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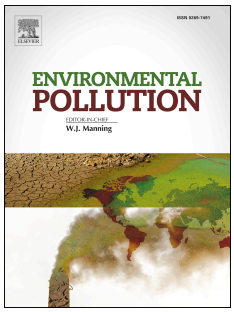

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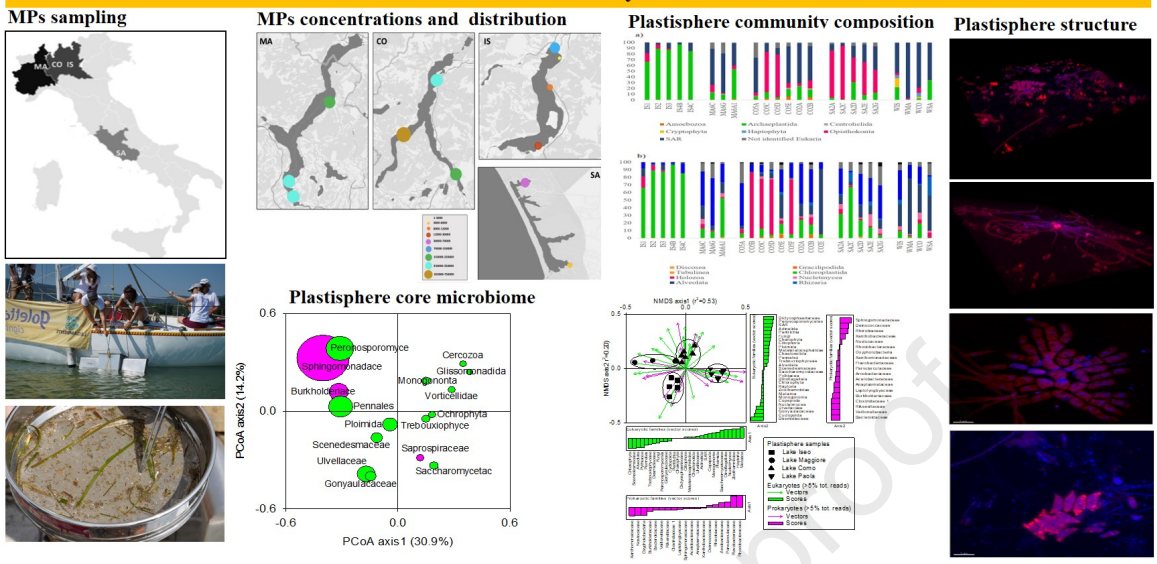

Plastisphere in lake waters: microbial diversity, biofilm structure, and potential implications for freshwater ecosystems

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Abstract

Once dispersed in water, microplastic (MP) particles are rapidly colonised by aquatic microbes, which can adhere and grow onto solid surfaces in the form of biofilms. This study provides new insights on microbial diversity and biofilm structure of plastisphere in lake waters. By combining Fourier Confocal Laser Scanning Microscopy (CLSM), Transform Infrared Spectroscopy (FT-IR) and high-throughput DNA sequencing, we investigated the microbial colonization patterns on floating MPs and, for the first time, the occurrence of eukaryotic core members and their possible relations with biofilm-forming bacterial taxa within the plastisphere of four different lakes. Through PCR-based methods (qPCR, LAMP-PCR), we also evaluated the role of lake plastisphere as long-term dispersal

vectors of potentially harmful organisms (including pathogens) and antibiotic resistance genes (ARGs) in freshwater ecosystems. Consistent variation patterns of the microbial community composition occurred between water and among the plastisphere samples of the different lakes. The eukaryotic core microbiome was mainly composed by typical freshwater biofilm colonizers, such as diatoms (Pennales, Bacillariophyceaea) and green algae (Chlorophyceae), which interact with eukaryotic and prokaryotic microbes of different trophic levels. Results also showed that MPs are suitable vectors of biofilm-forming opportunistic pathogens and a hotspot for horizontal gene transfer, likely facilitating antibiotic resistance spread in the environments.

Key words: Plastisphere; microplastics; freshwater; biofilms; eukaryotic community; antibiotic resistance; pathogens

1. Introduction

Global plastic production has increased constantly over the past 60 years and the lack of proper waste management practices of end-of-life plastic products is generating a widespread environmental contamination. Although the amount of discarded plastic sent to recycling has been rising since 2006, up to 25% of plastic waste is not yet properly disposed to landfills and are released into the natural environment (Geyer 2020). Once in water, the plastic debris may progressively break down into microscopic particles, known as microplastics (MPs) and considered as an emerging pollutant of aquatic ecosystems (Amaral-Zettler et al., 2021; Gall and Thompson 2015).

In marine environments, the abundance, composition, and source of MPs were assessed, along with the direct effects on aquatic organisms, ranging from zooplankton to mammals (Cole et al., 2011; Browne 2011; Andrady, 2011; Amelineau et al., 2016; Lambert and Wagner, 2018; Botterell et al., 2019). Only few studies focused on the role of MPs as dispersal vectors for environmental microbial communities (Amaral-Zettler et al., 2020; Oberbeckmann et al., 2014;).

While being transported by water flow, plastic debris and MPs provide a durable solid surface that can be colonized by planktonic microorganisms and transported for long distances, supporting the growth of microbial biofilms, multi-stratified microbial communities embedded in an exopolymeric matrix (Amaral-Zettler et al., 2020). Such new human-made ecosystem is referred to as the 'plastisphere' and numerous marine studies have demonstrated that these plastic-specific microbial communities are consistently different from those found in the surrounding waters (De Tender et al. 2015; Oberbeckmann et al. 2014; Harrison et al. 2014; Zettler et al. 2013). Several studies provide evidence of a common core of the plastisphere microbiome (Frère et al., 2018; Kirstein et al., 2019). Moreover, different factors, including location (e.g., biogeography and anthropogenic influences) and time of year (e.g., seasons) seem to influence the microbial community that develops on plastic surfaces in marine environments (Oberbeckmann et al. 2014; Oberbeckmann et al., 2016; Hoellein et al., 2017; McCormick et al., 2014, 2016).

A large body of the recent literature was dedicated to characterize the bacterial composition of plastisphere, while relatively few studies focused on the plastisphere eukaryotic biodiversity. Microeukaryotes are well represented on plastic debris and MPs, as found by microscope-based observations (Carson et al., 2013; Oberbeckmann et al., 2014; Bryant et al., 2016; Masó et al., 2016). Studies employing high-throughput sequencing of eukaryotic microbes on plastics are limited and only few were targeting prokaryotic and eukaryotic composition on the same MP samples (Kettner et al., 2017; Amaral-Zettler et al., 2021).

Unfolding the composition and diversity of eukaryotes and understanding their possible relations with prokaryotes is crucial to understand the role of plastisphere in the aquatic systems subjected to MP contamination. MPs may act as vectors of microorganisms and genes of ecosystem and human health concern. Examples include spreading of harmful algal bloom species, protozoan pathogens, and pathogenic bacterial microorganisms (Masò et al., 2003; Zettler et al. 2013; Kirstein et al., 2016; Dussud et al. 2018, Wang et al., 2020). MPs may also serve as hotspots of horizontal gene transfer, potentially facilitating pathogenicity and antibiotic resistance (AR) transfer in the environment (Eckert et al., 2018; Sathicq et al., 2021), with inherent risks for ecosystem and human health. Although recent investigations revealed the occurrence of MPs in freshwater ecosystems (Fisher et al., 2016; Pietrelli et al., 2017; Sighicelli et al., 2018; Di Pippo et al., 2020; Du et al., 2022), most studies were performed in marine environments.

Here we analysed the microbial composition of plastisphere collected from lake waters. We hypothesise that MPs will represent a newly pelagic freshwater habitat for benthic species with possible ecological and health implications, as previously highlighted for the neopelagic communities in the open ocean (Haram et al., 2021). More specifically, we analysed eukaryotic diversity and composition and we used bacterial taxon composition data (Di Pippo et al., 2020) to evaluate possible cross-kingdom interactions between eukaryotic and prokaryotic communities. Moreover, we evaluated possible implications of floating MPs and microbial colonization for freshwater ecosystems. The aims of this study were to (a) provide new insights into the lentic plastisphere

diversity, along with local variable factors (i.e., plastic type and degradation level), likely driving the colonization patterns and biofilm structure on freshwater MPs, (b) identify the occurrence of eukaryotic core members and their possible relations with biofilm-forming bacterial taxa within the freshwater plastisphere, (c) evaluate the role of MP-associated biofilms as possible vectors of harmful, parasitic and/or pathogenic organisms (and their associated ARGs) enabling their long-range dispersal in freshwaters.

2. Material and Methods

2.1. Study sites and sample collection

The targeted lakes are located in Northern and Central Italy and included Lake Maggiore (MA), Lake Como (CO), Lake Iseo (IS), and Lake Paola (PA) (Figure 1a). Lakes MA, CO, and IS are among the six Italian subalpine great lakes of glacial origin and represent an essential strategic water supply for human activities in a densely populated area. Lake PA, located in Central Italy, is a brackish water body, where freshwater and seawater combine in an area known for its wetland-type habitat. The four lakes are also popular touristic destinations due to high naturalistic and environmental values.

Sampling was carried out during the 14th edition of the field survey "Goletta dei Laghi" (July 2019), organised by Legambiente and intended to annually monitor the water quality of major Italian lakes. Four transects at Lake Iseo (IS1, IS2, IS3, IS4), three transects at Lake Como (CO2, CO5, CO6), Maggiore (MA1, MA5, MA6) and two transects at Lake Paola (SA1, SA2) were selected (Figure 1). Duplicate water samples (500 mL) for each transect were collected and filtered (Nucleopore polycarbonate filters with47 mm diameter and 0.2 mm pore size). Filters were maintained at −20 °C until further analyses to evaluate the taxa composition of planktonic communities. A manta trawl (40 × 20 cm opening and 330 μm mesh size) was used to collect samples for microplastic quantification, composition, and plastisphere characterization. The manta trawl was immersed 20 cm below the water surface and filtered a mean of 240 m^3 of water at an average trawling speed of 3 knots for 15 min. Three replicates were collected for each lake transect. Two replicates were sampled for quantification,

chemical composition and distribution of MPs in lakes and one replicate for plastisphere analysis. MPs collected for chemical analysis were stored in sterile bottles (30% hydrogen peroxide; T: 4 °C) until analysis. MPs for plastisphere analysis were gently washed with sterile saline solution (0.9%) to rinse off non-attached organisms and divided into different vials and stored at −20 °C. 61 microplastic particles was sampled for plastisphere analysis, grouped into 19 samples (i.e., 5 samples from Lake Iseo, 6 from Lake Como, 3 from Lake Maggiore, and 6 from Lake Paola) from which the DNA was extracted.

DNA extracted from MPs was used to (a) evaluate the eukaryotic composition by 18S rRNA gene sequencing in function of sample location, polymer type and degradation level and to evaluate possible association with bacterial taxa, (b) assess level of anthropogenic contamination and ARG presence by quantifying the indicator gene *IntI1* through quantitative PCR (qPCR), (c) evaluate the presence/absence of pathogenic bacteria by Loop-Mediated Isothermal Amplification (LAMP)-PCR. After DNA extraction, MPs were further analysed to determine their chemical composition.

MPs were also collected for CLSM observations, fixed with 5% final concentration formaldehyde and kept at –20 °C.

To guarantee a proper quality of research during the sampling campaign, we adopted contamination control measures, including (i) pre-filtration of all the work solution used, kept closed in clean glass bottles, (ii) cleaning of materials and equipment before use, (iii) use of cotton lab coat, (iv) covering of solutions and samples with aluminium foil, (v) use of metal tweezers.

2.2. Microplastic quantification and chemical composition

Sampled MPs were washed and separated from the organic matter using a stereomicroscope, with magnification up to 40 x. Samples were dried at 50° C and particles were sorted into categories based on shape (i.e., filament, pellet, ball, film, fragment,) and counted. MPs abundance was determined in all trawl samples and expressed as items km^{-2}.

Polymers were identified by using FT-IR (Fourier Transform Infrared Spectroscopy) (Hidalgo-Ruz et al. 2012). Attenuated total reflectance (ATR) mode was used to collect FT-IR spectra (Thermo scientific Nicolette 6700 spectrophotometer: 2 cm^{-1} resolution; 4000 - 400 cm^{-1} spectrum range). Since IR spectra of most of the samples showed polymer degradation, the Carbonyl Index (CI) for each polymer was also determined according to the equations previously reported (Guadagno et al., 2001; Mylläri et al., 2015).

A higher level of polymer degradation was indicated by a higher value of CI. CI values were divided into two degradation levels: Low Degradation Level (LDL: 0-0.5) and High Degradation Level (HDL: 0.6-1.0). Since degradation level is linked to MPs residence time in water, probably this can be mirrored by the biofilm age, with communities at initial phase of development associated to MPs with lower degradation level. Therefore, CI data were used to estimate the possible effect of biofilm aging on eukaryotic composition of plastisphere.

2.3. Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (FV1000, Olympus Corp., Tokyo, Japan) was used to visualise microbial micro-clusters within the structure of MPs. The DNA-specific fluorescent stain DAPI (1.5 μg ml^{-1}) (Vector Labs, Milano, Italy) was used to stain total viable cells. Lasers with excitation wavelengths used in this study were previously reported (Di Pippo et al. 2020). 2-D images (x–y plane) were captured at 0.5-μm intervals along the z-axis and 3-D images were reconstructed (Imaris 6.2.0 software: Bitplane AG, Zurich, Switzerland).

2.4. DNA extraction and High-Throughput sequencing of 18S rRNA gene

DNA from water and MP samples was extracted by using DNeasy PowerSoil Pro Kit (QIAGEN - Germantown, MD) following manufacturer's instructions.

An aliquot of the purified DNA was utilised as a template for amplifying the V4 region of 18S rRNA gene of eukaryotes (Eu565F: 5′-CCAGCASCYGCGGTAATTCC-3′; Eu981R: 5′

ACTTTCGTTCTTGATYRA-3′) (Crognale et al., 2021). Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Waltham, MA USA) was used to perform PCR reactions and Agencourt® AMpure XP bead protocol (Beckmann Coulter, USA) was used to purify amplicon libraries. A MiSeq platform was used to sequence samples using a MiSeq Reagent kit v3 (Illumina, San Diego, CA, USA) following guidelines to prepare and load samples. The Phix control library was used at a concentration of 15%. The intrinsic quality of the raw reads was firstly evaluated by using FastQC, then the sequences were analysed using QIIME2 software tools (2018.2 release). The QIIME2 plugins demux and cutadapt were used to demultiplex reads and remove primer sequences. The quality control for paired-end reads was performed by using DADA2 package (Callahan et al., 2016) and amplicon sequence variants (ASVs) were obtained. Taxonomy was then assigned to ASVs using a classifier based on the 18S rRNA gene database at a 99% similarity of the SILVA132 release (Quast et al., 2013).

The raw 18S rRNA gene sequences are available through the Sequence Read Archive (SRA) under accession number PRJNA855607. The raw 16S rRNA sequencing data published in Di Pippo et al. (2020) and used in this study are available under accession number PRJNA855619.

2.5. Quantitative PCR (qPCR)

qPCR was used to assess level of anthropogenic contamination and ARG presence in the DNA extracted from water and plastisphere samples by quantifying the Class I integron-integrase indicator gene *IntI1*, considered as proxy for anthropogenic ARG's contamination (Gillings et al. 2015). The amount of *intI1* was assessed by relative abundance after normalization to total bacterial load by 16S rRNA gene (Suzuki et al., 2000; Barraud et al., 2010).

In details, used annealing temperatures and primers/probes sequences were as follows:

for *IntI1* gene: IntI1fwd (5'-GCCTTGATGTTACCCGAGAG-3'), IntIrev (5'-GATCGGTCGAATGCGTGT-3') and IntIprobe (5'-FAM-ATTCCTGGCCGTGGTT CTGGGTTTT-BHQ1-3'), 60°C of annealing temperature;

for 16SrDNA gene: BAC1055F (5'-ATGGCTGTCG TCAGGT-3') and BAC1392(5'-ACGGGCGGTGTGTAC-3'), 55°C of annealing temperature.

CFX96^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) was used to perform qPCR assays in 96 well plates in 20 µL volume. Each reaction contained 10µL of 2X Mastermix (SsoAdvanced™ Universal Probes Supermix -Bio-Rad, USA) for *intI1* and SYBR Green Supermix (Bio-Rad USA) for 16S rDNA, 3 µl of DNA template, 6.25 µM probes and 10 µM primers. . Samples and no template controls (NTCs) were analysed in triplicates and data were analysed with the CFX ManagerTM software (version 3.1, Bio-Rad, Italy). A standard curve was obtained using serial dilutions of positive controls and the amount of target genes in samples was calculated. Positive controls for *intI1* were purchased from DSMZ, namely plasmid R388 (DSMZ 5189). Positive control for 16S rDNA were obtained by PCR amplification of 16S rRNA gene. The concentration of the purified plasmid or PCR amplified was determined using NanoDrop spectrophotometer and the copy number of *intI1* and 16S rDNA gene per µL of plasmid or genomic DNA solution was then calculated (Czekalski et al. 2012).

2.6. Loop-Mediated Isothermal Amplification (LAMP)-PCR

LAMP-PCR (Hara-Kudo et al., 2007) was used to evaluate the presence/absence of pathogenic enterobacteria (*Salmonella* spp) and opportunistic pathogens (*Legionella* spp, *Legionella pneumophila* and *Pseudomonas aeruginosa*) in the water and plastisphere samples. Each analysis was performed by using 3 µl of extracted DNA. AVANTECH LAMP-PCR pathogens detection kits (EBT-615 *Salmonella* screen GLOW; EBT-629 *Legionella* screen GLOW. EBT-621 *Legionella pneumophila* GLOW; EBT 626-*Pseudomonas aeruginosa* GLOW).

2.7. Statistical analyses

The non-parametric multivariate analysis of variance (PERMANOVA) was performed to test the difference in microbial community composition between environmental matrices (water *vs*

plastisphere), lakes (i.e., IS *vs* MA *vs* CO *vs* SA), polymer types (i.e., PE *vs* other polymers), and polymer degradation levels (LDL *vs* HDL). Similarity matrices of eukaryotic and bacterial community composition were calculated using sequencing data and applying the relative abundance-based Bray-Curtis index. A non-metric multi-dimensional scaling ordination plot (nMDS) was used to visualize the variation patterns of major eukaryotic and prokaryotic taxa at the family level (>5% of total reads). The values of relative abundance were incorporated into the nMDS analysis with a vector-fitting procedure, in which the length of the arrow is proportional to the contribution of each variable to the nMDS-axes. The Principal Coordinate Analysis (PCoA) ordination plot was used to visualize the relatively closer associations among the plastisphere core taxa (i.e., eukaryotic and prokaryotic families detected in all lakes and polymer samples). The non-parametric Kruskal-Wallis univariate test, with the Mann-Whitney pairwise comparison, was performed on the plastisphere core taxa in order to assess statistical differences between the sample groups (IS *vs* MA *vs* CO *vs* SA; PE *vs* other polymers; low *vs* high degradation level). All statistical analyses were performed by the PAST software package (PAlaeontological STatistics, ver. 4.04).

3. Results

3.1. Microplastic characterization

All samples contained MPs and showed great spatial variability, with particle counts ranging from 3,000 up to 750,000 per square kilometer. The microplastic shape distribution in all lakes showed the dominating occurrence of fragments with mean frequency of 73% (Figure 1). The microplastic chemical composition from all examined samples showed the dominating presence of polyethylene (PE), expanded polystyrene (EPS) and polypropylene (PP), respectively with mean value of 53%, 35% and 15% of total MPs.

3.2 Detection of biofilm-forming microorganisms and community architecture

Biofilm cells were distributed in patches of different thickness, with voids commonly present in

biofilm structure (Figure 2). Several diatom taxa were visualized in most of the analyzed samples, with pennate forms predominating (Figures 2i, 2j, 2k). Different filamentous and/or ramified green algae (Figure 2l) as well as members of *Scenedesmaceae* family (Figure 2i) also occurred in different biofilm communities. 3-D images confirmed the multi-stratified biofilm structure with bacterial microcolonies DAPI-stained closely associated with prokaryotic (Figure 2d) and eukaryotic (Figure 2h) autotrophs.

3.3 Microbial community composition and distribution in water and plastisphere

The high-throughput sequencing yielded between 69953 and 162532 high quality 18S rRNA gene reads. Considering the whole dataset, ASVs were affiliated to different taxa across almost all the eukaryotic tree of life, whose sequences were mainly attributed to members belonging to SAR supergroup (Alveolata and Stramenopiles), Holozoa, including Metazoa, Fungi and Chloroplastidia (Figure S1).

Planktonic communities in IS, CO, and SA were dominated by taxa affiliated to Alveolata, with Dinoflagellata as the main component (19.0-68.2%) (Figure S1a and S1b). *Peridinium* and *Ceratium* were the most common dinoflagellate genera retrieved in the target lakes, while ASVs affiliated to the genus *Gonyaulex* dominated the dinoflagellate community in PA. ASVs affiliated to Chloroplastidia and Stramenopiles were also common in water communities. In particular, within Chloroplastidia, most of the sequences were attributed to Chlorophyta (up to 34.2%) and Charophyta (up to 18.1%) members and within Stramenopiles, Bacillariophyta (up to 3.1%) were present (Figure S1a and S1b). Taxon composition of plastisphere eukaryotic communities was significantly different from that of planktonic eukaryotic communities (PERMANOVA, $p < 0.001$).

In plastisphere communities, most ASVs were affiliated to Archeoplastidia (up to 96.3%), Opistokonta (up to 92.7%), and SAR group (up to 69.0%) (Figure S1a). Among Archeoplastidia, Chloroplastidia members were prominent, while among Opistokonta, sequences were mainly affiliated to Holozoa (up to 86.7%). Within the SAR group, members belonging to Alveolata (up to

85.8%) and Stramenopiles (62.7%) were dominant (Figure S1a and S1b).

Taxon composition of the eukaryotic plastisphere varied significantly among lakes (PERMANOVA, $p < 0.001$). No significant differences in eukaryotic community composition were found either across polymer types (PE = others, $p > 0.05$ PERMANOVA) or MPs degradation levels (HDL = LDL, $p > 0.05$ PERMANOVA). Moreover, we performed nMDS analyses using both 18S rRNA and 16S rRNA sequencing data (published in Di Pippo et al. 2020) from all samples to evaluate the effect of sampling site on the plastisphere community as a whole. Results showed that MPs-associated communities clustered depending on lakes (Figure 3) and differences across samples were statistically significant (PERMANOVA, $p < 0.001$).

In particular, the plastisphere community of IS was characterised by most ASVs affiliated to Chloroplastidia (66.6 - 96.3%) (Figure S1b), with the highest contribution for members belonging to families Desmidiaceae (Charophyta, up to 91.3%) and Ulvellaceae (Chlorophyta, up to 47.2%) (Figure S2). Members belonging to Holozoa (up to 14.78%), Alveolata (up to 10.6%) and Stramenopiles (up to 12.7%) were also common, with the co-occurrence of bacteria families Nostocaceae and Burkolderaceae (Figures S3 and 3). Holozoa (0.7- 86.7%), Alveolata (0.2-85.8%) and Stramenopiles (1.6-55.9%) were the main taxa retrieved in almost all plastisphere samples in CO. Within Holozoa, most ASVs were affiliated to Metazoa organisms (Chaetonotida (up to 30.64%), Adinetida (up to 45.5%), Ploimida (up to 23.6%) (Figure S2). Within Alveolata, Ciliophora group was well represented (up to 24.1%), while Peronosporomycetes (up to 44.1%) and Bacillariophyceae (up to 17.4%) were the main components of Stramenopiles (Figure S2). Moreover, plastisphere in CO was characterized by the presence of Rhodobacteraceae and Flavobacteraceae members (Figures S3 and 3). In MA plastisphere, most ASVs were affiliated to Stramenopiles (24.3-62.7%) and Chloroplastidia (9.1 – 51.7%), with the highest contribution from Pennales (Bacillariophyceae, up to 60.3%) and Peronosporomycetes (up to 29.5%) within Stramenopiles, and Scenedesmaceae (up to 19.7%) and Ulvellaceae families (up to 47.2%) within Chloroplastidia (Figure S2). Metazoa (Holozoa, up to 5.5%) and Dinoflagellates (Alveolata, up to 4.2%) were also present. Among

Bacteria, Bacteroidaceae, Clostridiaceae and Rikenellaceae members mainly contributed to the Maggiore lake plastisphere composition (Figures S3 and 3). In PA, the plastisphere community was dominated by Metazoa (24.5 – 50.5%), Chlorophyta (1.2- 30.8) and Dinoflagellata (0.7- 32.4%). Within Metazoa, most ASVs were affiliated to members belonging to Ploimida (up to 23.7%), Copepoda (up to 2.1%) and Monogononta (up to 15.3%), while Ulvellaceae (Chlorophyceae, up to 29.8%) were the main component of Chlorophyta (Figure S2). Among Bacteria, members of Sphingomonadaceae, Deinococcaceae and Rhizobiaceae dominated plastisphere from Paola lake (Figures S3 and 3).

3.4 Microbial core taxa in lake plastisphere

ASVs affiliated to 13 eukaryotic families were present in the plastisphere of all lakes, regardless the polymer type and degradation level. Members of Peronosporomycetes (average 10.3% of total reads), Pennales (8.6%), and Ulvellaceae (5.7%) were the most abundant, although the relative abundance of each core taxon was highly variable across samples, with significant differences across lakes (Kruskal-Wallis test, $p < 0.05$) and no significant differences across polymer types (PE = others, $p > 0.05$. Kruskal-Wallis test for equal median) and PE degradation levels (HDL = LDL, $p > 0.05$).

Moreover, we computed PCoA analysis on the eukaryotic and bacterial core members (i.e. Sphingomonadaceae Burkholderiaceae and Saprospiraceae: Di Pippo et al. 2020) to evaluate the presence of possible associations among core taxa in sampled MPs (Figure 4). Among eukaryotic taxa, relatively closer associations were found between Trebouxiophyceae and families belonging to Ochrophyta and between the Chlorophyceaea families Ulvellaceae and Scenedesmaceae. Notably, inter-domain associations were retrieved between Burkholderiaceae (Gammaproteobacteria) and Pennales (Bacillariophyceae), Sphingomonadaceae (Alphaproteobacteria) and Peronosporomycetes, Saprospiraceae (Sphingobacteriales, Bacteroidetes) and Saccharomycetaceae (Figure 4).

3.5 Occurrence of gene IntI1 and potential pathogens

Class I integron-integrase gene intI1 was present in almost all the analyzed samples and its abundance widely varied across samples, ranging between 1 x 10^{-3} 16S rRNA gene copies (in CO) and 2.66x10^{-2}/16S rRNA gene copies (in MA). Moreover, intI1 values were higher in MPs (6.38 x 10^{-3} ± 1.80 x 10^{-2} 16S rRNA gene copies) than in water samples (1.69 x 10^{-3} ± 3.86 x 10^{-3} 16S rRNA gene copies) (Figure 5).

Except for IS, at least one of the screened pathogens was present in the lake plastisphere. *Legionella* spp. was present on MPs from CO (7.6% of the total screened samples) and PA (14.3%), while *Pseudomonas aeruginosa* occurred in CO (15.4%) and MA (11.1%). *Salmonella* spp. was present only in samples from PA (28.5%). In planktonic communities none of the screened pathogens was retrieved (Table 1).

4. DISCUSSION

The outcomes of this study showed a consistent occurrence of MPs in all samples, with concentrations values comparable to other lakes (Dusaucy et al., 2021; Li et al., 2018) and with a high spatial variability. The shape distribution and composition of retrieved MPs were in line with previous studies on MPs in either freshwater (Fischer et al., 2016; Dusaucy et al., 2021) or marine environments (Pietrelli et al., 2017).

4.1. Eukaryotic community in lake plastisphere

Eukaryotic communities on MPs differed from those in the surrounding water, as also found for prokaryotic communities in marine (Amaral-Zettler et al., 2015; Frere et al., 2018; Kirstein, 2018; Xu et al., 2019) and freshwater plastisphere (Mc Cormick et al., 2014; Mc Cormick et al., 2016; Hoellein et al., 2017; Di Pippo et al., 2020). Our results suggest that plastic particles may select eukaryotic microbes from surrounding water, likely affecting the taxa composition of the plastisphere community.

While primary producers (i.e., Chlorophyta, Charophyta and Bacillariophyta) and facultative mixotrophic microorganisms (Dinoflagellata) mainly composed the eukaryotic communities in water samples, eukaryotic microorganisms from different trophic levels constituted most of MP-associated biofilms. As primary or secondary consumers, different species belonging to Peritrichia and to Oligotrichia were found. Metazoan consumers were also present, regardless the small size of targeted microplastics (i.e., <5mm). The occurrence of members belonging to Monogononta and Bdelloidea (Rotifera), Chromadorea (Nematoda), Copepoda (Crustacea) classes indicate that MPs may offer suitable attachment sites for eggs, larvae and/or juveniles as previously suggested for marine plastisphere (Kettner et al. 2019). Chytridiomycota and Cryptomycota (Fungi), as well as fungal-like organisms, like members affiliated to Peronosperales (Oomycetes), which can have saprotrophic or parasitic life style, occurred in MP-associated biofilms. Members belonging to Dinophyceaea, which can have phototrophic and/or mixotrophic metabolisms, were also present in most of analysed samples.

4.2. Effect of location and substrate type on plastisphere communities

Beside the differences in eukaryotic composition between the plastisphere and planktonic communities, notable marked variations occurred in MP-associated communities between lakes. Geographic differences in bacterial composition of plastisphere have been observed in marine environments at various scales and, to less extent, in freshwater environments (Amaral-Zettler et al., 2015; McCormick et al., 2016; Oberbeckmann et al., 2018; Di Pippo et al. 2020; Barros and Seena 2021). No effect of the polymer type was instead observed on the eukaryotic composition of analysed MPs-associated biofilms. Previous studies reported that plastisphere bacterial diversity can be also affected by polymer types (Oberbeckmann et al., 2016; Kettner et al., 2019). However, the influence of different residence times in the water body and consequently different development age/stage of MPs communities might have masked the selective effect of polymer type on the eukaryotic community. In this study, no significant differences were found between plastic degradation levels,

likely indicating that eukaryotic composition may not be dependent on biofilm aging.

4.3. Plastisphere core microbiome and potential interactions among eukaryotic and bacterial taxa

The presence of a eukaryotic core microbiome in the studied freshwater plastisphere is highlighted in this study, despite the eukaryotic community composition was significantly different among lakes. While the existence of MP bacterial core microbiome was recognised in marine and freshwater ecosystems, eukaryotic core members are largely unknown. Corroborating the hypothesis that MPs may act as pelagic vectors of benthic species, most genera retrieved within the eukaryotic core members have benthic habitus and are known as typical freshwater biofilm colonizers (e.g., genera belonging to Pennales (Bacillariophyceaea, diatoms) and to Scenedesmaceae/Desmidiaceae, (Chlorophyceae, green algae) (Besemer et al., 2012; Battin et al., 2016; Besemer, 2016; Amaral-Zettler et al. 2020). Diatoms commonly occur in biofilms colonizing different plastic types exposed to sunlight in marine environments (Carson et al., 2013; Masò et al., 2016; Oberbeckmann et al., 2016; Amaral-Zettler et al. 2021). In particular, filamentous pennate diatoms (Pennales) were reported to colonize marine plastics at early stage of biofilm development, owing to their known capacity of producing complex adhesive exopolymeric substances, which facilitate the colonization process (Underwood et al., 2004).

Despite the significant impact of sampling site on the core microbiome composition, a number of inter and intra-domain associations between core members was retrieved even though a significant co-occurrence not prove for a microbial interaction. Many microorganisms can co-exist together because of similar environments preference by occupying the same niches (e.g., phototrophs belonging to the algal families Scedesmaceae and Ulvellaceae, Trebouxiophyceae, and Ochrophyta). However, the presence of the co-occurrence pattern of Burkolderaceae (Gammproteobacteria) and Pennales (Bacillariophyceae) may be explained by the capacity of Burkolderaceae of degrading complex organic matter (including exopolymeric substances), produced by oxygenic phototrophic microorganisms also in freshwater biofilms (Zancarini et al., 2017; Besemer, 2016). Moreover, the

association found among members of Saprospiraceae (Bacteroidetes) and Saccharomycetaceae (Fungi) could be due to the ability of bacterial biofilm members to break down complex carbon substrates, allowing available organic carbon to be utilised by fungal microorganisms (Raghukumar and Damare, 2011).

4.4. Relevance of MP colonization for aquatic ecosystems

The increasing interest on marine plastisphere is also due to the evidence that the colonization of MPs can have implications for aquatic ecosystems (Amaral-Zettler et al., 2021; Kettner et al., 2019). Recent investigations indicated MPs as a novel pelagic habitat for benthic microbes (Haram et al., 2021), by harbouring potential pathogenic species (Zhang et al. 2022). However, no data are available on the role of MP-associated biofilms as possible vectors of harmful, parasitic, and pathogenic organisms in freshwater ecosystems. As we have hypothesized, several ASVs recalling the occurrence of potential harmful microorganisms were found onto MPs found in lake waters.

Notably, HAB (Harmful Algal Bloom)-associated taxa were detected in PA, although they were present both in the planktonic and plastisphere communities. Members belonging to the genus *Gonyaulax* (Gonyaulacales, Dinophyceae) were found (up to 55.0% in water samples and up to 8.2% in biofilm communities). The marine planktonic genus *Gonyaulax* includes toxic species, frequently responsible for red tides and able to excrete saxitoxins. The unexpected occurrence of this planktonic dinoflagellate on MPs might be explained if they attach to MP as resting cysts. Many phytoplankton species, including many HAB species, survive long periods between blooms by forming benthic resting stages, that can germinate in response to the combination of favourable environmental conditions (Brosnahan, et al., 2020). The possibility that MPs can act as vectors for planktonic HAB dinoflagellates as cysts, transporting them over kilometres, may have serious ecological and human health implications. ASVs of genera affiliated to Gonyaulacales (i.e., *Ceratium* and *Peridinium*) were also found in the plastisphere colonizing MPs from IS and MA. Although allelopathy has been recognised for one *Peridinium* species (Rengefors and Legrand, 2001), both these taxa are generally

considered harmless. Allelopathy is also common in different planktonic and benthonic cyanobacterial species able of producing cyanotoxins that are among the most toxic naturally occurring compounds (Plaas and Paerl, 2021). ASVs affiliated to planktonic cyanobacteria species as *Planktothrix rubescens* were not found in water samples. On the other hand, different toxic cyanobacteria genera with benthic habitus were present on most of the lake MPs, including members of *Pseudoanabaena*, *Leptolyngbya*, *Calothrix* and *Phormidium* (Di Pippo et al. 2020). This finding may pose a serious concern since lakes are used as source of drinking water or for recreational activities. In any case, although it is not possible to prove that any of the reported microorganisms are effectively harmful since the production of both algal and cyanobacterial toxins depends on different environmental conditions (WHO, 2021), the presence of microbial taxa of health concern can indicate a potential risk.

ASVs affiliated to potentially parasitic eukaryotes, as Ascomycota, Fungi or fungus-like microorganisms belonging to Peronosporomycetes (Oomycota) were present on MPs. Different members of Saccharomycetaceae (e.g. *Candida* genera) Peronosporomycetes (*Pithium* and *Phytophthora* genera) are known human and/or plant parasite (Kettner et al. 2019).

Previous studies on marine plastisphere showed the presence of members of the genus *Vibrio* as well as other potentially pathogenic bacterial taxa (for example, members of Campylobacteraceae) in MPs samples, in both temperate and tropical marine environments, as well as in freshwater (Amaral-Zettler et al., 2020; Zhang et al. 2022). Additionally, MPs and their associated biofilms have also been described as hotspots of horizontal gene transfer, thus increasing genes exchange between different bacteria and potentially facilitating of antibiotic resistance transfer in the environments (Eckert et al., 2018; Imran et al. 2019).

Here, we showed the common presence of biofilm-forming opportunistic pathogens, such as *Legionella* spp., *Pseudomonas aeruginosa*, *Salmonella* spp. in the lake plastisphere. Therefore, MPs could be a vector of enteric and opportunistic pathogens transport in freshwaters. In line with studies on urban rivers MPs (Wang et al., 2020), we also showed that plastisphere samples harboured *intlI*

gene at higher level than water. Integron-integrase genes are considered important indicators of horizontal gene transfer and in particular, the class I integron-integrase gene represents a proxy for anthropogenic ARG's contamination (Gillings et al. 2015). Class I integrons commonly harbour gene cassettes associated with antibiotic resistance and have been often found on mobile genetic elements (MGEs) such as plasmids and transposons allowing their horizontal gene transfer (HGTs) (Gillings et al., 2008; Labuschagne et al., 2008). We hypothesized that plastisphere can constitute an environmental niche shared by environmental bacteria and pathogens were the physical barrier between donor and recipient bacteria is no longer present and HGT can more frequently occur. Further studies should be addressed to evaluate different ARGs presence and abundance of MPs occurring in lakes to have a clearer picture of the risks associated to MPs presence in lentic ecosystems. Moreover, it should be important to evaluate the contribution of extracellular DNA (eDNA) to the propagation of ARGs through MPs. Biofilm cells are indeed immersed in an exopolymeric matrix, where the polymer structure would favour the enrichment of eDNA-associated ARGs, and promote the acquisition and dissemination of ARGs (Gillings et al., 2009).

Conclusions

- The target lakes showed a significant presence of MPs, with concentrations, shape, and chemical composition similar to those found in other freshwater ecosystems.
- Lake plastisphere comprised microorganisms belonging to different trophic levels, with taxon composition differing from that of planktonic communities and among lakes.
- MPs-associated biofilms shared a core microbiome, constituted by eukaryotic and bacterial biofilm-formers and with inter and intra-domain associations.
- Plastisphere hosted a number of potential harmful, parasitic and pathogenic organisms, along with antibiotic resistance elements. Even though we cannot prove that any of the organisms we report are harmful, their presence can be considered an indication of potential ecological

and health risks. Future research should ascertain whether any of the potential pathogens and HAB taxa are truly harmful.

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Table 1. Pathogenic bacteria found in plastisphere and planktonic samples

	Salmonella spp.	*Legionella* spp.	*Legionella pneumophila*	*Pseudomonas aeruginosa*
IS	**ND**	**ND**	**ND**	**ND**
CO	**ND**	**7.6%**	**ND**	**15.4%**
MA	**ND**	**ND**	**ND**	**11.1%**
PA	**28.5%**	**14.3%**	**ND**	**ND**
W IS	**ND**	**ND**	**ND**	**ND**
W CO	**ND**	**ND**	**ND**	**ND**
W MA	**ND**	**ND**	**ND**	**ND**
W PA	**ND**	**ND**	**ND**	**ND**

ND: non-detected; Frequencies of detection in MPs and water (W) samples

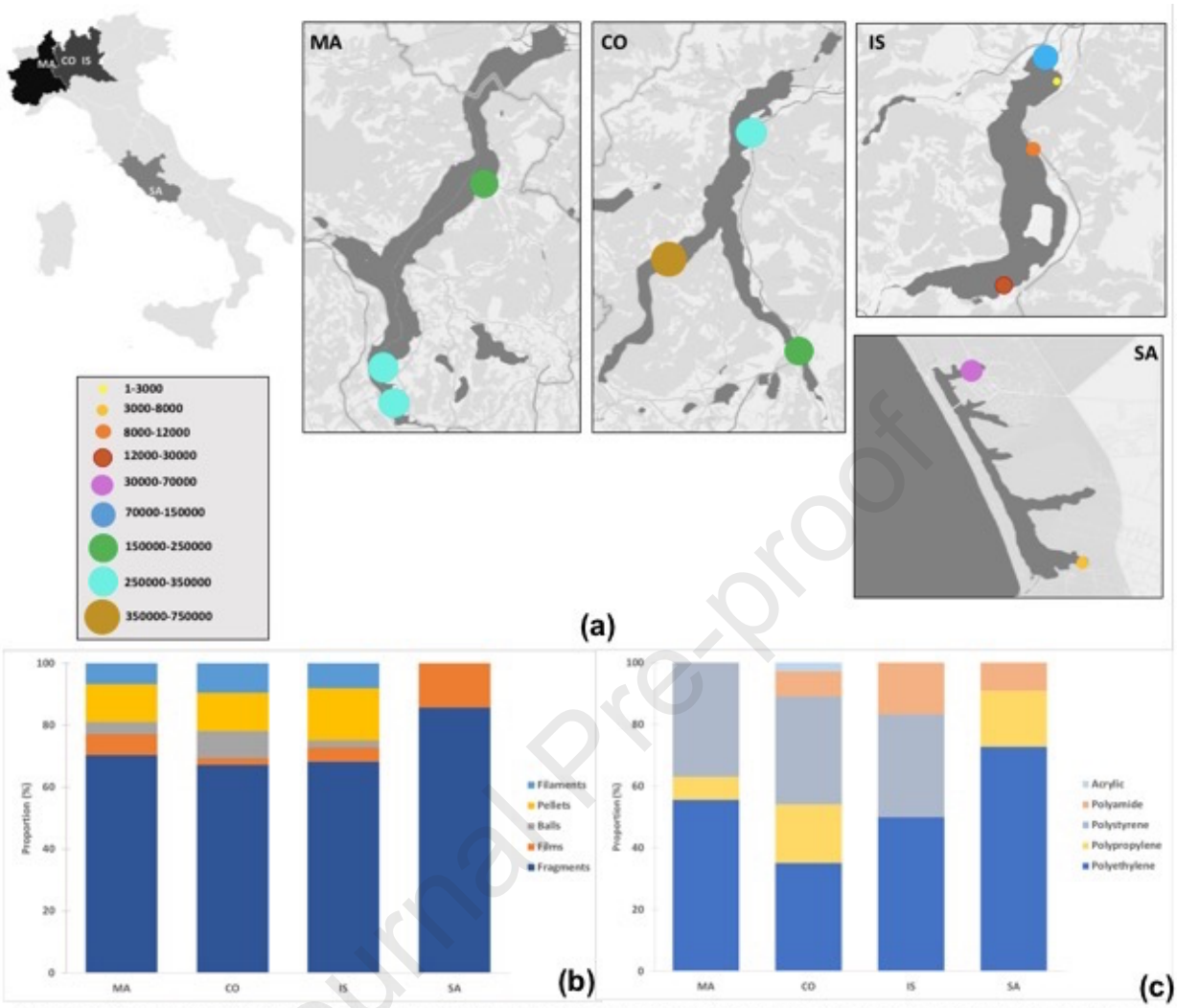

Figure 1. Abundance, distribution and types of MPs. (a) Map of the four lakes in Northern and Central Italy showing the location of sampling transects and MPs concentrations (items km^{-2}). The size and colour of the circles are proportional to measured concentration values. (b) Lake differences in the percentage of the 5 different categories of MPs. (c) Lake differences in the percentage of polymer types for MPs.

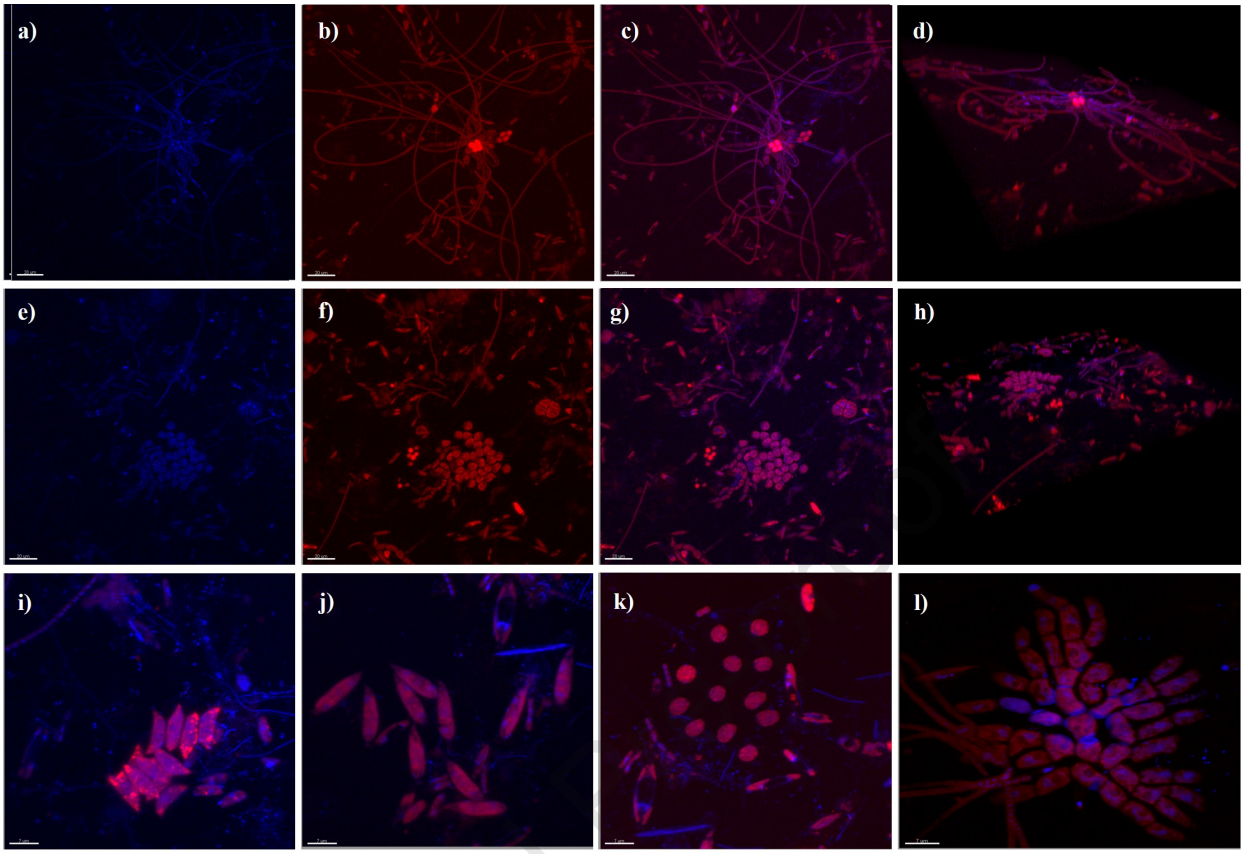

Figure 2. CLSM micrographs of different portions of plastic-associated biofilms sampled from Lake Iseo. a) Total DAPI stained cells (blue signal); b) Autofluorescence of chlorophyll *a* (red signal) of both filamentous Cyanobacteria and microalgal chloroplasts; c) overlapping of the two acquired images; d) 3-D reconstruction of image; e) Total DAPI stained cells (blue signal); f) Autofluorescence of chlorophyll *a* (red signal); g) overlapping of the two acquired images h) 3-D reconstruction of image g; i) total DAPI stained cells (blue signal) and autofluorescence of chlorophyll *a* (red signal) of members of Desmidiaceae family and of diatoms; j), k), l) members of Chlorophyceae and diatoms detected with autofluorescence of chlorophyll *a* (red signal) of chloroplasts.

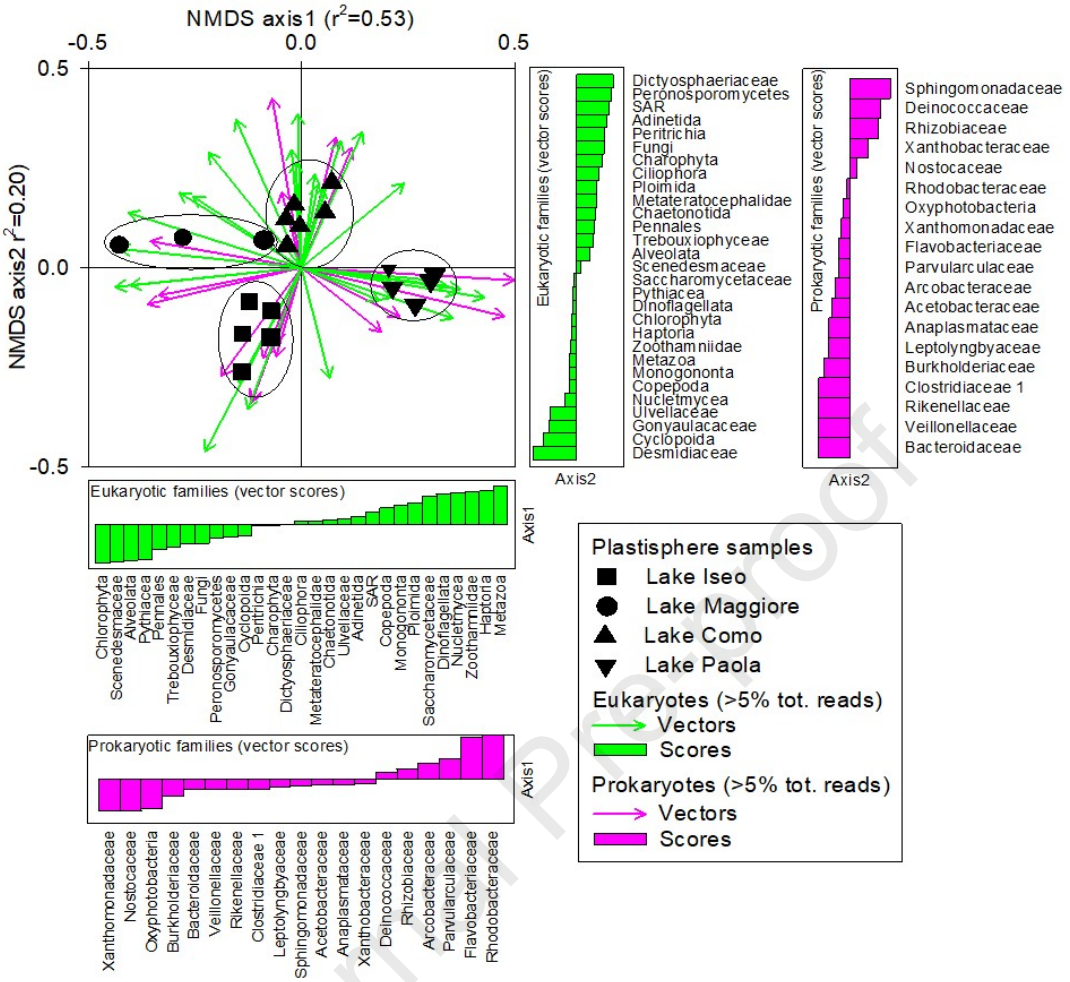

Figure 3. Nonmetric Multi-Dimensional Scaling ordination plot representing the related distribution of eukaryotic and prokaryotic communities at the family level in the four lakes. The vector length is proportional to the contribution of the relative abundance of each microbial taxon to the nMDS-axes.

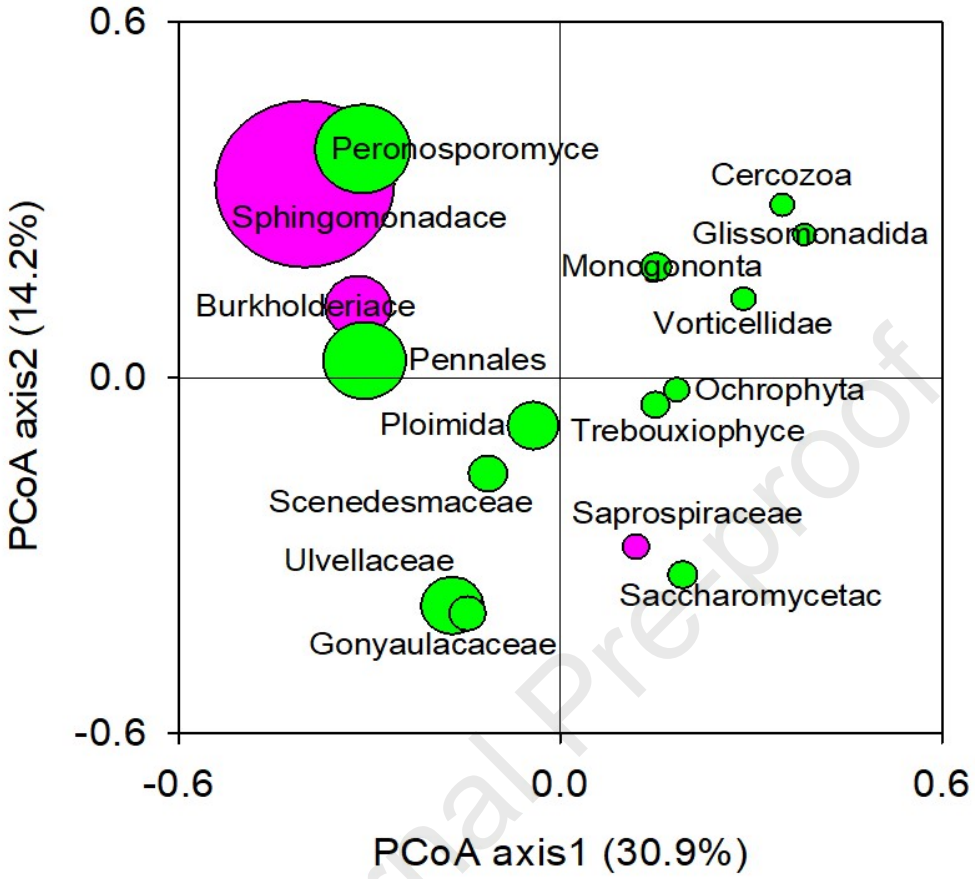

Figure 4. Principal Coordinate Analysis (PCoA), based on the Bray-Curtis similarity index, of eukaryotic (green dots) and prokaryotic (pink dots) core taxa at the family level. The size of dots is proportional to the relative abundance of each core taxa (average %) to the total reads. Taxa ordinated closer to one another showed more similar variation patterns than those ordinated further away.

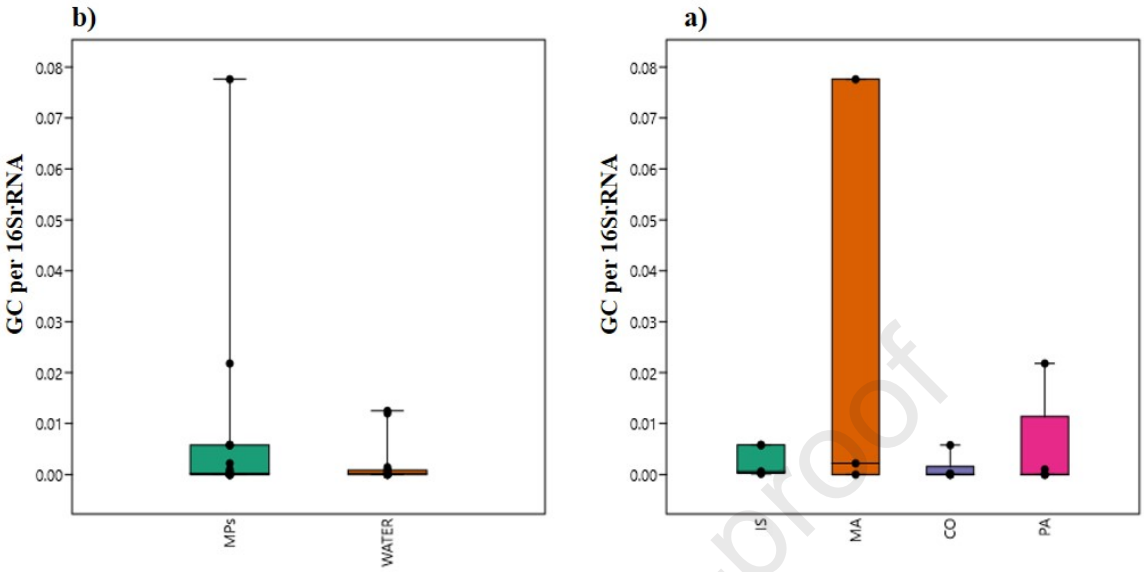

Figure 5. Relative abundances of *intI1* in water and microplastic samples.

Highlights

- Microorganisms of different trophic levels constituted plastisphere in lakes
- Eukaryotic plastisphere differed from planktonic communities and among lakes
- Plastisphere shared a core microbiome
- Plastisphere hosted potential harmful, parasitic and pathogenic organisms

Author statement

"Eukaryotic diversity in lake plastisphere: potential ecological and health implications"

All authors contributed to and approved the final form of this publication and take responsibility for the accuracy of the data and analysis.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: