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Microbial Diversity of Deep-Sea Ferromanganese Crust Field in the Rio Grande Rise, Southwestern Atlantic Ocean

Natascha Menezes Bergo[1] · Amanda Gonçalves Bendia[1] · Juliana Correa Neiva Ferreira[1] · Bramley J. Murton[2] · Frederico Pereira Brandini[1] · Vivian Helena Pellizari[1]

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Abstract

Seamounts are often covered with Fe and Mn oxides, known as ferromanganese (Fe–Mn) crusts. Future mining of these crusts is predicted to have significant effects on biodiversity in mined areas. Although microorganisms have been reported on Fe–Mn crusts, little is known about the role of crusts in shaping microbial communities. Here, we investigated microbial communities based on 16S rRNA gene sequences retrieved from Fe–Mn crusts, coral skeleton, calcarenite, and biofilm at crusts of the Rio Grande Rise (RGR). RGR is a prominent topographic feature in the deep southwestern Atlantic Ocean with Fe–Mn crusts. Our results revealed that crust field of the RGR harbors a usual deep-sea microbiome. No differences were observed on microbial community diversity among Fe–Mn substrates. Bacterial and archaeal groups related to oxidation of nitrogen compounds, such as Nitrospirae, Nitrospinae phyla, *Candidatus Nitrosopumilus* within Thaumarchaeota group, were present on those substrates. Additionally, we detected abundant assemblages belonging to methane oxidation, i.e., Methylomirabilales (NC10) and SAR324 (Deltaproteobacteria). The chemolithoautotrophs associated with ammonia-oxidizing archaea and nitrite-oxidizing bacteria potentially play an important role as primary producers in the Fe–Mn substrates from RGR. These results provide the first insights into the microbial diversity and potential ecological processes in Fe–Mn substrates from the Atlantic Ocean. This may also support draft regulations for deep-sea mining in the region.

Keywords Deep-sea ferromanganese crusts · Microbial community · Biogeochemical cycling · Rio Grande Rise · Geomicrobiology

Introduction

The process of biogenesis in Fe–Mn crusts formation is not well described at all. Microorganisms are suggested to be potentially involved in the elemental transition between seawater and Fe–Mn substrates [16, 45], and in the Mn oxidation and precipitation. Microbial diversity and abundance have been studied in the Fe–Mn crusts, and their associated sediment and water of Pacific Ocean seamounts [15, 22, 30, 31]. These studies have detected the potential for microbial chemosynthetic primary production supported by ammonia oxidation [12, 15, 31]. Wang and Muller (2009) proposed that free-living and biofilm-forming bacteria provide the matrix for manganese deposition, and coccolithophores are the dominant organisms that act as bio-seeds for an initial manganese deposition in the Fe–Mn crusts. Kato et al (2019) proposed a model of the microbial influence in the biogeochemical cycling of C, N, S, Fe, and Mn in Fe–Mn crusts, which indicates their contribution to crust development. In contrast, other authors suggested that these endolithic and epilithic microbial communities just live in/on Fe–Mn crust as a favorable physical substrate [12, 30].

Fe–Mn crust occurs on rocky substrates at seamounts globally and has a slow accretion rate of typically 1–5 mm per million years [10, 43, 47]. Seamounts and rises are most common in the Pacific Ocean where there are estimated to be over 50,000 [19, 47]. Fe–Mn crusts are economically important because they mainly contain cobalt and other rare and trace metals of high demand including copper, nickel, platinum,

✉ Vivian Helena Pellizari
vivianp@usp.br

[1] Instituto Oceanográfico, Universidade de São Paulo, São Paulo, Brazil

[2] National Oceanography Centre, Southampton, England

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and tellurium [10]. While crusts are potentially of economic value, seamounts also offer a habitat appropriate for sessile fauna, most corals, and sponges [9, 36]. However, the ecological roles of chemosynthetic and heterotrophic microbes in metal-rich benthic ecosystems remain unknown for the Atlantic Ocean.

Despite the increasing efforts that have been made using high-throughput DNA sequencing of microbes living on Fe–Mn crusts from the Pacific Ocean, similar studies from the Atlantic Ocean are still scarce. Considering the wide distribution of Fe–Mn crusts on seamounts globally and their potential for future mineral resource supplies [10], the study of their microbiome, function, and resilience to environmental impacts caused by its exploitation is essential [33].

To better understand how microbial community structure is shaped by metallic substrates in seamounts, we sampled Fe–Mn crust biofilm, crusts, encrusted coral skeletons, and calcarenite substrate from the Rio Grande Rise (RGR), Southwestern Atlantic Ocean. We used the sequencing of the 16S rRNA gene to determine the microbial diversity and community structure, and to predict microbial functions and ecological processes. We hypothesized that there is a core microbiome among the Fe–Mn substrates, despite the influence of different environmental conditions such as water masses, temperature, salinity, and depth.

Material and Methods

Field Sampling

The study area, RGR, is an extensive topographic feature of ~ 150,000 km^2 in the Southwestern Atlantic Ocean [7] (Fig. 1). RGR is approximately 1000–4000 m deep and is located ~ 1000 km to the east of the Brazil and Argentine basins [7, 26].

Fe–Mn crust biofilm, crust, encrusted coral skeletons, and calcarenite samples were collected during the Marine E-Tech RGR2 expedition DY094 onboard the Royal Research Ship (RRS) Discovery (NOC-NERC, UK) in October 2018 from the RGR (Fig. 1 and Table 1). Onboard the vessel, special care was taken to avoid cross-contamination among samples during the sample recovery and the material was aseptically collected from each dredge or from the ROV HyBIS (NOC-NERC, UK). The surface of the crust and encrusted coral samples was washed three times with seawater filtered through a 0.2 μm-pore polycarbonate membrane to remove loosely attached particles, possibly containing contaminants derived from sediments and seawater. Samples were immediately stored in sterile DNA/RNA-free Whirl-Pak bags. Surprisingly, some Fe–Mn crust samples had a shiny biofilm on their surface (Fig. 1d). The biofilms of the crust surface were peeled off using a sterile spatula and stored in sterile DNA/RNA-free crioval tubes. All samples were stored at − 80 °C until DNA extraction.

DNA Extraction, 16S rRNA Gene Amplification and Sequencing

The surface (~ 0–10 mm) of the Fe–Mn crust, encrusted coral skeletons, and calcarenite was subsampled using sterile hammers and chisels. The crust and encrusted coral skeletons pieces were crushed in a sterile agate mortar. DNA was extracted from 5 g of samples with the FastDNA™ SPIN Kit for Soil (MPBiomedical), according to the manufacturer's protocol [21, 39]. Negative (no sample) extraction controls were used to ensure the extraction quality. DNA integrity was determined after electrophoresis in 1% (v/v) agarose gel prepared with TAE 1X (Tris 0.04 M, glacial acetic acid 1 M, EDTA 50 mM, pH 8), and staining with Sybr Green (Thermo Fisher Scientific, São Paulo, Brazil). DNA concentration was determined using the Qubit dsDNA HS assay kit (Thermo Fisher Scientific, São Paulo, Brazil), according to the manufacturer's instructions.

Before sending samples for preparation of Illumina libraries and sequencing, the V3 and V4 region of the 16S rRNA gene was amplified with the primer set 515F (5′–GTGCCAGCMGCCGCGGTAA-3′) and 926R (5′–CCGYCAATTYMTTTRAGTTT-3′), [34] to check for the amplification of 16S using the extracted DNA. Negative (no sample) extraction controls were used for PCR amplification and the Illumina sequencing to check for the presence of possible environmental contamination [38]. Illumina DNA libraries and sequencing were performed at Biotika (www.biotika.com.br, Brazil) on a MiSeq platform in a paired-end read run (2 × 250 bp) following the manufacturer's guidelines. Sequencing outputs were the raw sequence data.

Sequencing Data Processing and Statistical Analyses

The demultiplexed sequences were analyzed with the software package Quantitative Insights Into Microbial Ecology (QIIME 2) version 2019.4 [3]. Sequences were denoised using DADA2 [6] with the following parameters: trim left-*f* = 19, trim left-*r* = 18, trunc-len-*f* = 287, trunc-len-*r* = 215. Amplicon sequence variants (ASVs) with sequences less than 10 occurrences were removed. The taxonomy was assigned to the representative sequences of ASVs using a Naive Bayes classifier pre-trained on SILVA release 132 clustered at 99% identity. FastTree and MAFFT [17] were used to create a rooted phylogenetic tree which was used in calculating phylogenetic diversity metrics.

Diversity and phylogenetic analyses were performed with the PhyloSeq [25], ggplot2 [48], and vegan [32] packages in the R software (Team, 2018). ASVs affiliated with chloroplasts, Eukarya, *Ralstonia* in Betaproteobacteria, and

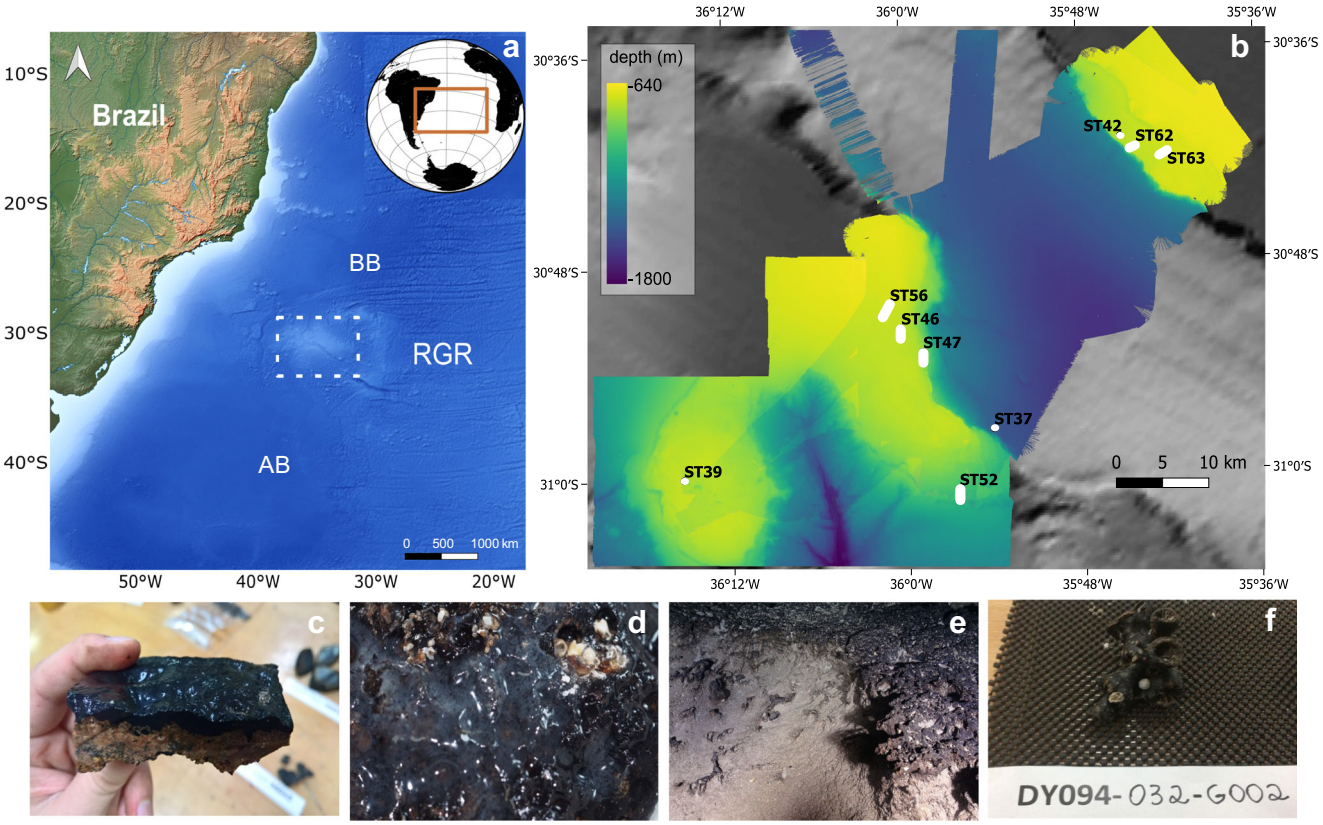

Fig. 1 The sampling map with the location of Rio Grande Rise, Atlantic Ocean (**a**). AB, Argentine Basin; BB, Brazilian Basin. Distribution of collection stations across Rio Grande Rise (**b**). Location of all samples collected with dredges (white lines) and with HyBIS (white circles). Numbers refer to stations. High resolution bathymetry (Jovane et al. 2019). Examples of (**c**) Fe–Mn crust , **d** Fe–Mn crust biofilm on Fe–Mn crust, **e** calcarenite, and **f** Fe–Mn encrusted coral skeletons collected during the scientific expedition on the RRS Discovery on the RGR

Bacillus in Firmicutes, originated from contaminants in clean laboratory water and/or the reagents used in DNA extraction for sequencing were removed from subsequent analyses [38]. Alpha-diversity metrics (e.g., observed sequence variants, Chao1, and Shannon diversity) were calculated based on ASV relative abundances for each sample. To determine if there were significant differences between alpha diversities, analysis of variance (Kruskal–Wallis one-way ANOVA on ranks test) test was performed in R. ASVs were normalized using the R package "*DESeq2*" by variance stabilizing transformation [24]. Beta diversity among sample groups was examined an ordinated weighted Unifrac normalized distance and visualized using principal coordinate analysis (PCoA, package Phyloseq). PERMANOVA analysis to compare groups in the PCoA plots was performed with the adonis function in the R package vegan. Betadisper from the same

Table 1 Sample list, sample type, latitude, longitude, depth from RGR and sampling equipment

Sample ID	Sample type	Water mass	Station	Lat	Long	Depth (m)	Sampler
DY094_63_01	Fe–Mn crust	SACW	63	30.704	35.704	663	Dredge
DY094_56_01	Fe–Mn crust	SACW	56	30.838	36.016	664	Dredge
DY094_47_01	Fe–Mn crust	SACW	47	30.875	35.981	762	Dredge
DY094_47_02	Fe–Mn encrusted coral skeletons	SACW	47	30.875	35.981	762	Dredge
DY094_52_02	Fe–Mn encrusted coral skeletons	AIA	52	31.009	35.943	903	Dredge
DY094_62_01	Calcarenite	SACW	62	30.698	35.742	661	Dredge
DY094_62_02	Calcarenite	SACW	62	30.698	35.742	661	Dredge
DY094_39_01	Fe–Mn crust biofilm	SACW	37	30.969	35.912	881	HyBIS
DY094_45_01	Fe–Mn crust biofilm	SACW	39	30.005	35.245	758	Dredge
DY094_46_03	Fe–Mn crust biofilm	SACW	46	30.854	35.005	685	Dredge
DY094_61_01	Fe–Mn crust biofilm	SACW	42	30.689	35.746	711	HyBIS

package was used to assess the differences in dispersions between sample groups. Relative abundance of taxonomic indicators was identified by the IndicSpecies [5]. The analysis was conducted on ASV counts excluding ASVS < 20 reads. Predicted microbial functional groups were identified by the Functional Annotation of Prokaryotic Taxa (FAPROTAX) database [23]. Statistical tests were considered significant at $p < 0.05$. FAPROTAX extrapolates functions of cultured prokaryotes (identified at the genus or species level) to the rest of the prokaryotic genus to estimate putative functions. The sequencing reads generated for this study can be found in the National Centre for Biotechnology Information (NCBI) database under the BioProject PRJNA638744.

Results

Alpha and Beta Diversity Estimates at Rio Grande Rise

A total of 666,782 sequences were retrieved from eleven samples and ranged from 35,166 to 131,475 per sample, (Supplementary Table 1). After removing chimeras, sequences were clustered into 1992 amplicon sequence variants (ASVs) (0.03 cut-off), and after filtering other contaminant groups, a total of 1874 ASVs were obtained. Alpha diversity indices were not statistically different among Fe–Mn substrates (Chao1, Kruskal–Wallis one-way ANOVA on ranks test, df = 4, $P = 0.06$; Shannon, Kruskal–Wallis one-way ANOVA on ranks test, df = 4, $P = 0.07$) (Fig. 2 and Supplementary Table 1). Beta diversity among the samples and substrates was tested using the weighted Unifrac distances and ordered by PCoA. The PCoA analysis captured 60.5% of the total variation of the prokaryotic community composition in the investigated samples. In the PCoA plot, samples were clustered by sample types, expected DY94_56_01, and DY94_46_03 (Fig. 3). However, there was no significant difference in beta diversity by sample types (PERMANOVA, df = 3, $F = 0.9$, $r^2 = 0.27$, $P = 0.6$), sampling location (PERMANOVA, df = 1, $F = 1.02$, $r^2 = 0.10$, $P = 0.4$), and depth (PERMANOVA, df = 1, $F = 1.01$, $r^2 = 0.10$, $P = 0.4$). The PCoA plot shows a scattering for sample depth (betadisper, df = 8, $F = 3.5e30$, $P = 0.001$) (Supplementary Fig. 1).

Microbial Community Composition at Rio Grande Rise

Bacterial and Archaeal composition varied in abundance or occurrence among substrates and samples (Fig. 4). For example, the microbial groups Proteobacteria (classes Gammaproteobacteria and Alphaproteobacteria), Thaumarchaeota (Nitrosopumilales), and Planctomycetes (classes Phycisphaerae) were abundant in all substrates, except the DY094_63_01, DY094_47_01, DY094_52_02, DY094_39_01, and DY094_46_03 samples (Fig. 4 and Fig. 5). ASVs affiliated to Verrucomicrobia, Marinimicrobia (SAR406 clade), Calditrichaeota, Cyanobacteria, and Firmicutes represented less than 1% of all sequences (Fig. 4a). Looking at class level, Nitrososphaeria was the most abundant group (23%), especially among the crust biofilm samples (Fig. 4b and Fig. 5). Gammaproteobacteria was the second most abundant class (22%), followed by Alphaproteobacteria (12%) (Fig. 4b and Fig. 5).

In Fe–Mn crust biofilm, the prevalent class was Nitrososphaeria (60-51%, Nitrosopumilales), Dehalococcoidia (20–7%, order SAR202 clade), Gammaproteobacteria (10–5%, orders Steroidobacterales and uncultivated UBA10353 marine group), Alphaproteobacteria (10–3%, orders Rhodovibrionales and SAR11), and Deltaproteobacteria (6–1%, orders SAR324 clade and NB1-j), except in the DY094_39_01 sample% (Fig. 4b and Fig. 5). The bacterial class Dehalococcoidia was not identified in DY094_39_01.

The prevalent classes in the crusts were Gammaproteobacteria (57–24%, order Steroidobacterales, MBMPE27, and SAR86), Alphaproteobacteria (17–16%, order Rhodovibrionales), and Nitrososphaeria (17–7%, order Nitrosopumilales) and NC10 (9%, order Methylomirabilales) (Fig. 4b). The uncultivated bacterial groups PAUC43f marine benthic group and Phycisphaerae were more abundant in DY094_47_01 and DY094_63_01 crust samples (Fig. 5). Otherwise, representatives of Dadabacteriia, Nitrospira, Deltaproteobacteria (order SAR324), uncultivated BD2-11 terrestrial group, uncultivated MBMPE27, and Calditrichia were more abundant in the DY094_63_01 crust (Fig. 5). ASV related to a Colwelliaceae family was only detected in the DY094_47_01 and DY094_63_01 crust samples.

In Fe–Mn encrusted coral skeletons, the prevalent class was related to Gammaproteobacteria (27% orders uncultivated MBMPE27, SAR86, and Steroidobacterales), Alphaproteobacteria (20%, orders Rhodovibrionales and SAR11), Planctomycetacia (7%, order Pirellulales), Nitrososphaeria (5%, Nitrosopumilales), and Deltaproteobacteria (5%, order NB1-j) (Fig. 4b and Fig. 5). The classes Entotheonellia (order Entotheonellales) and NC10 (order Methylomirabilales) were only detected in the DY094_52_02_MB sample (Fig. 5). Representatives of the classes PAUC43f marine benthic group and Geodermatophilaceae family were detected only in the DY094_47_02 sample (Fig. 5).

Calcarenite samples were dominated by ASVs affiliated to the class Nitrososphaeria (21%, Nitrosopumilales), Gammaproteobacteria (12%, orders SAR86 and Steroidobacterales), Alphaproteobacteria (10%, order Rhodovibrionales), Deltaproteobacteria (8%, orders Myxococcales and NB1-j), Planctomycetacia (6%, order Pirellulales), and NC10 (6%, order Methylomirabilales) (Fig.

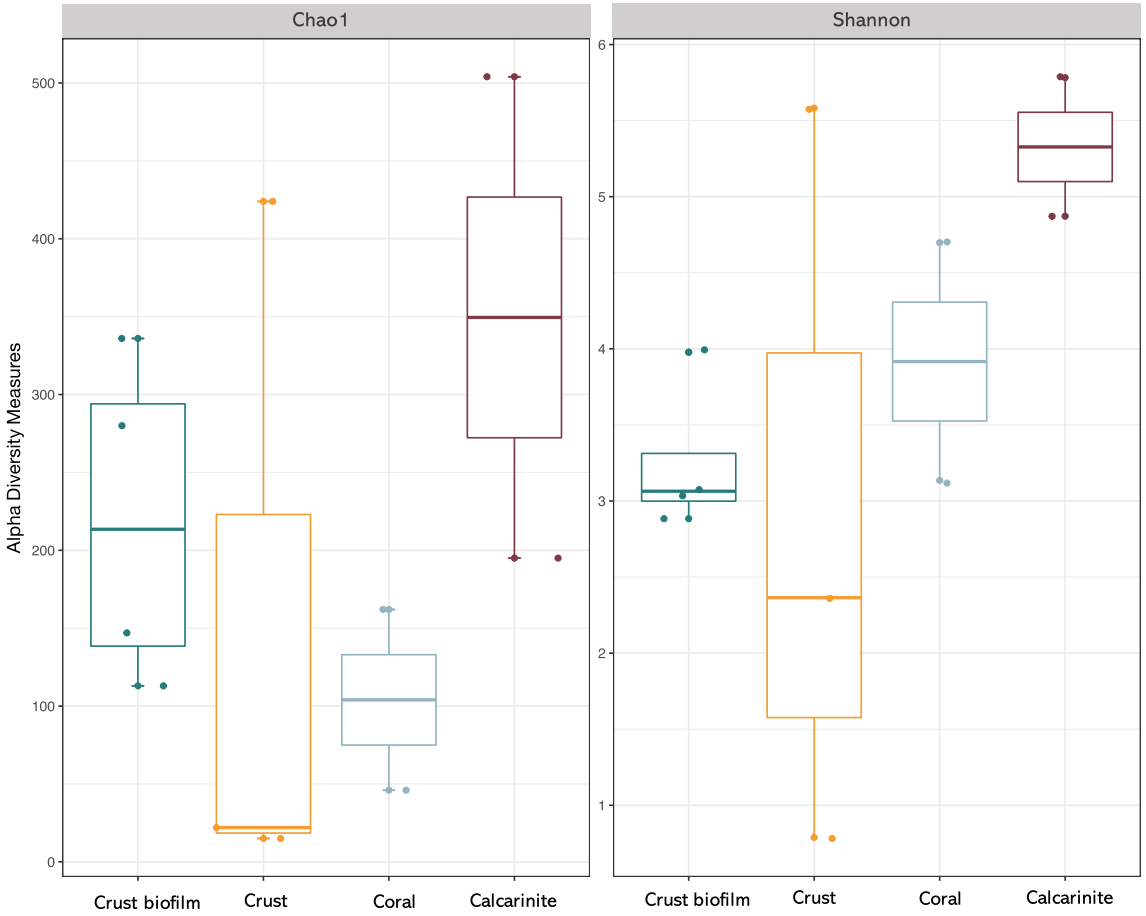

Fig. 2 Alpha diversity medians (number of ASVs, Chao1, and Shannon indexes) of microbial communities in the Fe–Mn crust biofilm-like, crusts, encrusted coral skeletons, and calcarenites on the RGR. There was no significant difference among alpha diversity by comparing the substrate medians

4b and Fig. 5). Representatives of uncultivated MBMPE27, Nitrospira, uncultivated PAUC43f marine benthic group, and SAR11 were more abundant in the DY094_62_02 sample (Fig. 5).

Microbial community composition among different substrates was further investigated by means of the indicator species analysis. We compared the relative abundance and relative frequency of each ASV to identify those specifically associated with only one substrate (unique) and those whose niche breadth encompasses several substrates (shared). Calcarinite harbored the highest set of unique ASVs (n = 24), mainly belong to the oligotypes Nitrosopumilales (n = 6, Thaumarchaeota), class of uncultured Alphaproteobacteria (n = 5), class Gammaproteobacteria (n = 3, families EPR3968-O8a-Bc78v and Nitrosococcaceae), class Phycisphaerae (n = 2, family Phycisphaeraceae), class Nitrospira (n = 2, family Nitrospiraceae), and class Gemmatimonadetes (n = 2, family Gemmatimonadaceae and uncultured class PAUC43f marine benthic group) (Supplementary Table 2). However, crusts, crust biofilm, and encrusted coral skeletons harbored fewer unique ASVs each (n = 1, n = 1, and n = 2, respectively). Those ASVs that are unique to crust and crust biofilms were ASV1055 and ASV0706, belonging to the class Gammaproteobacteria and Nitrosopumilales (Thaumarchaeota), respectively. The unique ASVs of encrusted coral skeletons are ASV0714 and ASV1608, from the family Pirellulaceae (phylum Planctomycetes) and the order Rhizobiales (class Alpaproteobacteria). Most of the unique ASVs belong to low abundance groups in the samples, except for Nitrosopumilales, Nitrospira, and PAUC43f marine benthic group (Fig. 5). Crust and Calcarenite share only one ASV that was related to the order Methylomirabilales (class NC10, ASV0807) (Supplementary Table 2).

Predicted Function Variation Among the Microbial Communities

We assigned 273 out of 1.874 microbial ASVs (14.6%) to at least one microbial functional group using the FAPROTAX database (Supplementary Table 3). Aerobic ammonia oxidation, aerobic nitrite oxidation and nitrification were functions predicted in all substrates with differences in relative abundance between samples (Fig. 6). Main functions associated with crust biofilm microbial communities were dark sulfide

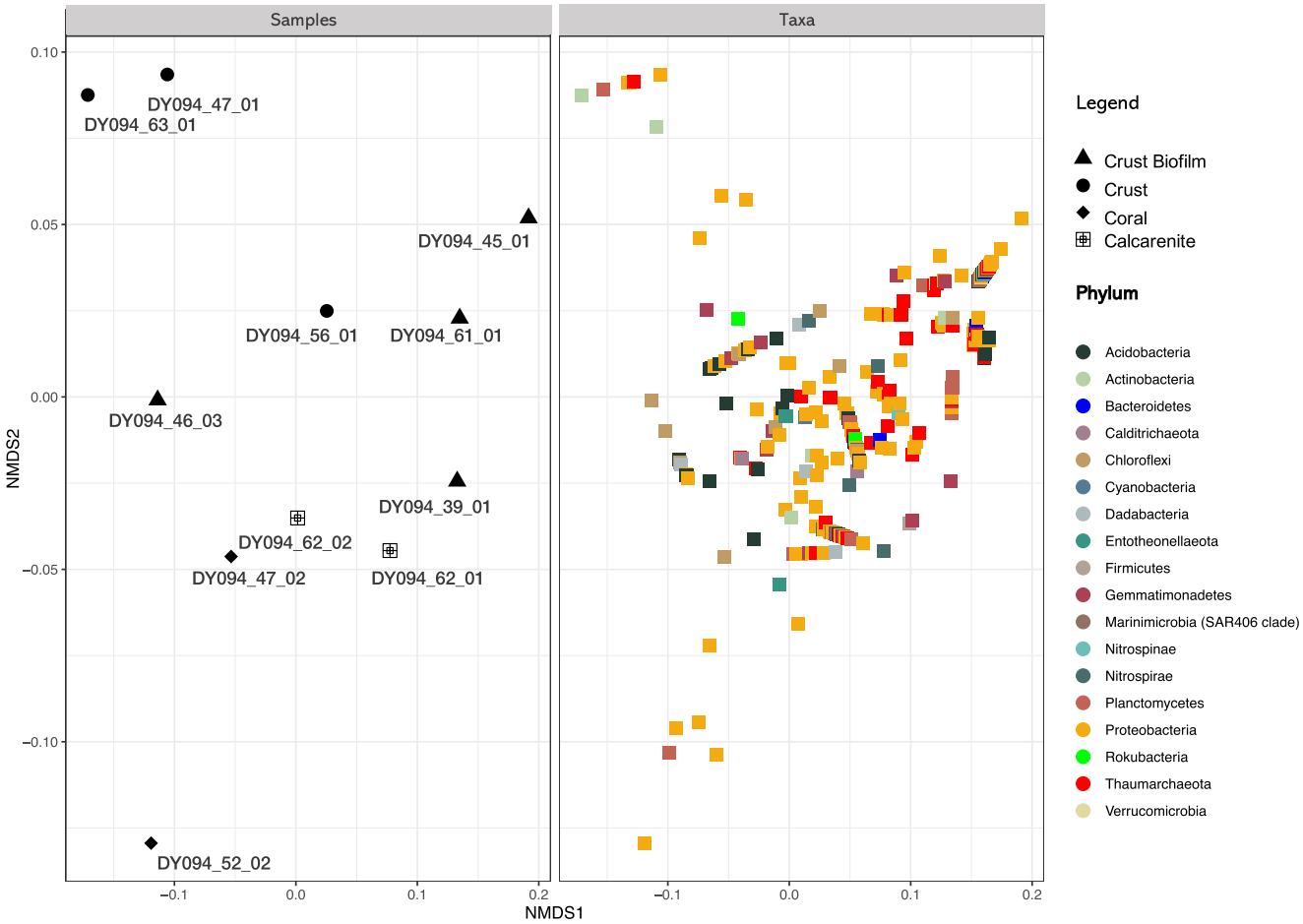

Fig. 3 Principal coordinate analysis (PCoA) of the microbial community of Fe–Mn crust biofilm, crusts, encrusted coral skeletons, and calcarenites from the RGR. The results were based on the amplicon sequencing data of the 16SrRNA genes using weighted Unifrac distance. The taxa correlating with the community differences (at phylum level) are also shown in the plot on the right (Taxa)

oxidation, dark oxidation of sulfur compounds, nitrate respiration, nitrate reduction, nitrogen respiration, and aerobic chemoheterotrophy. The predicted functions in crusts and calcarenite samples were fermentation and aerobic chemoheterotrophy. Manganese oxidation was predicted only in the DY094_47_02 coral sample (Fig. 6).

Discussion

Our results showed that there are no differences in bacterial and archaeal community structure in Fe–Mn crusts in comparison with other substrates: crust biofilm, encrusted coral skeleton, and calcarenite. We also observed a dispersion in microbial community structure between the sampling depth, as previously reported [15]. The water depth might be shaping the community structure and composition variance by samples from the RGR. Previous studies have shown that the concentrations of elements in the Fe–Mn crusts from the RGR change depending on the water depth [1] and consequently differences in the composition of the studied substrates can promote variations in the lifestyles of solid-surface attached microbes [15].

Microbial assemblages in samples from the RGR showed the dominance of the classes Gammaproteobacteria, Alphaproteobacteria, and Deltaproteobacteria, as described for others Fe–Mn crusts deposits [21, 40, 45, 49]. However, at higher taxonomic resolution, samples from the RGR showed higher abundance of the orders Steroidobacterales (family Woeseiaceae), uncultured MBMPE27, Methylomirabilales, and Nitrospirales [15, 22]. Besides that, SAR11 clade was detected in all samples from the RGR. The heterotrophic SAR11 was also reported previously in sediment samples from the South Atlantic Ocean [35]. The presence of these groups in our samples that are known to colonize water column is an indicative of a microbial transport and deposition on our substrates, or an influence of the surrounding seawater during the dredge sampling.

Previous studies have described that dissolved ammonia in seawater is an important nutrient in the Fe–Mn crusts [15, 16, 30, 31]. Even though there is no data from dissolved nutrients in seawater, nitrification (i.e., ammonia oxidation and nitrite

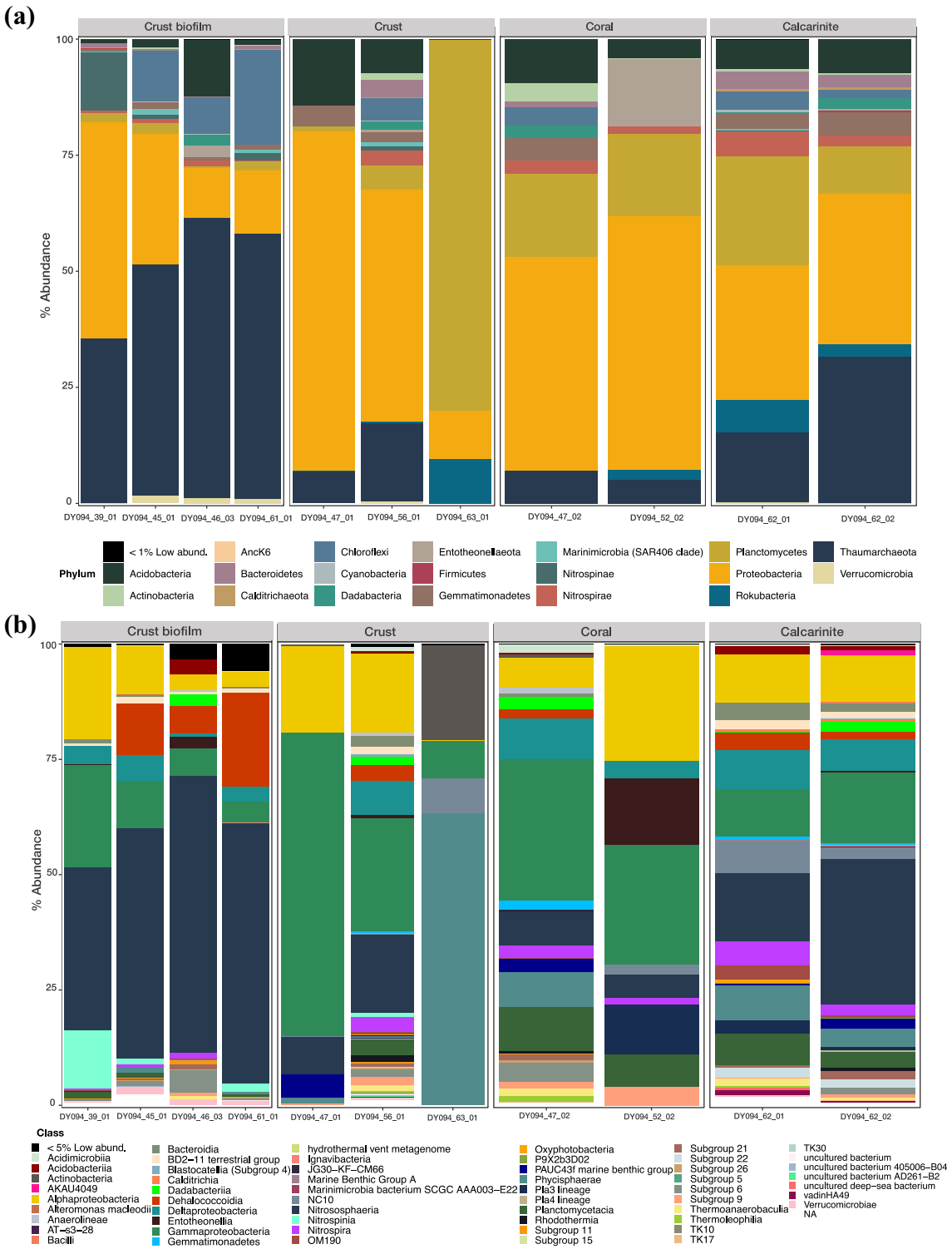

Fig. 4 The relative abundances of bacterial and archaeal taxonomic composition for **a** phylum and **b** class in the Fe–Mn crust biofilm, crusts, encrusted coral skeletons, and calcarenites from the RGR. Only phyla and classes with more than 0.1% of abundance are represented. Phyla with relative abundances below 1% and classes with relative abundances below < 5% were grouped together into low abundance groups. Gray boxes at the top indicate sample substrates, i.e., crust biofilm, crust, encrusted coral skeleton, and calcarenite

oxidation) might be an important process occurring in the Fe–Mn substrates from the RGR. Based on the recovered ASVs and the functional predictions performed in this study, a high proportion of chemolithoautotrophic ammonia-oxidizing Archaea (Nitrososphaeria class) was detected in the Fe–Mn substrates, especially in our Fe–Mn crust endolithic biofilm

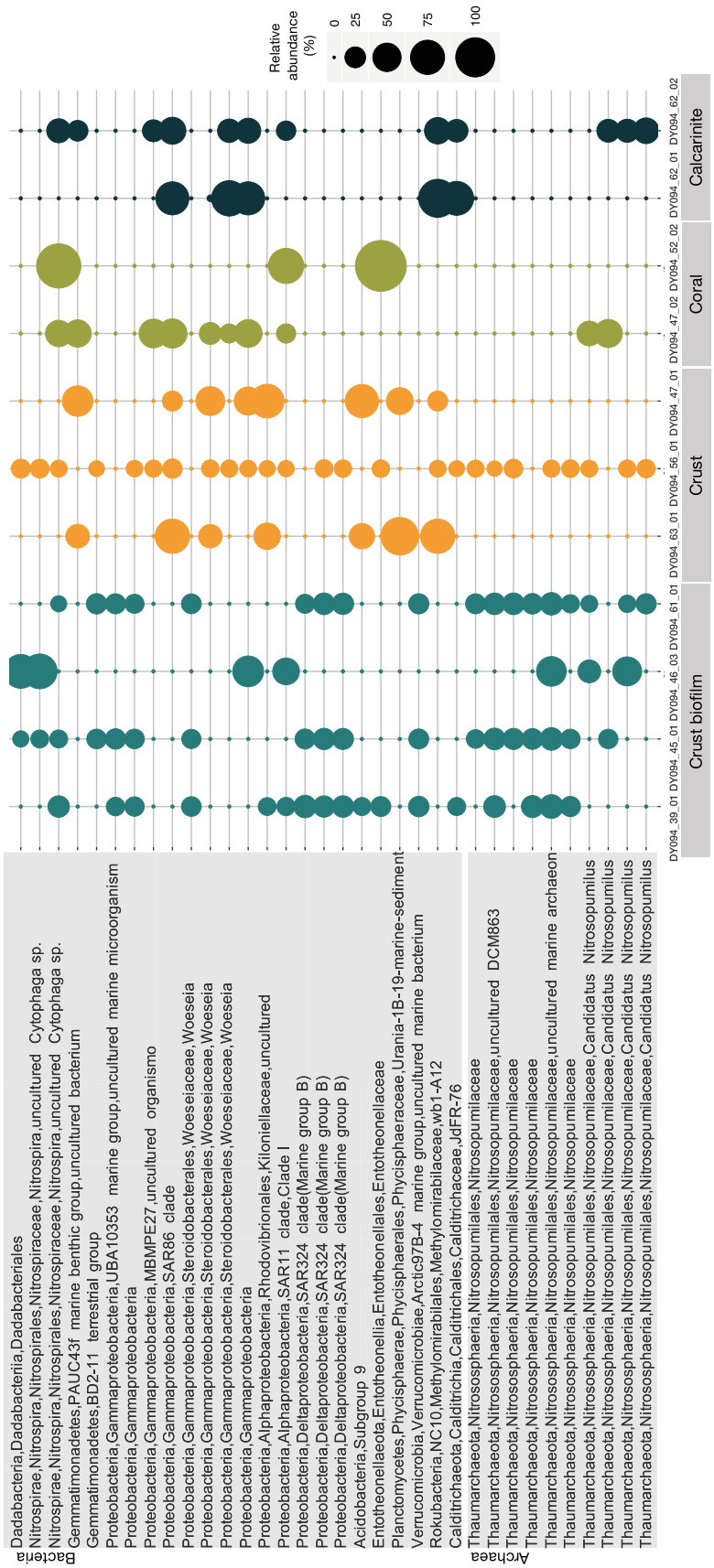

Fig. 5 Classification of the most abundant ASVs for archaea and bacteria (> 0.1% relative abundance) in the Fe–Mn crust biofilm, crusts, encrusted coral skeletons, and calcarenites. The size of circles is related to the relative abundance of each ASV. ASVs are organized by phylum. Gray boxes at the bottom indicate sample substrates, i.e., crust biofilm, crust, encrusted coral skeleton, and calcarenite

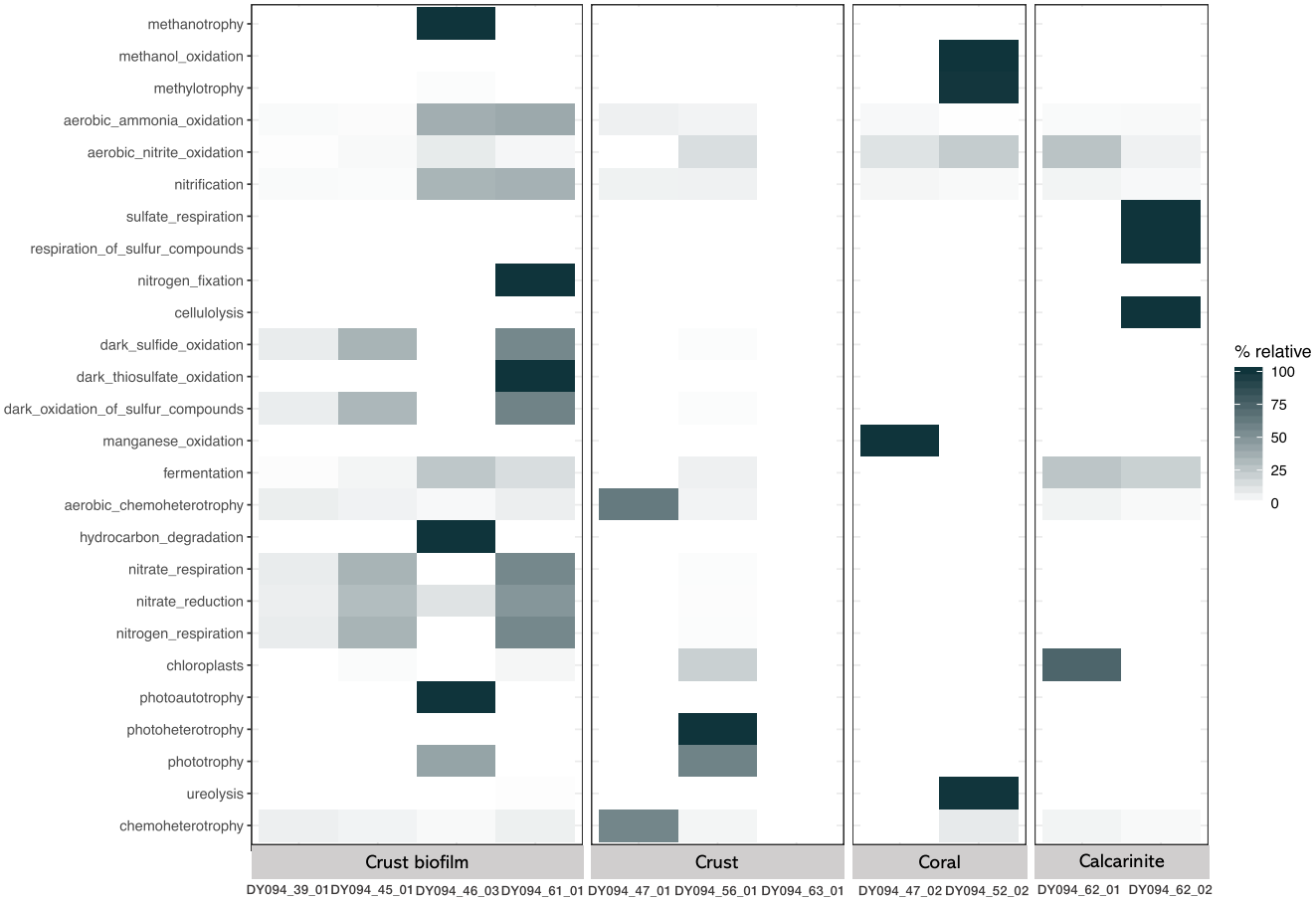

Fig. 6 Mean of relative abundances of microbial functional groups in Fe–Mn crust biofilm, crusts, encrusted coral skeletons, and calcarenites. Relative abundances are depicted in terms of color intensity from white (0) to dark green (100)

samples, as well as chemolithoautotrophic nitrate-oxidizing Bacteria (Nitrospinae class). Although there is a possibility of these ammonia oxidizers are contamination from the surrounding seawater during the sampling, a high proportions of these archaeal ammonia oxidizers (class Nitrososphaeria) and bacterial nitrate oxidizers (Nitrospinae) have also been reported in other Fe–Mn deposits [16, 27, 30, 31, 40]. Recently, scientists confirmed that chemolithoautotrophic bacteria from the phylum Nitrospirae use manganese as an energy source to grow and produce small nodules of manganese oxide [51]. In our samples, a higher proportion of ASVs related to Nitrospirae phylum was detected at Fe–Mn crust and Fe–Mn crust endolithic biofilm samples, being a potential group involved with the manganese oxide formation.

Shiraishi et al. (2016) suggested that Nitrososphaeria might be involved in manganese oxidation in Fe–Mn nodules, as they have multi-copper oxidase, a gene that is utilized by most known manganese oxidizers. Also, previous study proposed that Mn-reducing bacteria, such as species within *Shewanella* and *Colwellia*, are the major contributing microorganisms in the dissolution of Mn in Fe–Mn crusts [2]. Although we have not detected ASVs related to *Shewanella*, we identified ASVs in our results associated with acetate-oxidizing manganese reducers (Colwelliaceae) and manganese oxidizers (Geodermatophilaceae). These manganese oxidizers and reducers were in low abundance and detected only in Fe–Mn crusts and encrusted coral skeletons from stations 47 and 63. Furthermore, we detected a low abundance of ASVs in the Fe–Mn crust biofilm associated with iron reducers from the Magnetospiraceae family. Other possible mechanisms for Mn release on the Fe–Mn substrate surface are acidification through the production of nitrite by ammonia oxidizers and organic acid production by fermenting microorganisms [15]. Indeed, we detected ASVs related to ammonia oxidizers and fermenting microorganisms, including members of the orders Bacillales, Pseudomonadales, and Azospirillales.

Biofilms with filamentous microorganisms associated with micro-stromatolitic growth bands have been described in the Fe–Mn nodules and crust surfaces [4, 13, 42, 46]. Recently, Thaumarchaeota affiliated metagenome-assembled genome (MAG) was recovered from Fe–Mn crust and proteins associated with biofilm formation and surface adhesion capabilities were detected [16]. Kato et al (2019) suggested that these biofilms probably contribute to the Fe–Mn crust formation by adsorbing Fe and Mn oxide particles from seawater. Moreover, endolithic microorganisms within coral skeletons

have been previously reported [50], but those associated with Fe–Mn substrates are poorly studied. We found within the microbial community of our Fe–Mn encrusted coral skeleton an ASV belonging to the class Gammaproteobacteria, they are common members of coral microbiomes [18]. Our main contributing gammaproteobacterial family was related to the Woeseiaceae (order Steroidobacterales), in contrast to other studies of deep-sea corals [18, 50]. Members of the Woeseiaceae family were abundant in all Fe–Mn substrates in this study. The Woeseiaceae family is cosmopolitan in deep-sea sediments [29] and was also described across an Fe–Mn nodule field in the Peru basin [27]. The fact isolated Woeseiaceae members show a chemoorganoheterotrophic lifestyle [8], suggesting a role in organic carbon remineralization in the deep-sea [11].

In our Fe–Mn substrates, we also detected higher abundance of ASVs belonging to methane oxidation, i.e., SAR324 and Methylomirabilales. SAR324 of the Deltaproteobacteria was abundant in our crust biofilm and crusts and is known to have wide metabolic flexibility, including carbon fixation through Rubisco and an ability to oxidize alkanes, methane, and/or sulfur to generate energy [20, 37]. Members of the order Methylomirabilales, within phylum NC10, were abundant in our Fe–Mn crusts and calcarenite, and they are capable of nitrite-dependent anaerobic methane oxidation [44].

In addition, the functional prediction results indicate that carbon and nitrogen metabolism are likely important in Fe–Mn substrates from the RGR. This is probably because the RGR is in the oligotrophic South Atlantic Gyre, with a low concentration of organic carbon and high concentrations of nitrate, phosphate, and Fe–Mn substrates [28]. Organic carbon compounds in the deep-sea might be recalcitrant for microbial life [14], whereas Mn oxides are known to abiotically decompose recalcitrant organic carbon (i.e., humic substances) to simple carbon compounds, such as pyruvate, acetaldehyde, and formaldehyde [41]. These simple carbon compounds can be used as carbon sources by deep-sea microorganisms, as members of family Woeseiaceae and classes Methylomirabilales and SAR324 [41]. The majority of microbial groups detected in this study do not have known or inferred function described in the databases used by FAPROTAX and, for this reason, it is important to highlight the limitations of this functional prediction in describing microbial communities functioning. Further studies are needed to better understand the roles of these communities in the biogeochemical processes occurring on the Fe–Mn substrates in the RGR.

Finally, this study is the first to report microbial diversity in Fe–Mn substrates from the deep Atlantic Ocean. Our results reveal that (1) the microbial community diversity among Fe–Mn substrates are no different, (2) populations associated with nitrogen and carbon predicted metabolisms are likely important contributors for the ecological process occurring in Fe–Mn substrates, and (3) high number of unclassified sequences was recovered from the Fe–Mn substrates, indicating a large proportion of unknown species and hidden functions in RGR microbiome. Thus, further monitoring of the benthic-pelagic coupling microbiome, such as metagenomics and metatranscriptome, measurement of the oceanographic conditions, and chemical elements in the substrates from the RGR, is needed to better elucidate the ecological processes involved with the formation of Fe–Mn substrates.

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Data Availability The sequencing reads generated for this study can be found in the National Centre for Biotechnology Information (NCBI) database under the BioProject PRJNA638744.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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