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Attached and planktonic bacterial communities on bio-based plastic granules and micro-debris in seawater and freshwater

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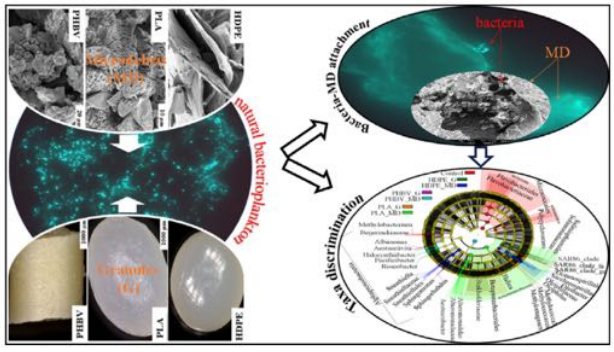
HIGHLIGHTS

- HDPE, PLA and PHBV bio-based plastics were exposed to freshwater and seawater bacterioplankton.
- Plastic granules and micro-debris hosted distinct bacterial communities.
- Higher biodiversity was found on HDPE and PLA granules.
- *Rhodobacteraceae* and *Comamonadaceae* were common in plastic biofilms.
- PET-degrading *Ideonella* (*Comamonadaceae*) was dominant in PHBV micro-debris.

GRAPHICAL ABSTRACT



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ABSTRACT

Bio-based plastics, produced from renewable biomass sources, may contribute to lowering greenhouse gases and the demand for fossil resources. However, their environmental fate is not well understood. Here, we compared the impacts of industrially produced granules (G) and micro-debris (MD) from three pristine bio-based plastics: high-density polyethylene (HDPE), polylactic acid (PLA) and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) on natural bacterial communities in seawater and freshwater using metagenomics. After one month, we found a dissimilarity between the microbial communities forming a biofilm on the plastics and planktonic bacteria. Further, different bacterial groups became dominant on different bio-based plastics, i.e. *Burkholderiaceae*, *Solimonadaceae*, *Oleiphilaceae*, and *Sneathiellaceae* on HDPE and *Alteromonadaceae* on PLA and *Rhodobacteraceae* on PHBV in seawater, and *Beijerinckiaceae* and *Chitinophagaceae* on HDPE, *Microtrichaceae* on PLA and *Caulobacteraceae* and *Sphingomonadaceae* on PHBV in freshwater. *Variovorax*, *Albimonas* and *Sphingomonas* genera were recorded on bio-based plastics in both seawater and freshwater. This study describes how different bio-based plastic materials and granule sizes influence the development of natural bacterial communities.

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1. Introduction

One of the biggest current world issues is plastic pollution. At the global level, it is estimated that approximately 80% of marine litter contains plastics that come from land-based sources through pathways such as run-off, sewage, wind flow, rivers and lakes (Li et al., 2016). Accumulation of plastics in both the ocean and freshwaters occurs due to their very slow degradation rate (Gewert et al., 2015; Lagarde et al., 2016). As they degrade, plastics break down into debris that can be divided into mesoplastics (~5–20 mm), large microplastics (~1–5 mm), small microplastics (~20–999 μm), and nanoplastics (<1 μm). This fragmentation process occurs due to multiple environmental factors, such as UV radiation, wind, waves, water chemistry, surface erosion, abrasion and microbial degradation (Barnes et al., 2009; Min et al., 2020). The hydrophobic surface of this plastic debris is almost immediately coated with inorganic and organic matter and biofilm, which profoundly influences its distribution, impact and fate.

Plastic debris presents a new ecological habitat for viruses, bacteria and other microorganisms in the aquatic environment (Zettler et al., 2013), known as the plastisphere. Freshwater and marine habitats share a number of features, but there are also differences between them, such as salinity, UV-light and pressure, which could affect the development of the plastisphere consortia (Harrison et al., 2018). The physical interactions of early microbial colonisation on plastic surfaces, and their reciprocal influences on weathering processes, sorption and the release of contaminants, are virtually unknown. The plastisphere may also affect interactions with other organisms, influencing fundamental ecological processes such as primary production (De Tender et al., 2015; Kominoski et al., 2009; Oberbeckmann et al., 2018). Microbial-plastic interactions could also give insights into the biodegradability of plastic litter and facilitate the development of new approaches to plastic disposal and/or recycling (Yoshida et al., 2016), e.g. through the isolation and examination of new polymer-degrading taxa in terms of their ability to biodegrade different plastic types (Yoshida et al., 2016). As such, detailed investigations on plastisphere communities in both marine and freshwater habitats, including their early-stage development, are of great importance (Harrison et al., 2018).

To date, most published studies have focused on the characterisation of microbial communities on plastic debris collected from different aquatic environments and their effect on invertebrate as well as vertebrate organisms (Amaral-Zettler et al., 2015; Al-Thawadi, 2020; Oberbeckmann and Labrenz, 2020; Oberbeckmann et al., 2014, 2015; Wu et al., 2019). While the composition of plastisphere assemblages has been long investigated, it is not known whether there are any general differences in the microbial colonisation of dominant types of plastic in marine, and especially freshwater, ecosystems. As such, it is important to study microbial interactions with pristine plastics before the final products or waste products end up in the environment. This is even more important for bio-based plastics if they are to become meaningful alternatives to conventional plastics.

In order to increase our knowledge of microbial interactions on bio-based plastics, this study aims to investigate the development and interaction of freshwater and seawater bacterial communities exposed to granules and micro-debris of three pristine bio-based plastics. To the best of our knowledge, this study is the first one on the interaction of freshwater and seawater bacterial communities on pristine bio-based plastics.

2. Materials and methods

2.1. Materials

Three different bioplastics were used in this study: high-density polyethylene (HDPE), representing the most common plastic waste, polylactic acid (PLA) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), both representing biodegradable plastics. All three plastics were tested in the form of both granules/pellets (G) and micro-debris (MD), the micro-size debris being produced by cryo-milling the granules using a 6775 Freezer/Mill® (SPEX SamplePrep, LLC, Germany).

For the HDPE component, we used sugarcane-based I'm Green™ SHA7260, purchased from Braskem S.A. (Brazil), which has a narrow molecular weight distribution, at least 94% bio-based content and a density of 0.96 g/cm^3. For the PLA, we used Ingeo 3001D, purchased from Nature Works (USA), a 100% bio-based and compostable resin (biodegraded through industrial composting conditions) derived by fermenting corn-based sugar. This grade has a weight average molecular weight (M_w) of 155,000 g/mol, a density of 1.24 g/cm^3 and a $_D$-lactide content of ~1.5%. For PHBV, we used ENMAT™ Y1000P, purchased from Tianan ENMAT (China), which has a density of 1.25 g/cm^3. This copolymer is reported as having a 3 HV (3-hydroxyvalerate) content of 3% and less than 0.5% additives based on boron nitride as a nucleating agent and Irganox 1010 as an antioxidant (Gontard et al., 2015), making the 3 HV content equivalent to 8 mol% (Corre et al., 2012).

2.2. Characterisation of plastics

Prior to testing, both the granules and micro-debris were inspected and visualised using a Leica DVM6 digital microscope (Leica, Germany) with a 16:1 zoom range and a 60° angle tilt function. The micro-debris was further characterised using a Zeiss Ultra Plus field-emission scanning electron microscope (SEM; Zeiss, Germany), the samples being fixed to aluminium stubs using double-sided carbon tape and cleaned with RF plasma (Evactron) for 10 min before SEM analysis. SEM images were acquired at an accelerating voltage of 5 kV at low probe current (about 15 pA) using an InLens secondary electron detector with SmartSEM software.

Roughness measurement, thermal analysis and non-isothermal crystallisation methods are described in the supplementary material.

2.3. Water collection

Freshwater for this study was collected from the Harcov reservoir near Liberec (50°46′12.8"N 15°04′32.1″E, Czech Republic) on 10 April 2019, at a point one metre from the reservoir bank and 10 cm below the surface. Seawater samples were obtained from the North Sea near the Norwegian Marine Institute Station at Tromsø (69°38′47.2"N 18°57′31.8″E, Norway) on 05 April 2019. All samples were passed through a stainless steel sieve (aperture size 1 mm) to remove large particles before storing in a cooled box for transport to the laboratory.

2.4. Experimental design

In the laboratory, all water samples (marine and freshwater) were passed through a 20 μm filter to remove algae, cyanobacteria and any other large microorganisms before adding the bio-based plastics. First, 250 mL of the filtered water was placed into a sterilised 500 mL Duran bottle, after which a sample of each plastic type (HDPE, PLA, PHBV as granule and micro-debris) was added to obtain a final concentration of 1 g/L. As the plastics floated and aggregated in the water, each sample was prepared in triplicate for each sampling point after 0, 7, 14 and 30 days. The experiment was performed under natural daylight at an average temperature of 25 °C. All bottles were manually shaken for 30 s and repositioned every day. At each sampling point, 250 mL samples were passed through a 20 μm filter to collect the plastic with its attached bacteria (AB), after which the water was passed through a 0.2 μm filter to collect any planktonic bacteria (PB). (For a summary of the experimental design, see Fig. 1.) The enrichment of bacterial communities on the plastics was then analysed by 16S rRNA sequencing and interactions between the plastics and associated bacteria observed under epifluorescence and SEM microscopy (see below).

At each sampling point, pH was measured using an XS instruments pH metre (Benchmetters, China) and turbidity using a TB 300 IR instrument

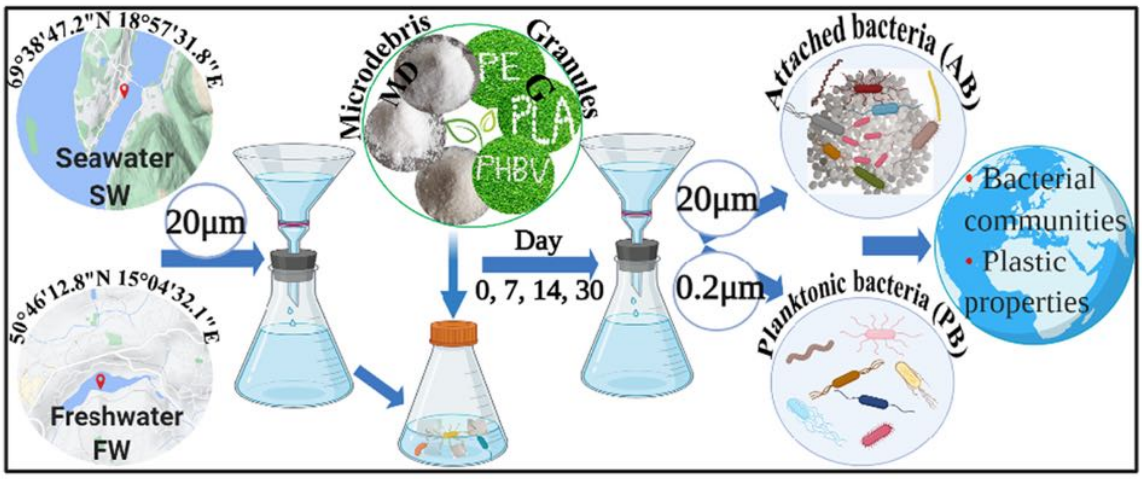

Fig. 1. Experimental design.

(Lovibond, Germany). Concentration of lactic acid released from PLA was detected by an AB Sciex 3200 QTRAP (Sciex, USA) connected to a Dionex Ultimate 3000 chromatograph (Thermo Scientific, USA).

2.5. Inspection of attached bacteria using epifluorescence and scanning electron microscopy

Microdebris bacteria were stained in the dark for 15 min using a Live/Dead BacLight kit (Life Technologies, USA) and then observed using an AxioImager epifluorescence microscope (Zeiss, Germany), with excitation set at 470 nm and emission at 490–700 nm. The bacteria were also observed under a Zeiss Ultra Plus field-emission SEM (Zeiss, Germany) (for settings, see 'characterisation of plastics' above).

2.6. DNA extraction and 16S rRNA sequencing

Samples from each sampling point (0, 7, 14 and 30 days) were immediately filtered through a 20 µm Whatman filter paper (Cytiva, UK), followed by a 0.22 µm Hydrophilic Durapore PVDF filter (Merck Millipore, Germany). The filters were then stored at −80 °C until DNA extraction. DNA was extracted from the filters using the FastDNA Spin Kit for Soil (MP Biomedicals, USA), according to the manufacturer's protocol, with the Bead Blaster 24 homogenisation unit (Benchmark Scientific, NJ, USA) employed for cell lysis. DNA concentration and purity were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and the *E*-Gel power-snap electrophoresis system (Invitrogen, USA).

All DNA concentrations were adjusted to obtain 1.66 ng/µL^{-1} of DNA. The targeted fragments amplified for sequencing were the V3-V4 region (466 bp) of the bacterial 16S rRNA gene, with the fragments barcoded using the modified primers B3Ft (TCG-TCG-GCA-GCG-TCA-GAT-GTG-TAT-AAG-AGA-CAG-NNN-NCC-TAC-GGG-AGG-CAG-CAG) and B3Rt (GTC-TCG-TGG-GCT-CGG-AGA-TGT-GTA-TAA-GAG-ACA-GGG-ACT-ACH-VGG-GTA-TCT-AAT) (Goux et al., 2015). The primers were modified by incorporating the Nextera XT® transposase sequence (Illumina Inc., USA) in the 5′ end of the forward and reverse primers and four additional random nucleotides in the forward primer to increase nucleotide diversity (Calusinska et al., 2019; Goux et al., 2015). Amplicons were generated using Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs Inc., USA), with the PCR reaction carried out according to the following thermal profile: 30 s at 98 °C, followed by 22 cycles of 5 s at 98 °C, 30 s at 50 °C, 30 s at 72 °C, and finally 2 min at 72 °C. The amplicons were purified with AMpure magnetic beads (Agencourt, Beckman Coulter Inc., USA) and then quantified and re-adjusted to 1.66 ng/µL^{-1} with the NanoDrop 1000 spectrophotometer, with 1.0 µL of each library then used as a template for a second PCR, where the Nextera XT® barcodes and the Illumina adapters necessary for hybridisation to the flow cell were added using the Nextera XT Index kit. The conditions for the second PCR were 30 s at 98 °C, followed by 8 cycles of 10 s at 98 °C, 30 s at 55 °C, 30 s at 72 °C, and final elongation at 72 °C for 2 min. The resulting amplicons were purified with AMpure magnetic beads and pooled in equimolar concentrations. The final concentration of the library pool was determined with the KAPA SYBR® FAST Universal qPCR Kit (Kapa Biosystems, USA). Libraries were mixed with Illumina-generated PhiX control libraries (5%) and sequenced with the MiSeq Reagent Kit V3–600 cycles (Illumina Inc., USA) (Calusinska et al., 2019).

2.7. Processing of sequencing data

Good quality sequences were obtained by de-multiplexing, trimming low quality sequences (limit = 0.03) and removing sequences with no ambiguous nucleotides or retaining less than 380 nucleotides using the Usearch pipeline (v7.0.1090_win64). Sequences with at least two reads were assigned to operational taxonomic units (OTUs) at 97% similarity (Calusinska et al., 2019). To obtain the taxonomy affiliation, the quality sequences were aligned with the SILVA database with a confidence threshold of 97% (Silva.nr_v123, https://www.arb-silva.de/). In order to compare the data from each sample, the high-throughput sequencing results were normalised to the sample with the lowest total count (20,000 reads) using Mothur software v.1.38.0 (Schloss et al., 2009). Statistical analysis was performed using Mothur (Team, 2008). Significant differences between different sample groups were set at a p-value of <0.05.

2.8. Bacterial community analysis

Bacterial diversity was performed using Chao, Shannon and Inverse Simpson alpha diversity and beta diversity (Bra-curtis) using the Jclass index and Bray-Curtis dissimilarity as distance measurements. Distance measurements were conducted using Non-Metric Multidimensional Scaling (NMDS) with Bray-Curtis Dissimilarity for a visual representation of bacterial community composition similarity between samples. To determine differences in the community composition of heterotrophic bacteria between treatments and plastic types, homogeneity of molecular variance (HOMOVA) and NMDS analyse was repeated after excluding *Cyanobacteria* using the Mothur pipeline.

The online Galaxy framework was used for discrimination of bacterial taxa on different plastics using all parameters for data formatting and linear discriminant analysis (LDA) effect size. Individual LDA size effects (LEfSe) were used to determine taxa associated with plastics after 30 days in each type of water. LEfSe analysis was also performed to identify bacterial genera discriminating for each of the plastics compared to a control without plastic.

The differences of bacterial community richness between controls and plastics were compared using ANOVA and Dunnett's test (GraphPad PRISM, USA).

3. Results

3.1. Plastic properties

Each bio-based plastic differed in outline area, surface area, shape, structure and roughness, as well as density and specific gravity (Fig. 2; for roughness measurements, thermal analysis and non-isothermal crystallisation, see supplementary Tables S1 & S2). The surface area of HDPE_MD and PHBV_MD was about 1.5 m^2/g, being around 10-times smaller compared with PLA_MD at 14.5 m^2/g. HDPE_MD (Fig. 1A & B) showed the largest outline area at 890 μm^2, while PLA_MD (Fig. 1D & E) and PHBV_MD (Fig. 1G & H) had a similar area at 15 and 20 μm^2, respectively (Table S1). PLA_G (Fig. 1D) had the largest outline area at 2030 μm^2, followed by PE_G (Fig. 1C) at 1700 μm^2 and PHBV_G (Fig. 1I) at 1300 μm^2. PE_G had a smoother surface than PLA_G and PHBV_G, which was in accordance with its higher maximal height (Fig. S1, Table S1). At a daily temperature ranging from 20 to 32 °C, both HDPE and PHBV were above their glass transition state (T_g) and had a viscoelastic character, the material having both viscous (liquid-like) and elastic (spring-like) properties (Figs. S2 & S3), while PLA was below its T_g with a glassy character (Fig. S4).

3.2. Interaction of bacteria and plastic micro-debris

Bacterial interaction with plastic MD was first detectable under epifluorescence after 14 days in both freshwater and seawater (Fig. S5), with many more AB cells visible after 30 days (Fig. 3). Likewise, bacteria were also observed for the first time after 14 days under SEM, with numbers increasing after 30 days (Fig. S6).

3.3. Bacterial community diversity and richness

At the beginning of the experiment, there were around 250 OTUs in seawater and 200 in freshwater. By day 30, OTUs had increased 1.5 times in seawater (350) and three-times in freshwater (600) (Fig. 4). In comparison with controls without plastic, all plastics in both seawater and freshwater showed slight differences in both AB and PB OTUs, with AB OTUs increasing in PE_G and PLA_G, but decreasing in PHBV_G and all MD samples after 30 days. PB OTUs showed only negligible changes, except for PE_G in seawater, and PLA_G, PHBV_G and PE_MD in freshwater (Fig. 4). While the Chao estimator displayed a similar trend as OTU number, the Inverse Simpson index showed diversity as increasing over time (Fig. 4).

A comparison of bacterial communities on different plastics based on NMDS analysis of the sequencing data obtained from days 0, 14 and 30 (samples from day 7 not sequenced as DNA yield was too low and no interactions were observed under the microscope) indicated that bacterial communities developed differently on different types and sizes of plastic (Fig. 5), with dissimilarity between AB and PB communities being significant (both $p < 0.001$) in both seawater (Fig. 5A) and freshwater (Fig. 5B). For MD samples, while there was no significant difference in PB composition for all plastics in seawater, AB composition was significantly different from the control for PLA_MD ($p < 0.05$) and PHBV_MD ($p < 0.05$). There was no significant difference in AB composition between PLA_G, PHBV_G and PE_G in seawater (Fig. 5A).

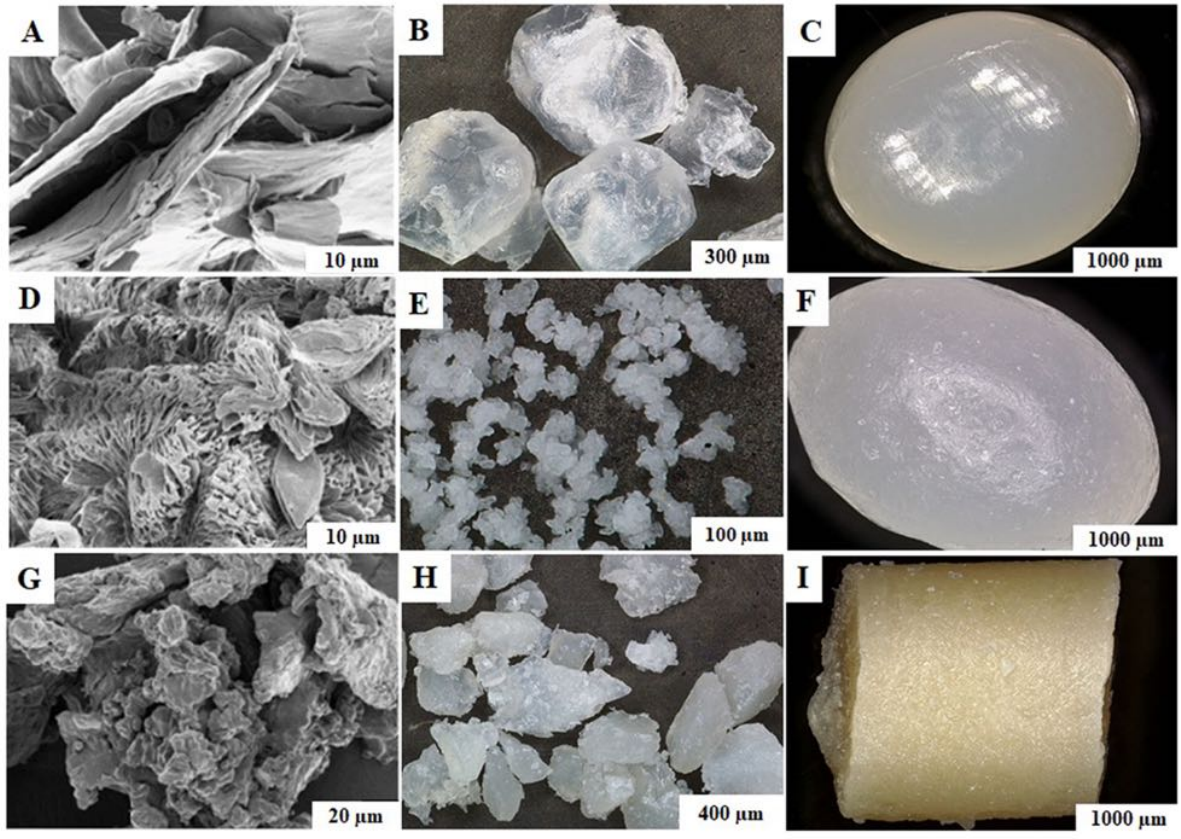

Fig. 2. HDPE, PLA and PHBV micro-debris (MD) under a scanning electron microscope and HDPE, PLA and PHBV granules (G) under a digital microscope: HDPE_MD (A, B), HDPE_G (C); PLA_MD (D, E), PLA_G (F); and PHBV_MD (G, H), PHBV_G (I).

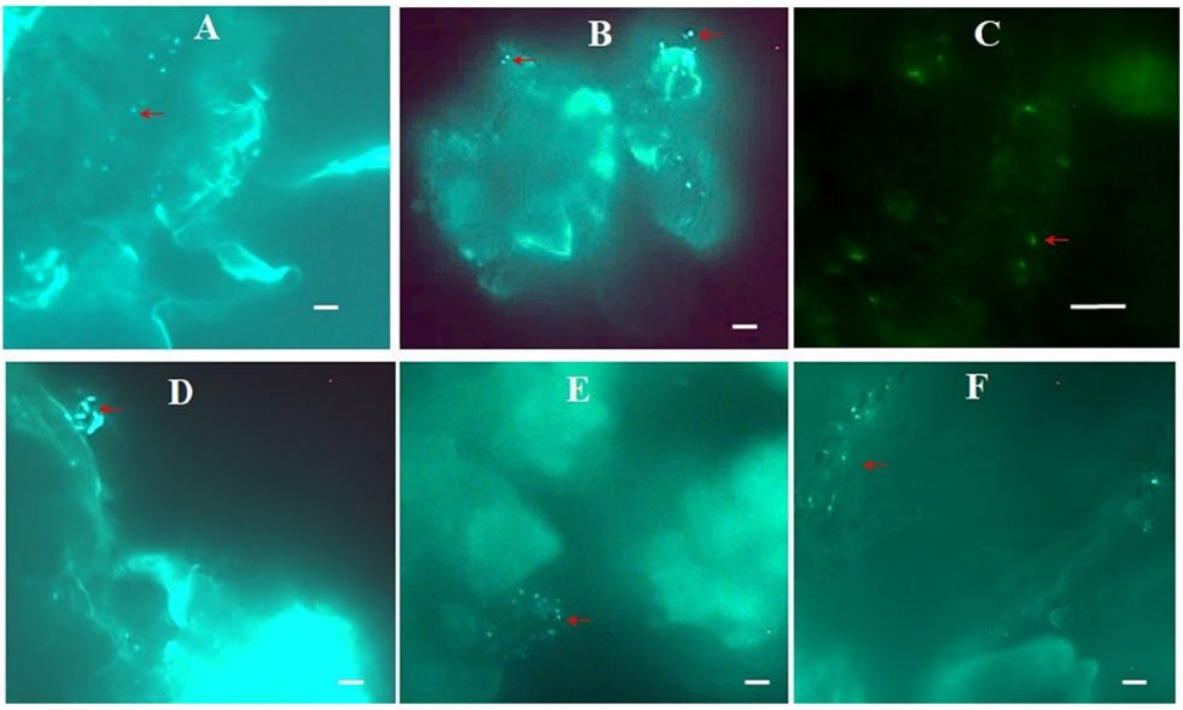

Fig. 3. Attached bacteria on micro-debris observed under epifluorescence microscopy after 30 days in seawater (upper images) and freshwater (bottom images). A, D = HDPE; B, E = PLA and C, F = PHBV. Red arrows indicate bacterial cells. Scale bar represents 5 μm.

In freshwater, there was a significant difference ($p < 0.05$) in AB composition between PLA_G and PHBV_MD, and a significant difference in PB composition between different sizes of PLA ($p < 0.005$), and between PHBV_G and PLA_G ($p < 0.05$) and PHBV_G and PLA_MD ($p < 0.02$) (Fig. 5B).

3.4. Effect of plastics on bacterial community composition

We identified bacteria belonging to three phyla in seawater and four phyla in freshwater (Fig. S7), with a higher number of phyla found on G than on MD and the number of phyla doubled and tripled over 30 days

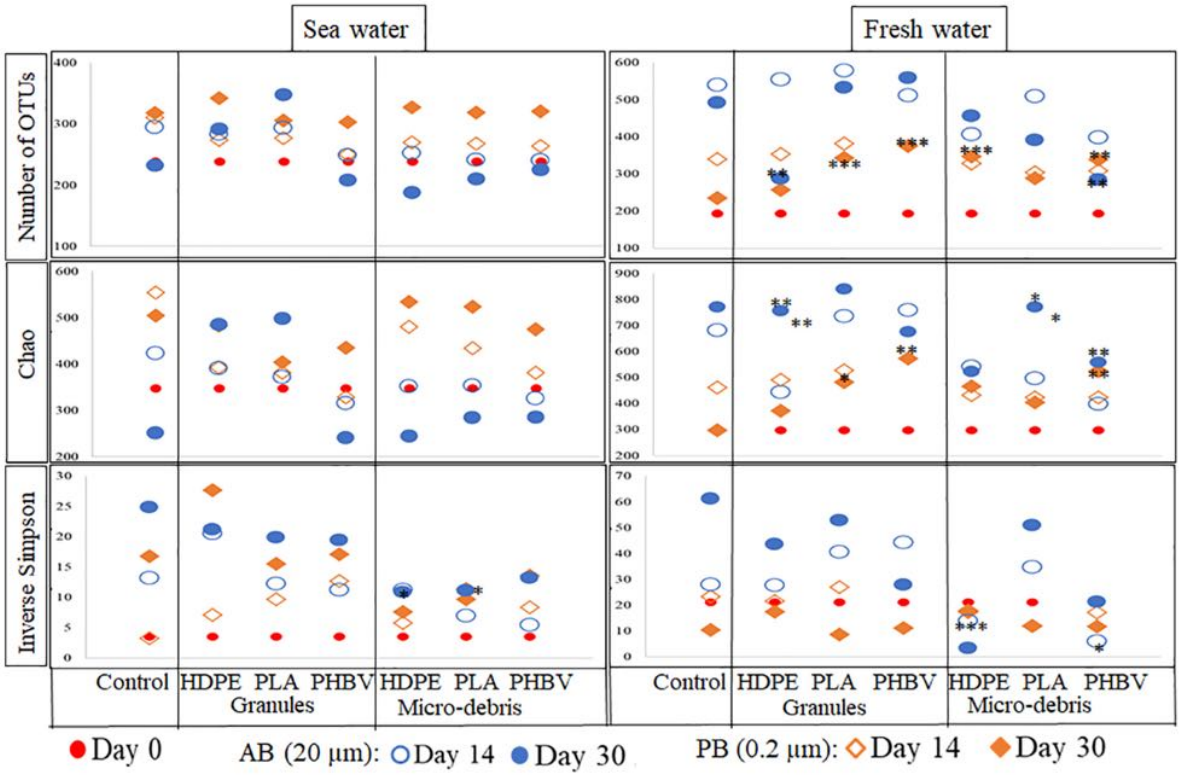

Fig. 4. Bacterial community richness (OTU) and diversity (Chao and Inverse Simpson indices) for plastic granules and micro-debris exposed to seawater and freshwater. Each symbol represents one sample. Duplicate samples are from day 0 and day 30. Asterisks show significant difference compared to controls without plastics $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

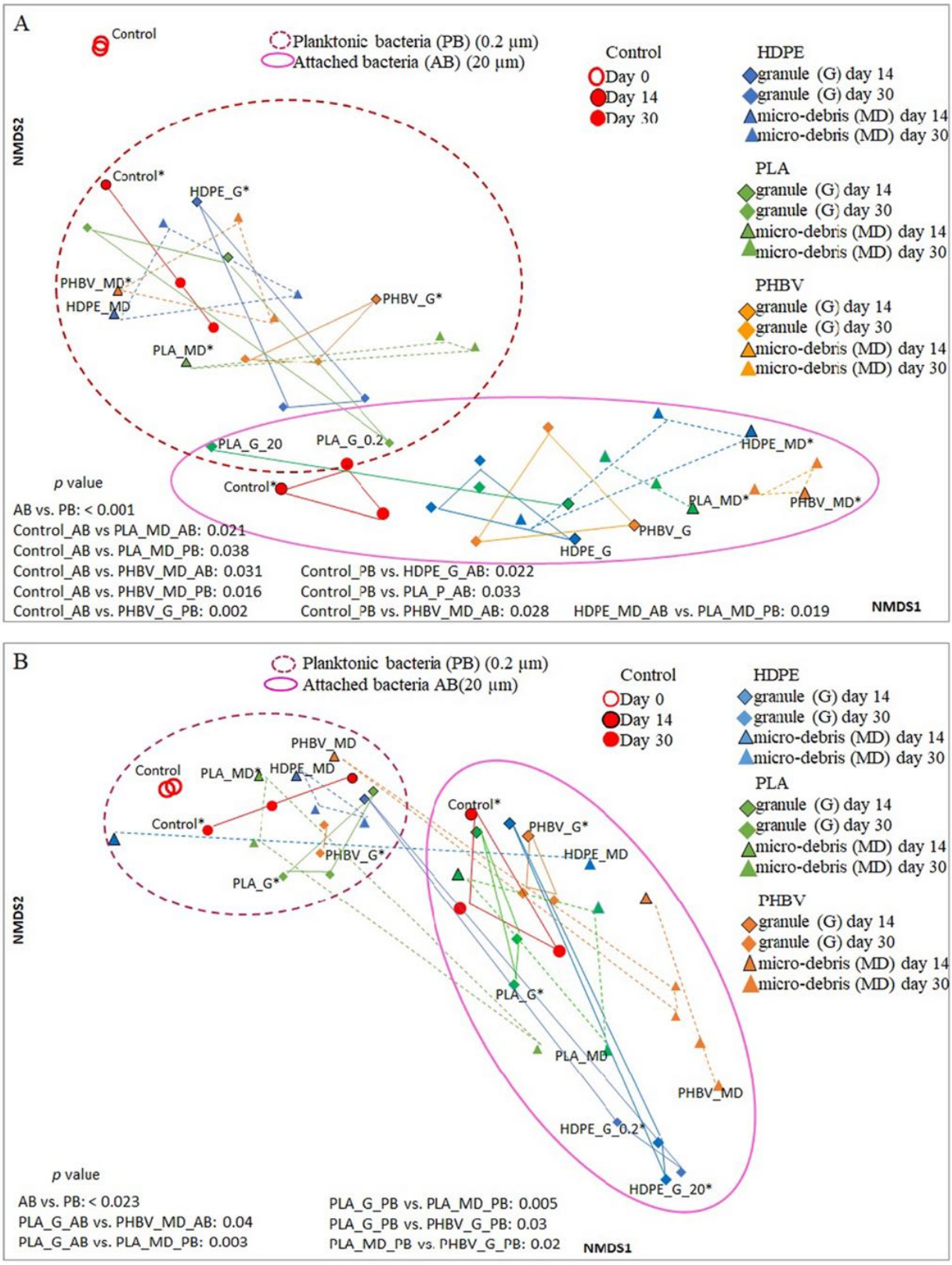

Fig. 5. Differences in bacterial community composition for bio-plastics exposed in seawater (A) and freshwater (B), using Bray-Curtis Dissimilarity as the NMDS distance measurement. Significant differences between bacterial communities of different plastics and the control without plastic (performed by HOMOVA) are marked by an asterisk *, with actual *p* values presented in the lower left corner. Each point represents one sample.

in seawater and freshwater, respectively. In both seawater and freshwater, PHBV_MD attracted the lowest number of bacterial phyla (Fig. S7).

In seawater, the two dominant phyla, *Proteobacteria* and *Bacteroidetes*, were present from the start of the experiment, with *Planctomycetes* only appearing after 30 days. *Proteobacteria* were dominant on both G and MD, while *Bacteroidetes* were more successful on G than on MD. *Actinobacteria* were only found on HDPE_G after 30 days in seawater (Fig. S7, seawater).

In freshwater, the control contained four phyla, with *Actinobacteria* and *Verrucomicrobia* dominant. *Proteobacteria* remained in similar abundance in the control and all plastic samples until the end of the experiment, while the abundance of *Bacteroidetes* decreased somewhat, which enhanced opportunists such as *Acidobacteria*, *Cyanobacteria*, *Verrucomicrobia* and *Planctomycetes* in the case of AB. In contrast, the abundance of *Acidobacteria* increased slightly in all samples except PLA_MD and PHBV_MD in the case of PB (Fig. S7, freshwater).

Taxa initially forming dominant populations on bio-based plastics were typically outcompeted by the end of the study (Table 1, Fig. S8). There was no clear pattern in species competition when inspecting the same material of different sizes in marine and freshwater environments. The only genus that was dominant on two different plastics (HDPE_G in seawater and PHBV_G in freshwater) was *Azospirillum* after 14 days (Table 1, Fig. S8).

Alpha- and *Gammaproteobacteria* were common on all plastic types and sizes in both marine and freshwater environments (Fig. 6A & B), while *Bacteroidia* was enriched in the control waters without plastics. The following bacteria were enriched on HDPE_G: *Polycyclovorans*, *Solimonadaceae*, *Salinisphaerales*, *Burkholderiaceae* and *Roseobacter* in seawater, and only *Rhodobacter* in freshwater. HDPE_MD enriched *Sneathiellaceae* and *Saccharospirillaceae* in seawater, and only *Caldimonas* (*Comamonadaceae*) in freshwater. PLA_G enriched *Methylococcaceae*, *Haliea* in seawater and *Microtrichaceae* and *Segetibacter* in freshwater. PLA_MD enriched *Alteromonadaceae*, *Haliela* and *Halocynthiibacter* in seawater and *Sphigobacteriales* and *Rhizobiales* in freshwater. *Oleiphilaceae* (*Oleiphilus*), *Sphingomonas* and *Pacificibacter* increased on PHBV_G and PHBV_MD in seawater, while *Comamonadaceae* (*Variovorax*, *Tibeticola*) on PHBV_G and *Ideonella* and *Caulobacter* were enriched on PHBV_MD in freshwater.

4. Discussion

We investigated the evolution of natural bacterial communities interacting with three different pristine bio-based plastics (HDPE, PLA, PHBV) in seawater and freshwater. In our study, the first AB were observed after 14 days for all three plastics in both types of water (Fig. 3). This is a similar time to that reported for biofilm formation on the surface of PE and a starch-based PE terephthalate blend in seawater (Eich et al., 2015) and for polyhydroxyalkanoate (PHA) and PLA in freshwater (Morohoshi et al., 2018). Interspecies interactions led to dynamic changes in biofilm structure, with early plastic colonisers always suppressed or outcompeted by other genera by the end of the study. Biofilm structure can influence the fate of microplastics in the water column and sediment, including potential biodegradation (Rogers et al., 2020); however, Oberbeckmann and Labrenz (2020) have expressed scepticism about microbial capability to metabolise plastics over a reasonable time frame.

Distinct bacterial communities were observed on G and MD, each differing from those in the original source seawater or freshwater and with specific AB communities depending on plastic polymer type (Fig. 5, Table 1). In general, we found a higher AB taxonomic diversity on G than MD, probably due to the larger total surface area of granules (Table S1), which could promote the adhesion of higher numbers of different taxa. Further, G hosted more AB populations in freshwater than seawater after 30-days exposure, reflecting higher PB diversity in freshwater than in seawater (Figs. 5 & S7). Moreover, bacterial taxa appeared to prefer HDPE_G and PLA_G to PHBV_G, which could be explained by distinct surface properties such as roughness (lowest in HDPE and highest in PHBV), hydrophobicity (highest in HDPE), topography, surface energy, charge and electrostatic interactions, which are known to influence the attachment of initial colonisers and biofilm development (Donlan, 2002; Rummel et al., 2017). In agreement with the results for our own PE sample, Das (2014) observed that a biofilm on the surface of hydrophobic PE comprised a high number of bacterial taxa, while very few were attached to hydrophilic materials such as PHBV and PLA. On the other hand, the hydrophilic PLA_G in our study hosted the highest number of bacterial taxa (Fig. 4). Alkaline environments (freshwater pH = 7.8, seawater = 8.3; Fig. S11) are known to promote abiotic degradation of PLA, with Xu et al. (2011) reporting more rapid

Table 1
Dominant genera (level 4 from the heatmap in Fig. S8) for attached bacteria in seawater and freshwater. Numbers in brackets represent the abundance level if the genus was not found in level 4. Note: planktonic bacteria (PB) are described in Table S3.

	Day	Seawater		Freshwater	
		G	MD	G	MD
HDPE	14	*Azospirillum*	*Saccharospirillum* *Cycloclasticus* *Cavicella* *Oleibacter*	*Pseudomonas*	*Aquabacterium* (3)
	30	*Salinirepens* *Haliea* *Sediminicola*	*Marinobacter*	*Candidatus_Megaira* *Lysobacter* *Curvibacter* *Gemmobacter* *Mycetocola*	*Obscuribacterales_ge* (3)
PLA	14	*Croceibacter* *Celeribacter*	*Halocynthiibacter* *Amphritea*	*Brevifollis*	*Bacteroidetes* *_VC2.1_Bac22_ge*
	30	*Thermorudis* *Parvularcula* *Limnobacter* *Roseobacter_clade* *Aureispira* *Marivita* *Albirhdobacter* *IS-44*	*Ahrensia (3)*	*Paraherbaspirillum* *SWB02* *OM190_ge*	*Neochlamydia* *oc32* *SD04E11* *37-13_ge*
PHBV	14	*Thalassotalea* *Fluvlicola* *Pseudopelagicola*	*Pseudoalteromonas*	*Azospirillum*	*Bldfi19_ge* *Paucibacter* *Ideonella* *OM27_clade*
	30	*Alliglaciecola* *Candidatus* *_Endobugula* *Aquibacter* *Bernardetla* *Lacinutrix*	*Roseovarius* *Hirschia* *Labrenzia* *Hyphomonas* *Spongiilbacter* *Congregibacter*	*Methyloversatilis* *Variovorax*	*Undibacterium* *Haliscomenobacter*

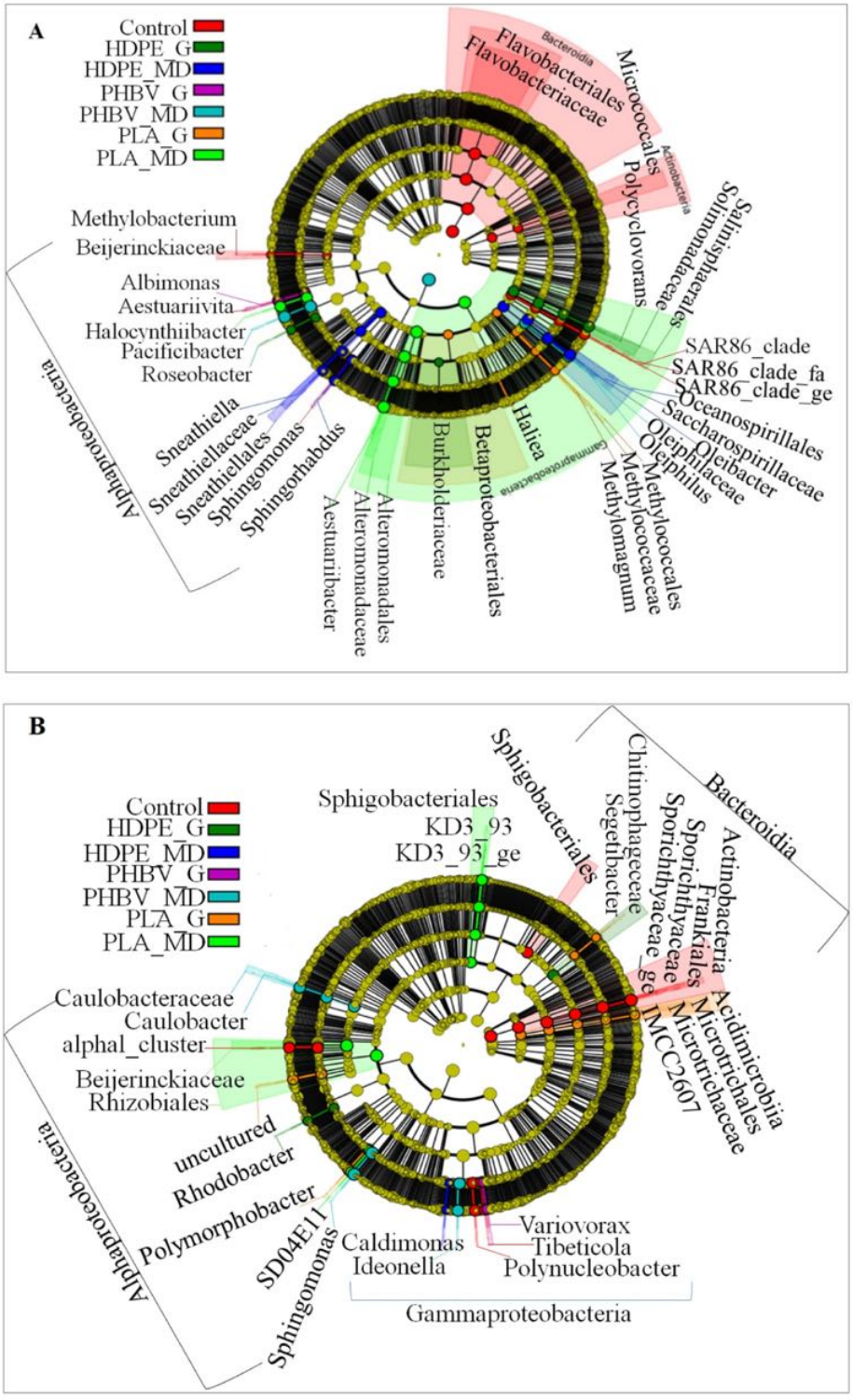

Fig. 6. Taxa discrimination summary for (A) attached bacteria in seawater and (B) attached bacteria in freshwater exposed to different plastics for 30 days (controls = SW or FW with no plastic). The microbial taxa cladogram differentiates significantly between materials, with the central point representing the root of the tree (bacteria) and each ring representing the next lowest taxonomic level (phylum to genus). Phylum and class are indicated by name on the cladogram, while the order, family or genera is provided in the key. Coloured circles and shading indicate the microbial lineage that was enriched in the corresponding plastic sample (see key in upper left), while yellow circles indicate non-significance. The diameter of each circle is proportional to taxon abundance. Note: taxa discrimination for planktonic bacteria (PB) are described in Fig. S9.

degradation after 16 days in water of pH 8. Correspondingly, increased turbidity in PLA_MD samples after 14 days (Fig. S10) could be due to lactic acid; however, we cannot prove that, because lactic acid concentrations in all our samples were below the detection limit (1 mg/L).

When looking at genera composition, early biofilms showed little or no clear pattern of development on the different plastics, the only exception being *Azospirillum*, which was dominant on HDPE_G in seawater and PHBV_G in freshwater after 14 days, but then decreased in

number by the end of the study (Table 1, Fig. S8). Though *Azospirillum* has been significantly associated with PET in marine debris (Debroas et al., 2017) and was found on degraded poly(3-hydroxybutyrate) in soil (Artsis et al., 2012), there is no direct evidence for its capability to biodegrade plastics. Opportunists from several bacterial families were found on the different plastic biofilms, such as *Rhodobacteraceae* on HDPE_G, PLA_G and PLA_MD, as well as both sizes of PHBV in seawater. *Rhodobacteraceae* are known to be early and abundant colonisers of different plastics, including PE (Oberbeckmann and Labrenz, 2020), and are capable of responding quickly to different carbon resources. We also found *Rhodobacter* attached to HDPE_G in freshwater (Fig. 6). This genus has also been reported in seawater microplastics (Pinto et al., 2019), suggesting that some genera are able to travel on plastic litter from lakes and rivers into marine environments. Similarly, *Sphingomonas* has been found on PHBV_MD in freshwater and PLA_G in seawater. *Members of the Sphingomonadaceae* attached to HDPE_MD in seawater have also been shown to form typical PE-associated communities in marine macro and microplastics (Debroas et al., 2017; Oberbeckmann and Labrenz, 2020), with the presence of *Sphingomonas* indicating degradation of PE plastics (Padmanabhan et al., 2019). Correspondingly, *Rhodobacterales*, *Sphingomonadales* and *Rhizobiales* represent important microbial associations within the riverine plastisphere community (Jiang et al., 2018). *Comamonadaceae*, commonly detected on marine and freshwater microplastics (Debroas et al., 2017; Goldstein et al., 2014; Jiang et al., 2018), was found in our study on HDPE_G (*Curvibacter*) and both PHBV_G (*Variovorax*) and PHBV_MD (*Ideonella*) in freshwater. Notably, freshwater *Ideonella* from the *Comamonadaceae* family was dominant on PHBV_MD in freshwater after 14 days (Table 1) and remained detectable after 30 days, despite their numbers decreasing (Fig. 6, Fig. S8). This genus recently gained attention for its ability to degrade PET (Yoshida et al., 2016). *Polycyclovorans was abundant on HDPE_G in our study, and was recorded in mature HDPE biofilm by* Kirstein et al. (2019). *Further*, *Rhizobiales* (freshwater) and *Alteromonadales* (seawater) were enriched in PLA_MD (Fig. 6A & B). *Rhizobiales* may act as key PE and PET plastic degraders (Debroas et al., 2017) and *Alteromonadales* also appears to interact with PET and PE marine microplastics (Rogers et al., 2020). In our study, *Variovorax* and *Albimonas* were both found on PHBV_G in freshwater and seawater (Fig. 6, Fig. S8). PHBV are known to be degraded by bacteria that excrete extracellular PHB depolymerases, such as members of the *Comamonadaceae* family, including *Comamonas testosterone* in seawater, *Acidovorax delafieldii* and *Variovorax paradoxus* in soil (Mergaert et al., 1993), and *Alcaligenes faecalis* and *Pseudomonas fluorescens* in activated sludge (Schirmer et al., 1993; Shirakura et al., 1986).

5. Conclusion

Our study highlighted the differential development of biofilms on three different pristine bio-based plastics (HDPE, PLA, PHBV) in the form of G and MD in both natural seawater and freshwater. In general, the early-stage biofilms hosted completely different dominant bacterial taxa than 30-day biofilms. Both G and MD hosted distinct bacterial communities compared with the source seawater and freshwater communities. Moreover, we observed a higher biodiversity on G compared to MD, most probably due to a larger surface area hosting more bacteria. The type of bio-plastic material undoubtedly shaped the microbial communities in biofilm, with biodiversity increasing on smoother surfaces and harder bio-plastics in freshwater, particularly as regards HDPE and PLA. While we observed no signs of biodegradation, we detected increased turbidity in PLA samples after 14-days, presumably associated with lactic acid that might be quickly utilised in bacterial metabolism. Opportunists belonging to *Rhodobacteraceae* and *Comamonadaceae* families were a common component of the plastic biofilms. Some genera appeared to be versatile, being recorded on bio-based plastics in both seawater and freshwater. These include *Variovorax* and *Albimonas* on PHBV_G and *Sphingomonas* on PHBV_MD in freshwater and PLA_G in seawater. Notably, the PET-degrading candidate *Ideonella* (*Comamonadaceae)* was dominant on PHBV_MD in freshwater.

Our study provides new information on the diverse formation of early-stage biofilms on pristine bio-based plastics, which could lead to changes in their mobility, persistency, or degradability in the freshwater and marine environment.

CRediT authorship contribution statement

NHAN, YEST and AŠ designed the original experiment. NHAN performed the experiment, analysed the results and wrote most of the manuscript. YSET, SC and PH helped perform the experiment. YSET and MP milled granules to obtain micro-debris, wrote part of the manuscript and commented on the final version. MB, PK and TB characterised the plastics. MC performed the bioinformatic analysis of the 16S rRNA sequences. AG advised on writing the text. AŠ advised on and corrected the text.

Declaration of competing interest

The authors declare no financial competition or interests in relation to the work described.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.147413.

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