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RESEARCH ARTICLE

Evaluation of prokaryotic and eukaryotic microbial communities on microplastic-associated biofilms in marine and freshwater environments

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Abstract

Microplastics (MPs) are major concern due to their potential harm to ecosystems and most research has focused on their presence and fate, with limited attention to their biodegradation in aquatic ecosystems. Nevertheless, MPs act as hotspots for the colonization by a diverse range of microorganisms that can adhere to plastic surfaces, resulting in the subsequent formation of biofilms—a potential threat especially in terms of pathogenicity. This study employed 16S rRNA and 18S rRNA sequencing metagenomic analyses to investigate microbial communities within biofilms on plastic materials exposed to long-term marine and freshwater environments. Three *Arcobacter* species *(Arcobacter nitrofigilis, Arcobacter acticola*, and *Arcobacter suis)* emerged as dominant species in M_MP sample, while *Flavobacterium tructae* was the predominant species within the F_MP sample. The 18S rRNA sequencing revealed the presence of the fungal phylum *Ascomycota* and the microalgal species *Pseudocharaciopsis ovalis* in F_MP. Although, the primary species detected on M_MP and F_MP samples include bacteria previously implicated as pathogen, the predominant species identified in this study were unconnected to MP-associated biofilms or MP degradation. Their presence constitutes a novel discovery, opening promising avenues for the exploration of their potential involvement in the biodegradation of MPs within aquatic environments.

KEYWORDS
community structure, metagenomics, microplastics, polyethylene, polylactic acid

1 | INTRODUCTION

Microplastics (MPs), which are particles with a diameter smaller than 5 mm, have been regarded as a substantial concern for aquatic ecosystems due to their deleterious effects on different aquatic organisms, ranging from phytoplankton and zooplankton to fish and cetaceans. The presence of MPs is at extensive rates in marine environments, leading to numerous investigations into their dissemination, fate, and ecological consequences. Recent studies have also revealed the existence of MPs in freshwater systems, including rivers, and lakes [1]. Irrespective of their source, MPs have become prevalent across global ecosystems, extending diverse systems from aquatic to terrestrial regions and watersheds. This emergence mountains concerns regarding potential risks for human health through the consumption of plastic-contaminated seafood and fish, in addition to the effects on aquatic organisms [2]. This highlights the necessity for further research into the fate and potential effects of MPs within freshwater contexts. The widespread distribution of plastic particles across aquatic ecosystems offers a substrate that microorganisms can adhere to and colonize, resulting in the formation of a biofilm—a cooperative consortium referred to as the *plastisphere*—on the plastic surface. Biofilms are intricate communities comprised of various microorganisms that coexist and mutually benefit as symbiotic entities [3]. In this synergy, MPs serve a dual role: they provide surfaces conducive to the formation of biofilms and concurrently release dissolved organic carbon (DOC) as the slowly degrading polymers break down into the surrounding water. Released DOC augments the proliferation and activity of heterotrophic microorganisms within the plastisphere [4]. The coexistence of bacteria and MPs transforms MPs into hotspots of microbial pathogens and hubs for horizontal gene transfer (HGT). HGT facilitates the exchange of antibiotic resistance genes (ARGs) between biofilm-forming microorganisms and surrounding bacteria via mobile genetic elements, expanding the potential for MP-associated biofilms to acquire ARGs from distant ecosystems. Subsequently, this dynamic relationship induces the transfer of pathogenic traits, fostering the emergence of a more antibiotic-resistant bacterial community within the MP-associated biofilm [5].

Recently, attention has been directed toward the investigation of microbial colonization, composition, and structural attributes of MP-associated biofilms. Microbial communities can exhibit notable divergence from the surrounding aquatic environment as the composition on natural substrates generally differ from those of free-living microbes and their assemblages, a phenomenon attributed to the distinct activities of sessile organisms [6]. Environmental conditions such as temperature, salinity,

PRACTICAL APPLICATION
- Community composition varied between biofilms on marine and freshwater samples after long-term months exposure.
- Alpha diversity was higher on microplastic-associated biofilm of freshwater sample.
- Species including *A. nitrofigilis*, *A. acticola*, *A. suis*, *Poseidonibacter lekithochrous*, and *Arenimonas maotaiensis* not previously associated with microplastics were detected.
- Three pathogenic Arcobacter species were identified as dominant in marine plastisphere, indicating their likelihood potential involvement in microplastic degradation.
- No significant eukaryotic microorganisms were detected to persist after long-term exposure.

pressure, and the presence of light and oxygen, and transport within environmental matrices may have a substantial role, leading to variations in community structures within MP-associated biofilms between marine and freshwater systems [7]. Studies predominantly focus on prokaryotic microorganisms to elucidate the microbial interactions within MP-associated biofilms. Meanwhile, the role of eukaryotic microorganisms remains to be uncovered. Prokaryotic biofilms attract eukaryotic predators such as fungi, algae, small metazoans and protozoa to be secondary colonizers of MPs. The incorporation of eukaryotes adds intricacy to the structure of the biofilm community, strengthen its overall complexity [8]. Hence, a comprehensive elucidation of the role of eukaryotic microorganisms is pivotal to comprehend the intricate process of MP-associated biofilm formation, the dynamics of community evolution, and their collective influence on the fate of MPs and the encompassing ecosystem.

The current study investigated the community dynamics of both prokaryotic and eukaryotic microorganisms in biofilms of polylactic (PLA) and polyethylene (PE) MP materials in marine and freshwater environments. In addition to the non-degradable polymer PE, PLA was selected as the MP material to be exposed to marine environment. Recently, PLA has a pioneering role due to its well-established reputation as an environmentally friendly, biodegradable, biocompatible, and biobased polyester [9]. Through this approach, the study aims to enrich the understanding of the intricate structure characterizing MP-associated biofilms across different aquatic settings. Given the likelihood that species persisting on MP surfaces throughout the long exposure duration may be utilizing

MP particles as the sole carbon source, this study aimed to identify novel species for potential use as bioaugmentation agents in future MP degradation studies. The results from the metagenomic sequencing analysis revealed species that colonized both marine and freshwater MP surfaces but have not been previously linked to MP degradation.

2 | MATERIALS AND METHODS

2.1 | Sample collection and acquisition of biofilm layers

Polylactic acid (PLA) biodegradable plate and polyethylene (PE) transparent film were obtained from commercial sources. In March 2022, PLA sample was immersed in the marine environment of Gölcük Kavacık Marina, Kocaeli city, Marmara Sea (40°43′32.6″N, 29°50′19.3″E). In November 2022, PE sample was immersed in freshwater, Poyrazlar Lake, Kocaeli city, Marmara region (40°51′18.2″N, 30°18′00.3″E). Immersions took place at depths ranging from 50 cm to 100 cm below the water surface. After an exposure period of 8 months, the PE samples deployed in the freshwater lake setting were collected while the PLA samples from the marine environment were retrieved after 16 months. In the 16th month, the water quality parameters for Gölcük Kavacık Marina in Kocaeli (Marmara Sea) were recorded as follows: conductivity 34,300 μS/cm (WTW 3210), pH 7.75 (Thermo Orion Star A325), temperature 26.3°C, DO 7.46 mg/L (Thermo Scientific Orion Star A223). Also, for Poyrazlar Lake in the 8th month, the recorded parameters were as follows: conductivity 173 μS/cm (WTW 3210), pH 7.92 (Thermo Orion Star A325), temperature 27.2°C, DO 7.8 mg/L (Thermo Scientific Orion Star A223). The biofilm layers formed on PLA and PE samples was carefully scraped from the plastic surfaces to be used for subsequent metagenomic analysis.

2.2 | Physical and chemical characterization of microbial biofilms and detection of microplastics

Plastic sample thickness was measured with a Mitutoyo micrometer before and after the exposure. Initial thickness for PLA plate was 290 μm, and PE transparent film was 150 μm. For the physical characterization, a light microscope (Olympus BX51 T, Olympus Corp., Tokyo, Japan) set at 4X magnification was used to observe the basic morphology of PLA and PE samples. The microscope was equipped with a digital camera (Olympus, DP20) to obtain microscopic images of the MPs [10]. For the chemical structure characterization of the polymers, attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was applied and spectra were obtained. This analytical technique provides insights into the chemical bonds within molecules. Specifically, carbon-containing polymers can be unequivocally identified through FTIR, wherein distinctive bond configurations yield unique infrared (IR) spectra, enabling the differentiation of plastics from other organic and inorganic interferences. Bruker, ATR-FTIR spectroscopy instrument (ALPHA, Germany) at 4 cm^{-1} resolution, 4000–600 cm^{-1} range, and 32 scan conditions were used to obtain the infrared spectrum of polymers. The spectra obtained were also matched with those in the Bruker polymer library and the ATR-FT-IR polymer library collections to identify the polymer type [11].

Various points on the surfaces of PLA and PE samples within the film structure may exhibit distinctions. Therefore, the carbonyl index (CI) was calculated by using the average of at least 10 spectrum values obtained from different points on the samples. For polymers like PE and PLA, the CI is computed by dividing the absorbance of the carbonyl peak by the absorbance of a reference peak. The CI values for PE [12] and PLA [13] materials are calculated as follows, respectively:

$$CI_{PE} = Abs(1794)/Abs(1471) \qquad (1)$$

$$CI_{PLA} = Abs(1754)/Abs(1452) \qquad (2)$$

CI value results are evaluated as follows.

1) low (CI = 0–0.15)
2) medium (CI = 0.16–0.30)
3) high (CI > 0.31)

Furthermore, Scanning Electron Microscopy (SEM) analysis was performed to obtain high-magnification images of the MPs and to determine the surface structure at the end of exposure in the natural environment. To scrutinize the surface structure of MPs, samples of PLA and PE were affixed to double-sided adhesive carbon tabs on aluminum SEM stubs. FEI Quanta FEG 450 model Field Emission Scanning Electron microscope was used for morphological analyses [14].

2.3 | Genetic characterization of prokaryotic and eukaryotic microbial communities

Genomic DNA from biofilms of marine (M_MP) and freshwater (F_MP) samples was extracted using the Genomic DNA Isolation Kit (Norgen Biotek Corp.,

Canada) following the manufacturer's instructions. The chosen primer pair for amplicon library construction targeted a segment spanning 1500 bp, encompassing the V1–V9 region of the 16S rRNA gene, while the primer pair targeted a 600 bp region of the 18S rRNA gene [15]. Furthermore, Nanopore barcode DNA sequences were appended to the 5′ terminus of these target-specific primers. For the 16S targeted analysis, the sequence of the forward primer was TTTCTGTTGGTGCTGATATTGC-AGRGTTTGATYHTGGCTCAG-3′, and the sequence of the reverse primer was 5′- ACTTGCCTGTCGCTCT-ATCTTC-TACCTTGTTAYGACTT −3′. For the 18S targeted metagenomic analysis, the sequence of the forward primer was 18S-566F 5′-CAGCAGCCGCGGTAATTCC-3′, and the sequence of the reverse primer was 18S-1289R 5′-ACTAAGAACGGCCATGCACC-3′. Initial primer proofreading involved the use of DNA polymerase with a 2x Reaction Mix and 200 nM of each primer. The PCR conditions included an initial denaturation step at 95°C for 3 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 seconds, extension at 72°C for 90 s, and a final extension at 72°C for 5 min.

The amplicon library was prepared using the Ligation sequencing kit 1D (SQK-LSK108) from Oxford Nanopore Technologies and loaded into the MinION device, along with 45 µL of barcoded DNA mixture and 5 µL of lambda phage DNA as a positive control. DNA end repair and dA-tailing were carried out using the NEBNext End Repair/dA-tailing Module and purification with Agencourt AMPure XP beads. For adapter ligation, 0.2 pmol tips were prepared, and DNA was mixed with Blunt/TA ligase master mix and adapter mixture. After incubation, a final purification step was performed using an Adapter Bead Binding buffer and 0.5X Agencourt AMPure XP beads. The sequencing mix (14 µL of DNA library) was combined with Loading beads (25.5 µL) and Running Buffer mix (35.5 µL) and loaded onto a primed R9.4 flow cell. A 48-h (R9.4) sequencing protocol was carried out using MinION™ control software (MinKNOW™ v. 0.46.1.9).

2.4 | Data and statistical analyses

Following the sequencing, fast5 results were converted to fastq using Guppy software, incorporating base-calling and de-multiplexing (https://github.com/nanoporetech/qcat). Given the average length of the 16S rRNA region at 1500 bp, reads falling within the 1250–1750 bp range were filtered using Trimmomatic. Additionally, as the 18S rRNA region was targeted with an average length of 600 bp, reads within the range of 300–1000 bp were selected. Curated reads underwent a specialized Python-based analytical workflow involving BLAST alignment to construct Operational Taxonomic Units (OTUs) [16]. Taxonomic information

was extracted from sequences with over 60% coverage and 80% pairwise similarity. Phylogenetic analyses, alpha and beta diversity assessments, PCA, PCoA, biomarker, phenotype analyses, and dynamic Krono charts were executed using Qiime2 (v.2019.7) (https://qiime2.org/) [17], while Mothur (v.1.48.0) organized taxonomic classifications (https://mothur.org/wiki/miseq_sop/). Graphs and tables used Python libraries. Linear Discriminant Analysis Effect Size (LEfSe) identified taxonomic groups explaining bacterial community variations. Functional annotations were made by using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/). Statistical data, presented as mean ± standard deviation (SD), underwent one-way ANOVA to assess statistically significant disparities among sample groups.

3 | RESULTS AND DISCUSSION

3.1 | Taxonomic annotation and alpha diversity on plastics

In the 16S rRNA sequencing, M_MP samples yielded 31,647 sequences, forming an equal number of OTUs in the bacterial community after quality filtering. In the freshwater ecosystem, F_MP samples produced 16,163 sequences, assigned to an equal number of OTUs in the bacterial community after quality filtering. For the 18S rRNA sequencing, M_MP exhibited 12,893 sequences in the eukaryotic communities, while F_MP contained 5880 sequences within the eukaryotic communities. Alpha diversity analysis, assessing bacterial community complexity, revealed substantial differences in species richness, Pielou's evenness, and Shannon indices between M_MP and F_MP samples, with the latter exhibiting higher richness, evenness, and diversity (Supplementary material 1a–1c). The evaluation of eukaryotic community complexity also demonstrated significant differences in Chao1 index, Pielou's evenness, and Shannon indices, emphasizing distinctions between M_MP and F_MP samples (Supplementary material 2a-2c). Remarkably, F_MP samples displayed superior fungal and microalgal species richness, evenness, and diversity compared to M_MP samples. The 18S rRNA sequencing for M_MP revealed a notable absence of data on fungal and microalgal species, potentially linked to challenges in acclimating to the marine environment during extended exposure.

3.2 | Physical and chemical characterization of microplastics

Following the specified exposure period, notable changes in the thickness of the plastic samples were not observed.
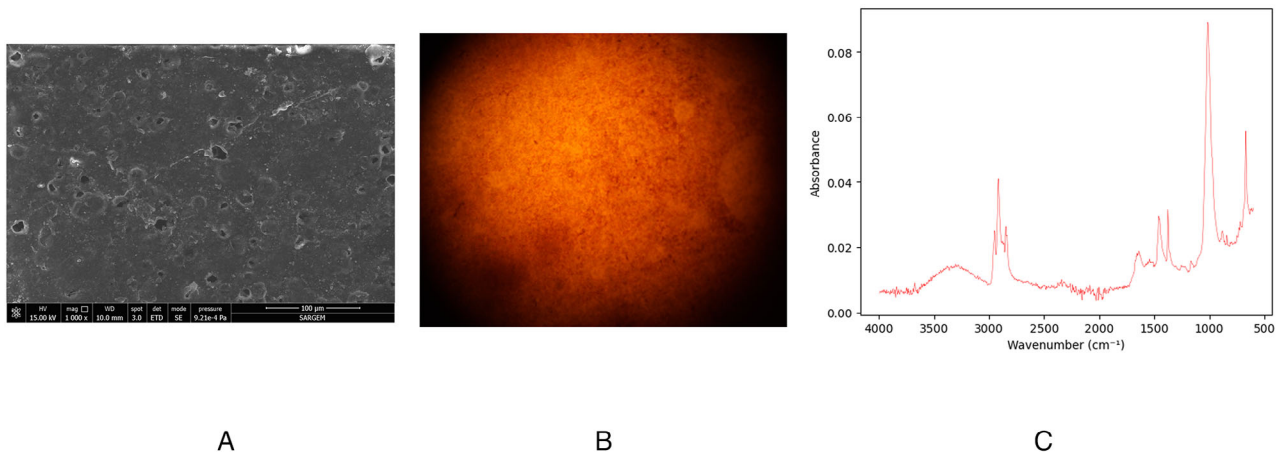
FIGURE 1 PLA biodegradable plastic after the exposure to the marine environment: SEM image (A), Microscope image at 4x (B), ATR-FT-IR spectra (C).

The samples underwent collection, followed by a washing and cleaning processes, and were then dried under standard room conditions. Post-exposure measurements demonstrated that the thickness of PLA particles remained constant at 280 μm, whereas the thickness of PE particles retained its initial value of 150 μm. In addition, examining the ATR spectra of samples obtained at the end of the exposure period and evaluating the carbonyl index (CI = 0.1) for PE reveals minimal wear and tear on the PE samples. Given the inherent durability of conventional plastics like PE, insignificant changes are expected over an eight-month waiting period, as observed in this study. In contrast, the calculated CI for PLA is 0.38, indicating significant degradation of PLA material in the marine environment over a sixteen-month period. This degradation, coupled with the aging of the PLA film surface, is also evident in the images obtained through SEM analysis (Figures 1A–C, 2A–C).

3.3 | Investigating the composition and distribution of prokaryotic and eukaryotic microbial communities on microplastic-associated biofilms in the marine environment

Bacterial community composition was investigated through 16S rRNA targeted-metagenomic analysis at phylum, class, and species levels. Major bacteria were determined as either >1% average abundance in one sample; as a result, 5 phyla, 8 classes, and 13 species were chosen as major bacteria. The results of the phylum level obtained from M_MP, illustrated in Figure 3A, demonstrate that *Campylobacterota* is the dominant phylum, comprising 60.5% of the total. Previous studies have shown that the phylum *Campylobacterota* is among those microorganisms that may possess the capability to degrade MPs, potentially utilizing the plastic particles
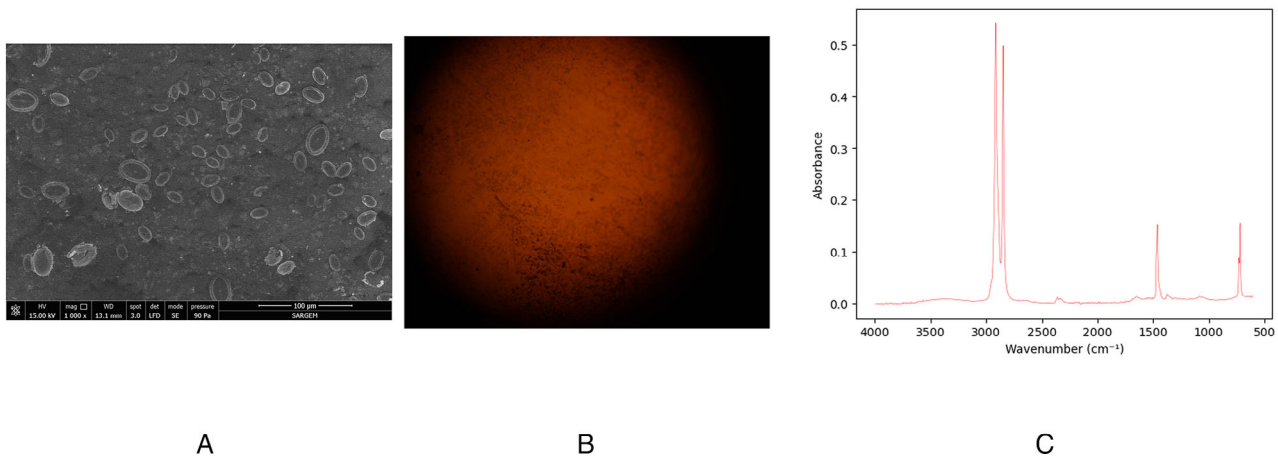

FIGURE 2 PE transparent film after the exposure to the freshwater environment: SEM image (A), Microscope image at 4x (B), ATR-FT-IR spectra (C).
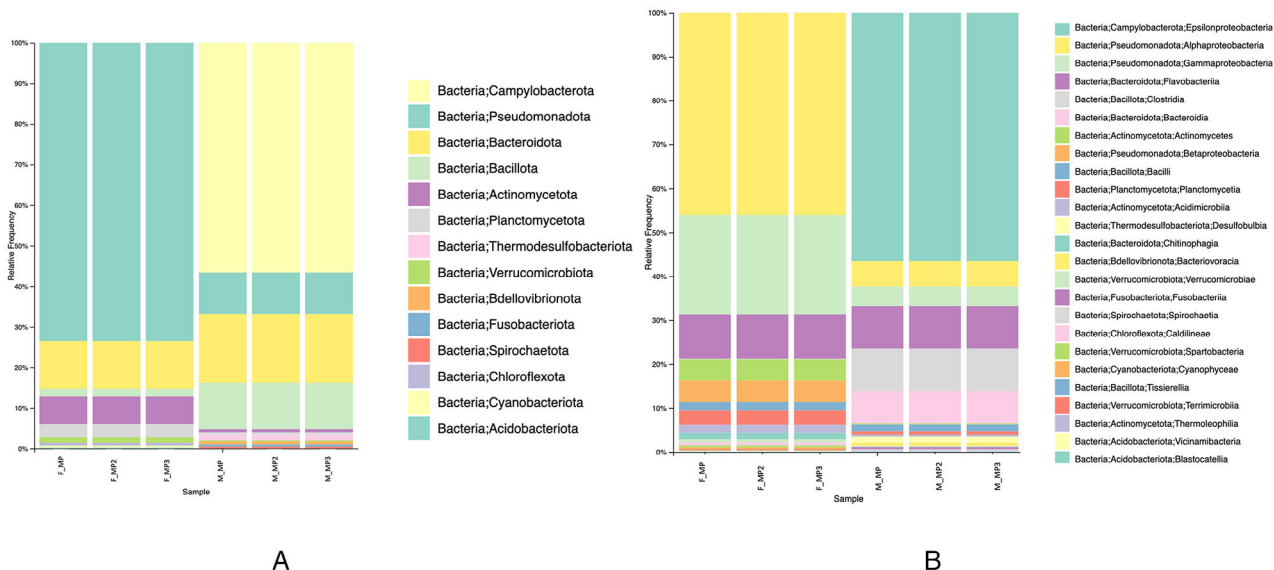
FIGURE 3 Taxonomy assignment of prokaryotic community of M_MP and F_MP samples at phylum (A) and class (B) levels.

as a growth substrate. The other abundant phyla were found as *Bacteroidetes* at 15.72%, *Firmicutes* at 11.76%, and *Proteobacteria* at 7.95%, this finding is consistent with previous investigations that have characterized microbial communities inhabiting the surfaces of marine MPs [18, 19]. In addition, species within the phyla *Proteobacteria* and *Bacteroidetes* have been identified as pivotal group in the initial colonization of plastic surfaces in marine [20]. Figure 3B illustrates the distribution of bacterial abundance at the class level within the biofilm colonizing the surface of M_MP. The predominant bacterial group within the biofilm consisted of *Epsilonproteobacteria*, representing a majority proportion of 60.47% of the total. Subsequently, classes of *Clostridia* (10.02%), *Flavobacteria* (8.63%), *Bacteroidia* (7.08%), *Alphaproteobacteria* (4.45%), *Gammaproteobacteria* (3.49%), *Bacilli* (1.56%), and *Desulfobulbia* (1.1%) were the other predominant microbial colonizers on M_MP, consist of which is consistent with findings from a previous study [21] *Alphaproteobacteria* and *Gammaproteobacteria* were reported as the primary colonizing groups in the biofilms on the MPs surfaces [22], hence, the continued presence of these two bacterial groups after long-term exposure indicates their potential as invasive species, demonstrating their capacity to effectively utilize MP particles as a resource to support their development.

At the species level, *Arcobacter nitrofigilis* was identified as the most abundant, accounting for 13% of the microbial composition, as represented in Figure 4A. The subsequent notable species in descending order of abundance were as follows: *Arcobacter acticola* (12%), *Poseidonibacter lekithochrous* (11%), *Arcobacter suis* (9%), *Fusibacter paucivorans* (6%). Although a recent study has reported the potential of *Arcobacter spp.* to exhibit sig-

nificant biofilm-forming activity, the current scientific literature lacks comprehensive insights into the relationship about *Arcobacter* species and their colonization on MP-associated biofilms or MP degradation [23]. However, considering the extended duration of PLA in the marine environment, it is reasonable to hypothesize that the species composing the biofilm layer at the end of long period possess the capability to utilize MP particles as a substrate for their sustained growth. These species are among the limited microorganisms capable of enduring on the MP surface for the long-term exposure and are therefore likely to possess the potential to exploit PLA particles for their growth through degradation. Therefore, it is crucial to investigate these species further as potential agents for bioaugmentation in future studies focusing on MP degradation. 18S rRNA sequencing did not yield any detection of fungal or microalgal species. This suggests that eukaryotic microorganisms may face significant challenges in adapting to and colonizing the marine ecosystem and the prolonged exposure of PLA particles to marine conditions did not provide suitable conditions for a notable detection in the diversity and abundance of these microorganisms.

3.4 | Investigating the composition and distribution of prokaryotic and eukaryotic microbial communities on microplastic-associated biofilms in freshwater environment

In current study, notable change in salinity had a pronounced effect on community structures, resulting in corresponding shifts in the microbiota's composition.
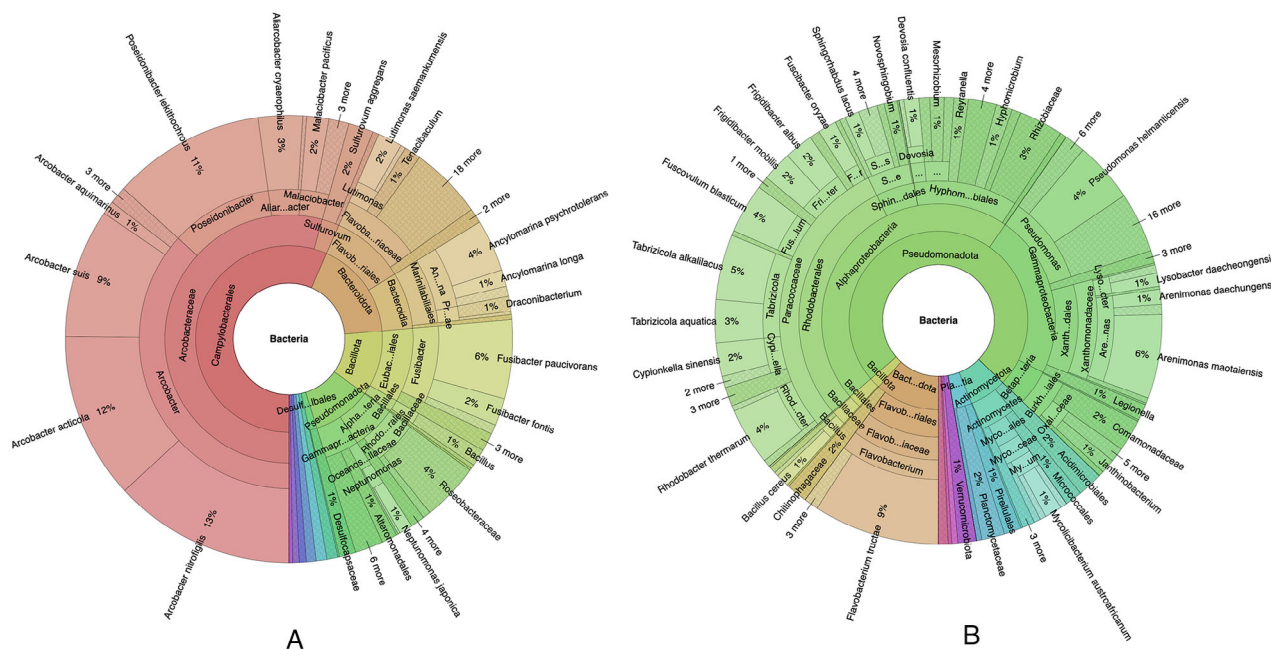
FIGURE 4 Taxonomy assignment at species level of MP-associated biofilm community in marine (A) and freshwater (B) environments.

These differences have been clearly revealed in metagenomic analyses utilizing 16S and 18S sequencing, providing insights into the distinct community structures within these two aquatic environments. The top 6 phyla with the highest relative abundance (chosen according to their >1% average abundance) were shown in Figure 3A. In contrast to phylum *Proteobacteria* (7.95%) of the biofilm on M_MP, *Proteobacteria* was the most dominant phylum of the plastisphere with a substantial abundance of 74.35% in F_MP. *Bacteroidetes* (10.75%), *Actinobacteria* (6.5%), *Planctomycetota* (3.88%), *Firmicutes* (2%), and *Verrucomicrobiota* (1.33%), consistent with previous studies investigating microbial diversity of biofilms on MPs on freshwater [19, 24]. The prevalence of these phyla was anticipated, as they are commonly distributed throughout the environment. They can be found in various ecological niches, including the intestinal tracts of both humans and animals, as well as in soil, sediments, and water.

At the class taxonomic level, the plastisphere exhibited a predominant presence of *Alphaproteobacteria*, constituting 48.5% of the total population. Subsequently, *Gammaproteobacteria* (21.5%), *Flavobacteria* (9.2%), *Actinobacteria* (4.6%), *Betaproteobacteria* (4.3%), *Planctomycetia* (3.88%), and *Bacilli* (2%) represented other dominant taxonomic groups within the plastisphere Figure 3B. A variety of bacterial isolates previously obtained from cultures where polyethylene terephthalate (PET) or polypropylene served as the sole carbon source was reported to encompass different phyla, including *Gammaproteobacteria*, *Actinobacteria* and *Bacilli* [25]. It has been also previously indicated that during the later stage of biofilm formation on MPs (after 135 days), there

is a notable shift in the dominant family of polyethylene colonizers towards *Flavobacteria* while a notable increment in the population of *Alphaproteobacteria,* *Gammaproteobacteria* and *Planctomycetia* is observed [26]. Considering this phenomenon, the predominance of these taxonomic groups after an extended period is logically consistent, and there is a likelihood that these taxa may have utilized the PE particles as their sole carbon source. At the species level, 17 predominant bacterial species are presented in Figure 4B, constituting approximately 50% of the total plastisphere and each exceeding 1% in average abundance in one sample. Recently confirmed as a pathogenic agent, particularly affecting fish, *Flavobacterium tructae* with an abundance of 9%, was found to be the most prevalent species among the plastisphere community [27]. *Arenimonas maotaiensis*, a freshwater bacterium, made the second-largest contribution to the plastisphere community with an abundance of 6%. *Tabrizicola alkalilacus*, a recently discovered species belonging to the *Alphaproteobacteria* class, also exhibited relatively high predominance with an abundance of 5%. *Rhodobacter thermarum*, constituting 4%, and psychrophilic *Pseudomonas helmanticensis*, with a 4% abundance, emerged as the subsequent dominant species within the plastisphere. Another noteworthy species, despite its lower abundance, is *Bacillus cereus* (1%). It has demonstrated the capability to degrade multiple types of plastic, making it a significant contributor to the plastisphere community. In a study MP degradation was analyzed using two species of *Bacillus* bacteria, one of which was *B. cereus*. Various types of MPs, including PE (polyethylene), PET (polyethylene terephthalate), PP

(polypropylene), and PS (polystyrene), all with a size of less than 250 μm, were utilized as carbon sources and energy substrates for the growth of these bacteria. The study indicated that *B. cereus* achieved a degradation rate of up to 7.4% for PS within a 40-day experimental timeframe. Additionally, it exhibited varying degrees of degradation for the other types of plastics, each at distinct ratios [28]. These findings indicate that the plastisphere's bacterial community exhibited notable shifts in response to environmental factors such as salinity and pH, affecting phylum, class, and especially species levels, comparing with the biofilm community on marine MPs. Similarly, alterations in bacterial community composition were identified in the study conducted by Dudek and Neuer. These variations were attributed to both exposure time and geographic location [29].

18S rRNA sequencing results indicate that the F_MP plastisphere displayed a notable diversity and abundance of eukaryotic organisms compared to the M_MP plastisphere. Within this community, *Ascomycota*, a fungal phylum, was a significant predominant, constituting 3% of all eukaryotes present. The presence of *Ascomycota* aligns with previous studies, which have also highlighted its prevalence in plastisphere communities [25]. Microalgal species *Pseudocharaciopsis ovalis* (1.19%) was also found in F_MP. The low diversity and quantity of eukaryotes in the F_MP plastisphere may be attributed to the prolonged presence of plastic material in the freshwater environment. Over time, MP may not have provided the necessary environmental and nutritional conditions for the eukaryotes initially attached to the MP surfaces to thrive and persist for an extended duration. In other words, the extended exposure of MPs to the aquatic environment may have limited the ability of eukaryotic organisms to establish and maintain viable populations on the MP surfaces.

3.5 | Comparison of microplastic-associated biofilms with other biofilm formation

3.5.1 | Pathogenicity in microplastic-associated biofilms

The potential risks associated with MPs and their associated microbial communities, some of which may be pathogenic, are a matter of concern. Pathogenic microorganisms, possibly adhering to MPs originating from various sources such as wastewater treatment plants and animal gastrointestinal tracts, can be introduced into natural environments [30]. Plastic samples from diverse environments, including marine and freshwater ecosystems, have reported the presence of various potential pathogens. As plastic debris transits through wastewater

treatment facilities and into aquatic systems, there is a significant opportunity for human pathogens to adhere to its surfaces. A recent study has demonstrated a notable temporal increase in biofilm development on MP surfaces exhibiting more substantial biofilm formation compared to stone. Correlation analysis indicated the possibility of a tetracycline-resistant bacterium (WPS-2) within the biofilm on MP surfaces, while no such correlation was observed in the biofilm on stone [31]. Nevertheless, it is crucial to emphasize that the function of plastics as carriers for the transportation of pathogenic microbial communities necessitates further in-depth investigations.

In current study, the identification of microbial species associated with MPs through metagenomic sequencing enabled the assessment of zones of microbial colonization and enhanced the understanding of the dispersion of non-culturable pathogenic microbial species. In M_MP, the predominant class of the plastisphere, *Epsilonproteobacteria*, includes *Arcobacter* spp., which hold significance due to foodborne enteric pathogen members. *Arcobacter* spp. are widely distributed in environmental and animal contexts, displaying a broad host range and inhabiting diverse habitats. They have been identified in various aquatic environments, including sewage, and various freshwater sources [32]. Moreover, in the current study, *A. suis*, which was observed to have a relatively high abundance of 10.1% within the M_MP plastisphere, has also been identified to possess virulency, besides another foodborne pathogen *Aliarcobacter cryaerophilus* (1%) [33]. Furthermore, *F. tructae*, identified at a prevalence of 9% within the F_MP plastisphere, has been reported as a pathogen for fish. Microbial community changes can have a notable influence on the diversity of metabolic functions. The 16S rRNA profiling data for M_MP and F_MP samples following an extended exposure period were subjected to annotation using the KEGG database for the purpose of assessing the functional content, as illustrated in Figure 5. The observation of substantial levels of ansamycin biosynthesis, a group of bacterial secondary metabolites renowned for their antimicrobial properties, and the biosynthesis of antibiotics belonging to the vancomycin group within both MP samples lends robust support to the hypothesis that MP-associated biofilms may harbor bacteria with pathogenic potential and heightened resistance to antimicrobial agents [34]. This convergence of microbial pathways, collectively indicative of antibacterial production and antibiotic resistance, underscores the potential public health concerns associated with MP pollution and the necessity for further investigation into the microbial ecology of MP-contaminated environments.

The potential role of MP-associated biofilms in the dissemination of ARGs in aquatic environments is another concern. Biofilms can form on the surfaces of MPs, providing a conducive layer for microbial attachment and
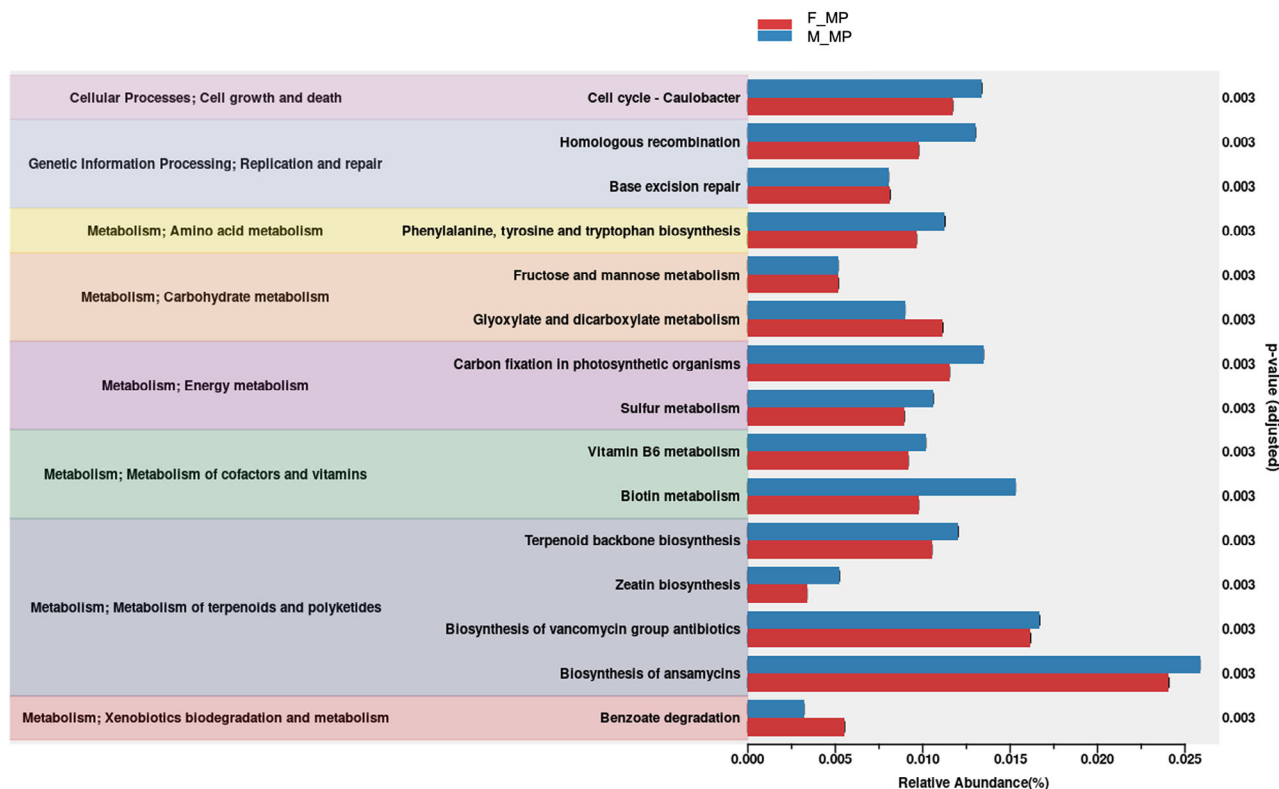
FIGURE 5 The KEGG pathways on M_MP and F_MP samples.

growth [35]. Within biofilms, the proximity of bacteria allows for interactions that may facilitate HGT, potentially transferring ARGs from one bacterium to another. MPs can adsorb and accumulate antibiotics, creating selective pressures that could favor the proliferation of antibiotic-resistant bacteria within biofilms. Additionally, microbial communities in biofilms demonstrate a significant capacity to tolerate toxic organic chemicals, such as antibiotics and antimicrobials, as well as inorganic substances like heavy metals, making them more resilient compared to microorganisms in external environments [30]. It is important to note that the knowledge of the extent of microbial tolerance to these substances is currently limited. The prevalence of *bla* resistance genes has been documented to be notably high in α-*Proteobacteria* (31.4%), *Firmicutes* (30.4%), *Actinobacteria* (27.1%), and *Bacteroides* (25.7%), all of which exhibited substantial abundance in the current findings from both marine and freshwater samples. Additionally, *tet* and *dfr* genes were previously observed to be distributed within these taxonomic groups at varying ratios [36].

3.5.2 | Microplastics as novel microbial niches

Irrespective of their origins, MPs have become ubiquitous in nearly all global environments. They are accumulating at an accelerating rate within aquatic ecosystems, including oceans, lakes, and rivers, which serve as repositories for plastics transported from terrestrial regions. This accumulation raises substantial concern regarding potential human health risks stemming from the ingestion of contaminated fish and seafood, while aquatic life exposures the danger at least as much as human life. Consequently, the fate and behavior of MPs in various environments, encompassing different aquatic ecosystems, are the recent subject of comprehensive research efforts [2]. MPs, serving as a novel marine and freshwater habitats for microbial populations, have the capacity to selectively create a niche for the colonization of aquatic microorganisms and the subsequent development of biofilms in a couple of ways. MPs can expedite the dispersion of microorganisms within the environment due to their substantial mobility, facilitating the drifting and prolonged persistence of surface-dwelling microorganisms in aquatic environments. These particles can transport microbial communities to novel habitats through ocean currents, potentially including coral pathogens found in various plastics and debris [21]. Furthermore, the current study indicates a notable abundance of pathogens on MPs, which can serve as vectors for the dissemination of specific bacterial communities. MPs may offer protection and shelter to colonizers, bolstering their resilience against environmental condition and given suitable conditions, a substantial influx of alien species, particularly toxic and pathogenic bacteria, may invade new

habitats and multiply fast over a short period. This can lead alterations and disruptions of the local community structure, resulting in biological invasions that have the potential to impact water quality safety and pose threats to both human health and ecosystems [37]. Additionally, MPs can facilitate gene exchange among microorganisms within biofilm communities and/or between these communities and the external environment. This increased genetic exchange is attributed to the substantial variability in bacterial genes and ARGs, which may often occur via HGT in the environment. Notably, pathogenic and antibiotic-resistant bacteria carry a wealth of pathogenic and ARGs, and these genes can be transferred through multiple pathways between biofilm communities. It is noteworthy that such microbial groups are surprisingly prevalent, a phenomenon that has also been observed in the present study [38].

4 | CONCLUDING REMARKS

The results indicate that MP-associated biofilms exhibit substantial genetic diversity in both marine and freshwater environments. MP samples showed dominance of the bacterial phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*. Significantly, *Arcobacter nitrofigilis* and *Arcobacter acticola* were found in substantial abundance and this was the first identification of their existence in plastisphere. Furthermore, *B. cereus* species, capable of MP degradation, and pathogenic *A. suis* and *F. tructae* were also determined in M_MP and F_MP samples, respectively. The low abundance of eukaryotic microorganisms may be attributed to the unsuitability of the extended 6-month period for their development. The predominant bacterial species observed in both M_MP and F_MP samples, especially *A. nitrofigilis*, *A. acticola*, *A. maotaiensis*, *T. alkalilacus*, and *P. lekithochrous* have not previously been associated with MPs, indicating their potential exploitation in future research aimed at MP biodegradation.

CONFLICT OF INTEREST STATEMENT
The authors have declared no conflicts of interest.

DATA AVAILABILITY STATEMENT
Data available on request from the authors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.