, *Desulfobulbus*, *Eudoraea*, and *Paeniclostridium* genera (Fig. 7C). In agreement with previous study reporting that these genera



**Fig. 7.** Co-occurrence network between specific energy metabolism marker genes and the 50 most abundant prokaryotic genera at SB. The networks were inferred from the relative abundances of the 50 most abundant prokaryotic genera that inhabit SB sediments (Moreira et al., 2023) and the relative proportions of specific marker genes belonging to (a) methanogenesis, (b) methanotrophy, and (c) denitrification energy metabolisms. The analysis focused on highly significant interactions (Spearman;  $p < 0.01$ ), resulting in a restricted set of 150 edges. The positive correlations are indicated in green lines and mutual exclusions in red lines. Methanogenesis, methanotrophy, and denitrification marker genes are represented by red, orange, and green circles, respectively.

were identified as bioindicators of reduced pollution/eutrophication in the external SB sector (Moreira et al., 2023), this observation suggested that they are probably responsible for  $N_2O$  emissions at external SB sector. Interestingly, the correlation of *Desulfobulbus*, sulfate-reducing bacteria, with denitrification genes highlight the interaction between biogeochemical cycles in marine ecosystems (Zhang et al., 2023).

Finally, the relative abundance of *nosZ* gene, responsible for  $N_2O$  reduction to  $N_2$  (Kim et al., 2022), was positively correlated (Spearman;  $p < 0.01$ ) with the relative abundance of *Andersenella*, *uncultured Anaerolineaceae*, *Robignitalea*, and *Spirochaeta-2* (Fig. 7C). It has been shown that some members of the *Hyphomicrobiale* order (e.g. genus *Andersenella*) are able to grow anaerobically using nitrate as an electron acceptor (Oren and Xu, 2014), further confirming the biogeochemical cycles interaction at SB.

#### 4. Conclusions

The Sepetiba Bay (SB) presents two contrasted sectors, the internal SB sector exhibiting contaminated sediments caused by the excess of nutrients and pollutants conveyed by the major tributary rivers that flow into the bay, while the external SB sector sediment presents moderate pollution because of the oceanic currents' influence.

The relative abundances of metabolisms involved in methanogenesis, nitrate-reduction, denitrification,  $N_2$ -fixation, and thiosulfate oxidation were found higher in the internal SB sector. The chronic metal-contamination and the high organic content, as well as redox conditions may explain the higher relative abundance of these metabolisms in the internal SB sector, which probably play a crucial role in the natural attenuation of contaminants. Particularly,  $N_2$ -fixation and thiosulfate-oxidation were identified as energy metabolism indicators of the polluted internal SB sector. The  $N_2$ -fixation was also found to be associated with methanogenesis, *Methanofastidiosales* being the main taxa involved in this process. Thus, the internal SB sector holds higher  $CH_4$  emission and higher  $N_2O$  consumption than the external sector. In contrast, the external sector exhibited a higher relative abundance of predicted *pmo* marker genes (methane oxidation), and higher relative abundance of *norB/C* marker genes involved in  $N_2O$  emission. Noteworthy, both SB sectors exhibited the potential for  $N_2O$  emission, the relative abundance of *norB/C* genes being 3 to 4 times higher than that of *nosZ* gene. Thus, SB should be considered as a bay where the potential of  $N_2O$  emission dominates.

Our study revealed that SB own microbial communities exhibiting the potential to produce GHG, the main concern being the production of  $N_2O$ . However, further studies are required to determine the comprehensive actual balance of GHG emissions. Nevertheless, we recommend that research efforts should be made to identify and map the key

microbial taxa associated with GHG emissions in coastal areas impacted by human activities. By unveiling the pivotal players within coastal microbial communities, such investigations will pave the way for targeted strategies aimed at mitigating greenhouse gas emissions and advancing sustainable environmental practices worldwide.

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#### CRediT authorship contribution statement

**Vanessa Almeida Moreira:** Writing – review & editing, Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Cristiana Cravo-Laureau:** Writing – review & editing, Supervision, Conceptualization. **Angelo Cezar Borges de Carvalho:** Methodology. **Alice Baldy:** Methodology. **Edison Dausacker Bidone:** Writing – review & editing, Supervision. **Elisamara Sabadini-Santos:** Writing – review & editing, Supervision. **Robert Duran:** Writing – review & editing, Writing – original draft, Validation, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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