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Deep-sea plastisphere: Long-term colonization by plastic-associated bacterial and archaeal communities in the Southwest Atlantic Ocean

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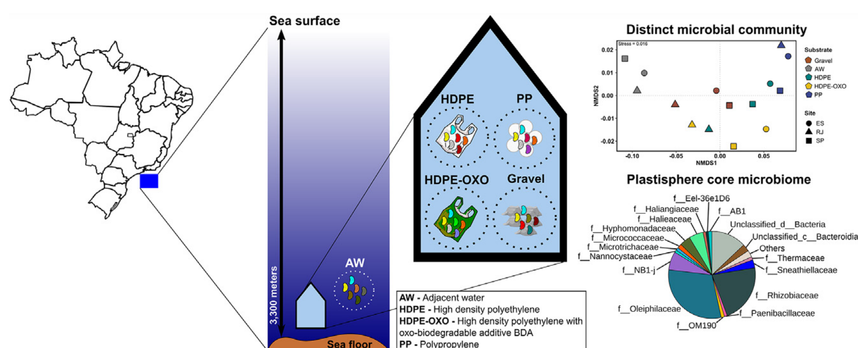
HIGHLIGHTS

- Long-term colonization experiment in situ of plastic substrates by deep-sea microbes
- Microbial community is distinct among plastics, but not related to site.
- The deep-sea plastisphere core microbiome is composed by low abundance taxa.
- The core microbiome includes some taxa not previously observed in association with plastics.

GRAPHICAL ABSTRACT



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ABSTRACT

Marine plastic pollution is a global concern because of continuous release into the oceans over the last several decades. Although recent studies have made efforts to characterize the so-called plastisphere, or microbial community inhabiting plastic substrates, it is not clear whether the plastisphere is defined as a core community or as a random attachment of microbial cells. Likewise, little is known about the influence of the deep-sea environment on the plastisphere. In our experimental study, we evaluated the microbial colonization on polypropylene pellets and two types of plastic bags: regular high density polyethylene (HDPE) and HDPE with the oxo-biodegradable additive BDA. Gravel was used as control. Samples were deployed at three sites at 3300 m depth in the Southwest Atlantic Ocean and left for microbial colonization for 719 days. For microbial communities analysis, DNA was extracted from the biofilm on plastic and gravel substrates, and then the 16S rRNA was sequenced through the Illumina Miseq platform. Cultivation was performed to isolate strains from the plastic and gravel substrates. Substrate type strongly influenced the microbial composition and structure, while no difference between sites was detected. Although several taxa were shared among plastics, we observed some groups specific for each plastic substrate. These communities comprised taxa previously reported from both epipelagic zones and deep-sea benthic ecosystems. The core microbiome (microbial taxa shared by all plastic substrates) was exclusively composed by low abundance taxa, with some members well-described in the plastisphere and with known plastic-degradation capabilities. Additionally, we obtained bacterial strains that have been previously reported inhabiting plastic substrates and/or degrading hydrocarbon compounds, which corroborates our metabarcoding data and suggests the presence of microbial members potentially active and involved with degradation of these plastics in the deep sea.

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1. Introduction

Plastic waste has become a global challenge. Although many countries are involved in public policies to mitigate this problem, tons of plastic waste continue to enter the oceans annually (GESAMP, 2019; Jambeck et al., 2015; PlasticEurope, 2019; Schmidt et al., 2017) . In 2019, 368 million tons of plastics were produced around the world (PlasticEurope, 2020). As a large majority of these materials are used and disposed of quickly, due to the lack of correct management of these residues, they reach all environments globally. Oceanographic models estimate that more than 5 trillion plastic particles are floating in the ocean (Eriksen et al., 2014).

Once in the marine environment, plastic waste is exposed to physical and chemical factors, such as ultraviolet radiation, physical abrasion and chemical oxidation, which contribute to the degradation and fragmentation of this material into microplastics (i.e. plastic objects < 5 mm in diameter) (Roager and Sonnenschein, 2019; Liu et al., 2020) . Microplastics are ubiquitous in marine environments, including below the sea surface. Microplastic chemical composition (and thereby density) and shape determine whether it is more likely to float or sink, which will influence the distribution in pelagic or benthic ecosystems (Cole et al., 2011; Pierdomenico et al., 2019; Pinnell and Turner, 2019; Van Cauwenberghe et al., 2013; Woodall et al., 2014) . Furthermore, plastics that are buoyant at the sea surface, after fouling by organisms and adherence of particles, can sink over time (Woodall et al., 2014). As a consequence, marine plastic contamination is ubiquitous, including in remote marine environments such as the deep ocean (Woodall et al., 2014, Chiba et al., 2018).

Although both macro- and microplastics have already been reported in deep-ocean regions, knowledge regarding plastic colonization by deep-sea prokaryotic communities in both pelagic and benthic ecosystems is incipient (Bergmann and Klages, 2012; Chiba et al., 2018; Galgani et al., 1996; Krause et al., 2020; Pierdomenico et al., 2019; Schlining et al., 2013; Woodall et al., 2018). Plastics can be used as substrata and be rapidly colonized by microorganisms, which form biofilms on the plastic surface, a community we refer to as the “plastisphere” (Zettler et al., 2013). The biofilms give microorganisms protection from adverse environmental conditions, establishing a favorable environment, facilitating the cycling of nutrients and genetic exchanges between individuals, making the biofilm community more efficient than planktonic communities (Dang and Lovell, 2016; Xue et al., 2020). The ability to colonize and metabolize substrate surfaces is a mechanism that promotes advantages for microorganisms in situations with nutritional limitation (Dang and Lovell, 2016), as can be the case in deep-sea regions. Plastic substrates in the deep ocean may also offer a new hotspot of colonization, as well as a relevant source of carbon to support the microbial community, proportionally magnifying the abundance of potentially plastic degrading microorganisms within the plastisphere. A growing number of studies have reported microorganisms capable of degrading hydrocarbons (Didier et al., 2017; Oberbeckmann et al., 2017; Oberbeckmann and Labrenz, 2020; Saygin and Baysal, 2020a, 2021), raising the hypothesis that they would be consuming this material in nutritionally limited conditions, such as those found in deep-sea ecosystems. Some recent studies have identified the presence of pathogenic microorganisms in the plastisphere, causing concern regarding the dispersion of antimicrobial-resistant organisms in the environment (Laverty et al., 2020; Xue et al., 2020; Zhang et al., 2020).

In addition to the influence of the environment (which can be prone to microbial colonization or not), polymer composition is suggested to modulate the structure and composition of the plastisphere (Dussud et al., 2018a; Dussud et al., 2018b; Kirstein et al., 2018, 2019; McCormick et al., 2014; Pinto et al., 2019; Zettler et al., 2013). Moreover, geographic locations have also been indicated to exert influence on the microbial community of the plastisphere (Oberbeckmann et al., 2016). In contrast, independent of environmental factors or plastic substrate type, some microbial taxa have been reported in multiple plastispheres (Tu et al., 2020; Zettler et al., 2013) and they are now referred to as the “core microbiome”. The core microbiome members comprise taxa with high occupancy across a dataset that are hypothesized to reflect functional relationships with the host (or substrate) (Shade and Handelsman, 2012). In the plastisphere, these members are thought to be involved in biofilm formation and/or metabolizing compounds from the plastic substrate (Didier et al., 2017; Tu et al., 2020).

In the last decade, the number of studies on the plastisphere has increased (Amaral-Zettler et al., 2020; Oberbeckmann and Labrenz, 2020). However, efforts have been concentrated mainly on epipelagic (e.g. Bryant et al., 2016; Carson et al., 2013; Zettler et al., 2013) or shallow benthic systems (Pinnell and Turner, 2019). Only a few studies, however, analyzed the plastisphere in deep-sea habitats (Krause et al., 2020; Woodall et al., 2018). Studies that assessed marine plastisphere microbial communities can be classified into three basic groups: those that randomly collected plastic marine debris (PMD) or microplastics (Bryant et al., 2016; De Tender et al., 2015; Zettler et al., 2013); studies that deployed plastic substrates in the ocean (Oberbeckmann et al., 2016; Tu et al., 2020) and those conducted in laboratory conditions (Kirstein et al., 2018; Ogonowski et al., 2018). The microbial community structure inhabiting deployed plastic substrates for long periods is as yet poorly studied (Kirstein et al., 2018; Oberbeckmann et al., 2016, 2018; Tu et al., 2020), even less so in deep sea environments, assumed to be one of the final destinations for plastic particles present in the ocean (Woodall et al., 2014). Deep sea environments are characterized by low temperatures, high pressure, absence of light and the consequent absence of photosynthetic primary production (Corinaldesi, 2015). Together with a general reduction of organic matter input, these extreme conditions promote high selective pressures on the microbial community. The input of anthropogenically generated substrates (i.e., sources of carbon) into the deep sea, such as plastic substrates, creates new habitats (or food sources) to be colonized by microorganisms. However, the composition of these substrata may select pelagic microorganisms with specific features that allow them to colonize and metabolize this carbon source (Dussud et al., 2018a; Dussud et al., 2018b).

Here, we conducted the first in situ experimental study that characterized the structure and composition of the microbial community (the bacterial and archaeal) associated with different types of plastic substrates deployed for 719 days at three sites in deep waters in the Southwest (SW) Atlantic Ocean (3300 m). Our objectives in this study were to understand (i) if there are differences in microbial communities inhabiting plastics polymers as opposed to control samples and adjacent seawater, and differences among multiple sites, (ii) if there is a core microbiome among plastics that can contribute to the description of the plastisphere in the deep SW Atlantic Ocean (different from shallow waters), and (iii) if it is possible to isolate viable bacteria through cultivation of plastic substrates that are potentially related to plastic degradation. For this, we performed a high-throughput sequencing of the 16S rRNA gene to access bacterial and archaeal communities and used traditional culturing methods to assess the plastics as substrates for microbial growth.

2. Materials and methods

2.1. Studied area and deployment methods

The experimental sites were located in a region of the sea floor on the Brazilian continental shelf that encompasses the transition between the North Atlantic Deep Water (NADW) and the Lower Circumpolar Deep Water (LCDW). This region is characterized by water temperature between 3 °C and 4 °C, salinity between 34.6 and 35.0, oxygen concentration above 5 mL L^{-1} and low nutrient levels (i.e. oligotrophic) (Gonzalez-Silvera et al., 2004).

Autonomous aluminum structures that housed multiple experiments, called landers, were deployed at three sites along the southeastern Brazilian continental margin, and between 21°S and 28°S. The sites

were named using the initials of the nearest neighboring state: ES (22°50′27.24″S; 38°24′58.68″W), RJ (25°20′17.88″S; 39°38′28.32″W) and SP (28°1′42.24″S; 43°31′43.32″W), with distances of 304 km between ES and RJ, 786 km between ES and SP, and 496 km between RJ and SP (Fig. 1). Landers were deployed on June 2–6, 2013 at 3300 m depth, using the R/V Alpha Crucis from the Oceanographic Institute at the Universidade de São Paulo (IO-USP). On each lander, single use plastic bags of two different polymers, 60 g of pristine polypropylene (PP) pellets (Braskem) and 60 g of commercial aquarium gravel, natural quartz, as an inorganic control were deployed. Plastic pellets were cylindrical, ~3 mm × 4 mm, and gravel fragments selected for comparison analyses were chosen to be the same approximate size. Plastic bags included: (i) regular high density polyethylene (HDPE) grocery bag material (Valbags, ValGroup Brasil), and (ii) biodegradable grocery bag material made from HDPE with the oxo-biodegradable additive BDA, HDPE-OXO, (Willow Ridge Plastics, Inc.). Polymer identities were either confirmed by the manufacturers or printed directly on the material supplied. All types of substrates were placed inside fiberglass mesh bags (~8 × 15 cm; mesh size, 1 mm), attached to the metal lander frame and secured with nylon fishing line and rope before deployment (Fig. S1).

After 719 days in all cases, 23.6 months (on May 22–25, 2015), samples were collected with the support of NPo Almirante Maximiano (H-41, Brazilian Navy). For DNA extraction, 30 pellets of gravel, 30 plastic pellets and cut strips of plastic bag material were rinsed lightly with autoclaved distilled water. These were then placed into vials (10 per vial for pellets or sufficient to occupy approximately the same vial volume as pellets for bags, n = 3 replicate vials), filled with RNAlater buffer solution (Thermo Fisher Scientific, Waltham, MA, USA), and stored at −20 °C until analysis. For live culturing, an additional 5 pellets of plastic, 5 pellets of gravel and an equivalent volume of cut strips of plastic bag material were placed directly into separate Eppendorf tubes and filled with sterilized seawater without rinsing. Eppendorf tubes were shaken gently, and stored at 4 °C until immediate analysis upon return to the laboratory. All handling materials were sterilized between each step.

Before retrieving each lander, water samples from the same depth and current as the plastic samples were collected using a Rosette water sampler equipped with Niskin bottles. These water samples (adjacent water - AW) were collected to analyze the structure of the microbial communities in the environment where plastics were deployed. Each water sample (~10 L) was immediately filtered through a 0.22 μm polycarbonate membrane (diameter 45 mm; Millipore, Bedford, MA, USA) using a peristaltic pump, and stored at −80 °C.

2.2. DNA extraction, 16S rRNA gene amplification and sequencing

Samples were processed at LECOM, the Microbial Ecology Laboratory at the Oceanographic Institute (IO-USP) of the University of São Paulo. Extraction of the total DNA from the plastics was performed in triplicate using the PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA). Extraction of DNA from the water was performed using a PowerWater DNA Isolation Kit (MoBio, Carlsbad, CA, USA) according to the manufacturer's specifications. Extracted DNA was quantified with a Qubit 1.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA); integrity was verified using agarose gel electrophoresis 1% (v/v).

Six PCR reactions from each sample were pooled and purified with the DNA Clean & Concentrator™ kit (Zymo Research, Irvine, CA, USA), and quantified with Qubit 1.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). PCR was conducted following the Earth Microbiome 16S Illumina Amplicon Protocol. Amplicons were sent to the Molecular Research - MR. DNA company (Texas, USA) for sequencing on the Illumina Miseq platform in a 2 × 300 bp paired-end system. The V4 hypervariable region of the 16S rRNA gene from Bacteria and Archaea was amplified with the universal primers 515F and 806R (Caporaso et al., 2010) with specific adapters for the Illumina Miseq platform. Sequence data (raw .fastq files) have been submitted to the GenBank under accession number PRJNA692207.

2.3. Bioinformatics and statistical analyses

Processing and quality control of reads was performed using QIIME2 version 2019.10 (Bolyen et al., 2019). After graphic inspection of quality profiles, raw reads were subjected to trimming and filtering, then clustered into Amplicon Sequence Variants (ASV) with DADA2

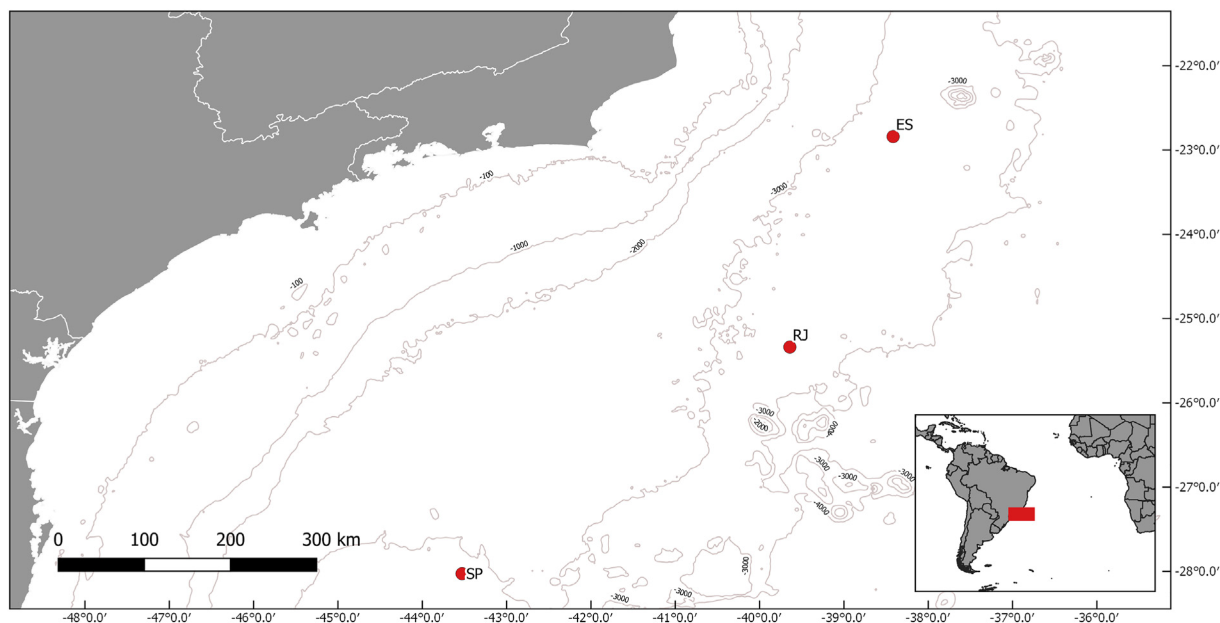

Fig. 1. Map of the study region (Southwestern Atlantic) indicating the three experimental sites where the landers were deployed (red dots), all placed along the 3300 meter bathymetric line. Sites: ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo.
Source: GeoMapApp®.

denoising (Callahan et al., 2016) using the QIIME 2 package (Bolyen et al., 2019). Sequence counts were rarefied to 45,020 reads per sample across all samples to mitigate uneven sequencing depth.

The ASV richness, Shannon and InvSimpson diversity indexes were calculated using *phyloseq* and *vegan* packages. Normality and Homogeneity of variances was assessed by Shapiro-Wilk normality and Levene's test, respectively. If the data showed a normal distribution and the variances were homogeneous, differences between groups were assessed by one-way analysis of variance (ANOVA) and subsequent post-hoc Tukey's tests, which were performed using *stats* and *agricolae* packages in R (v.3.6.1) to assess differences in diversity indexes among substrates and sites. To analyze statistical differences in the richness and alpha diversity by the site, the Chao1, Shannon and evenness indexes of the different substrate types were grouped. To test the differences among substrate types, the data from the sites were grouped. To compare the structure of the bacterial communities among substrates and sites, non-metric multidimensional scaling (NMDS) ordination was performed, based on weighted UniFrac dissimilarities among all samples. Differences in the microbial community structure among substrates and sites were tested by performing a permutational multivariate analysis of variance (PERMANOVA) on the community matrix (Anderson, 2001).

To identify an ASV that was significantly more abundant among substrate types, we performed DESeq2, Differential Expression analysis for Sequence count data (Love et al., 2014). The DESeq2 input was a rarefied microbial dataset previously treated using the Prevalence Interval for Microbiome Evaluation (PIME) package (Roesch et al., 2020). PIME uses machine-learning to generate ASV prevalence among samples, and validate it by comparison with control Monte Carlo simulations with randomized variations of sequences (Roesch et al., 2020). The full rarefied dataset consisting of 5199 ASVs was filtered using the PIME R package (Roesch et al., 2020). PIME removes the within-group variations and captures only biologically significant differences which have high sample prevalence levels. PIME employs a supervised machine-learning algorithm to predict random forests and estimates out-of-bag (OOB) errors for each ASV prevalence dataset at 5% intervals. High OOB errors indicate that a given prevalence dataset interval is noisy, while the minimal OOB errors (OBB error = zero) represent the absence of noise. Here, the minimal OOB errors occurred with a 70% prevalence interval, which represented 471,078 sequences distributed among 535 ASVs. This 70% prevalence dataset was used for DESeq2 subsequent analyses.

To observe the occurrence of ASVs among substrate types, the samples were grouped by substrate type and the taxa abundance table transformed to presence/absence. The unique and shared ASVs were then visualized using an UpSet plot, *UpSetR* package (Conway et al., 2017). The ASVs shared by all plastic types were considered the core microbiome.

2.4. Cultivable plastic-associated bacteria

In sterile Petri dishes, samples of plastic substrates were inoculated directly into the mineral culture medium adapted from Sekiguchi et al. (2010). The medium was prepared to contain per liter of distilled water: 1.87% of Marine Broth (Difco), 1.5% NaCl, 0.35% KCl, 5.4% $MgCl_2 \cdot 6H_2O$, 2.7% $MgSO_4 \cdot 7H_2O$, 0.5% $CaCl_2 \cdot 2H_2O$, 1.2% agar with 0.25% poly-β-hydroxybutyrate (PHB) granules added. The samples were incubated for 15 days at 13 °C, or until the growth of colonies around the plastic samples was observed. All morphologically different macroscopic colonies were selected using the depletion technique two to three times until pure colonies for sequencing were obtained. The isolates were preserved in 20% glycerol in an ultra-freezer at −80 °C.

The genomic DNA of 22 isolates was extracted using the Purelink Genomic DNA kit (Invitrogen by Thermo Fisher Scientific, Carlsbad, USA), according to the manufacturer's specifications. Extracted DNA was quantified with a Qubit 1.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and the Qubit®� dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), integrity was verified using agarose gel electrophoresis 1% (v/v). Amplification of the RNAr 16S gene was conducted using primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 1401R (5′-CGGTGTGTACAAGGCCCGGGA-3′). The polymerase chain reaction (25 μL reaction) was performed using Gotaq Mix Hot Start, 0.25 μL of each primer and 2 μL of DNA template. The PCR conditions were: initial denaturation temperature of 95 °C, 3 min; followed by 30 cycles of 94 °C, 1 min; 53 °C, 30 s; 72 °C, 1 min; and a final extension at 72 °C for 10 min. The PCR product was purified using the DNA Clean & Concentrator kit (Zymo Research, Irvine, USA) according to the manufacturer's specifications and sent for sequencing at Genomic Engenharia Molecular, where they were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Palo Alto, USA) with the 515F primer.

Sequence analysis was initially performed using CodonCodeAligner Software (CodonCode Corporation, Dedham, MA, USA). Through this software, the sequences were checked for quality and treated. After obtaining the treated sequences, the SILVA v138 database (High-Quality Ribosomal RNA Databases) was used to align the sequences, to identify the isolates and to construct the phylogenetic trees through MEGA X software (Kumar et al., 2018), using the maximum-likelihood method (999 bootstraps). All sequencing data was deposited in GenBank (National Center for Biotechnology Information Sequence Read Archives) under accession numbers between MW216888 and MW216902.

3. Results

3.1. ASV richness and alpha diversity among substrata and sites

From the 15 samples sequenced, a total of 1,086,988 valid sequences (i.e. reads) were obtained, representing an average of 72,465 ± 12,068 (SD) reads per sample. The obtained reads were clustered into 5310 ASVs, representing an average of 702 ± 149 ASVs per sample. Rarefaction curves indicated a stationary phase, suggesting sufficient depth of sequencing to account for the diversity of the microbial community on the plastic substrates, gravel, and seawater samples (Fig. S2).

Overall, among substrate type, the ASV richness measured by the Chao1 estimator was significantly higher on gravel samples (898.33 ± 84.77) (ANOVA; $F = 4.85$; $df = 4$; $p = 0.027$), while the microbial richness from AW samples and plastic substrates (HDPE and PP) was lower (631 ± 106, 616 ± 80 and 583 ± 141, respectively) (Fig. S3A). Although the Shannon diversity was not affected by substrate types (ANOVA; $F = 2.77$; $df = 4$; $p = 0.102$), the diversity observed on gravel samples was 1.05, 1.07, and 1.2-fold higher than PP, HDPE-OXO, and HDPE samples, respectively (Fig. S3B). The evenness was also not affected significantly by substrate type (ANOVA; $F = 1.65$; $df = 4$; $p = 0.111$), but a lower mean evenness was observed on HDPE (0.66), AW (0.68), and HDPE-OXO (0.71), while the PP samples showed the higher evenness (0.76) (Fig. S3C).

The site did not exhibit a significant effect on any diversity indexes measured (substrates grouped). However, the ASV richness measured by the Chao1 estimator was higher from RJ (741.09 ± 159.06) (ANOVA; $F = 0.61$; $df = 2$; $p = 0.564$), while SP and ES showed similar richness (675.54 ± 81.16; 681.38 ± 203.29, respectively) (Fig. S3D). Shannon index averages were high for RJ (4.78 ± 0.55) and SP (4.71 ± 0.20), while ES showed the lowest mean values (4.51 ± 0.55) (ANOVA; $F = 0.38$; $df = 2$; $p = 0.517$) (Fig. S3E). Finally, the lowest evenness was observed at ES (0.69 ± 0.07) and was similar between RJ and SP (0.72 ± 0.06 and 0.72 ± 0.02, respectively) (ANOVA; $F = 0.61$; $df = 2$; $p = 0.565$) (Fig. S3F).

3.2. Microbial community structure among substrates and sites

Non-metric multidimensional scaling (NMDS) based on weighted Unifrac dissimilarities revealed that the global pattern of microbial

diversity was significantly explained by the substrate type (Fig. 2). Based on Permutational Multivariate Analysis of Variance (PERMANOVA) the microbial community structure was highly dependent on the substrate type ($R^2 = 0.78$, $p = 0.001$), while the site effect was lower and not significant ($R^2 = 0.11$, $p = 0.054$). Adjacent water (AW) samples were particularly distinct, while gravel samples showed more similarities to plastic samples.

3.3. Microbial composition and taxa differential abundance between plastic and control samples

The microbial community of all substrates were composed in majority by the phylum Proteobacteria (40 to 77%), while other phyla showed different colonization patterns among plastic substrates (HDPE, HDPE-OXO and PP), gravel and AW, including both presence/absence and differences in relative proportions of the phyla (Fig. 3A). For example, Firmicutes was abundant in all PP samples (average 20 ± 6%), while found in smaller proportions in HDPE (6.6 ± 6.2%), HDPE-OXO (6.1 ± 5.6%) and gravel samples (3.8 ± 1.9%). Among plastics, NB1-j was more abundant in HDPE (4.6 ± 5.9%) and archaeal phyla (such as Crenarchaeota and Nanoarchaeota) were prevalent in HDPE and HDPE-OXO (5.1 ± 4 and 7.3 ± 4.7%, respectively). The lowest proportions of those archaeal phyla were observed in PP (0.7 ± 0.4%). Chloroflexi, Marinimicrobia (SAR406 clade) and SAR324 (Marine group B) were mainly present in AW samples (7.1 ± 1.6, 5.6 ± 0.28 and 6.9 ± 0.1%, respectively) but not in the plastic substrates used in this study.

Differences between plastics and gravel samples were strongly evident when we examined deeper taxonomic levels. We observed ASVs from a total of 37 families that were significantly more abundant in plastic substrates when compared with gravel samples; these include families, such as Nitrincolaceae, Rhodobacteraceae, Beijerinckiaceae, Flavobacteriaceae, Bacillaceae, Saccharospirillaceae, Sporolactobacillaceae, Microtrichaeae, Micavibrionaceae, Caulobacteraceae, Rhizobiaceae, Oleiphilaceae, Halieceaea, Micrococcaceae, Haliangiaceae, Pseudomonadaceae, Colwelliaceae, Methylologellaceae, among others (Fig. 3B). Seven families showed ASVs with differential abundances for both plastic and gravel samples, such as Cyclobacteriaceae, OM190, Spongiibacteraceae, NB1-j, Cryomorphaceae, Nitrosopumilaceae and unclassified Bacteroidia. Gravel samples exhibited 16 families significantly more abundant in comparison with plastics (e.g. Kangiellaceae, OM182 clade, Crocinitomicaceae, Sphingomonadaceae and Bacteriovoracaceae) (Fig. 3B).

When we observed the microbial families that were significantly more abundant in the plastic substrate than AW samples, 63 families were identified (Fig. S4A). These families are coincident to those described above, when we compare the plastic substrates with gravel. These results, associated with those obtained from the comparison between plastic substrates and gravel, support our findings that a selected group of microbial families are capable of attaching and colonizing plastic substrates in deep waters.

Furthermore, when we compare gravel with AW samples, microbial families such as Rhodobacteraceae, Beijerinckiaceae, Bacillaceae, Saccharospirillaceae, Sporolactobacillaceae, Rhizobiaceae, Oleiphilaceae, Halieceaea, Pseudomonadaceae and Colwelliaceae were identified as significantly more abundant on gravel samples, while Flavobacteriaceae and Microtrichaeae exhibited more abundance in the gravel and AW samples (Fig. S4B). These results indicate that microbial families in the water are more likely to attach to substrates than remain in the water column. However, besides being identified as attached both to gravel and plastic substrates, our analysis reveals that these families were significantly more abundant on the plastic substrate.

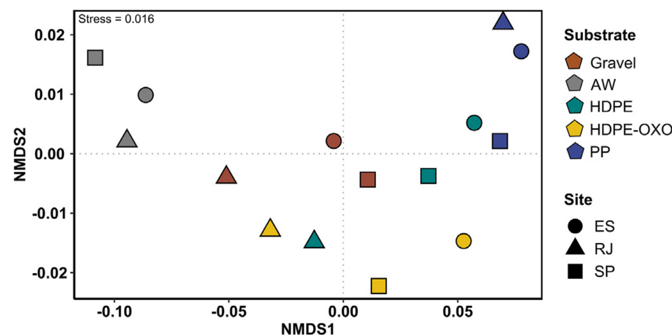

Fig. 2. Non-metric multidimensional scaling (NMDS) ordination of weighted unifrac dissimilarities in microbial community structures. Substrate: Gravel; AW, adjacent water; HDPE, High density polyethylene bag (film); HDPE-OXO, High density polyethylene bag (film) with oxo-biodegradable additive BDA; PP, Polypropylene pellets. Sites: ES, Espírito Santo; RJ, Rio de Janeiro; SP, São Paulo.

3.4. Microbial composition and taxa differential abundance among plastic types

Among each substrate type, the largest number of unique ASVs was found on gravel samples (1096 ASVs), followed by AW, PP, HDPE-OXO and HDPE, with respective values of 954, 736, 732 and 475 ASVs. Grouping those ASVs into families to examine abundance, we identified a predominance of low abundance families on all substrates deployed (relative abundance < 2% of total community) on PP (71.9%), HDPE-OXO (63.9%), gravel (56.8%) and HDPE (40.2%). The percentage of low abundance families in AW samples was lower (21.4%) (Fig. 4).

Overall, the number of common ASVs among substrates deployed (pairwise combinations among HDPE, HDPE-OXO, PP and gravel) was higher than common ASVs between AW and substrates deployed (Fig. 4). These results may confirm taxa effectively colonizing the substrates over long periods. Another piece of evidence to support this idea is the high number of ASVs (201) shared among all substrates deployed, while only 91 ASVs were observed shared among the substrates deployed and the adjacent water (AW) (Fig. 4).

Similar polymer composition was also suggested as exerting influence on the microbial taxa composition. We identified 163 ASVs shared between HDPE and HDPE-OXO, plastic substrates composed basically by HDPE. In contrast, a lower number of ASVs were shared between PP and HDPE-OXO (68) and PP and HDPE (46), substrates composed of different polymers (Fig. 4).

Based on the 44 microbial families with ASVs significantly more abundant in plastic substrates than in gravel samples (Fig. 2B), we checked the distributions of these families among plastic substrates types (Fig. 5). Results showed us three major family groups: (i) the generalists, found with significant abundance in all plastic types (HDPE, HDPE-OXO, and PP), (ii) the plastic HDPE generalists, found with significant abundance in plastic HDPE substrates (HDPE and HDPE-OXO), and finally, (iii) the specialists group, composed by families found in differential abundances in specific plastic substrates.

Fourteen families were classified into a generalist group, which included Bdellovibrionaceae, Halieaceae, Microtrichaceae, Pseudomonadaceae, and uncultured families. The HDPE plastic generalists were composed of 13 families, including CCM11a, Cryomorphaceae, Oleiphilaceae, Rhizobiaceae, Flavobacteriaceae, and Nitrosopumilaceae. Among specialist families, the Beijerinckiaceae and Staphylococcaceae were significantly more abundant in PP samples, whereas NB1-j, OM190, Saccharospirillaceae, Spongiibacteraceae, Micavibrionaceae and an unclassified class of Alphaproteobacteria were more abundant in HDPE-OXO samples. We did not identify any families specifically associated with HDPE samples.

3.5. Core microbiome of the plastisphere community

To identify the core microbiome of the plastisphere from the deep Southwest Atlantic Ocean, the shared ASVs among plastic substrate types were examined (Fig. 4). A total of 28 ASVs were shared among plastic types (Table S1), comprising the core microbiome of the

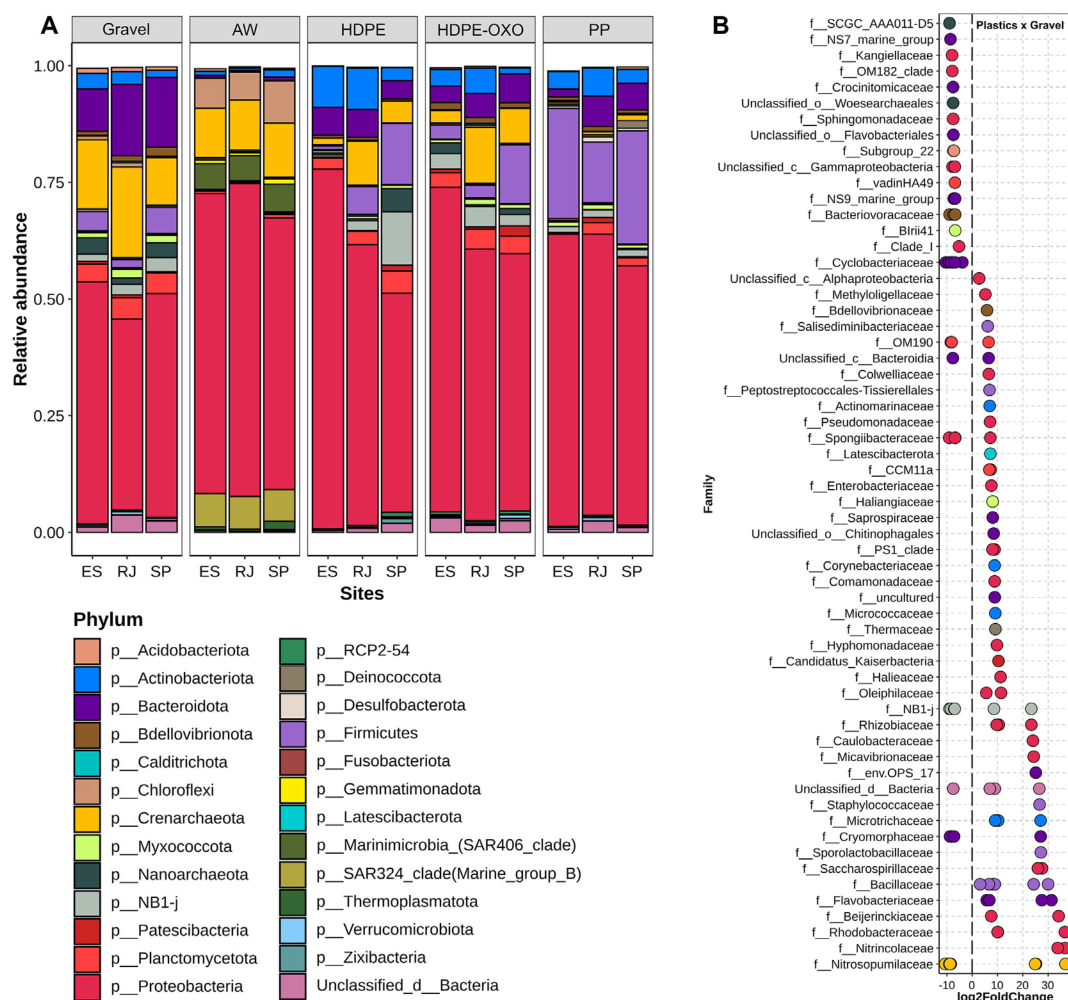

Fig. 3. (A) Relative abundance of the microbial community at the phylum level (Bacteria and Archaea) among sites. (B) Differentially abundant Amplicon Sequence Variant (ASV) comparing plastic substrates (HDPE, HDPE-OXO and PP merged) and gravel. Substrate: Gravel; AW, adjacent water; HDPE, High density polyethylene; HDPE-OXO, High density polyethylene with oxo-biodegradable additive BDA; PP, Polypropylene. Significant ASVs (padj < 0.05) are represented by single data points, grouped by family on the y-axis and by color according to the taxonomic phylum from which the ASV originates. Positive values (log2FoldChange) indicate ASVs significantly more abundant in plastic substrates; Negative values indicate the opposite. Unclassified taxons were represented by the prefix Unassigned_.

plastisphere from the deep Southwest Atlantic Ocean. These ASVs were classified within 23 bacterial families, while 17 families included 97% of the total core microbiome (Fig. 4). This families were composed by Oleiphilaceae (30% of core microbiome), Rhizobiaceae (22%), unclassified Bacteria (13%), NB1-j (7%), Halieaceae (5%), Hyphomonadaceae (4%), Sneathiellaceae (3%), unclassified Bacteroidia class (3%), Micrococcaceae (2%), AB1 (1%), OM190 (1%), Thermaceae (1%), Paenibacillaceae (1%), Haliangiaceae (1%), Microtrichaceae (1%), Nannocystaceae (1%) and Eel-36e1D6 (1%). Six other families represented approximately 3% of the core microbiome (Fig. 4).

3.6. Cultivable plastic-associated bacteria

To identify microorganisms potentially related to plastic degradation, bacterial strains were cultivated from all substrates, including all plastic types and gravel (Table S2). Overall, 15 strains were affiliated according to their 16S rRNA gene sequences to a bacterial genus. The most dominant family identified among the strains was Halomonadaceae, recovered from all substrates. This family was represented by two genera, *Salinicola* and *Halomonas*. Four families were isolated only from plastic substrates, Flavobacteriaceae (HDPE and HDPE-OXO), Pseudoalteromonadaceae (HDPE), Marinobacteraceae (HDPE-OXO) and Rhodobacteraceae (PP). In contrast, Micrococcaceae and Bacillaceae were isolated only from gravel. The comparison of the sequences of all isolates with the SILVA rRNA database confirmed the identities of the isolates. A phylogenetic tree was constructed with the bacterial sequences deposited in the database most closely related to our isolates (Fig. S5). All the bacterial genera isolated in this study have been reported associated with or degrading hydrocarbon compounds (Table S2).

4. Discussion

Despite the high variability among samples and a limited number of replicates, our results indicate that the substrate effect on microbial communities is reflected by taxonomic composition rather than by richness and alpha-diversity indexes. To the best of our knowledge, there is no previous study that has experimentally addressed the microbial colonization on plastic substrates in deep waters. Several previous studies (either from laboratory or field experiments) have reported lower richness and diversity on plastic substrates in comparison with the surrounding waters near the ocean surface, suggesting that plastic substrates are selected by a specific and less diverse microbial community (McCormick et al., 2014; Ogonowski et al., 2018; Zettler et al., 2013). Nevertheless, deep water is generally oligotrophic, while surface waters have a constant input of organic matter from primary production by the phytoplanktonic community that could support high microbial diversity. These general oligotrophic conditions could also be attributed to the lack of significant differences in microbial richness and diversity

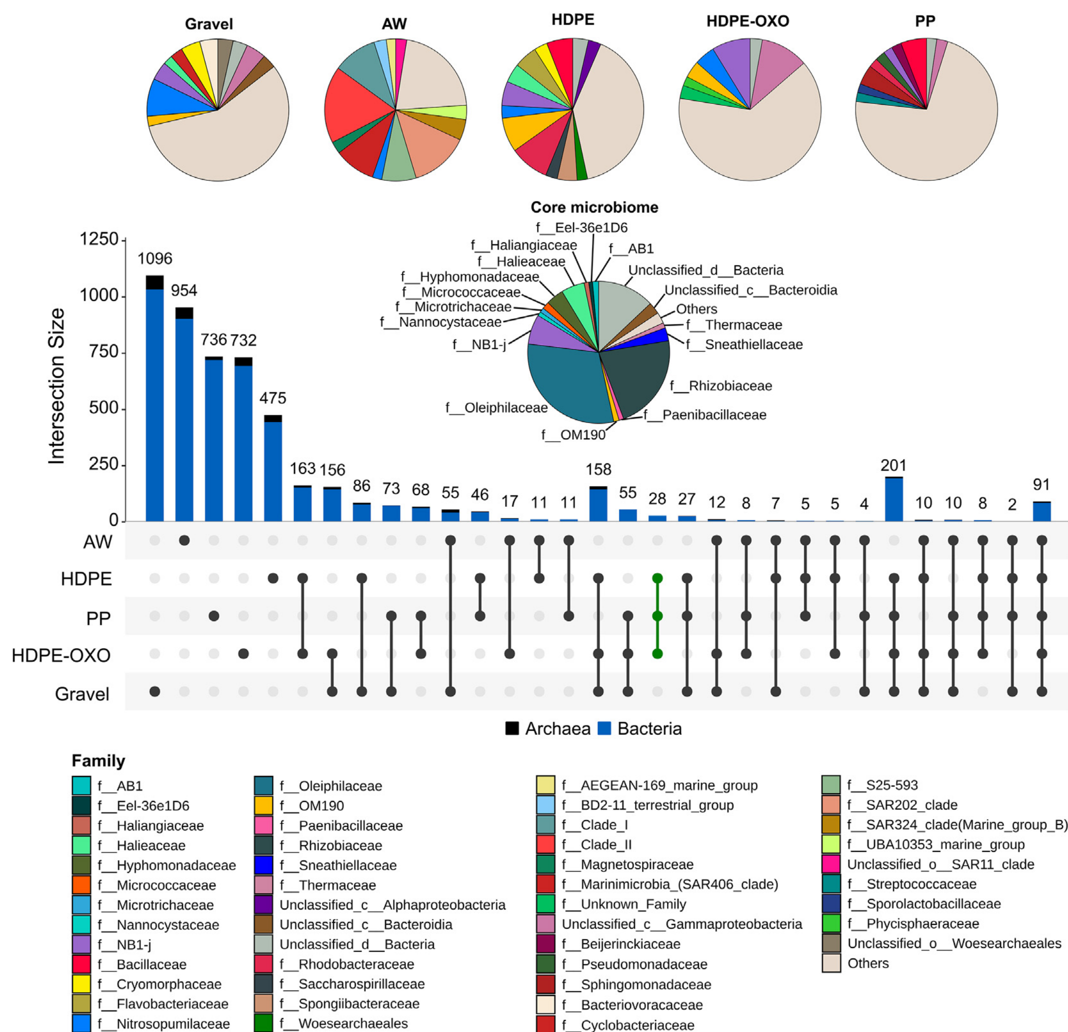

Fig. 4. Upset plot composed by ASVs identified among substrates. Circles indicate substrates. Black lines connecting circles indicate shared ASVs. Vertical bars indicate intersection size (number of ASVs) on each set. Blue and black bars represent Bacteria and Archaea ASVs, respectively. The green line represents ASVs shared by all plastic samples. Substrates: AW, adjacent water; HDPE, High density polyethylene; HDPE-OXO, High density polyethylene with biodegradable additive BDA; PP, Polypropylene. Pie charts show microbial composition specific to each substrate (families with abundance > 2%) and those shared among plastics types (core microbiome). Substrate: Gravel; AW, adjacent water; HDPE, high density polyethylene; HDPE-OXO, high density polyethylene with oxo-biodegradable additive BDA; PP, polypropylene.

among sites, which are exposed to the same depths and water mass. Abiotic parameters associated with water mass are suggested to be the main driver of the microbial community in the pelagic system. The similarity of drivers observed among the sites could thus explain the lack of significant differences found in our study, as well as the high similarity among AW samples from different sites (Fig. 2). Although at different sites, all samples were deployed at the same depth (3300 m), under similar temperatures (1–2.44 °C) and salinities (34.75–34.9 psu) at deployment time (unpublished data), similar to the ranges 3–4 °C and 34.6–35 psu reported in another study in the region along with similar oxygen concentrations (above 5 mL L^{-1}) and low nutrient levels (oligotrophic) (Gonzalez-Silvera et al., 2004).

Our results showing a strong effect from substrate type on microbial community structure (PERMANOVA, $R^2 = 0.78$, $p < 0.001$) indicate niche partitioning of microbial communities among substrates (Dussud et al., 2018a). Substrate-dependence has been reported by studies regarding environmental and controlled conditions (Dussud et al., 2018a; Dussud et al., 2018b; Kirstein et al., 2019, 2018; McCormick et al., 2014; Zettler et al., 2013; Saygin and Baysal, 2020a; Saygin and Baysal, 2020b), showing evidence of the selective effect of plastic substrates. Under natural environmental conditions, substrate-dependence has also been reported in studies that randomly collected PMD (Plastic marine debris) (Didier et al., 2017; Dussud et al., 2018a; Ogonowski et al., 2018; Zettler et al., 2013) or that deployed plastic substrates (Kirstein et al., 2019). This shows microbial communities attached to a plastic substrate are distinct from free-living seawater communities or those attached to other hard substrata. The chemical structure of plastics substrates is determinant to microbial colonization; studies indicate that microplastics inhibit bacterial growth (Ustabasi and Baysal, 2020; Saygin and Baysal, 2020b; Sun et al., 2018), which can directly impact the structure and composition of the microbial community of the plastisphere. Besides the substrate composition, the shape of the plastic particle could also act as a driver of the microbial diversity. For example, it is possible that the differences in the microbial structure and composition found between HDPE and PP substrates could also be attributed to shape differences in the plastics, not only to differences in composition. The HDPE substrate used in our study was in the form of a film, while the PP was a pellet, thus the isolated effect of plastic shape on microbial diversity was not evaluated. However, while some recent studies have indicated that particle shape affects the microbial biofilm thickness (Wright et al., 2020), if this is reflected in changes in the microbial community composition is as yet unknown.

The microbial communities from gravel samples were more similar to the plastic substrates, while AW samples were particularly distinct

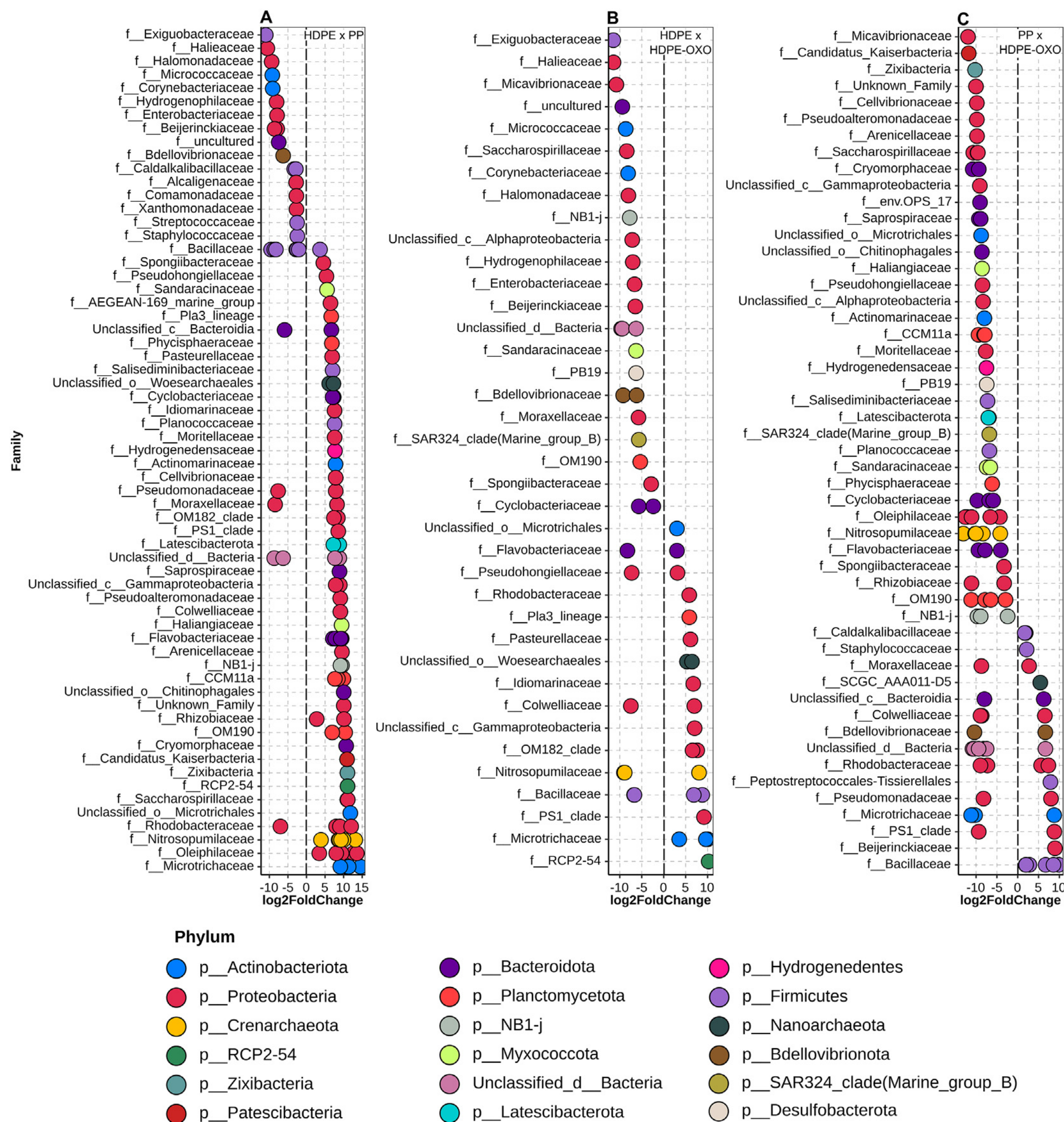

Fig. 5. Differentially abundant Amplicon Sequence Variant (ASV) comparing HDPE and PP (A), HDPE and HDPE-OXO (B); PP and HDPE-OXO (C). Significant ASVs (padj < 0.05) are represented by single data points, grouped by family on the y-axis and by color according to the taxonomic phylum from which the ASV originates. Positive values (log2FoldChange) indicate ASVs that were significantly more abundant in HDPE (A and B) and PP (C) plastic substrates; negative values indicate the opposite. Substrate: Gravel; AW, adjacent water; HDPE, high density polyethylene; HDPE-OXO, high density polyethylene with oxo-biodegradable additive BDA; PP, polypropylene.

(Fig. 2). These results were expected, as the plastic samples and gravel were deployed for 719 days; while water samples represented only a single moment in time, the plastic and gravel samples are the results of cell deposition and dynamic succession over a long period. The AW samples are therefore a type of control, providing information on the microbial taxa present in the water column before retrieving the substrates. Corroborating our results, Oberbeckmann et al. (2016) demonstrated significant differences for multiple taxonomic groups when comparing plastic biofilm communities and the surrounding seawater communities; although the bacterial communities attached to PET bottles were distinct from the free-living seawater communities, the authors also found that PET-associated communities were similar to other types of particle-associated or glass-bound communities collected in the surrounding seawater. Those results confirm the ability of pelagic microorganisms to colonize a range of substrates without specificity (Dussud et al., 2018a).

At deeper taxonomic levels, the differences between the plastic and gravel samples were strongly evident. We identified 37 families significantly more abundant in plastic substrates than on gravel samples (Fig. 2B), such as Methylologellaceae, Colwelliaceae, Pseudomonadaceae, Haliangiaceae, Micrococcaceae, Halieceaea, Oleiphilaceae, Rhizobiaceae, Microtrichaeaed, Flavobacteriaceae, Rhodobacteraceae and unclassified families of Alpha- and Gammaproteobacteria. Corroborating our results, previous studies have shown that microplastics were mainly colonized by Alpha- and Gammaproteobacteria, which were shown to act as primary colonizers, and Flavobacteria (Bacteroidetes), which appeared to act as secondary colonizers (Lee et al., 2008). Additionally, bacterial families classified as Flavobacteriaceae, Pirellulaceae, Rhodobacteraceae (Alphaproteobacteria) and Microtrichaceae (Acidimicrobiia) were identified as the most dominant families on microplastic (PE) biofilms exposed for 135 days to the marine environment at 12 m depth (Tu et al., 2020).

When comparing our microbial taxa from plastic substrates with previous studies related to the plastisphere in epipelagic ecosystems, we found several families in common, such as Microtrichaceae, Rhizobiaceae, Halieaceae, Spongiibacteraceae, Rhodobacteraceae, Micavibrionaceae, Flavobacteriaceae, Halomonadaceae, Kangiellaceae, Hyphomonadaceae, Comamonadaceae, Oleiphilaceae and Bacillaceae (Amaral-Zettler et al., 2020; Feng et al., 2020; Pinto et al., 2019; Rogers et al., 2020). The family Oleiphilaceae comprises members that obligately utilize hydrocarbons through the alkane hydroxylase (*alkB*)

pathway (Golyshin et al., 2002); its detection in our plastic samples likely indicates their potential role in degrading plastic substrates in deep-sea ecosystems. Further, among the families described by these authors, Rhodospirillaceae members were not detected in our samples, which is in agreement with their photosynthetic capacity and thus their prevalence in epipelagic ecosystems. We detected a few families in our plastic samples which were not identified in these previous studies, such as Nitrosopumilaceae. Nitrosopumilaceae members are widely distributed in several deep-sea environments and have an important role as primary producers through ammonia oxidation (Zhong et al., 2020). Their presence in our plastic substrates likely reflects the high abundance of this family in these ecosystems, which might favor their attachment to a variety of substrates available for colonization.

Remarkable differences were observed when we grouped the microbial families by their distributions among plastic types as generalists (significantly abundant on all plastic types, HDPE, HDPE-OXO and PP), HDPE plastic generalists (significantly abundant on HDPE and HDPE-OXO), and specialists (significantly abundant on specific plastic substrates). Members of the Bdellovibrionaceae, Halieaceae, Microtrichaceae and Pseudomonadaceae families were identified as generalists. The ability to colonize and potentially metabolize the carbon from plastic polymers of different substrates in deep environments, under oligotrophic conditions, confers advantages on these microbes in comparison to the entire microbial community. Some of these families have been previously described in association with different types of microplastics from several locations (e.g. Dussud et al., 2018a; Jiang et al., 2018; Tu et al., 2020). Tu et al. (2020) found a high abundance of Microtrichaceae members within biofilms of polyethylene microplastics from coastal seawater in the Yellow Sea, China, with increasing abundance according to longer exposure periods (135 days). Halieaceae members were detected in polyethylene, polypropylene and polystyrene microplastics from the Yangtze estuary (Jiang et al., 2018) and polyethylene microplastics from the Yellow Sea (Tu et al., 2020), both in China. This family is composed of marine bacteria that are capable of assimilating propylene through alkene monooxygenase genes (Suzuki et al., 2019). In addition, we observed microbial families associated specifically to HDPE samples (with and without biodegradable additives). Those microbes, in contrast to the generalist group, are suggested to be more adaptive to colonizing HDPE polymers, with weak or no influence from biodegradable additives. The influence of biodegradable additives was observed in the specialist taxa group (i.e. those microbes more adapted to a specific polymer type). The presence of additives in the polymer compositions may support microbial dynamics over time (Dussud et al., 2018b). Additionally, those additives could be an extra source of nutrients that may reflect in the multiplication of the different microorganisms. Similar results were reported by Dussud et al. (2018b) that suggested a strong effect of the polymer type on the bacterial community, because the composition of microbial biofilm on LDPE and LDPE-OXO (PE with pro-oxidant additives) was completely distinct, while AA-LDPE-OXO (artificially aged LDPE-OXO) and PHBV (poly(3-hydroxybutyrate-co-3-hydroxyvalerate)) showed higher similarity, all under controlled conditions. As observed by these authors for shallow waters, we also observed an influence of the plastic types HDPE and HDPE-OXO on selecting specific microbial taxa in deep waters.

A total of 28 ASVs were identified as core microbiome members in the plastisphere. Defining a common core microbiome in the plastisphere across different studies may be difficult, because variations between experimental designs make it difficult to compare studies directly, as do the variety of study-specific approaches used to define the core (Didier et al., 2017; Tu et al., 2020; Zettler et al., 2013). However, some taxa observed in our study were widely described by previous studies, which provides evidence of common core members of the plastisphere from both surface and deep waters. ASVs from the bacterial families Oleiphilaceae and Hyphomonadaceae were found as members of the core microbiome in our plastic samples. These families have members known to degrade hydrocarbons (Golyshin et al., 2002; Ozaki et al., 2007) or are frequently associated to plastic substrates in the marine environment (Bryant et al., 2016; De Tender et al., 2017; Oberbeckmann et al., 2018; Pinto et al., 2019; Zettler et al., 2013).

In addition, taxa reported from plastic substrates, but not in the core microbiome were also identified. For instance, Microtrichaceae were reported as a dominant taxon on a PE surface during the early phase of biofilm formation (Tu et al., 2020) and Sneathiellaceae colonized plastic debris along a transect through the North Pacific Subtropical Gyre (Bryant et al., 2016). Notably, taxa not previously described from plastic substrates were also identified. Micrococcaceae is a well-documented bacterial family inhabiting deep-sea sediments (Chen et al., 2005, 2016; Sass et al., 2001); their members were already identified in sediments from the Southwest Indian Ridge at depths ranging from 1662 to 4000 m (Chen et al., 2016), in a hypersaline 3500 m depth site in the Mediterranean Sea (Sass et al., 2001), and were isolated from an Antarctic lake and deep-sea sediments from the tropical West Pacific (Chen et al., 2005). *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium* spp. (Rhizobiaceae), a taxon reported as nitrogen-fixing (Franche et al., 2009), was an abundant member of the core microbiome. The family Rhizobiaceae is commonly involved in plant-microbe interactions and was reported recently in marine environments (Kimes et al., 2015). In deep-sea environments, the species of Rhizobiaceae *Georhizobium profundi* was isolated from sediment collected at 4524 m depth (Cao et al., 2020), but its association with plastic substrata were only described in freshwater environments (Wang et al., 2020; Wen et al., 2020). Moreover, another taxa that comprised our core microbiome was NB1-j, an uncultivated bacterial family that was previously found in Japan Trench sediment at 6292 m depth (Yanagibayashi et al., 1999), and in 800 to 1450 m depth sediments heavily impacted by an oil spill in the northern Gulf of Mexico (Hamdan et al., 2018). Finally, taxa from AB1 family (previously assigned as unclassified Alphaproteobacteria) and Eel-36e1D6 (previously assigned as unclassified environmental clone groups), which also comprised the core microbiome, were reported in deep-sea hydrothermal fields, as well as in ferromanganese crusts (Nitahara et al., 2011). Overall, these results highlight a significant number of deep-sea taxonomic groups that were not described by previous studies inhabiting the plastic substrates but were found inhabiting our plastic substrates in the deep SW Atlantic Ocean.

We identified some taxa in the core microbiome that might be potentially related to plastic degradation, according to previous studies. For example, *Arthrobacter* spp. (Micrococcaceae) isolated from plastic waste in the Gulf of Mannar, India, was reported degrading high-density polyethylene (HDPE); after 30 days incubation, they had reduced the weight of the substrate by 12% (Balasubramanian et al., 2010). In addition, members of the Halieaceae family that have known capabilities of assimilating propylene through alkene monooxygenase genes (Suzuki et al., 2019) were described in plastic substrates from the Yangtze estuary (Jiang et al., 2018) and Yellow Sea (Tu et al., 2020), both in China. Members of the Paenibacillaceae family, such as *Paenibacillus* spp. have shown high potential to degrade LPDE and HDPE when in consortia with *Pseudomonas* spp., *Stenotrophomonas* spp. and *Bacillus* spp. (Skariyachan et al., 2017). Pure cultures of *Bacillus* spp. strains also have been demonstrated to have a high potential to degrade PE, polyethylene terephthalate (PET), PP, and polystyrene (PS). Strains (*Bacillus cereus* and *Bacillus gottheilii*) isolated from mangrove sediments, were reported growing on a synthetic medium containing different microplastic polymers as the sole carbon source (Auta et al., 2017; Auta et al., 2018). Furthermore, we were able to isolate bacteria from our plastic substrates that comprise families and genera previously described as colonizing or degrading hydrocarbon substrates (Table S2). The isolation of these bacteria indicates that degradation may be occurring, suggested by the presence of bacteria known to degrade plastics. Our results demonstrated that viable strains can be recovered from deep-sea conditions, which should be further explored in future studies to reveal their plastic-degradation capacity.

Information about microbial communities associated with the plastic substrata in the deep-ocean is scarce in published research studies (Krause et al., 2020; Woodall et al., 2018). To date, no results from other studies deploying samples for a long period in deep sea environments have been published. Our pioneer study showed that several taxonomic groups previously described as plastic colonizers in surface waters seem to also colonize the plastic substrates in the deep sea. However, we also identified some groups in the plastisphere that are typically found inhabiting deep-sea sediments, such as NB1-j, Rhizobiaceae and Eel-36e1D6 members, most of them still poorly characterized and not yet cultivated. In addition, 13% of taxa in the core microbiome were not classified to any microorganism previously deposited in the taxonomy reference databases, which might indicate sequencing artefacts or that we identified potential novel groups not yet described. Our study addresses the gap in the knowledge of microbial colonization in plastics deployed for a long period in the deep sea, highlighting the presence of microbial taxa reported by early studies as involved with plastic degradation processes. However, further studies are needed to better understand their role in plastic colonization and degradation in deep-sea ecosystems.

5. Conclusion

In summary, the microbial communities colonizing the plastic surfaces were distinct and dependent on polymer type. The site along the Brazilian coastline where the samples were deployed had no effect on the microbial community. Our results demonstrated a core microbiome exclusively composed of low abundance taxa; some members were not previously described as associated with plastic substrates, while other bacterial families had previously been described as degrading plastics, but not in deep-sea environments. Additionally, we were able to cultivate and isolate bacterial strains from our plastic substrates, which are known hydrocarbon degraders. It is important to note that some microbial taxa detected in our study were previously reported degrading plastics in controlled conditions, while their ability to degrade the plastic compounds under deep-water conditions remains unknown. We provide the first evidence of an unexplored microbial community composing the deep-sea plastisphere, which may be used as a baseline for future studies on the functionality and plastic degradation capacity under oligotrophic conditions, high pressure, low temperatures and darkness.

CRediT authorship contribution statement

Luana Agostini: Conceptualization, Methodology, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Julio Cezar Fornazier Moreira:** Formal analysis, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Amanda Gonçalves Bendia:** Formal analysis, Writing – original draft, Writing – review & editing, Visualization. **Maria Carolina Pezzo Kmit:** Writing – original draft, Writing – review & editing. **Linda Gwen Waters:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Marina Ferreira Mourão Santana:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Paulo Yukio Gomes Sumida:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition. **Alexander Turra:** Conceptualization, Methodology, Writing – review & editing, Project administration, Funding acquisition. **Vivian Helena Pellizari:** Conceptualization, Methodology, Writing – review & editing, Supervision.

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148335.

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