Marine Pollution Bulletin 208 (2024) 116961

Contents lists available at ScienceDirect

Marine Pollution Bulletin

journal homepage: www.elsevier.com/locate/marpolbul

Plastisphere in an Antarctic environment: A microcosm approach

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ARTICLE INFO

Keywords:
Plastic biofilms
Plastic pollution
Maritime Antarctica
Metabarcoding
qPCR
Microplastics

ABSTRACT

Microplastics are present even in remote regions like the Southern Ocean. Once in the water, they are rapidly colonised by marine microorganisms, forming the *plastisphere*. To address this issue in Antarctic waters, we conducted a microcosm experiment by incubating polypropylene, polyethylene, polystyrene microplastic pellets, and quartz for 33 days on Livingston Island, South Shetland Islands, Antarctica. We analysed plastic colonisation and plastisphere dynamics using scanning electron microscopy, flow cytometry, bacterial cultivation, qPCR, and 16S rRNA gene metabarcoding. Our results show rapid and consistent colonisation, although biomass formation was slightly slower than in other oceans, indicating unique environmental constraints. Time was the main factor influencing biofilm communities, while plastic polymer types had little effect. We observed a transition in microbial communities from early- to late-biofilm stages between days 12 and 19. Additionally, we described the bacterial plastisphere composition in this Antarctic environment, including the presence of hydrocarbon-degrading bacteria.

1. Introduction

The widespread usage and mismanagement of plastics has led to the presence of plastic pollution in ecosystems worldwide. Each year, the world generates around 360 million tons of plastic, yet merely 7 % of it is recycled, leaving most of the waste to accumulate in the environment (Thacharodi et al., 2024). The Southern Ocean is not exempt, and microplastics have been reported in both biotic and abiotic matrices, but especially in seawater (Rota et al., 2022). In the marine environment, these microplastics represent a novel ecological niche, providing a surface that is rapidly colonised by microorganisms, forming a biofilm referred to as the *plastisphere* (Zettler et al., 2013; Dang and Lovell, 2016; Amaral-Zettler et al., 2020). Remarkably, previous studies indicated that the surface of one plastic pellet contained around 25 times the bacteria found in one ml of water (Schlundt et al., 2020; Liang et al., 2023; Ballesté et al., 2024). In that way, microplastics can act as both shelters and vectors, facilitating the widespread dispersion of many microorganisms, including potential microbial pathogens and bacteria harbouring antibiotic resistance genes (Jacquin et al., 2019; Wu et al., 2019; Liang et al., 2023). Furthermore, microplastics may potentially impact composition, structure and diversity of microbial communities, which in turn may lead to an alteration of the biogeochemical cycles, greenhouse gas fluxes, and atmospheric chemistry (Rogers et al., 2020; Agathokleous et al., 2021). Thus, considering the increasing abundance of floating plastics, their surface and the abundance of bacteria thriving on them, the plastisphere may have a global effect at different levels. Consequently, understanding microbial colonisation of plastics and the associated risks is of extreme importance.

Micro- and macroorganisms gradually colonise the surfaces of plastic particles in a classical ecological succession, starting from rapid biofilm formation and progressing to the development of complex biofouling communities over time (Bryant et al., 2016; Jacquin et al., 2019; Rogers et al., 2020). The colonisation of microplastics is affected by both abiotic and biotic drivers. However, the most significant drivers shaping the plastisphere community include geographic location (Amaral-Zettler et al., 2020; Caruso, 2020; Coons et al., 2021), together with seasonality and time variability (Oberbeckmann et al., 2014; Jacquin et al., 2019). Furthermore, it is clear that the microbial community associated with microplastics is significantly different from that of the surrounding water. Whether the substrate's nature affects the biofilm community is still unclear, as there is no general consensus (Zettler et al., 2013; Caruso, 2020; Rogers et al., 2020). Currently, most studies either describe

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https://doi.org/10.1016/j.marpolbul.2024.116961
Received 9 August 2024; Received in revised form 5 September 2024; Accepted 6 September 2024

various stages of biofilm formation under marine conditions or provide new taxonomic insights into bacteria colonising plastics (Jacquin et al., 2019). Yet, absolute microbial densities are sparsely reported, complicating comparisons among different study sites. Only a few studies from different oceans reported absolute bacterial abundances by employing distinct approaches and methodologies, mainly epifluorescence microscopy and flow cytometry. Furthermore, scanning electron microscopy (SEM) is usually performed for visualisation of the biofilm (Dussud et al., 2018a, 2018b; Schlundt et al., 2020; Liang et al., 2023; Stevenson et al., 2023).

Understanding the factors shaping the composition of the plastisphere is crucial, especially in the Southern Ocean, where there are remarkably few studies analysing the microbial biofilms associated with plastics. An initial overview of the Antarctic plastisphere, especially focusing on diatoms, was reported in sea surface waters around the Antarctic Peninsula (Lacerda et al., 2019). In King George Island (South Shetlands, Antarctica), there is a preliminary report on prokaryotic communities colonising two plastic fragments (Cappello et al., 2021), while at the Ross Sea, a study on microbial colonisation and metabolic activity on polyvinylchloride (PVC) and polyethylene (PE) was conducted (Caroppo et al., 2022). Additionally, a first approach to the community-level physiological profile of the plastisphere has also been reported (Caruso et al., 2023) and recently the temporal dynamics of biofilm community after 3, 9 and 12 months have been investigated (Papale et al., 2024). To contribute to filling knowledge gaps on plastisphere in Antarctic environments, we conducted a microcosm study over 33 days, analysing for the first time, the settlement and colonisation of microbial biofilm on various types of microplastics. The experiment took place at Livingston Island (South Shetland Islands, Antarctica), where a recent study reported a concentration of 0.264 ± 0.185 marine microdebris items per m^3 in the surface water (Monràs-Riera et al., 2023) and another reported the presence of microplastics in three bivalve species (Gonzalez-Pineda et al., 2024). Overall, recent studies point to local activities such as tourism, fisheries, and research, as the main contributors to microplastic pollution in the Antarctic environment (Caruso et al., 2022; Rota et al., 2022).

Throughout our colonisation experiment, we monitored both the dynamics of bacterial abundances of the biofilm and the microbial community composition, combining several techniques, including SEM, flow cytometry, cultures, and molecular methods (such as quantitative PCR). This study aimed to enhance our understanding of plastisphere colonisation and community composition, especially within the Antarctic environment. Here, we intended not only to help fill knowledge gaps but also to enable robust inter-study comparisons. We also aimed to contribute to the methodological landscape of plastisphere research, facilitating more comprehensive assessments of plastic pollution impacts globally. The insights gained from our study have the potential to aid in risk assessment, management, and conservation strategies for mitigating the adverse effects of plastic pollution, especially in polar environments.

2. Materials and methods

2.1. Microcosm experiment

Three independent microcosm experiments were conducted at the Antarctic Spanish Research Station in Livingston Island (South Shetlands, Antarctica) in January–March 2022. Each microcosm was set on compartmentalized twenty-litre glass aquariums, previously sterilized and rinsed with seawater (Fig. S1). Each contained 100 pellets of polyethylene (PE; 2–4 mm nominal granule size; density: 0.950 g cm^{-3}; floating), polypropylene (PP; 3 mm nominal granule size; density: 0.9 g cm^{-3}; floating), and polystyrene (PS; 3–5 mm nominal granule size; density: 1.050 g cm^{-3}; sinking), and 100 quartz fragments (quartz; 2–4 mm nominal granule size; density: 2.2 g cm^{-3}; sinking) as a control. Substrates were placed in the sections randomly in each aquarium to avoid location effects. All substrates were purchased from Goodfellow Cambridge Limited (Huntingdon, England; PP: PP306306/1; PE: ET326310/10; PS: ST316311/1; quartz: SI616304/1). They were previously weathered and sterilized with hydrogen peroxide (7.5 %) for 6 h, kept under UV light for 30 min, and washed with sterile water to eliminate potential organic matter, and microorganisms. A representative sample of substrates was scanned with an HP G4050 flatbed scanner at high resolution (1200 dpi; 47.2 pixels mm^{-1}) and the images were processed with ImageJ v1.54d software to measure the surface area. For pellets the surface area was estimated using the formula of the sphere surface area: $4\,\pi\,r^2$, where 'r' represents the mean radius of each plastic pellet. For quartz fragments, the scan surface area was multiplied by two to account for both faces. Aquariums were filled with sea water collected next to the station. A water pump maintained the water flowing and two air bubblers per aquarium ensured proper aeration. Microcosms were kept for 33 days outdoors, under cover to avoid the inputs of rain and snow, and at an environmental temperature of 0 ± 2 °C. Substrates were collected after 2, 5, 9, 12, 19, 26, and 33 days of immersion. Water was monitored at the beginning (day 0) and the end of the experiment (day 33) to evaluate potential natural changes. Five hundred ml of water was filtered through 0.22 μm pore size cellulose ester membrane (SO-PAK, Millipore, Darmstadt, Germany), and filters were immediately frozen for further DNA extraction. For the quantification of bacterial abundance in water by flow cytometry, 2 ml were collected and immediately fixed with glutaraldehyde 0.5 % for further examination during 15 min at 4 °C. Physicochemical characteristics, total carbon and total nitrogen, were measured at the "Centres Científics i Tecnològics" of the Universitat de Barcelona (CCiT-UB) with a total organic carbon (TOC) analyser multi N/C 3100 (Jena).

2.2. Biofilm structural analysis by Scanning Electron Microscope imaging

SEM was used to observe the structure of the biofilm during colonisation. For this analysis, one substrate from each aquarium ($n = 3$) was picked on each sampling day. Three non-incubated items of each substrate were used as controls. Before fixation, items were first rinsed with sterile seawater and with phosphate buffer (PB) 0.1 M pH 7.4 to remove bacteria not attached to biofilms. We fixed the biofilm covering the pellets with a solution of glutaraldehyde 2.5 % in PB at pH 7.4. Samples were preserved at 4 °C until analysis and imaging at CCiT-UB. Samples were fixed with 1 % of osmium tetroxide, washed with Milli-Q water (4 × 10 min), and dehydrated with different ethanol (EtOH) solutions in water: 50 % (1 × 10 min), 70 % (ON), 80 % (1 × 10 min), 90 % (3 × 10 min), 96 % (3 × 10 min), and 100 % EtOH (3 × 10 min). Finally, the samples were dried using Emitech K850 critical point dryer, mounted on double-coated carbon conductive tape and carbon coated to improve their conductivity. SEM observation was done with a JEOL JSM 7001F at the CCiT-UB.

2.3. Determination of marine bacteria by culture media and flow cytometry

Bacterial density was measured for the PP pellets by culture on marine agar and by flow cytometry on each sampling day, whereas for the other substrates (PE, PS, and quartz), these parameters were only measured at the end of the experiment (day 33). We pooled five randomly picked items from the three microcosms ($n = 5$) to consider potential variability among pellets and aquaria. Substrates were gently agitated in a tube containing 5 ml of filtered seawater to remove any unattached organisms. Afterwards, they were transferred to a tube containing 5 ml of sterile seawater and sonicated using an ultrasound bath for 1 min twice to detach biofilm and disaggregate bacteria. Of the remaining solution, 1 ml was used for culturing and 4 ml for analysis by flow cytometry.

To follow up the colonisation and assess the bacterial growth during the experiment, culturable bacteria were quantified using Marine Agar

2216 (Difco, Madrid, Spain) after incubation for 48 h at 20 °C. A volume of 0.1 ml of water or detached bacteria from substrates was inoculated in marine agar; when a high concentration of bacteria was expected, different dilutions using sterile water were assayed (1:10; 1:100; 1:1000). Results were expressed as cfu mm^{-2} for substrates and cfu ml^{-1} for water.

Flow cytometry analysis was used to determine the number of total cells in the biofilm during the colonisation. From the remaining solution, 4 ml were fixed with glutaraldehyde 0.5 %, kept at 4 °C for 15 min, and frozen at −80 °C for further analyses at CCiT-UB. In the laboratory, samples were thawed and homogenised using a vortex, 500 μl of each sample was heated for 5 min at 60 °C to enhance staining. Afterwards, 10 μl of SYBR Green I (Sigma-Aldrich, Saint Louis, MO, USA) (1:100 distilled water diluted) was added and incubated in the dark for 15 min at 60 °C. Flow cytometry analysis was performed using an Aurora (Cytek) spectral flow cytometer. The emitting fluorescence signal excited by the blue laser was of 488 nm. The flow cytometry results were analysed using FlowJo™ v10.8 Software (BD Life Sciences). Total cell concentration (TCC) results were expressed as cell count mm^{-2} in the case of substrates, and cell count ml^{-1} for water.

2.4. DNA extraction

DNA was extracted to characterize the bacterial communities of the substrates and the surrounding water, and further determine the bacterial abundance in the biofilm during the colonisation. For each substrate five items were picked at random from the three aquaria and pooled together to minimize the potential variability among both the three aquaria and the collected items. Extraction was carried out using DNeasy PowerBiofilm® Extraction Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The DNA extracted was eluted to a final volume of 100 μl. Extraction controls were run together with the samples using previously weathered and sterilized pellets with hydrogen peroxide (7.5 %) for 6 h, exposed to UV light for 30 min, and washed with sterile water to eliminate potential organic matter and microorganisms.

2.5. Quantitative real-time polymerase chain reaction

The biofilm density during colonisation was measured using quantitative real-time PCR (qPCR) targeting a fragment of the 16S rRNA gene as previously described (Baker et al., 2003). The total 16S rRNA gene was quantified by a StepOne Real Time PCR System (Applied Biosystems, Foster City, CA, USA) using the primers 341F and 534R (Table S3) (Muyzer et al., 1995, 1996). PCR amplification was performed in a total 20 μl reaction mixture using PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA). The mixture contained 1 μl of DNA template, 10 μl of SYBR Green (Applied Biosystems), 1 μl of each primer, and 7 μl of ultrapure water to reach the final volume. The PCR program was initiated at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 60 °C for 15 s, and extension at 60 °C for 1 min. The limit of detection was 80 gene copies per reaction. Four points of the standard curves were included and generated from 10^2, 10^4, 10^6, and 10^8 dilutions of a customized gBlock gene fragment (Integrated DNA Technologies, Coralville, IA, USA). Controls with no template were run for all assays. All samples, standards, and controls were run in duplicate. Only amplification efficiencies between 90 and 110 % were considered as acceptable for quantification. The results of the molecular methods of microbial quantification in substrates and water samples were respectively expressed as gc mm^{-2} in the case of substrates, and gc ml^{-1} for water.

2.6. Microbial diversity analysis

2.6.1. Illumina 16S rRNA amplicon sequencing

Sequencing of each sample was performed using the Illumina MiSeq platform at the Genomics Unit of Centre for Genomic Regulation Core Facilities (CGR, Barcelona). The V4 region was amplified from DNA sample extracts using the primers from the Earth Microbiome Project [515F (Parada et al., 2016) (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (Apprill et al., 2015) (5′-GGACTACNVGGGTWTCTAAT-3′)]. The PCR process included a primer concentration of 0.2 mM and KAPA HiFi HotStart ReadyMix (Roche) in a final volume of 25 μl. Cycling conditions were as follows: an initial denaturation step of 3 min at 95 °C, followed by 25 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, concluding with a final elongation step of 5 min at 72 °C. Subsequent to this initial PCR step, water was added to reach a total volume of 50 μl, and the reaction mixture underwent purification using AgenCourt AMPure XP beads (Beckman Coulter). The initial PCR primers contained overhangs that facilitated the addition of full-length Nextera adapters with barcodes for multiplex sequencing in a second PCR step. This resulted in sequencing-ready libraries with an approximate insert size of 450 bp. To achieve this, 5 μl of the first amplification were employed as a template for the second PCR with Nextera XT v2 adaptor primers, again in a final volume of 50 μl, using the same PCR mix and thermal profile as the first PCR, but with only eight cycles. Following the second PCR, 25 μl of the final product was utilized for purification and normalization using the SequalPrep normalization kit (ThermoFisher Scientific), following the manufacturer's guidelines. The libraries were then eluted and pooled for sequencing. To estimate quantity and verify size distribution, the final pooled libraries underwent analysis using Agilent Bioanalyzer or Fragment analyser High Sensitivity assay. Prior to sequencing with Illumina's MiSeq 2x300bp Sequencing, quantification was performed through qPCR employing the KAPA Library Quantification Kit (Kapa-Biosystems). Sequencing included negative controls including blanks from the DNA extraction process, as well as from the DNA amplification. The data is available at Mendeley Data public repository (doi: 10.17632/747grvg49y.1).

2.6.2. Bioinformatic analyses

We employed FastQC (0.12.1) and bbduck trimming (39.01) within the bioconda environment to process and analyse the raw sequence data. This involved the removal of adapters, primers, barcodes, and leading Ns from the sequencing reads. Subsequently, we utilized DADA2 (Divisive Amplicon Denoising Algorithm 2, version 1.22.0) within RStudio (version 4.1.2) to process the sequences into amplicon sequence variants (ASVs) using default parameters (Callahan et al., 2015). Firstly, quality filtering and the trimming of sequences was set to 220 bp (for forward reads) and 175 bp (for reverse reads) setting the maximum number of expected errors at two (EE = 2). This parameter is a better filter than simply averaging quality scores (Edgar and Flyvbjerg, 2015). Thereafter, we performed sequence dereplication, alignment, and merging of forward and reverse reads, culminating in the removal of chimeras and the generation of an Amplicon Sequence Variants (ASV) table. Taxonomy was assigned to the resulting ASVs table using the SILVA 138 reference database and was imported to the Phyloseq R package (version 1.38.0) for microbiome analyses.

2.6.3. Statistics and downstream data analyses

Alpha and beta diversity were assessed using the Phyloseq R package (version 1.42.0) (McMurdie and Holmes, 2013) and the vegan R package (version 2.6.4) (Oksanen et al., 2022), with a significance level of $p < 0.05$. Alpha diversity was measured for richness and diversity by Shannon index after rarefying the ASV table. Differences in alpha diversity indices were evaluated independently for matrices (plastic; quartz; water), substrates (PP; PE; PS; quartz; water), time (days 2, 5, 12, 19, 26, 33), and biofilm stages (Early-biofilm, comprising days 2, 5, and 12; Late-biofilm, comprising days 19, 26, and 33) as factors by means of a one-way analyses of variance (ANOVA) and Tukey's honestly significant difference (HSD) test to identify the significant differences. For the beta diversity analysis, we transformed the number of reads of each ASV into relative abundance and calculated the Bray Curtis dissimilarity. To

assess differences in community composition between groups, a non-parametric permutational multivariate analysis of variance (PERMANOVA) test with fixed factors and 9999 permutations was performed. A pairwaise PERMANOVA test was conducted using the pairwiseAdonis package v.0.4 (Martinez Arbizu, 2020) to examine differences between groups. Furthermore, for the dissimilarity visualisation we performed Principal Coordinates Analysis (PCoA) and hierarchical clustering.

3. Results

3.1. Characterization of the microcosm conditions

Seawater samples had an initial TOC concentration of 15.7 ppm, while the total nitrogen concentration remained below 0.5 ppm. These concentrations remained quite stable, with a TOC concentration of 24.92 ± 18.84 ppm and a total nitrogen concentration below 0.5 ppm at the end of the experiment. The surface area of plastic pellets was estimated to 65.784 mm^2 for PP, 53.068 mm^2 for PE, 46.084 mm^2 for PS, and 24.38 mm^2 for quartz, which allowed us to calculate the abundance of bacteria per surface unit (mm^2).

In seawater, the abundance of the bacteria community was of $1.26{\cdot}10^6$ gene copies (gc) of 16S rRNA ml^{-1}. While flow cytometry showed an abundance of $6.69{\cdot}10^5$ cell count ml^{-1} and the culturable bacteria on marine agar a concentration of $3.29{\cdot}10^3$ cfu ml^{-1}. The bacterial concentration in seawater increased at the end of the experiment as it was detected with 16S rRNA, flow cytometry, and culture methods (Table 1) ($5.72{\cdot}10^6$ gc ml^{-1}; $1.20{\cdot}10^6$ cell count ml^{-1}; and $9.70{\cdot}10^3$ cfu ml^{-1}, respectively).

3.2. Structure of microbial biofilm by SEM

SEM images confirmed that colonisation was initiated at least on day 2, as the first single bacterial settlers were found on the sample's surface. In general, by day 5, the formation of a microbial exopolysaccharide matrix was already observed, leading to the development of a complex microbial matrix in the subsequent days. On sinking substrates (PS and quartz), choanoflagellates and diatoms were found to establish from day 12, while on floating substrates (PP and PE) this was observed from day 19 onwards. Overall, various microorganisms and shapes were visible, including cocci and bacilli, which dominated the microbial community during the initial days (days 2, 5, and 12). By days 19, 26, and 33, we observed an increasing presence of choanoflagellates and several diatom morphotypes (Fig. 2).

3.3. Bacterial dynamics on substrates over time

The bacterial abundance for the substrates presented an initial concentration on day 2 of $5.91{\cdot}10^4$ 16S rRNA gc mm^{-2} for PP, $5.11{\cdot}10^4$ gc mm^{-2} for PE, $1.04{\cdot}10^4$ gc mm^{-2} for PS, and $8.53{\cdot}10^4$ gc mm^{-2} for quartz. The abundance of the 16S rRNA gene copies remained relatively stable throughout the experiment, reaching a final concentration at day 33 of $1.90{\cdot}10^5$ gc mm^{-2} for PP, $3.81{\cdot}10^4$ gc mm^{-2} for PE, $6.30{\cdot}10^5$ gc mm^{-2} for PS, and $1.99{\cdot}10^6$ gc mm^{-2} for quartz (Table 1; Fig. 1). Overall, we observed a range between $3.32{\cdot}10^4$–$2.51{\cdot}10^5$ gc mm^{-2} for PP, $2.18{\cdot}10^4$–$9.20{\cdot}10^4$ gc mm^{-2} for PE, $1.04{\cdot}10^4$–$6.30{\cdot}10^5$ gc mm^{-2} for PS, and $8.53{\cdot}10^4$–$1.99{\cdot}10^6$ gc mm^{-2} for quartz, from day 2 to day 33. The same trend was observed over the course of the experiment for the two other techniques used to monitor the bacterial abundance: flow cytometry, and culturable bacteria on marine agar, albeit specifically for the PP (Fig. S2). Within 48 h, flow cytometry revealed an initial abundance of $7.92{\cdot}10^2$ cell count mm^{-2}, which slightly increased to a concentration of and $2.87{\cdot}10^3$ cell count mm^{-2} at the end of the experiment on PP (Table 1). On the marine agar plates, we detected a concentration of $2.28{\cdot}10^1$ cfu mm^{-2} which increased to $3.62{\cdot}10^2$ cfu mm^{-2} at day 33. Overall, rapid colonisation followed by sustained bacterial abundance was observed. We further estimated the final densities of the remaining substrates (PE, PS, and quartz) on day 33. Using flow cytometry, we encountered densities of $2.31{\cdot}10^3$ cell count mm^{-2} for PE, $4.12{\cdot}10^3$ cell count mm^{-2} for PS, and $7.42{\cdot}10^3$ cell count mm^{-2} for quartz. Regarding culturable bacteria on marine agar, we observed abundances of $3.39{\cdot}10^2$ cfu mm^{-2} for PE, $8.25{\cdot}10^2$ cfu mm^{-2} for PS, and $3.86{\cdot}10^2$ cfu mm^{-2} for quartz (Fig. S3). The number of 16S rRNA gene copies were around 70 times higher than flow cytometry counts. Meanwhile, the number of culturable cells represented between 3 and 12 % of the total cells counts by flow cytometry.

3.4. Diversity and taxonomic composition of the microbial community

The microbial communities were analysed using Illumina sequencing of the 16S rRNA gene obtaining 4,099,209 reads. The sample PS day 2 was removed from further analysis due to a low number of reads. A total of 842,707 reads were obtained after all previously reported processing steps, with an average of 33,708.280 ± 6543.733 (SD) reads per sample. The reads were clustered into 1087 ASVs, with Archaeal taxa accounting for just 10 ASVs, while the remaining ASVs were attributed to Bacteria.

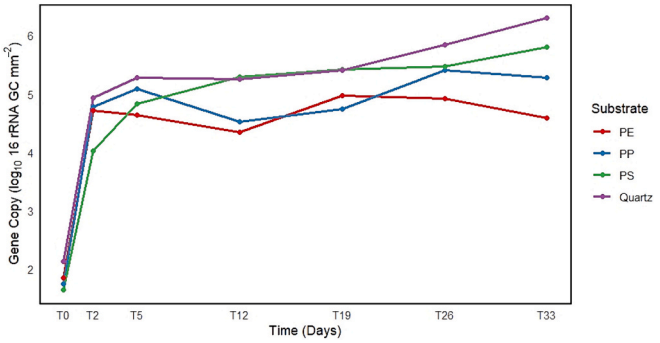

Fig. 1. Density of bacteria quantified by qPCR of the 16S rRNA gene in polyethylene (PE; red), polypropylene (PP; blue), polystyrene (PS; green), and quartz (purple). Data shown as log_{10} of the 16S rRNA gene copies per mm^2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Abundance of bacteria detected on plastic pellets (PP; PE; PS), quartz, and water, at different times (days 2, 5, 12, 19, 26, 33) by heterotrophic culturable marine bacteria, flow cytometry, and quantification of the 16S rRNA gene.

Time (day)	Culture (cfu mm^{-2}/ml^{-1})		Cytometry (cell count mm^{-2}/ml^{-1})		qPCR (16S rRNA gc mm^{-2}/ml^{-1})				
	Water	PP	Water	PP	Water	PP	PE	PS	Quartz
T0	$3.29{\cdot}10^3 \pm 1.70{\cdot}10^2$	x	$6.69{\cdot}10^5$	x	$1.26{\cdot}10^6$	$5.59{\cdot}10^1$	$7.00{\cdot}10^1$	$4.36{\cdot}10^1$	$1.35{\cdot}10^2$
T2		$2.28{\cdot}10^1 \pm 8.60{\cdot}10^{-1}$		$7.92{\cdot}10^2$		$5.91{\cdot}10^4$	$5.11{\cdot}10^4$	$1.04{\cdot}10^4$	$8.53{\cdot}10^4$
T5		$8.21{\cdot}10^1 \pm 4.30$		$1.57{\cdot}10^3$		$1.19{\cdot}10^5$	$4.35{\cdot}10^4$	$6.64{\cdot}10^4$	$1.87{\cdot}10^5$
T12		$2.08{\cdot}10^2 \pm 1.40{\cdot}10^1$		$1.83{\cdot}10^3$		$3.32{\cdot}10^4$	$2.18{\cdot}10^4$	$1.92{\cdot}10^5$	$1.78{\cdot}10^5$
T19		$4.71{\cdot}10^1 \pm 4.30{\cdot}10^1$		$2.95{\cdot}10^3$		$5.54{\cdot}10^4$	$9.20{\cdot}10^4$	$2.61{\cdot}10^5$	$2.50{\cdot}10^5$
T26		$4.06{\cdot}10^3 \pm 1.03{\cdot}10^3$		$3.70{\cdot}10^3$		$2.51{\cdot}10^5$	$8.23{\cdot}10^4$	$2.90{\cdot}10^5$	$6.85{\cdot}10^5$
T33	$9.70{\cdot}10^3 \pm 1.56{\cdot}10^2$	$3.62{\cdot}10^2 \pm 1.50{\cdot}10^1$	$1.20{\cdot}10^6$	$2.87{\cdot}10^3$	$5.72{\cdot}10^6$	$1.90{\cdot}10^5$	$3.81{\cdot}10^4$	$6.30{\cdot}10^5$	$1.99{\cdot}10^6$

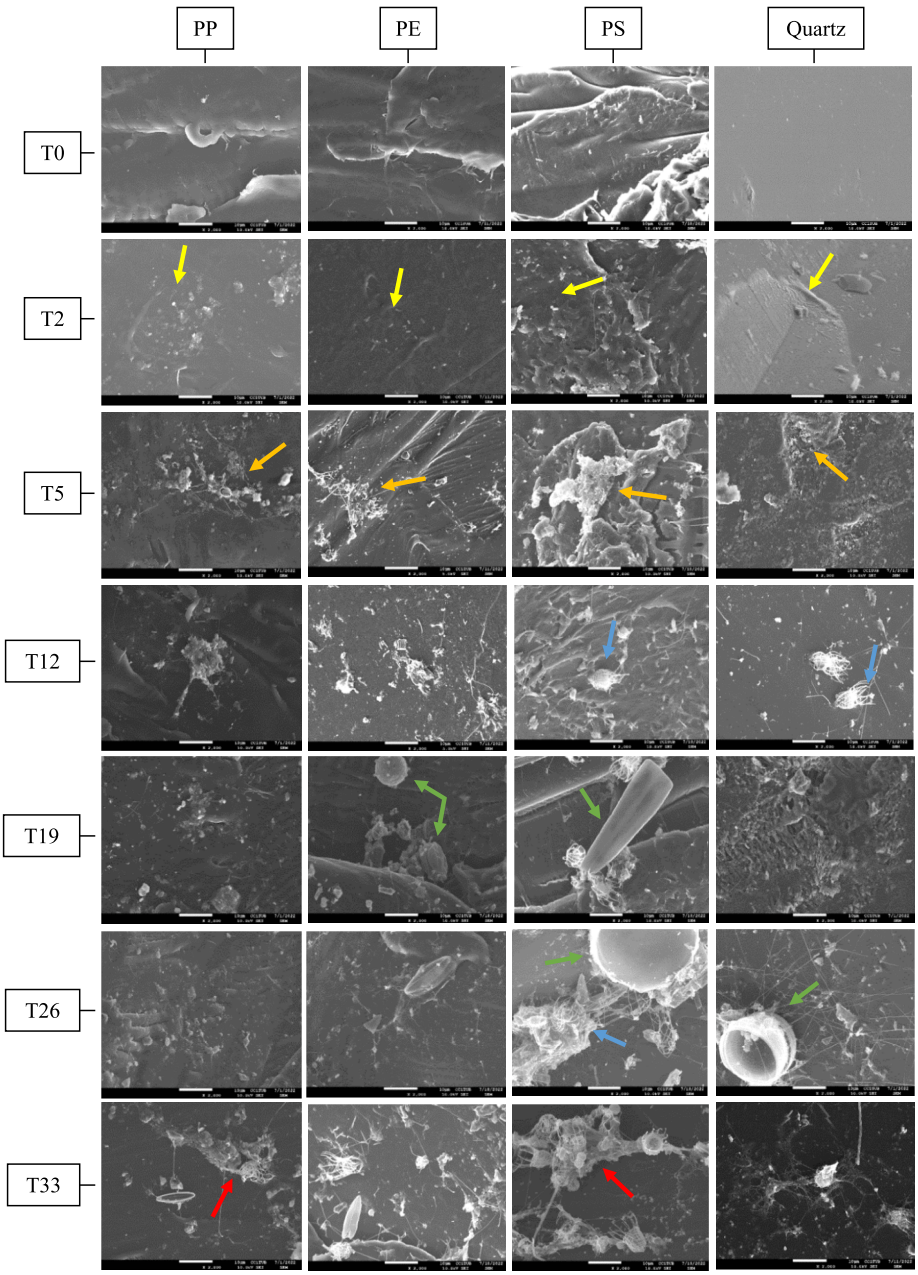

Fig. 2. Scanning electron microscopy micrographs showing the colonisation process on polyethylene (PE), polypropylene (PP), polystyrene (PS), and quartz at different times (days 2, 5, 12, 19, 26, 33). Yellow arrows indicate the first settlers. Orange arrows indicate the microbial exopolysaccharide matrix. Blue arrows indicate choanoflagellates. Green arrows indicate diatoms. Red arrows indicate the red arrows indicate an amalgamation of prokaryotes and eukaryotes in polysaccharide mucus. Scale bars: 10 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

These were grouped into 5 phyla, 17 classes, 40 orders, 57 families, and 87 genera. Our investigation was focused on analysing sequences associated with the Bacteria domain, given the limited identification of Archaea within the plastic biofilms in our analysis.

Microbial α-diversity richness was estimated using the Shannon and Chao1 indices finding no significant differences between the community diversity across matrices (water, quartz and plastic polymers), substrates (PP, PE, PS, quartz and water), or time (days 2, 5, 12, 19, 26, 33) for both estimators (Tukey's HSD test; p-value >0.05; Table S1). However, early- and late-biofilm did show a significant difference for Shannon Index (Tukey's HSD test; p-value = 0.0311; Table S1), with the latter showing higher levels of diversity (Fig. S4). Microbial β-diversity was estimated by Bray Curtis dissimilarity analysis and visualized by a principal coordinate analysis (PCoA; Fig. 3) and a hierarchical cluster (Fig. S5). PCoA showed a noticeable gradual differentiation due to time along the Axis 1 (33.6 %), progressing from the earliest sampling times to the latest. Whereas Axis 2 (12.2 %) exhibited a clear differentiation between water and the substrates. The clustering analysis supported these results and clearly defined two clusters: i) an initial biofilm cluster encompassing the earlier sampling times (day 2 to day 12), remarkably including PE day 19, and ii) a late-biofilm cluster comprising the later sampling times (day 19 to day 33).

Further analysis of the community composition by PERMANOVA test confirmed significant differences (p-value <0.05) within matrices, substrates, and time. In this regard, these variables were analysed by a pairwise PERMANOVA test for multiple comparisons. For matrices, significant differences (p-value <0.05) were observed between plastic and water and between quartz and water. However, no significant

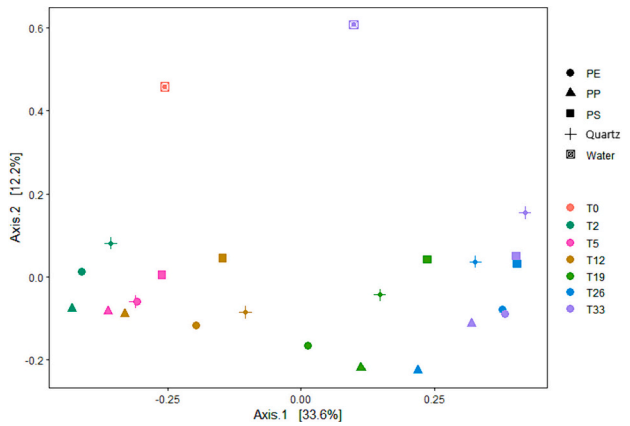

Fig. 3. Principal coordinate analyses (PCoA) of the samples: polyethylene (PE), polypropylene (PP), polystyrene (PS), quartz, and water, at different times (days 2, 5, 12, 19, 26, 33).

differences (p-value >0.05) were observed between plastic and quartz. For single comparisons between substrates and water, all plastic polymers (PP; PE; PS) and quartz showed significant differences (p-value <0.05) compared to water. Meanwhile, no significant differences (p-value >0.05) were found within plastic polymers nor when each was compared to quartz (Table S2). Regarding time, significant differences (p-value <0.05) were observed only between days 2, 5, and 12 when compared to days 19, 26, and 33, except for the comparison between days 12 and 19, suggesting a gradual transition (Table S2).

3.4.1. *Bacterial community of water*

The initial taxonomic composition of the water bacterial community predominantly consisted of the phyla Bacteroidota and Pseudomonadota (51 % and 47 % of the reads respectively). Within Bacteroidota, the Flavobacteriales order, and specifically the Flavobacteriaceae family, dominated (49 % of the reads) with genus *Polaribacter* as the main genus (41 % of the reads). In relation to Pseudomonadota, Alphaproteobacteria (20 % of the reads) and Gammaproteobacteria (27 % of the reads) were the main classes. Among Alphaproteobacteria, *Rhodobacterales* was its main order (14 % of the reads) with genus including *Sulfitobacter* and *Loktanella*, whereas for Gammaproteobacteria, *Enterobacteriales* was the main order (15 % of the reads) including the genus *Colwellia* and *Psychromonas* (Figs. 4, S6, and S7). The genus *Oleispira* represented 1 % of the Bacteria. By the end of the experiment (day 33), the water bacterial community slightly shifted. The abundance of the phylum Bacteroidota increased (63 % of the reads from 51 %), while Pseudomonadota decreased (37 % of the reads from 47 %). Within Bacteroidota, the Flavobacterial order, particularly the genus *Polaribacter* exhibited an increase (59 % of the reads from 41 %). Concerning Pseudomonadota, the class Alphaproteobacteria increased (25 % of the reads from 20 %) whereas the Gammaproteobacteria decreased (12 % of the reads from 27 %). Among Alphaproteobacteria, *Rhodobacterales* was also the main order (24 % of the reads from 14 %), principally represented by the *Sulfitobacter* genus (17 % of the reads). Within Gammaproteobacteria, *Enterobacteriales* was still the main order (9 % of the reads from 15 %) although their abundance decreased. In this case, the main genus shifted to *Glaciecola* (6 % of the reads) and *Pseudoalteromonas* (1 % of the reads) (Figs. 4, S6, and S7).

3.4.2. *Bacterial communities of the plastisphere*

The composition of the bacterial communities associated with both plastic pellets and quartz exhibited substantial similarity in the higher taxonomic ranks throughout the experiment. The predominant phylum was Pseudomonadota (82 ± 6 % of the reads), followed by Bacteroidota (18 ± 6 % of the reads). Within the Pseudomonadota phylum, the predominant class were Gammaproteobacteria (68 ± 11 % of the reads), consistently exhibiting abundant presence throughout the experiment and Alphaproteobacteria (14 ± 11 % of the reads), increasing notably its presence since day 19. In the case of Bacteroidota, the main class was Flavobacteria (13 ± 7 % of the reads), and the number of reads

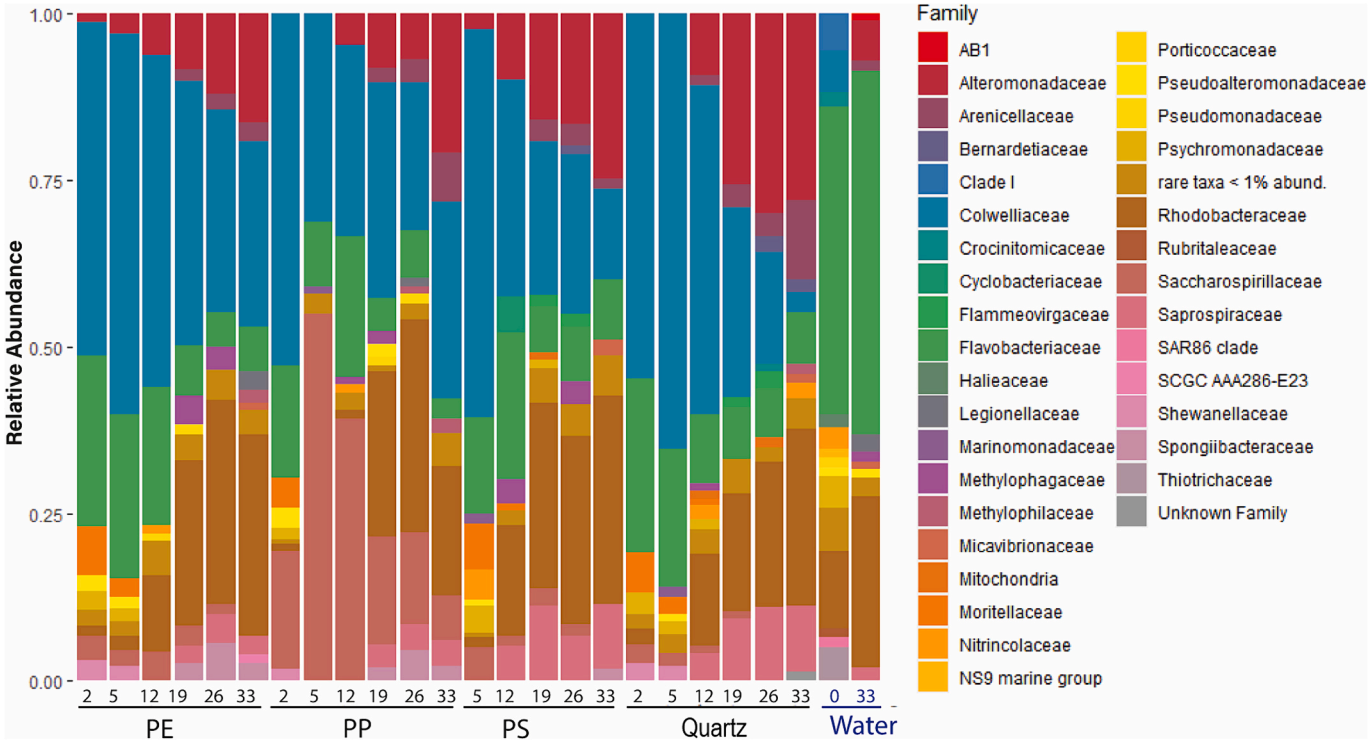

Fig. 4. Relative abundances at family level for polyethylene (PE), polypropylene (PP), polystyrene (PS), quartz, and water, at different times (days 2, 5, 12, 19, 26, 33).

remained relatively stable over the experiment. Gammaproteobacteria, Alphaproteobacteria, and Flavobacteria represented most of the total reads during the experiment (95 ± 5 %) (Figs. 4, S6, S7, S8). The genus *Colwellia* prevailed during the early-biofilm stage (50.64 ± 11.93 % of the reads), decreasing in the late-biofilm stage (27.83 ± 11.30 % of the reads) for all the substrates.

At day 33, higher diversity was observed for the biofilm communities across all substrates. For PP, although the genus *Colwellia* dominated the plastisphere (33 % of the reads from 55 %), the genus *Glaciecola* (16 % of the reads from 0 %), *Sulfitobacter* (10 % of the reads from 1 %), *Arenicella* (8 % of the reads from 0 %), *Oleispira* (8 % of the reads from 17 %), and *Paraglaciecola* (7 % of the reads from 0 %) increased. PE was also mainly composed by *Colwellia* (33 % of the reads from 55 %), followed by *Sulfitobacter* (24 % of the reads from 2 %), *Glaciecola* (13 % reads from 0 %), and *Paraglaciecola* (6 % of the reads from 1 %). Conversely, for PS, *Sulfitobacter* (23 % of the reads from 0 %) was the most abundant genus, followed by *Colwellia* (16 % of the reads from 59 %), *Glaciecola* (14 % of the reads from 0 %), *Paraglaciecola* (14 % of the reads from 2 %), and *Lewinella* (10 % of the reads from 0 %). For quartz, the genus *Colwellia* decreased to become residual (3 % of the reads from 57 %), while *Sulfitobacter* (16 % of the reads from 2 %), *Glaciecola* (16 % of the reads from 0 %), *Paraglaciecola* (15 % of the reads from 0 %), *Arenicella* (13 % of the reads from 0 %), and *Lewinella* (10 % of the reads from 0 %) increased becoming the most abundant genera. Notably, during the first 12 days, the genus *Oleispira* was the second dominant in PP representing the 17, 56 and 38 % of the reads at days 2, 5 and 12 respectively. In contrast, *Polaribacter* was the second most abundant genus in PE, PS, and quartz. For a more detailed taxonomy for all samples at different hierarchy levels, see the Krona diagrams (Fig. S8).

4. Discussion

We studied here for the first time the process of plastic colonisation by marine bacteria in a polar environment using a microcosm over a controlled time series and comparing different plastic polymers. This information represents an initial step in understanding the Antarctic plastisphere assemblages and their time- and substrate-related variability. Microplastics in remote regions like the Southern Ocean contribute to the formation of plastisphere communities, potentially representing changes in the ocean microbial assemblage balance and posing environmental risks. Although numerous studies focusing on this issue are emerging, there are still significant knowledge gaps, particularly in Antarctic environment.

In our experiment, bacteria rapidly colonised all substrates, settling in at least within the first 48 h, and afterwards maintaining relatively stable abundances over time. Generally, the higher densities found here for all polymers were an order of magnitude lower than those reported for plastic debris on the Mediterranean coast ($1.9{\cdot}10^{6}$ ± $2.4{\cdot}10^{6}$ gc mm^{-2}; $1.1{\cdot}10^{4} \pm 1.3{\cdot}10^{4}$ cfu mm^{-2}) (Liang et al., 2023). Specifically for PE, bacterial cell count at day 33 ($2.31{\cdot}10^{3}$ cell count mm^{-2}) was an order of magnitude lower than in PE deployed at the coastal North Atlantic for one and two weeks ($4.0{\cdot}10^{4}$–$1.07{\cdot}10^{5}$ cells mm^{-2} measured by CLASI-FISH analysis) (Schlundt et al., 2020). Conversely, a study conducted in the Ross Sea (Antarctica) reported similar densities not only in PE but also in PVC during a 12-month colonisation experiment (PE: $1.08{\cdot}10^{3}$–$1.82{\cdot}10^{3}$ cells mm^{-2}; PVC: $1.57{\cdot}10^{3}$–$4.36{\cdot}10^{4}$ cells mm^{-2}) (Caroppo et al., 2022). Therefore, considering that Antarctic bacteria exhibit slower growth rates in these colder temperatures due to reduced biochemical activity (Van Gestel et al., 2020; Parrilli et al., 2021), our results suggest that also the plastisphere in Antarctic conditions may develop slower than in other oceans. However, it should be noted that an incubation experiment testing different plastics in Mediterranean conditions showed densities of the same order of magnitude throughout the course of that experiment, despite exhibiting higher densities from day 15 to 45 (range of $3.4 \pm 0.51{\cdot}10^{3}$ to $9.05 \pm 1.23{\cdot}10^{3}$ cell count mm^{-2}) (Dussud et al., 2018a). Broadly, we highlight a certain variability among studies and methods, which should be addressed in future research.

Notably, the highest bacterial abundances in our experiment were found in PS and quartz, while lower and more variable abundances were observed in PP and PE. This difference may be attributed to their densities: PS and quartz have higher densities than water, causing them to settle at the bottom of the aquaria. In contrast, PP and PE, with lower densities, floated. This observation was further corroborated by SEM micrographs, which showed a progression from initial settlers to complex microbial assemblages on all plastics and quartz. On the sinking substrates, choanoflagellates and diatoms began establishing from day 12, whereas on floating substrates, this occurs from day 19 onwards. Remarkably, diatoms were the most frequently observed type of eukaryote, consistent with findings from previous studies (Carson et al., 2013; Oberbeckmann et al., 2016; Lacerda et al., 2019; Caruso, 2020). Overall, our results indicate that sunken items undergo a more straightforward and stable colonisation process over time. This may be because they are constantly submerged in water and in contact with microbial communities from various surfaces, such as sediments, rocks, or, in this case, the bottom of the aquaria.

To obtain complementary information regarding bacterial colonisation, we employed culturable marine heterotrophic bacteria and flow cytometry exclusively for PP, and for all substrates on day 33. We observed that qPCR reported higher density values since it targets the 16S rRNA gene, which lacks discrimination between live and dead bacteria and amplifies multiple gene copies within each organism. The number of 16S rRNA gene copies is around 1 to 15 copies in bacteria, so a mean of 7 to 8 copies of the 16S gene can be assumed for bacteria (Větrovský and Baldrian, 2013). Flow cytometry, by targeting well-conserved bacterial DNA, provided evidence of live bacteria, while culture methods report only bacteria capable of growing in the media used. Both methods are known to underestimate bacterial densities: flow cytometry may have difficulties in counting aggregated bacteria, in this case, due to the exopolysaccharide matrix (Dussud et al., 2018a), and by culture bacteria method, only 0.1–1 % is cultivable. Here, the fact that culturable cells increased over time suggests that part of this increment is due to viable bacteria and not just the accumulation of DNA from dead bacteria. Overall, flow cytometry and culture methods may provide results closer to the actual abundance of the living fraction. However, qPCR remains the reference method for monitoring colonisation as it encompasses the majority of sampled bacteria.

In this study, the microbial assemblages were found to have similar bacterial richness over the course of the experiment for both matrices and substrates, as no significant differences in terms of alpha-diversity were observed. In contrast, most research suggest that the plastisphere typically demonstrates greater richness than communities from surrounding water and other substrates, especially in the biofilm early stages (Dussud et al., 2018a; Dudek et al., 2020; González-Pleiter et al., 2021; Zhao et al., 2021). Also, some studies have reported differences among plastic polymers (Dussud et al., 2018a). Here, significant changes in alpha-diversity were only observed for Shannon Index when categorizing our samples in two groups: early- and late-biofilm stage, with the latter exhibiting higher richness. This could be attributed to a shift from a poor early plastisphere, to a diverse mature plastisphere. In this line, β-diversity analysis revealed significant differences not only in the microbial community composition between the surrounding water compared to the plastisphere and quartz but also over time, defining two groups: the early-biofilm from day 2 to day 12 and the late-biofilm from day 19 to day 33. Several studies have shown that the plastisphere community shares similarities with communities on other substrates like glass, metal, and organic particulated matter. However, it notably diverges from the microbial community present in the water column (Bryant et al., 2016; Dussud et al., 2018a; Caruso, 2020; Amaral-Zettler et al., 2020; Papale et al., 2024). Remarkably, a study conducted in the Ross Sea (Antarctica) shows that the sampling site did not play a significant role as a microbial community driver, with the matrix as the

more relevant factor defining the community assemblages (Caruso et al., 2023). Moreover, current studies agree that in the early stages of the biofilm formation there is a shift in the composition of bacterial communities, from an early plastisphere community to a mature plastisphere community (Caruso, 2020; Dudek et al., 2020; Wright et al., 2021). However, in our study the early-biofilm microbial community lasted longer than, for example, in the Caribbean (Bocas del Toro, Panama) and the Mediterranean (Banyuls bay, France) waters (Dussud et al., 2018a; Dudek et al., 2020). Thus, our results may indicate a slow colonisation succession in the Southern Ocean waters. Note that bacterial plastisphere communities were not significantly driven by substrate type as no significant differences were observed among different substrates, thus pointing to general processes as main shapers of the biofilm community like most previous studies (Caruso, 2020; Rogers et al., 2020; Coons et al., 2021). However, some particular genus like *Oleispira* has been more associated to PP. Generally, our results indicate that in our experiment, time emerges as the primary driver of bacterial community dynamics, which is consistent with findings from previous studies (Oberbeckmann et al., 2018; Amaral-Zettler et al., 2020; Caruso, 2020; Rogers et al., 2020).

To the best of our knowledge, in this study, we report for the first time the taxonomy of the Antarctic bacterial plastisphere along the early colonisation process. The phyla Pseudomonadota, with the main classes Gammaproteobacteria and Alphaproteobacteria, and phyla Bacteroidota, with the main class Flavobacteriia, were the dominant groups among microplastics, in line with findings in most previous plastisphere studies (Oberbeckmann et al., 2016, 2018; Ogonowski et al., 2018; Dussud et al., 2018a; Dudek et al., 2020; Martínez-Campos et al., 2021; Papale et al., 2024). These bacterial classes are typical Southern Ocean early colonisers (Abell and Bowman, 2005; Cavicchioli, 2015), known for extracellular polymeric substance production (Dang and Lovell, 2000) and association with diatoms (Amin et al., 2012). Furthermore, studies of bacterial assemblages conducted at the same study site (Livingston Island) found Flavobacteria and Gammaproteobacteria to be the dominant classes in the surface waters (Martinez-Varela et al., 2020). Additionally, Pseudomonadota and Bacteroidota dominated also the bacterial assemblages of Antarctic sponges (*Mycale acerata* and *Dendrilla antarctica*) and their surrounding water (De Castro-Fernández et al., 2023). Generally, during the early-biofilm formation, the predominant genus was *Colwellia*, which also exhibited high representation in the bacterial assemblages of Livingston Island surface water (Martinez-Varela et al., 2020). Notably, *Colwellia* is a generalist strain adapted to cold environments with the potential to degrade hydrocarbons (Martinez-Varela et al., 2020; Mason et al., 2014). During the early stages of biofilm formation on PE, PS, and quartz surfaces, the genus *Polaribacter* also exhibited a certain level of abundance. *Polaribacter* is a typical bacteria from polar surface waters with ability to form polysaccharides and contributing to the microbial loop and blue carbon sequestration (Bowman, 2018). Conversely, during the early-biofilm stage, PP was characterized by the genus *Oleispira*, which is a known hydrocarbon degrader adapted to cold temperatures (Kube et al., 2013).

Late-biofilm exhibited higher abundances of several genera in all substrates. We observed an increase in the *Glaciecola* genus, which typically thrives in sea-ice algal assemblages associated with early diatom blooms, as it has low abundance in the water body (Bowman et al., 1998; Van Trappen et al., 2004; Von Scheibner et al., 2017). The *Sulfitobacter* genus also exhibited high abundances. This genus is reported to exhibit algicidal effects against microalgae and to possess adaptations to cold temperatures (Barak-Gavish et al., 2018; Zeng et al., 2020). It belongs to the Rhodobacteraceae family, which is known to produce exopolymeric substances, converting a low-nutrient status biofilm to a high-nutrient status (Dang et al., 2008). In fact, the enrichment of this family in 2–3 weeks biofilm has also been observed in the North Atlantic Ocean (Schlundt et al., 2020). Conversely, *Polaribacter* became residual, unlike in the water community, showing no persistence on biofilms. The main difference between plastic polymers and quartz biofilm was the *Colwellia* genus, which remained the most abundant genus on plastics but not on quartz, where it became residual by day 33. Thus, it appears that plastics may serve as a suitable substrate for this genus, while it loses competitiveness on quartz, maybe due to the lack of an hydrocarbon source. Overall, for the substrates, our results demonstrate the succession from early generalist colonisers, which possess the capability to enhance biofilm formation, to a more biofilm-specialized bacterial community throughout the experiment. Furthermore, we observed certain bacteria of particular interest, especially for their potential in hydrocarbon bioremediation and other biotechnological applications. It is noteworthy that no notable pathogenic strain was identified throughout the experiment, which contrasts with studies in other areas with a strong human impact (Liang et al., 2023; Ballesté et al., 2024).

Our results have to be interpreted within the context of a microcosm experiment under specific conditions. The experiment was incubated outdoors, under a 8 m roof, with temperature ($\pm$ 2 °C) and sunlight (18 h at the beginning and 13 h at the end of the experiment) conditions reflecting the environmental parameters plastics would be exposed to during the summer polar period. However, conditions differ across different polar seasons, with larger temperature fluctuations and reduced sunlight in winter, which will shape the water bacterial communities and ultimately affect bacterial colonisation. Additionally, since microcosms are closed systems, there was no introduction of new microorganisms through water circulation, and we observed also a slight increase in salinity. Consequently, the development of the plastisphere may have been constrained or affected by these factors. Despite this, it is important to note that biofilm bacteria are typically more resilient to environmental fluctuations than planktonic bacteria.

Microcosms simplify the complexity and scale of natural ecosystems by controlling environmental conditions. This controlled environment facilitates the identification of factors shaping colonisation. However, to address their limitations in future studies, we advocate to combining microcosm experiments with field incubations and the collection of environmental samples to capture more realistic plastisphere dynamics. In this context, our study provides a baseline for future research on the Antarctic plastisphere, offering methodological advancements and new data.

5. Conclusions

This study aimed to understand the colonisation and microbial community dynamics of the Antarctic plastisphere by a microcosm experiment deployed for 33 days using different plastic polymer types. Our results show that while colonisation occurs rapidly and consistently, growth rates seem to be slower compared to other oceanic regions, suggesting unique environmental constraints. Our results also reveal a transition in microbial communities from early- to late-biofilm stages between day 12 to 19, with time being the main driver of the biofilm community. The different materials: plastic polymers and quartz, did not play any significant role in defining the bacterial assemblage community. Over time, there was a shift from generalist colonisers to more specific biofilm bacteria. Additionally, we found some bacteria of particular interest regarding potential biotechnological applications such as the genus *Oleispira*, which was notably associated to polypropylene. Plastic associated pathogenic microorganisms were not found. More extensive research reporting absolute bacterial densities during the colonisation is required to better understand these processes. Moreover, we further recommend to use more than one analytical method to approach the bacterial abundance quantification. We believe that the results of this study will have implications for environmental monitoring, risk assessment, and the formulation of strategies to mitigate the emerging issue of plastic pollution in the Southern Ocean.

Funding

This work was supported by BACT&PLAST (PID2019-108957GA-I00), CHALLENGE (PID2019-107979RB-100), CHALLENGE-2 (PID2022-141628NB-I00), and the Marine Biodiversity and Evolution group (MBE; 2021SGR01271). P.M.R acknowledges financial support from PREDOCS-UB Grant (2020) of the University of Barcelona.

CRediT authorship contribution statement

Pere Monràs-Riera: Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Data curation, Conceptualization. **Conxita Avila:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Elisenda Ballesté:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Data curation, 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.

Acknowledgments

We thank all members of the CHALLENGE-1 Antarctic cruise (2021-2022) and the staff of the Livingston Island Antarctic Station for their support. We thank the CCiT-UB of the UB for providing expert technical advice, in particular Eva Prats and Rosa Rivera for the SEM analysis, Jaume Comas for the flow cytometry analysis, the CRG-Genomics for the sequencing analysis, and Ignasi Jarne Sanz from Josep Carreras Leukaemia Research Institute for the mentoring in bioinformatics. This work was supported by BACT&PLAST (PID2019-108957GA-I00), CHALLENGE (PID2019-107979RB-100), CHALLENGE-2 (PID2022-141628NB-I00), and the Marine Biodiversity and Evolution group (MBE; 2021SGR01271). P.M.R acknowledges financial support from PREDOCS-UB Grant (2020) of the University of Barcelona. This study is part of the 'Integrated Science to Inform Antarctic and Southern Ocean Conservation' (Ant-ICON) research programme of the Scientific Committee on Antarctic Research (SCAR), and the Plastic Working Group of SCAR.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.marpolbul.2024.116961.

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