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Biodegradation of microplastic in freshwaters: A long-lasting process affected by the lake microbiome

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Abstract

Plastics have been produced for over a century, but definitive evidence of complete plastic biodegradation in different habitats, particularly freshwater ecosystems, is still missing. Using ^{13}C-labelled polyethylene microplastics (PE-MP) and stable isotope analysis of produced gas and microbial membrane lipids, we determined the biodegradation rate and fate of carbon in PE-MP in different freshwater types. The biodegradation rate in the humic-lake waters was much higher (0.45% ± 0.21% per year) than in the clear-lake waters (0.07% ± 0.06% per year) or the artificial freshwater medium (0.02% ± 0.02% per year). Complete biodegradation of PE-MP was calculated to last 100–200 years in humic-lake waters, 300–4000 years in clear-lake waters, and 2000–20,000 years in the artificial freshwater medium. The concentration of 18:1ω7, characteristic phospholipid fatty acid in Alpha- and Gammaproteobacteria, was a predictor of faster biodegradation of PE. Uncultured Acetobacteraceae and Comamonadaceae among Alpha- and Gammaproteobacteria, respectively, were major bacteria related to the biodegradation of PE-MP. Overall, it appears that microorganisms in humic lakes with naturally occurring refractory polymers are more adept at decomposing PE than those in other waters.

INTRODUCTION

Plastic particles are found globally in oceans, lakes, and sediments (Cózar et al., 2014; Driedger et al., 2015; Tanentzap et al., 2021). As a result of high-molecular weight, hydrophobicity, and the absence of functional groups, microbial degradation of the plastic backbone is considered a slow process, so slow that it is difficult to measure using conventional methods like the evolution of CO_2 or weight loss. Physical degradation, chemical degradation, and photodegradation can speed up microbial degradation (Chamas et al., 2020). Unlike in terrestrial environments, the thermal degradation of plastics is not crucial in marine or freshwater ecosystems. However, the degradation process is usually initiated by photooxidation (UV radiation) or hydrolysis, which is eventually followed by chemical oxidation (Andrady, 2011; Wagner & Lambert, 2018). Degradation ultimately ends with microbial carbon uptaken into biomass and/or carbon dioxide (CO_2) respiration. Our previous study showed biodegradation and utilization of polyethylene carbon chains for building microbial fatty acids and further upgrading these products into essential ω-3 and ω-6 polyunsaturated fatty acids (Taipale et al., 2019). Thus, some simple and monotonous plastic polymer parts can be biotransformed to nutritionally most valuable biomolecules. Moreover, a recent study by Sheridan et al. (2022) showed that dissolved organic carbon (DOC) leachate from plastic could support higher microbial biomass than natural DOC. Therefore, it seems microplastic surfaces and extracts can support different microbiomes;

meanwhile, biodegradation speed of plastic backbone and direct utilizers of plastic are still unclear.

Biodegradation, depolymerization, and mineralization of microplastic compost, soil, and sediments are estimated to vary from hundreds of years to thousands of years (Chamas et al., 2020). However, biodegradation speed in freshwaters and marine waters is still lacking, even though the problem is biggest in aquatic environments. Some plastic degrading bacterial pure cultures (Auta et al., 2018; Yoshida et al., 2016), and even one marine fungus species (Paço et al., 2017), have been isolated, but most studies on microbial microplastic communities from freshwater and marine environments have focused on microbes on the surface of the microplastics, often called plastisphere (Debroas et al., 2017; Miao et al., 2020). Surface-adhering microbes are, however, not necessarily the key members of the biodegradation processes of microplastic in nature.

Tools for studying the slow degradation rate of recalcitrant materials such as microplastic are limited (Chamas et al., 2020). Traditionally plastic decomposing has been studied by visual observation of degraded film and change in the mass (Andrady, 2011; Kaplan et al., 1994). Yoshida et al. (2016) detected polyethylene terephthalate (PET) degradation under microscopy and analysed the activity of different genes related to the degradation process. Finally, the authors isolated the PET-degrading strain *Ideonella sakaiensis*, which belongs to the Comamonadaceae family of the Betaproteobacteria. However, the used methods need long incubation times and do not enable studying MP biodegradation by microbial communities in different environments. Recently, the ^{13}C-isotope-labelling experiments have been used for studying the decomposition of polystyrene by plastic-eating mealworms (Yang et al., 2015) and the fate of the MP carbon in the freshwater food web (Taipale et al., 2019). The ^{13}C-isotope-labelling and the carbon isotope measurements of CO_2 and biomass (e.g. phospholipid fatty acids [PLFAs]) enable the calculation of the exact degradation rate for recalcitrant materials and allow to determine of which part of the MP carbon is respired and which part is used for building new biomass. For example, Yang et al. (2015) noted that mealworms excreted most carbon from polystyrene and used only a small proportion to build new biomass. Furthermore, using ^{13}C-PLFA techniques, characteristics of MP utilizing microbial groups can be identified, which can, in the best cases, give information about the key microbial groups of the process (Taipale et al., 2019).

In this study, we aimed to determine the fate of MP polyethylene (PE-MP) in freshwaters by calculating the mass balance of respired and assimilated carbon. Since microbial communities can vary by the availability of natural organic matter and lake physio-chemical parameters (Sheridan et al., 2022), we exploited the ^{13}C-isotope-labelling technique and studied lakes of two different humic levels to cover the variation in the boreal lakes. Furthermore, to understand the role of microbes in the mineralization and assimilation of PE-MP, we determined the MP biodegradation rate (mineralization + assimilation) not only for humic-lake water and clear-lake water but also using an artificial freshwater medium. We used ^{13}C-labelled PE-MPs and measured carbon isotopes from gas and phospholipid fatty acids to calculate mass balance. Additionally, we determine potential PE-MP decomposers by combining the fatty acid results and the 16S rRNA gene sequencing.

EXPERIMENTAL PROCEDURES

Microplastic incubations in natural waters and artificial medium

We studied complete biodegradation of polyethylene (99 atom % ^{13}C, Sigma-Aldrich, product number 493279; size range 1.2–40 μm; Figure 1A) in humic-lake water (Lake Haukijärvi, 61.13°N and 25.80°E, surface area = 0.022 km^2, max depth = 5 m, DOC = 16 ± 0.1 mg C L^{-1}, pH = 6.4 ± 0.2), clear-lake water (Lake Vesijärvi, 61.04°N and 25.32°E, surface area = 107.57 km^2, max depth = 42 m, DOC = 6 ± 0.1 mg C L^{-1}, pH = 7.8 ± 0.2), and artificial MWC medium (pH = 7.4 ± 0.1) (Guillard & Lorenzen, 1972). Water from Lake Haukijärvi was taken from the surface (0–0.5 m) close to the littoral zone (2–3 m from the shoreline), and water from Lake Vesijärvi was taken from surface water (0–5 m) Enonselkä Basin (Lankiluoto). Prior experiment, waters were incubated at room temperature for 4 weeks to stabilize microbial communities and impoverish organic carbon sources in the bottles. We weighed 2.0 mg of polyethylene into 250 ml glass serum bottles with a gas-tight, which after 150 ml of lake water or artificial medium was added to serum bottles (100 ml headspace). Each treatment contained three replicates with ^{13}C-labelled polyethylene and two control bottles without polyethylene to measure the δ^{13}C background of waters during the incubation in the dark. Serum bottles were incubated for 8 weeks at constantly shaking (110 rpm) in an orbital shaker (Heidolp Unimax 2010) at room temperature (21 ± 1°C). Microbial biomass and size of particles were measured at the end of the experiment using the CASY cell counter (Omni Life Science) and 60 μm capillary (measurement range 1.2–40 μm).

Analysis of the concentration and δ^{13}C of dissolved inorganic carbon

DIC concentration and carbon isotope measurements were taken from the headspace using sterile plastic

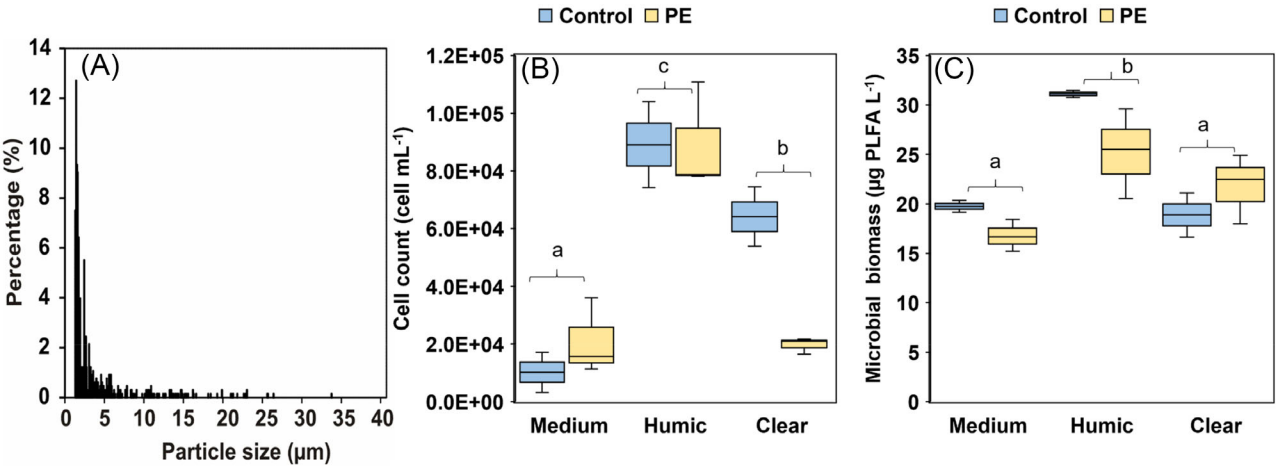

FIGURE 1 The size-frequency distribution of MP-PE and microbial biomass. (A) The size distribution of used PE-MP in the experiments. (B) The number of cells/particles and (C) and microbial biomass based on phospholipid fatty acids (PLFA) after the 8-week incubation of polyethylene (PE, $n = 3$) and controls (no-PE, $n = 2$) in the artificial freshwater medium (medium), humic-lake water (humic), and clear-lake water eutrophic (clear). Letters cite the difference between waters (PE + control), which explained most of the variation among treatments.

syringes and 25-gauge metal needle weekly and from the water after incubation. Agilent 7890B GC (Agilent Technologies, Palo Alto, CA, USA) was used to analyse CO_2/DIC. The equipment configuration included two separate channels with stainless steel packed columns (HayeSep Q 80/100, length 8 feet x inner diameter 1/8″) using three detectors (flame-ionization [FID], thermal conductivity [TCD], and micro electron catching [µ-ECD]). The TCD and methanizer-FID were connected in series to measure CH_4 and CO_2. Low CO_2 level samples were converted to CH_4 through the methanizer and measured by FID. The TCD was solely used for the measurements of high CO_2 concentrations. Column and valve box temperatures in isothermal runs were 60°C.

A 5 ml of gas or water was taken from each treatment and injected into helium-washed 12 ml Exetainers with 0.5 ml of H_3PO_4 (ortho-H_3PO_4, 85%, Merck, Darmstadt, Germany) (Taipale & Sonninen, 2009). The $\delta^{13}C$ of DIC was measured with Isoprime100 IRMS (Elementar UK Ltd., Cheadle, UK) coupled to an Isoprime TraceGas pre-concentration unit. Isotopic enrichment (Δ) of $\delta^{13}C$ was calculated between microplastic treatments and control, and thus positive values (‰) show mineralization of ^{13}C-polyethylene into carbon dioxide.

For the mineralization calculations, the $\delta^{13}C$ value of CO_2 was first converted to the atom% by Equation (1) and then average of controls was deducted from the treatment (increase of ^{13}C). The $\delta^{13}C$ value of CO_2 was turned to atom% (AP) by the following equation:

$$AP = \frac{\delta^{13}C + 1000}{\delta^{13}C + 1000 + \frac{1000}{R_{standard}}} \times 100 \quad (1)$$

where $R_{standard}$ value is 0.01118 (VPDB) (Fry, 2006).

We then calculated the mass% (MP) of polyethylene-C mineralized to CO_2 by the following equation:

$$MP = \frac{\Delta AP \times m_{CO_2}}{m_{PE}} \quad (2)$$

where ΔAP is the difference between treatment and control as atom% (average of two replicates), m_{CO_2} is the mass of CO_2-C (mg) in the serum bottle at the end of the incubation and m_{PE} is the mass of fully labelled polyethylene-C (mg) added into the bottle in the beginning of the incubation.

The total mineralized carbon was calculated by combining the $^{13}CO_2$ in the headspace and water.

The mineralization rate per day based on the respiration of CO_2 was calculated using the following equation:

$$\text{Mineralization rate}(\%\text{per day}) = \left(\frac{\text{Equation 2 } (\textit{headspace} + \textit{water})}{\text{duration of the experiment in days}}\right) \quad (3)$$

where the experiment duration was 56 days. Due to the slow mineralization rate, final results are presented for the year (Equation 3× 365.25 days).

Lipid extraction, fractionation, and transesterification

After 8 weeks of incubation, 60 ml of lake water and artificial medium was filtered through cellulose nitrate membrane filters (Whatman, 0.2 µm pore size). Lipids were extracted from the filters using the Folch protocol (Folch et al., 1957) and 1,2-dinonadecanoyl-sn-glycero-

3-phosphatidylcholine (Larodan) as the internal standard. Briefly, lipids were extracted with 3 ml of chloroform: methanol 2:1 mixture and then sonicated for 10 min, after which 0.75 ml of distilled water was added. Samples were mixed in the vortex and centrifuged (2000 rpm) in Kimax glass tubes, after which the lower phase was transferred to a new Kimax tube. The solvent was evaporated to dryness. Lipids were fractionated into neutral lipids (NLs), glycolipids (GLs), and phospholipids (PLs) using a Bond Elut (0.5 mg) Silica cartridge. First, the resin of the cartridges was conditioned using 5 ml of chloroform. Subsequently, the total lipids (1 ml) were applied to the resin, and rinsed using chloroform. NLs were collected under vacuum using 10 ml of chloroform, and glycolipids were washed by adding 10 ml of acetone. PLs (membrane fatty acids) were collected after the final resin was washed using 10 ml of methanol and evaporated to dryness.

Fatty acids of phospholipid fraction were methylated using acidic conditions. Toluene and sulfuric acid were used to transesterify fatty acid methyl esters (FAMEs) at 50°C for 10 h. The FAMEs were analysed with a gas chromatograph (Shimadzu Ultra, Japan) equipped with a mass detector (GC–MS) and using helium as a carrier gas and a Phenomenex® (California, USA) ZB-FAME capillary GC column (30 m × 0.25 mm × 0.15 μm). The temperature was held for 1 min at 50°C and then increased by 10°C per minute to the 130°C, which after the temperature was increased by 7°C per minute to the 180°C. The temperature was then raised by 2°C per minute to 200°C, and the final temperature increase was made to the 260°C at the rate of 7°C per minute. The total running time was 35.14. Identification and quantification of FAME followed the previously published method (Taipale et al., 2016).

Gas chromatography combustion stable isotope ratio mass spectrometry

The $\delta^{13}C$ values of FAs were determined using a GC-C TA III connected to an Isotope Ratio Mass Spectrometer (IRMS, DELTAPLUSXP, Thermo Co.) at the Department of Environmental Sciences of the University of Eastern Finland, Kuopio, Finland. Fatty acids were separated using a 30 m DB-23 column (0.25 mm × 0.15 mm) and then oxidized to carbon dioxide in an oxidation reactor at a temperature of 940°C with the reduction reactor kept at 630°C. The temperature program of the GC column started at 50°C and was kept for 1 min at 50°C, after which the temperature was raised by 30°C min^{-1} to 140°C, and then by 1°C min^{-1} to 220°C, and finally by 15°C min^{-1} to 300°C. The total run time was 49.3 min. The injector temperature was kept at 270°C. The samples were run against an internal standard, 1,2-dinonadecanoyl-sn-glycero-3-phosphatidylcholine (Larodan, $\delta^{13}C = -28.43‰$), which was used for drift and linear correction. The calculated precision for standard FAME was ±0.4‰, and the accuracy was ±0.3‰. Only peaks with a total height of 50 mV at mass 44 were counted. The $\delta^{13}C$ value of individual FAs was manually calculated using individual background values.

The $\delta^{13}C$ values of individual PLFA were converted to atom% using Equation (1). We then calculated the mass % of polyethylene-C converted to microbial biomass (PLFA) using Equation (2), where ΔAP is the difference between treatment and control as atom%, and m_{PLFA} is the mass of individual PLFA-C (PLFA content of samples was multiplied by carbon proportion of individual fatty acids) at the end of the incubation. The accumulation of ^{13}C-PE into microbial biomass was calculated by using Equation (2), but for ΔAP we used content weighted ΔAP of microbial PLFA (i15:0, a15:0, i17:0, a17:0, 15:0, 17:0, 16:1ω7c, 16:1ω9c, 18:1ω7c, 18:1ω9c, 18:2ω6) and total organic carbon was based on PLFA. Total organic carbon in microbes was calculated based on the fact that PLFAs represent an averagely 5% of all organic carbon in heterotrophic bacteria (Taipale et al., 2015). The assimilation rate on the biomass was then calculated using Equation (3). Biodegradation rate is the sum of mineralization and assimilation on the biomass.

Bacterial growth efficiency

We calculated bacterial growth efficiency (BGE) for PE-MP in different waters by the following equations (del Giorgio & Cole, 1998):

$$BGE = \frac{\text{bacterial production (BP)}}{BP + \text{bacterial respiration (BR)}}$$

in where bacterial production is ^{13}C-PE-MP assimilated per day (mg C per day) and bacterial respiration is $^{13}CO_2$ respiration per day (mg C per day).

DNA extraction, amplification, and sequencing

At the end of the experiments, 10 ml of sample water was filtered (Supor® 0.2 μm/25 mm, PES, Pall Corporation), and the filter was transferred into a bashing bead lysis tube (ZR BashingBead™ Lysis Tubes 0.1 and 0.5 mm, Nordic BioSite, USA) and stored at −80°C until DNA extraction. DNA was extracted using ZymoBIOMICS™ DNA Miniprep Kit and following the manufacturer's instructions and stored at −20°C.

PCR reactions contained DreamTaq Green PCR Master Mix (ThermoFisher Scientific), 1 μl of 10 μM primers M13-515FY and P1-806R (Mäki et al., 2016;

Parada et al., 2016), 2.5 μl of bovine serum album (BSA, 1 mg/ml ThermoFisher Scientific) and 3 μl of template DNA in a total of 25 μl. PCR was initialized at 95°C for 3 min, followed by 35 cycles of 95°C for 45 s, 50°C for 1 min, and 72°C for 90 s. The final step was at 72°C for 10 min before cooling to 4°C. PCR amplicons were checked by agarose gel electrophoresis on 1% agarose gel (120 V for 45 min).

Sample barcoding was performed as described above, but using 1 μl of PCR amplicons from the 1st reaction as template, and with barcoded fusion primer IonA-M13F and 806R-P1 (Mäki et al., 2016), and with 10 PCR cycles only. PCR amplicons were checked by agarose gel electrophoresis, and samples with low DNA content were purified and concentrated with SparQ Quanta purification beads (SparQ PureMag Beads, Quantabio) according to the manufacturer's protocol. Amplicons were quantified with Qubit (Qubit Fluorometric Quantification, Thermo Fischer Scientific) and pooled equimolarly. Pooled library was purified by SparQ Quanta using 1.3× ratio (v/v), quantified by 2200 TapeStation system (Agilent Technologies, USA), and sequenced by Ion Torrent Personal Genome Machine (PGM) (Life Technologies, USA) using IonPGM Hi-Q View OT2 400 kit and Sequencing kit with a 318 IonChip (Thermo Fisher Scientific).

Sequence data processing

The sequence data from the IonTorrent server was processed by CLC Genomic Workbench 12 with a microbial genomics module (Qiagen, Denmark). Low-quality sequences lacking forward or reverse primers, shorter than 250 bp, or longer than 450 were discarded, and primer sequences with flanking sequences were trimmed. The samples were subsampled to 25,000 reads. For reference-based OTU clustering, the SILVA 16S v132 database at 99% resolution was used, with OTUs clustered at 97% identity. The similarity percentage to be used when annotating novel OTUs was 80%. We used SILVA ribosomal RNA gene database version 132 for taxonomic classifications.

Statistical testing

Permutational multivariate analysis of variance (PERMANOVA) with Euclidean distance as a resemblance matrix was used for univariate analysis (Anderson et al., 2017). Univariate analysis was used to compare BGE, degradation rate, and time for complete biodegradation among treatments ($n = 3$ for each treatment). We used two crossed factor design in PERMANOVA for testing differences in cell density, microbial biomass based on phospholipid fatty acids, CO_2 concentration, and $\delta^{13}C$ value of the water and headspace of bottles, in which the first factor was the presence or absence of plastic (PE vs. Control; $n = 3$ for PE, but $n = 2$ for control) and second factor was water type (Medium vs. Humic vs. Clear, $n = 3$ for each treatment).

Main OTUs with >0.5% average sequence abundance in the sequenced samples (humic-water lake, clear-water lake and artificial freshwater medium, $n = 3$ for each) were used for statistical analysis. Bray Curtis similarity matrix of % of OTU, isotopic enrichment (Δ, treatment-control; ‰) of bacterial phospholipid fatty acids (i14:0, i15:0, 15:0, 16:1ω9, 16:1ω7, i17:0, a17:0, 17:0, 18:1ω9, 18:1ω7, 18:2ω6) and biodegradation rate was created using Primer 7 (Clarke & Gorley, 2015), which was used to create non-metric multidimensional scaling (nMDS) plot (Clarke, 1993). The bubble plot tool was used to visualize the biodegradation rate of different samples. The interactions between nMDS1 and nMDS2 and variables were analysed with Spearman correlation analysis. Hierarchical cluster analysis was used to create similarity groups in nMDS. We used Pearson linear regression analysis to create a connection between biodegradation speed and the concentration of $PLFA_{18:1\omega7}$ or percentage of uncultured Acetobacteraceae and Comamonadaceae to identify potential PE decomposers.

RESULTS AND DISCUSSION

Biodegradation of polyethylene-microplastics in freshwaters

We employed ^{13}C-isotope labelling of polyethylene-MP (PE-MP; 99.9% <20 μm, Figure 1A) to determine MP carbon's complete biodegradation time and fate. The participation of bacteria in PE-MP biodegradation was investigated using lake waters from humic and clear-water lakes. In addition, we employed a sterilized artificial freshwater medium, thus the microorganisms in this treatment originated only from the PE surface. Cell and particle density and microbial biomass based on the PLFA were mainly (65%–73% of all variation) determined by the water (PERMANOVA (cell/PLFA): Pseudo-$F_{2,14}$ = 32.9/14.4, P(MC) = 0.001/0.004; Figure 1B) and PE addition did not have a statistically significant impact on the cell density or PLFA biomass (PERMANOVA (cell/PLFA): Pseudo-$F_{1,14}$ = 2.2/1.6, P(MC) = 0.152/0.242; Figure 1C).

Microbial activity in the control (no PE-MP) and PE-MP bottles during our 8-week incubation was higher in the humic-water lake treatment than in the clear-water lake or the artificial freshwater medium treatment when measured as CO_2 from the headspace of incubation bottles (PERMANOVA: Pseudo-$F_{5,14}$ = 207.6, P(MC) = 0.001; Figure 2A). Microbes in the control bottle probably utilized terrestrial dissolved organic carbon,

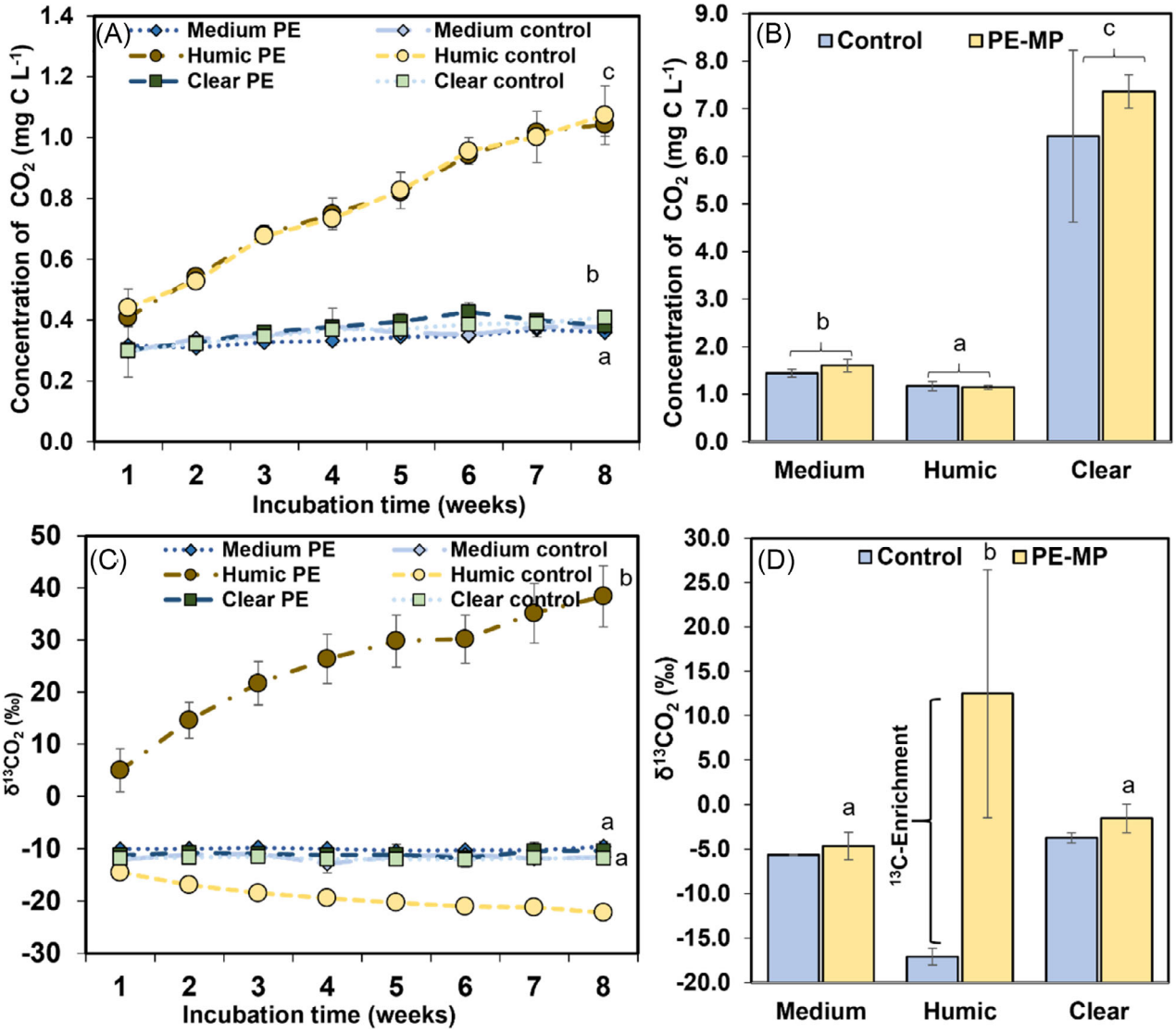

FIGURE 2 The concentration of CO_2 and respiration of ^{13}C-PE into CO_2. (A) The concentration of carbon dioxide (respiration) in the headspace and (B) the water during the 8-week incubation of polyethylene (PE, $n = 3$) and controls (no-PE, $n = 2$) in the artificial medium (medium), clear eutrophic (clear) and humic mesotrophic (humic) lake water. The development of δ^{13}C value of carbon dioxide in the (C) headspace and (D) water during the 8-week incubation in the artificial freshwater medium (medium), humic-lake water (humic), and clear-lake water eutrophic (clear). Letters in A and C cite the statistical difference between waters (PE treatments), c > b > a, $p < 0.05$. Letters in B and D cite the statistical differences between waters (PE + control), which explained most of the variation among treatments, c > b > a, $p < 0.05$.

detritus, and recalcitrant natural polymers (Jones, 1992; Wiegner et al., 2015), as evidenced by higher particle density than in the clear-water lake or artificial medium. During 8 weeks of incubation, the content of CO_2 in the headspace of artificial freshwater medium or clear-water lake bottles did not significantly increase. However, the CO_2 concentration of water was higher in the clear-water lake than in any other bottle (PERMANOVA: Pseudo-$F_{5,14}$ = 207.6, P(MC) = 0.001; Figure 2B), but we cannot conclude microbial activity in these bottles because we did not measure CO_2 concentration of water from the start. The lower CO_2 concentration of water in humic-lake water treatment (pH = 6.4 ± 0.2) than in the clear-lake water treatment (pH = 7.8 ± 0.2) can be explained by the fact that CO_2 has limited solubility in the acidic water (Dodds, 2002). The ^{13}C-labelling of CO_2 in headspace and water (measured as the difference between PE and control) increased clearly and steadily in the humic-lake water treatment showing higher microbial rework with the carbon from PE-MP in the humic-lake water than in the clear-lake water or artificial medium (PERMANOVA: Pseudo-$F_{2,8}$ = 11.8, P(MC) = 0.007; Figure 2C, D). Moreover, ^{13}C-enrichment of CO_2 did not differ between clear-lake water and artificial medium treatment.

Bacteria in the humic-lake water treatment assimilated ^{13}C from PE-MP into their membrane lipids

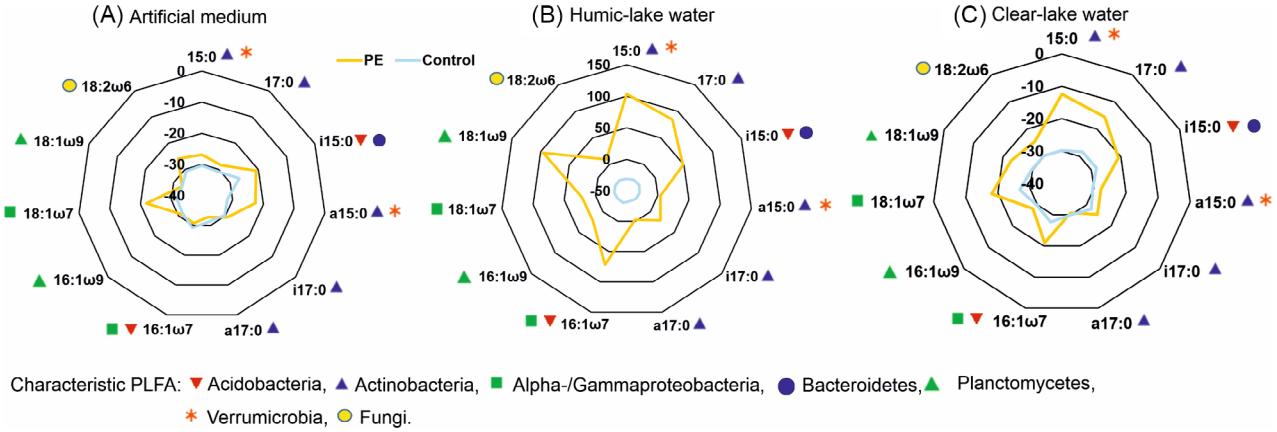

FIGURE 3 ^{13}C-PE-MP accumulation into the microbial membrane lipids. The average δ^{13}C values of individual phospholipid fatty acids of the microbiome in the artificial medium (A), humic-lake water (B), and clear-lake water (C) after 8-week incubation ($n = 2$ for controls and $n = 3$ for MP-PE)

(average ^{13}C-enrichment of PLFAs $= 77 \pm 44$‰) at higher rates than bacteria in the clear-water lakes (average ^{13}C-enrichment $= 17 \pm 24$‰) or artificial medium (average ^{13}C-enrichment $= 1 \pm 7$‰; PERMANOVA: Pseudo-$F_{2,8} = 16.7$, P(MC) $= 0.002$; Figure 3). We also detected assimilation of ^{13}C from PE-MP into microbial PLFAs in artificial medium and clear-lake water bottles, reflecting some utilization of MP carbon from PE. Different ^{13}C-enrichment patterns of microbial PLFAs in different water types suggest different microbial communities in the mineralizing PE-MP (Figure 2). Moreover, the ^{13}C-labelling of microbial PLFAs in the humic water was lower than in our previous study (Taipale et al., 2019) (average ^{13}C-enrichment of PLFAs $= 377 \pm 364$‰). One potential explanation could be the lower availability of nutrients in the humic-water lake in our present study than in the earlier study (TP $= 26$ μg L^{-1} vs. 136 μg L^{-1}) since nutrients are well known to limit bacterial growth in humic lakes (Hessen et al., 1994). Additionally, here we pre-incubated lake waters to reduce the availability of alternative organic carbon sources outside MP carbon, which may have reduced the initial microbial biomass. Our results reveal that the ^{13}C-labeling of different PLFAs was relatively equal in humic-lake water, implying that different bacterial groups were involved in the utilization of PE-MP at early stages. The fairly similar ^{13}C-enrichment of different PLFAs contradicts our previous study, in which only two bacterial PLFAs were heavily ^{13}C-enriched (Taipale et al., 2019). Here the most ^{13}C-enriched PLFAs in humic-lake water were 16:1ω7, 18:1ω9, 15:0, and 17:0, which are typical PLFAs for Planctomycetes, Acidobacteria, and Alpha- and Gammaproteobacteria (Elshahed et al., 2007; Kulichevskaya et al., 2014; Ratledge & Wilkinson, 1988). Linoleic acid (LIN; 18:2ω6) is a typical PLFA in heterotrophic nanoflagellates and fungi (Stahl & Klug, 1996; Véra et al., 2001) that was also lightly ^{13}C-enriched in the humic-lake water treatment, however, less than bacterial PLFAs.

We determined MP carbon fate and biodegradation speed using carbon concentration data and a calculated increased amount of ^{13}C in CO_2 and microbial biomass. MP carbon has a similar fate in humic-lake and clear-lake water treatments (Figure 4A), wherein microbes utilized carbon primarily (80%–90%) to construct new biomass, and only a small portion of MP carbon was used for energy (CO_2 production). BGE describes the efficiency in which substrate carbon is turned into new biomass (del Giorgio & Cole, 1998). Our results showed that BGE in humic-water lakes was higher than in the artificial medium (PERMANOVA: Pseudo-F2, 8 $= 8.8$, P(MC) $= 0.019$; Figure 4B). Our BGE values with PE were similarly high with excreted organic carbon of phytoplankton that has high usability for bacteria to build new biomass (del Giorgio & Cole, 1998). However, our BGE values are only seemingly high since microbes most likely used (terrestrial) dissolved organic carbon in the bottles for energy, as seen in high respiration also in the controls of humic-water treatment. Actually, previous studies have found that the degradation of lignin, which is a recalcitrant natural polymer, requires readily available energy and carbon sources (Klotzbücher et al., 2011). Our result suggests that PE is majorly used for building new biomass and microbes decomposing PE also requires labile carbon sources for energy.

The biodegradation rate of PE-MP in the humic-lake water was 5–30 times higher than in the clear-lake water or artificial medium (PERMANOVA: Pseudo-$F_{2,8} = 52.5$, P(MC) $= 0.001$, Figure 4C), resulting in an extensive time range for complete biodegradation of PE-MP (Figure 4D). PE-MP would have been entirely decomposed in humic-lake water in 180 ± 20 years, but the completed biodegradation time varied from 700 to 6000 years in the clear-lake water and 5000 to

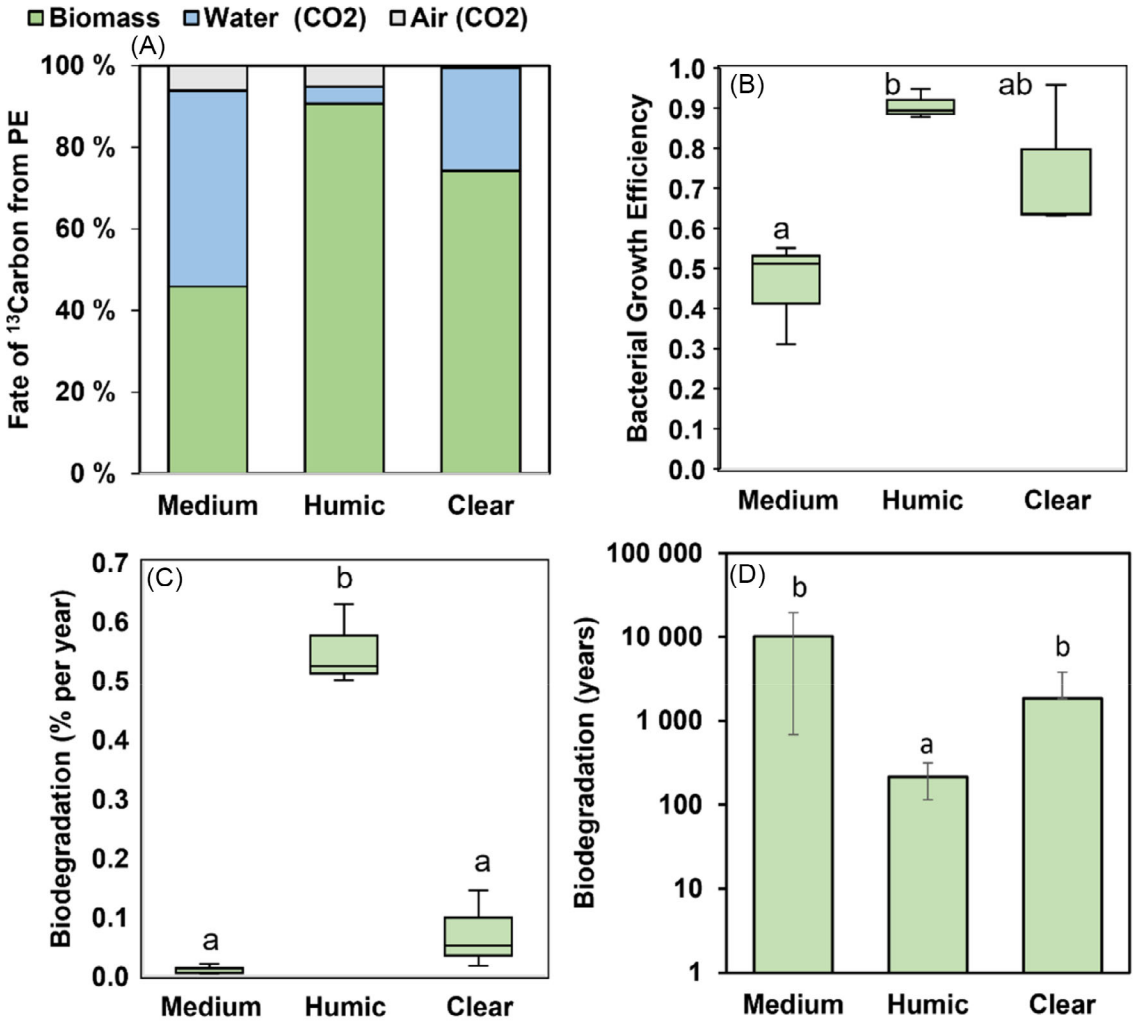

FIGURE 4 The fate of PE-MP carbon and biodegradation rate of PE-MP. (A) The fate of ^{13}carbon of PE-MP into microbial biomass and CO_2 (water or air), (B) bacterial growth efficiency (BGE) for PE, (C) biodegradation rate (% in a year), and (D) complete biodegradation time in years in the artificial freshwater medium, humic-lake water, and clear-lake water ($n = 3$ for all). Letters cite the statistical difference between treatments (PERMANOVA, $p < 0.05$)

30,000 years in the artificial freshwater medium. Altogether, our result showed the water-type's key role, which explained 94% of the variation in the PE-MP biodegradation. Our results in humic and clear-lake water indicated similarly and slower, respectively, biodegradation than Chamas et al. (2020), who suggested complete biodegradation of PE bottles to take 100 years in sea and 400 years on the land without UV radiation. However, our results do not include sun radiation (UV–VIS), which is a key mechanism that initiates microplastic breakdown in both marine and freshwater environments and can halve estimated degradation time (Chamas et al., 2020). Our findings also revealed high variation among clear-lake water replicates, implying that the mineralization process might be easily disrupted if microorganisms are given more labile organic molecules to mineralize. Overall, MP is biodegradable in lakes, and lake type, chemical, and the microbiological environment play a key role in determining the speed at which MP is degraded. Effects on the biota need to be still investigated in more detail.

Microbial communities in the biodegradation of polyethylene-microplastics in freshwaters

The results of 16S rRNA gene analysis of microbial communities from various treatments were paired with ^{13}C-enrichment of phospholipid fatty acids and biodegradation rate. At the same time, our findings with potential PE-MP decomposers support and differ from the previous results with the microbial community on the plastisphere in marine, brackish, and freshwaters (Dussud et al., 2018; Oberbeckmann et al., 2014; Szabó et al., 2021). Bacilli and Actinobacteria were the most common bacterial groups in the artificial medium (Figure 5). Previous research has found Actinobacteria

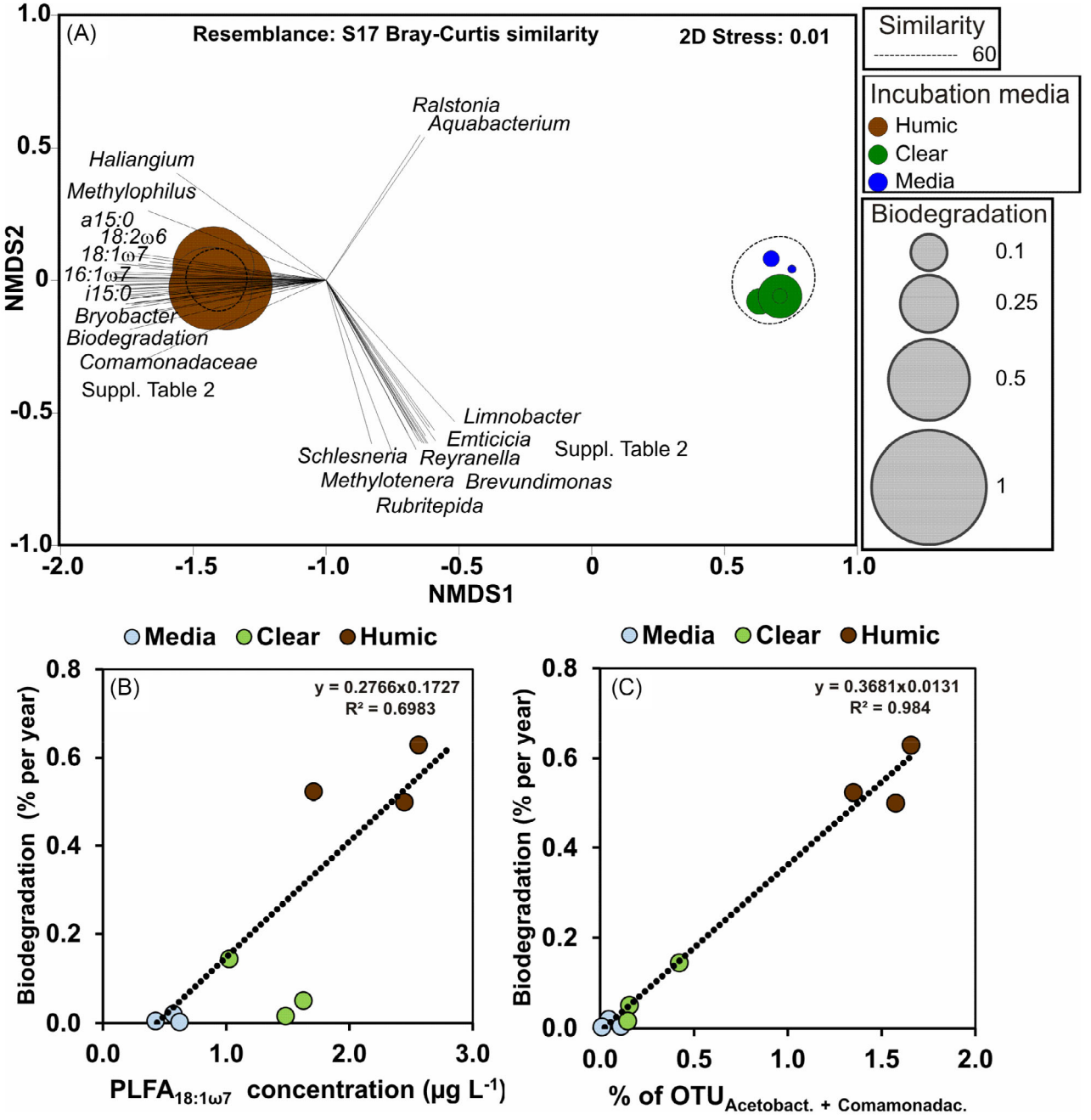

FIGURE 5 PE-MP biodegradation-related microbial community. (A) Non-metric multidimensional scaling plots of Bray Curtis similarity of OTU data (>0.5% of all rRNA sequences) at the genus level, ^{13}C-enrichment of microbial phospholipid fatty acids and biodegradation rate (loadings on nMDS1 and nMDS2 see Supplemental Table S2). The size of the bubble plot stands for faster biodegradation (% in a year) which was highest with humic-lake waters (humic) than in the clear-lake waters (clear) or artificial freshwater medium (medium). Dashed lines show 60% similarity according to cluster analysis. (B) Regression between biodegradation rate (% per year) and the concentration of 18:1ω7c that is typical in Alpha- and Gammaproteobacteria and (C) percentage of uncultured Acetobacteraceae and Comamonadaceae which strongly correlated with NMDS1

to be common bacteria in the plastisphere in soils (Rüthi et al., 2020), but Zettler et al. (2013) discovered that they are more common in seawater than in the plastisphere, supporting our hypothesis that Actinobacteria play a minor role in mineralizing PE-MPs.

NMDS analysis at the class level identified ^{13}C-enrichment of PLFA and biodegradation rate with NMDS 1 axis that closely correlated with Acidomicrobiia and other non-common bacterial classes (Figure S1). However, NMDS analysis of bacterial OTUs at the genus level indicated a substantial correlation with the genus of *Bryobacteria*, *Candidatus Solibacter*, and uncultured Acidomicrobiia from Acidobacteria, which can explain the high ^{13}C-

enrichment of 16:1ω7 (Kulichevskaya et al., 2014). Acidobacteria is common water bacteria that digest bacterial polysaccharide gellan gum and tolerate a variety of contaminants and heavy metals, suggesting that they may decompose some pollutants (Baik et al., 2013; Kielak et al., 2016). Our findings strongly suggest that Acidobacteria are involved in the breakdown of PE-MP. Furthermore, two species of acetic acid bacteria, assigned to the genus *Rhodovastum* and uncultured Acetobacteraceae, are possible PE-MP decomposers and can explain the ^{13}C-enrichment of 18:1ω7 (Figures 3 and 5A). These bacteria normally oxidize carbohydrates or ethanol during fermentation and create acetic acid. *Rhodovastum* can also grow phototrophically in addition to heterotrophic growth (Okamura et al., 2009). We also discovered a substantial correlation between the biodegradation rate and the clade CL500-3 (Phycisphaerae), the most abundant Planctomycetes in freshwaters (Andrei et al., 2019) and can explain the ^{13}C-enrichment of 18:1ω9 (Elshahed et al., 2007).

The first PET-degrading bacterium, *Ideonella sakaiensis*, was identified as a member of the Comamonadaceae family (Yoshida et al., 2016). Here, carbon from PE-MP was utilized by uncultured Comamonadaceae, which has 100% similarity with OTU previously found in a polluted coastal lagoon (Salloto et al., 2012). Furthermore, our findings revealed that the concentration of $PLFA_{18:1\omega7}$, a fatty acid prevalent in Alpha- and Gammaproteobacteria, including the previously mentioned Acetobacteraceae and Comamonadaceae, gave the best explanation for the biodegradation speed of PE-MP (Figure 5B,C). In our previous study (Taipale et al., 2019) with the biodegradation of PE-MP in a highly humic lake, this PLFA was substantially ^{13}C-enriched. Lignin-degrading Alpha- and Gammaproteobacteria have been shown to produce extracellular peroxidases, which may explain their essential role in the biodegradation of plastics as well (Bugg, Ahmad, Hardiman, & Singh, 2011; Bugg, Ahmad, Hardiman, & Rahmanpour, 2011). The increased quantity of plastic-degrading enzymes in plastic-polluted areas has been interpreted as a microbe's response to plastic pollution (Zrimec et al., 2021). However, our findings imply that microbes in humic lakes can biodegrade PE-MP employing the same enzymes as natural refractory polymers. This resulted in facilitated biodegradation time of MP in humic-lake waters compared to clear-lake waters and artificial medium. Our findings show that aliphatic MPs persist in freshwater for a long period, meaning that they will be passed on from generation to generation.

AUTHOR CONTRIBUTIONS

S. J. Taipale, P. Kautonen and J. V. K Kukkonen designed the study. P. Kautonen carried out experiments and analysed CO_2 concentration and phospholipid fatty acids. J. Vesamäki analysed carbon isotopes from gas samples. S. J. Taipale and C. Biasi analysed δ^{13}C values of phospholipid fatty acids, and J. Vesamäki and R. Nissinen made molecular biology analysis and bioinformatics. S. J. Taipale made statistics and S. J. Taipale and M. Tiirola calculated together mineralization rates. S. J. Taipale wrote the initial draft of the paper. All authors discussed the results and commented on the manuscript.

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CONFLICT OF INTEREST

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

DATA AVAILABILITY STATEMENT

Sequence and sample data obtained during this work have been submitted to NCBI repositories (BioProject PRJNA813857).

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SUPPORTING INFORMATION

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

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