A Pan-European study of the bacterial plastisphere diversity along river-to-sea continuums

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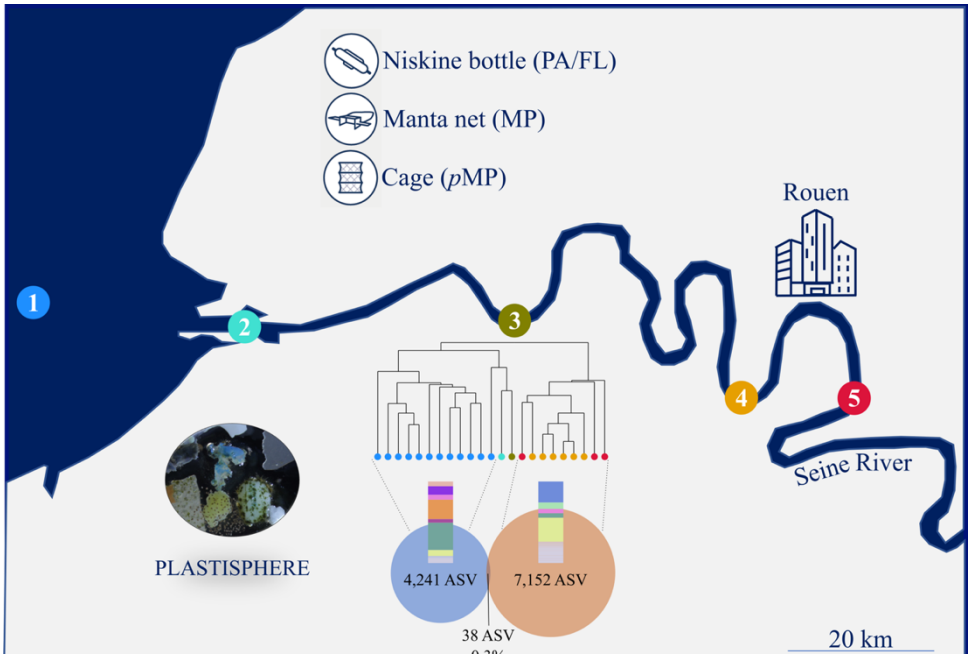
Abstract :

Microplastics provide a persistent substrate that can facilitate the transport of microbes from one ecosystem to another. Since most marine plastic debris originates from land and is carried to the ocean by rivers, a significant concern about the plastisphere is the potential dispersal of freshwater bacteria into the sea. To address this question, we explored the plastisphere on microplastic debris (MPs) and on pristine microplastics (*p*MPs) as well as the bacteria living in surrounding waters, along the river-sea continuum in nine major European rivers sampled during the seven months of the *Tara Microplastics* mission. In both marine and riverine waters, we found a clear niche partitioning among MPs and *p*MPs plastispheres when compared to the bacteria living in the surrounding waters. Among the large dataset, we found a clear gradient of bacterial community structure from the freshwater to the sea, with a complete segregation in plastisphere composition between the two ecosystems. We also described for the first time a virulent human pathogenic bacteria on MPs (*Shewanella putrefaciens*) able to infect human intestinal epithelial cells, that was only detected in river. Our results reinforce the major role played by the environmental conditions in shaping plastisphere biodiversity, that is not consistent with a critical transfer of pathogens between freshwater and seawater ecosystems.

Graphical abstract:



Highlights :

- Almost complete segregation between seawater and freshwater plastispheres
- Salinity is the main driver of plastisphere communities in the river-to-sea continuum
- Evidence of human bacterial pathogen on microplastics in river
- Plastisphere niche partitioning is a common feature in the river-to-sea continuum

1. Introduction

Microorganisms living on microplastic debris (MPs) have received a growing attention since the characterization of a distinct and very diverse community, called the ‘plastisphere’, as compared to the microorganisms living in the surrounding seawater (Zettler et al., 2013). MPs released into the marine environment provides a new habitat that is rapidly colonized by microorganisms (Harrison et al., 2014). The microbial biomass harbored on MPs can be significant, up to 6 % of the total mass of the MP (Morét-Ferguson et al., 2010). The biomass of the known plastisphere has been previously estimated from approximately 1,000 to 15,000 metric tons, corresponding to a range of 0.01 to 0.2 % of the microbial biomass in the open ocean surface waters (Mincer et al., 2016). This number is probably underestimated given recent estimates of microplastic concentrations in the ocean surface that have been reassessed from 5 to 24.4 trillion pieces (Isobe et al., 2021).

The ecological impact of this anthropogenic microbial niche is still largely unknown. Trace nutrients are concentrated onto the plastic surface, making them more bioavailable for microbial phototrophs and heterotrophs that play a crucial role in the carbon biogeochemical

cycle (Conan et al., 2022). Interactions between bacteria and the large diversity of parasitic and saprophytic microeukaryotes may also impact the carbon processing within the new plastic habitat (Kettner et al., 2019, Amaral-Zettler et al., 2021). MPs provide a durable substrate for marine life, facilitating the transport of microorganisms over long distances. They also trigger colonization and dissemination of harmful species such as toxic algal species causing harmful blooms (Masó et al., 2003) or putative human pathogenic bacteria (Laverty et al. 2020) and fungi (Ormsby et al. 2023). Physical oceanographic models showed that plastic can migrate over 1,000 km in less than 2 months (Law et al., 2010). However, the colonization dynamic and resilience to environmental changes and stressors with time and across large geographical regions remain largely unknown (Amaral-Zettler et al., 2020). Environmental changes have been shown to drastically affect the plastisphere composition (Basili et al., 2020), suggesting that plastic-attached communities could undergo drastic changes when transitioning from one ecosystem to another.

Research efforts have mainly focused on investigating the plastisphere in the marine environment, under the assumption that the ocean is the ultimate sink for plastic pollution (Martin et al., 2020). Less attention has been given to riverine waters despite the fact that about 80% of marine plastic debris are believed to originate from rivers, with an annual transfer of 500 kilotons over the year 2020 (Kaandorp et al., 2023). There is now increasing evidence that many rivers across the globe exhibit significant higher microplastics concentrations than the marine environment (Eriksen et al., 2013; Weiss et al., 2021). The plastisphere in freshwater ecosystems was also found as a specific niche for microorganisms when compared to the surrounding riverine waters (Yang et al., 2020). In a review paper, Barros and Seena (2021) indicated that some plastisphere microbes, including pathogenic bacteria, are detectable in both freshwater and marine systems. The potential transfer of pathogens between the two ecosystems has been hypothesized and emphasized to the shelter provided by the biofilm growing on plastics. This has been evidenced under laboratory conditions (mesocosm) by the detection of human pathogens bound to microplastics, highlighting their survival during the transition from freshwater to marine conditions (Metcalf et al., 2023).

Most studies have focused on incubation experiments using pristine microplastics (pMPs) with known polymer types, and relatively few have examined communities on environmentally collected microplastic debris (MPs) in freshwater or marine environments (Amaral-Zettler et al., 2020; Barros and Seena, 2021). Some studies used postconsumer plastics, such as PET bottles or plastic bags (Muthukrishnan et al., 2019; Oberbeckmann et

al., 2016), whereas others used industrial *p*MPs from known manufacturing sources such as industrial primary microplastic pellets (Metcalf et al., 2023). The diversity of experimental designs makes it difficult to directly compare studies, albeit some of them compared results with environmentally collected MPs. For example, a minimum of one-month incubation in seawater corresponded to the development of a mature biofilm that presented similarities to environmentally collected MPs (Dussud et al., 2018a, Dussud et al., 2018b, Odobel et al., 2021).

In this study, we explored the plastisphere bacterial communities along river-to-sea continuums in nine major European rivers sampled during the seven months of the *Tara* Microplastics mission (Ghiglione et al., 2023). We tested the hypothesis of a transfer of microorganisms (including putative pathogens) together with MPs rafting along a salinity gradient from the sea, the outer estuary, and downstream and upstream of the first heavily populated city. We also incubated pristine microplastics (*p*MPs) at each sampling site during one month prior the mission, in order to compare bacteria living on MPs and *p*MPs to the free-living (FL) and organic particle-attached (PA) bacteria living in the surrounding water at the same sampling site.

2. Material and methods

2.1. Sampling design

The *Tara* Microplastics mission was conducted for 7 months along nine major rivers in Europe (Ghiglione et al., 2023). Harmonized sampling methodologies were used over 45 sampling sites in nine European rivers. Four to five sampling sites were selected along a salinity gradient from the sea (station 1) and the outer estuary (station 2) to intermediate salinity (station 3), and downstream (station 4) and upstream (station 5) of the first heavily populated city located on each river, including London on the Thames, Hamburg on the Elbe, Rotterdam on the Rhine, Rouen on the Seine, Nantes on the Loire, Bordeaux on the Garonne, Tortosa on the Ebro, Arles on the Rhone, and Rome on the Tiber (**Suppl. Table 1**). Only 4 stations were sampled in the Thames and Rhine rivers, with the intermediate-salinity station missing. Water samples and MPs were taken onboard the French research vessel (RV) *Tara* or from a semi-rigid boat in shallow waters.

Water sampling was performed at each sampling station and just prior to the concomitant 330-µm manta trawl deployments. An 8-L Niskin bottle was triggered just below the surface and water subsamples were transferred to a set of specific devices for nutrients, particulate matter and bacterial diversity analyses. For the latter, one or two liters (depending

on turbidity) of 25 µm prefiltered water (Nylon mesh) were successively filtered onto 3 µm and 0.2 µm-pore size polycarbonate filters (47 mm diameter, Nucleopore) and the filters were stored at -80°C before DNA extraction of both organic-particle-attached bacteria (PA) and free-living bacteria (FL), respectively.

Sampling for MPs was conducted using a 330-µm mesh size manta trawl (aperture of 30 × 80 cm, 2.5 m long nylon net, and 30 × 10 cm^2 weighted cod end). The manta trawl was deployed at an approximate speed of 2.0 knots for 60 min in seawater and 10 min in rivers, in order to avoid clogging (especially in rivers). After carefully rinsing of the net with water from the sampling site, macro-debris of natural origin (*e.g.*, algae, branches, leaves) were eliminated through rinsing above the collector. MPs of approximately 1 to 5 mm in size accumulated in the final volume of 1.0 L of the collector were transferred into glass Petri dishes, observed under a binocular magnifying glass, sorted using alcohol and flame-sterilized forceps and immediately frozen in liquid nitrogen and stored at -80°C until further DNA extraction and chemical identification by attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR).

A team on land was dispatched one month before the arrival of *Tara* for site reconnaissance and for the deployment of cage structures (30 cm x 10 cm cylinder) containing pristine microplastics (*p*MPs) for the *in-situ* colonization experiment. Around 10g of pellets made of polyethylene (PE), polyoxymethylene (POM), and Nylon mesh (polyamide-6,6; NYL) were separately immersed during one month at the same sampling site as water collection and manta trawl deployments, sorted using alcohol/flame sterilized forceps and immediately frozen in liquid nitrogen and stored at -80°C until further DNA extraction.

2.2. Temperature, salinity, nutrients, suspended particulate matter and particulate organic matter

A thermosalinograph (TSG, Seabird SBE45) was installed onboard the RV *Tara* for surface temperature and conductivity measurements at a sampling frequency of 0.1 Hz. Discrete vertical measurements from 0 to 30 m depth (or less in shallow waters) were also made at each sampling station using a portable Sontek CastAway CTD probe (ADCPro, France) attached to the rope holding the 8-L Niskin bottle. The analytical precision was 0.01°C for temperature and 0.01 to 0.05 for salinity.

Nutrients were analyzed from a 18 mL subsample of water filtered through a glass syringe fitted with a Whatman AnoDisc-Paradisc 0.45 µm filter and placed in a 20 mL polyethylene scintillation vial. Another 8 mL were placed in a 20 mL polyethylene

scintillation vial for ammonium (NH_4+) analysis. Nitrate (NO_3^-), nitrite (NO_2^-), phosphate (PO_4^{3-}), and dissolved silica ($Si(OH)_4^-$) concentrations were measured on a continuous flow Seal-Bran luebbe® AutoAnalyzer III, whereas NH_4+ determinations were performed by fluorimetry on a Jasco FP-2020 fluorimeter (Holmes et al. 1999). The analytical precision of NO_3^-, NO_2^-, PO_4^{3-}, and $Si(OH)_4^-$ is ± 0.02 µM, ± 0.01 µM, 0.02 µM, and ± 0.05 µM, respectively, and ± 5 nM for NH_4^+.

Suspended particulate matter (SPM) was determined from water subsamples (from 100 to 500 mL) filtered on pre-combusted glass fiber filters (Whatman GF/F, 47 mm, precombusted at 450°C during 12 h), dried at 60 °C and stored in a desiccator until further analysis. SPM concentrations were determined by differences between the dry weights of the respective filters before and after filtration. The POC measurements were performed using a high-combustion procedure with a Leco CN 2000 elemental analyser (detection limit: 0.1 mg of C) after decarbonatization through repeated additions of 100 µL of HCl 25 %, separated by 60 °C drying steps until no effervescence was observed.

2.3. DNA extractions, PCR, sequencing and sequences analysis

DNA extractions were performed using a classical phenol-chloroform method with slight modifications, as previously described (Rodriguez-Blanco et al., 2009). A preliminary amplification of the full length 16S rRNA gene was performed with the Phusion High Fidelity Polymerase Chain Reaction (PCR) Master Mix with GC buffer (ThermoFisher Scientific) using 27F and 1492R primers, followed by an amplification of the V4-V5 region using 515Y and 926R primers (Parada et al., 2016) with Illumina-specific barcodes.

Sequencing was performed on Illumina Novaseq by Genoscope (Evry, France). Raw FASTA files were deposited at NCBI with accession numbers PRJEB72022 (ERX11897590-ERX11897459). Sequences analysis was done using the DADA2 pipeline (Callahan et al., 2016) for ASV establishment and taxonomy assignment. Taxonomic assignment was done using the SILVA 138 SSU database. ASV that did not belong to the Bacteria kingdom as well as ASV from chloroplasts and mitochondria were removed from the dataset. The number of sequences per sample was normalized by rarefaction (n=14,706) for sample comparison. All further analyses were performed in the resampled ASV table containing 137,948 ASV in 316 samples.

2.4. ATR-FTIR analysis

An attenuated total reflectance-Fourier transform infrared spectrometer (ATR-FTIR Vertex70v, Bruker, ATR Golden Gate) was used to determine the polymer composition and chemical characteristics of the sorted MPs. FTIR spectra were identified using POSEIDON software (Kedzierski et al., 2019). Analyses were performed using the following parameters: 32 scans, 4 of resolution and large scale from 4000 to 600 cm^{-1}.

2.5. Bacterial culture, bacterial virulence, biofilm formation and taxonomic affiliation

For some sampling stations in the Rhine (stations 1 and 4) and Loire (stations 1 and 5) river-to-sea continuum, supplemental manta trawls were deployed at the same sampling sites described above in order to collect MPs for bacterial culture. Each sorted MP was rinsed and transferred into 5 mL of L1+Si medium (Guillard and Hargraves, 1993) containing 30 $g.L^{-1}$ of red salt (L1-RS30) for the marine stations or no red salt for the freshwater stations. Samples were incubated at 18°C for 24h after addition of 1 mL of Marine Broth /100 medium (0.05 $g.L^{-1}$ bactopeptone, 0.01 $g.L^{-1}$ yeast extract with or not 30 $g.L^{-1}$ of red salt) and then plated on Marine Agar /100 (MB/100 with 15 $g.L^{-1}$ bactoagar) with the appropriate amount of salt (RS30 for station 1 or RS0 for station 5) and incubated at 18°C for one week. Different morphotypes (around 25 per rivers) were selected for further colony isolation.

Bacterial virulence of the selected strains was tested by tissue culture assays using the Caco-2 cell line, as previously described (Dias et al., 2019). Briefly, bacterial adhesion/invasion assays were performed by incubating a mix of 10^8 $CFU.mL^{-1}$ of the tested bacteria (OD 0,6) with monolayers of confluent Caco-2 cells (7.5 10^5 cells per well) in sterile 12-well plate containing 1mL of Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher) for 1h at 37°C, under 5% of CO_2. To assess the number of adhesive and invasive bacteria, cells were then lysed with Triton 0.5% (ThermoFisher) after three washing steps with 1 mL of Phosphate Buffer Saline (PBS) 1X in order to get rid of non-adhesive bacteria and the colony forming units (CFU) were counted by serially dilution and plating on lysogeny broth (LB) agar medium. To assess only the invasive bacteria, cells were treated with 50 $\mu g.mL^{-1}$ of gentamycin for 1h at 37°C and CFU were counted as above on LB agar plates. Four biological replicates were tested with three measures per condition (n=12) for further statistical tests using Fisher T-test analysis. Control experiments were done in parallel and in the same conditions by using the reference strain *Shewanella putrefaciens* previously isolated from red deer faeces kindly provided by Maria José Saavedra (Dias et al., 2019).

Biofilm formation capability of the selected strains was assessed by using crystal violet dye, as previously described (Couvigny et al., 2015). Incubation was done using 100 µL of LB culture (initial concentration of 10^8 CFU.mL^{-1}) in polypropylene (PP) or polystyrene (PS) 96 well plates for 48 h at 37°C. Bacteria were dumped, washed and incubated with 0.1% of crystal violet dye (Sigma Aldrich, V5265) and washed again three times to remove the unbound crystal violet. The 96 well plates were then air-dried under chemical hood at room temperature before adding 30% acetic acid in each well to solubilize crystal violet and optical density was measured at 550 nm with microplate reader (SpectraMax, Molecular devices, LLC, USA). Four biological replicates were done with 16 measures per condition (n=64). Statistical significance was assessed by comparing the mean of each condition using one-way ANOVA analysis.

Genomic DNA was extracted from selected colonies with Wizard® Genomic DNA Purification Kit (Promega, France). Purified PCR products (AmpliClean™ Cleanup Kit) of the amplified 16S rRNA genes (27Fmod and 1492Rmod primers, Eurofins MWG Operon, Ebersberg, Germany) were sequenced by Sanger technology on the Bio2Mar platform (Observatoire Océanologique, Banyuls-sur-Mer, France) using primer 907R (Eurofins MWG Operon, Ebersberg, Germany) with ABI 3130 genetic analyzer (Life Technologies, Carlsbad, California, USA), as previously described (Tourneroche et al. 2019). The quality of each sequence was checked manually and the closest match in NCBI databases was determined by BLAST (Altschul et al., 1990). Further, sequences were aligned in Muscle, as implemented in MEGA 7.0 (Kumar et al., 2016). Alignments were reviewed manually to verify mismatches, and a phylogenetic tree was constructed by maximum likelihood using the K2, G+I model. The reliability of each node in the tree was assessed by bootstrapping over 500 replicates.

2.6. Data management and statistical analysis

Data were treated with R version 4.3.1. Graphical representations were done using the *ggplot2* version 3.4.2 (Wickham, 2016) and the *vegan* version 2.6.1 (Dixon, 2003) packages. Kruskal-Wallis and Dunn tests (Holm-Bonferroni correction) were done with the *stats* version 4.3.1 and *rstatix* version 0.7.2 packages respectively. Resampling and calculation of alpha-diversity indexes were done using the *phyloseq* package version 1.44.0 (McMurdie and Holmes, 2013). The Bray-Curtis dissimilarity matrix, NMDS and PERMANOVA tests were done using the *vegan* package. Post-hoc pairwise analyses were done using the *pairwiseAdonis* package version 0.4.1 with Holm-Bonferroni correction. Similarity Percentage analysis (SIMPER) was performed to identify the contribution of each ASV, using

PRIMER 6. Clustering was done using UPGMA method with the *stats* package version 4.3.1. Venn diagrams were done using the *MicEco* package version 0.0.19.

3. Results

3.1. Chemical nature of MPs and environmental variables

We visually sorted 115 MPs (ranging size 1 to 5mm) in order to obtain 0 to 12 pieces per sampling station (mean = 2.6, SD=3.4, n=44 sampling sites). ATR-FTIR analysis revealed that polyethylene (PE) dominated the composition of the MPs (45.4%), followed by polypropylene (PP; 11.7%) and polystyrene (PS; 8.4%), Polyethylene vinyl acetate (PEVA), Polyacrylic (PA) and polyvinyl chloride (PVC) were minority (3.1%, 2.1% and 0.4%, respectively). Unidentified polymers represented 29% of the collected pieces and were categorized as "unknown" (**Suppl. Fig. 1**). The same tendency was found in between marine (station S01; PE=60.0%; PP=26.6%; PS=1.7%) and freshwater stations (S04 and S05; PE=39.5%; PP=18.1%; PS=13.1%). Exception were found for Ebre (S01), Seine (S04), Tiber (S02) and Garonne (S01 and S04) with abundant PS pieces (100%, 29%, 57%, 25% and 30%, respectively).

Environmental variables were followed in all rivers, except for nutrients (NO_3^-, NO_2^-, NH_4^+, PO_4^{2-},) and silica that were not measured in the Garonne, Loire and Tiber rivers (**Suppl. Table 1**). Salinity was from 30.0 to 34.0 in Atlantic seawaters and from 37.1 to 38.3 in Mediterranean seawaters. Salinity gradient was marked in all rivers until less than 2.0 for downstream and upstream stations, except for downstream London (Thames) that was still influenced by Atlantic seawater (19.3). Temperature remained stable in each river, with relatively low difference between seawater and freshwater (Δ=3.4°C), except for the Seine river (Δ=9.2°C). The evolutions of the particulate organic carbon (POC) in the river-to-sea continuum were more chaotic, being sometimes higher in seawater (Elbe, Rhine, Seine) and sometimes higher in intermediate of freshwaters. Correlation was found between silica and total nitrogen (Σ NO_3^-, NO_2^-, NH_4^+) (Spearman rank, $p<0.05$). Nutrients and silica, as well as suspended particulate matter (SPM) were always one to two orders of magnitude lower in seawater as compared to the corresponding river (including estuarine, intermediate and freshwater).

3.2. Bacterial alpha-diversity

Alpha-diversity was assessed by calculation of Chao1, Pielou (appendix 2.2) and Shannon indexes (**Fig. 1 and Suppl. Fig. 2**). Overall, free-living bacteria (FL) present the lowest Shannon diversity values (median = 4.8 ± 0.58, n=42) as compared to the other samples (median = 5.4 ± 0.78, n = 39 and median = 5.4 ± 0.86, n = 104, for PA and *p*MPs, respectively), except for MPs sampled at sea (stations 1) (median = 4.0 ± 1.2, n = 28). Regardless of the sampling site, highest Chao1 values were systematically found for the PA bacteria samples (median values between 1,323 and 3,013, n = 39) as compared to other samples (median values between 602 for MPs at station 1 and 2,177 for *p*MPs at station 5, n = 28 and n = 11 respectively).

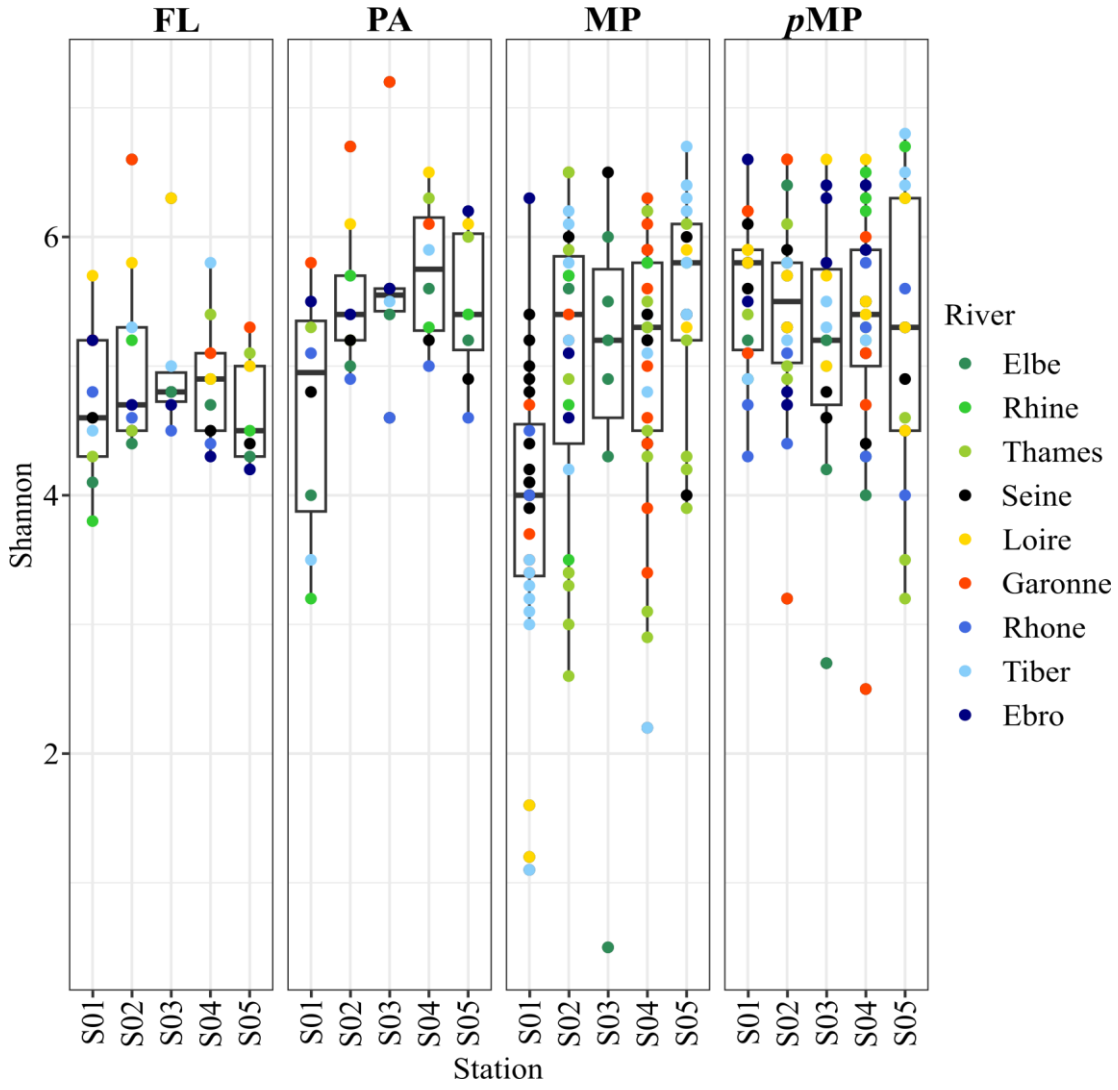


Figure 1. Shannon indexes at each station (S01 to S05), all rivers considered (with different colors). PA refers to the particle-attached bacteria, FL to free-living bacteria, MP to floating microplastics and *p*MP to one-month plastispheres growing on pristine plastics.

Nonparametric pairwise multiple comparisons in independent groups using Dunn's test showed no significant difference in all diversity indexes when compiling water (FL and PA) and plastic (MPs and *p*MPs) samples at any of the stations (Holm-Bonferroni corrections, $p > 0.5$). All diversity indexes were significantly lower for MPs sampled at sea (stations 1) as compared to those sampled in freshwater, upstream from the city (station 5) (Dunn tests with Holm correction; *p*-value = 2.2×10^{-4}, *p*-value = 1.7×10^{-3} and *p*-value = 3.9×10^{-4} for Chao1, Pielou and Shannon indexes, respectively). This was not the case for *p*MPs samples, which did not show significant difference of diversity indexes within and between rivers. No significant difference in diversity indexes was found between MPs and *p*MPs groups, except for sea samples (station 1), for which significantly lower Chao1, Pielou and Shannon diversity were observed for MPs (*p*-value = 0.021, *p*-value = 2.1×10^{-6} and *p*-value = 7.9×10^{-6} respectively).

3.3. Bacterial beta-diversity

NMDS based on Bray Curtis similarity showed clear distinction in community structure between samples taken at sea (station 1) and freshwater samples (stations 4 and 5), with intermediate similarities for samples originating from the estuarine (station 2) and intermediate salinity stations (station 3) **(Fig. 2)**. A PERMANOVA test confirmed that the sampling station/ river-to-sea continuum drove the entire dataset of bacterial community structure ($R^2 = 0.050$, *p*-value = 0.001), with significantly lower difference between stations 4 and 5. Bacterial communities associated with plastics (both MPs and *p*MPs) differed significantly from the surrounding water communities (both FL and PA fractions) at each station. No significant difference was found between FL and PA community structures at each different station ($p > 0.05$) and within the rivers ($p > 0.05$). Another driving factor was the river origin that significantly explained the community structures ($R^2 = 0.087$, *p*-value = 0.001).

Bacterial communities associated with plastics (both MPs and *p*MPs) differed significantly from the surrounding water communities (both FL and PA fractions) at each station. Significantly higher dissimilarity was found in the freshwater (post-hoc multiple comparisons from stations 4 and 5; $R^2 = 0.12$, *p*-value= 0.001 and $R^2 = 0.17$, *p*-value= 0.001 respectively) as compared to seawater samples (post-hoc multiple comparisons from station 1; $R^2 = 0.084$, *p*-value= 0.001). No significant difference was found between FL and PA community structures at each different station (*p*-value> 0.05) and within the rivers (*p*-value > 0.05).

Some differences were highlighted between communities associated to MPs compared to *p*MPs at different sampling sites, when the number of samples allowed the statistical comparison. In particular, significant differences were identified between MP and *p*MP plastispheres sampled at seawater station 1 of the Seine and the Garonne rivers (post-hoc multiple comparisons with Holm-Bonferroni correction; $R^2 = 0.23$, *p*-value = 0.004 and $R^2 = 0.27$, *p*-value = 0.022, respectively). Except for some stations in the Seine (station 5) and Garonne (station 2) rivers, significant dissimilarities were always found between MPs and pMPs plastisphere community structures at the same sampling station (post-hoc multiple comparisons with Holm-Bonferroni correction, $p<0.05$). Finally, the composition of the polymers was also not significantly driving the community structures, since no difference was found among MPs made of PE, PP and PS communities ($p > 0.05$) and among *p*MPs made of PE, POM or NYL ($p > 0.05$) within the same stations of all rivers.

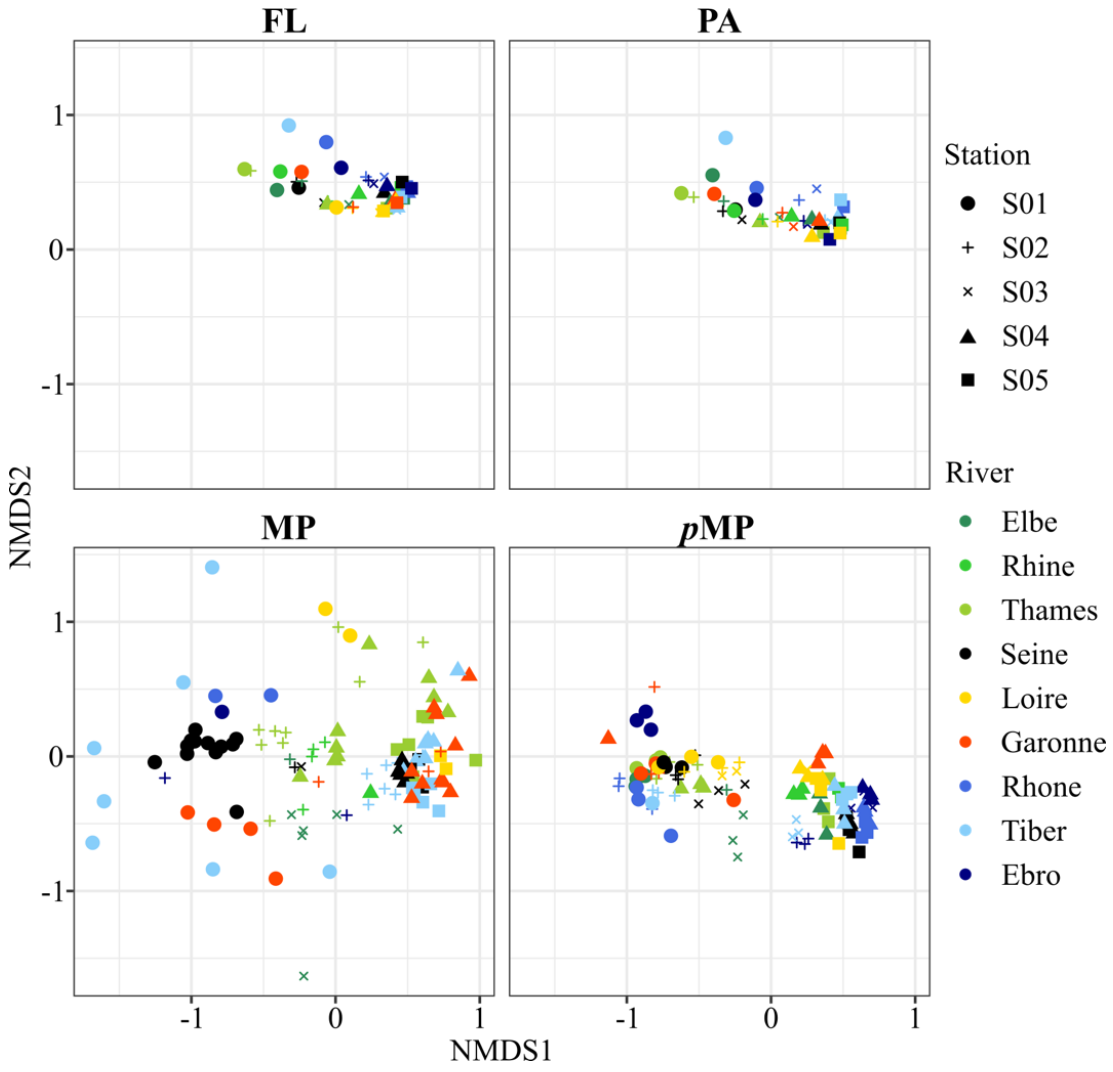


Figure 2. Nonmetric multidimensional scaling (NMDS) plot showing dissimilarities among FL, PA, MPs and *p*MPs communities. Bray-Curtis distances were calculated using the whole dataset, as well as the NMDS, which was then separated in four panels according to the sample type, for better visualization (stress = 0.19). Colors indicate the river, dot shapes correspond to the sampling station.

3.4. Focus on MPs plastisphere communities in the Seine river

We decided to focus on the bacterial diversity associated to MPs samples for the Seine River, because sufficient MPs sequencing data were available for all sampling stations. As previously mentioned for the entire dataset, MPs plastispheres in the Seine River differed significantly when sampled in seawater (station 1) or freshwater environments (stations 4 and 5) (post-hoc multiple comparisons with Holm-Bonferroni correction; R^2 = 0.34, *p*-value = 0.001 and r^2 = 0.23, *p*-value = 0.003 respectively). Unweighted pair group method with arithmetic mean (UPGMA) dendrogram based on Bray Curtis dissimilarities confirmed the sample organization into two clusters: the first one including samples from MPs communities from freshwater stations (stations 4 and 5), and the second comprising samples from seawater (station 1) separated from the estuarine and intermediate sampling stations (stations 2 and 3) (**Fig. 3**). Taxonomy associated to MPs from stations 4 and 5 showed high relative abundance of Burkholderiales and Deinococcales (mean relative abundance of 21 % and 12 %, respectively), whereas these groups had minor contributions at station 1 (only 0.14 % and 0.05 %, respectively). In the later station, samples presented high abundance of bacterial taxa belonging to Chitinophagales, Flavobacteriales and Rhodobacterales (mean relative abundance of 17 %, 26 % and 23 % respectively, against 1.2 %, 5.7 % and 3.3 % at stations 4 and 5, respectively) (**Fig. 3**).

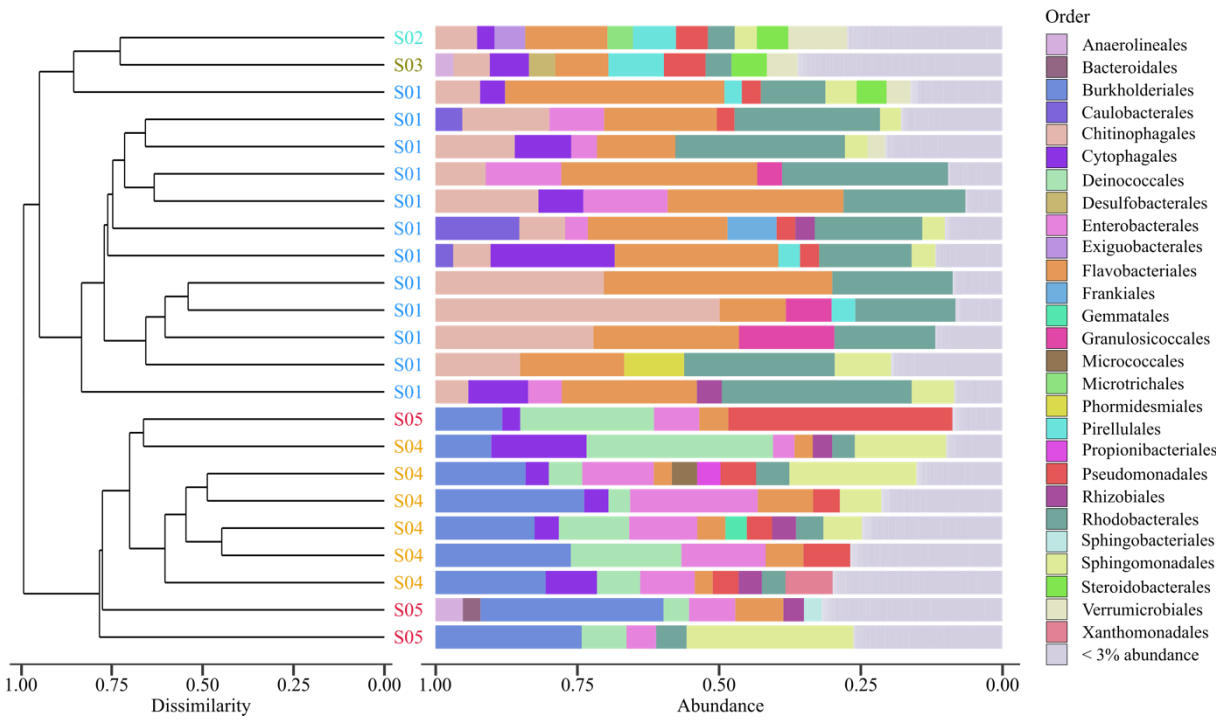


Figure 3. UPGMA dendrogram based on a Bray-Curtis dissimilarities among bacterial communities associated with MPs along the Seine river sampling stations, and the associates taxonomic relative abundances. Stations 1 (S01) are represented in blue, station 2 (S02) is represented in cyan, station 3 (S03) is represented in green, stations 4 (S04) are represented in orange, and stations 5 (S05) are represented in red. Bar charts represent the cumulative abundances of taxa at the order level.

Venn diagram showed that only 0.3 % of the total ASVs (38 ASVs) were shared between sea and fresh waters MPs communities (**Fig. 4**), whereas all the other ASVs were unique to one or the other aquatic compartment (river or sea). The shared ASV happened to be either abundant in samples from freshwater stations, or belonging to the rare biosphere (average relative abundance < 0.01 of the total ASVs) of sea or freshwater samples. Among 30 genera identified as containing pathogenic taxa, only 3 were found in these 38 common ASV, *e.g., Psychrobacter, Massilia* and *Acinetobacter* genera, corresponding to 5 ASVs with mean relative abundances below 0.01% in sea samples (MPs collected at station 1). SIMPER analysis highlighted 18 dominant ASVs contributing to a cumulative 30 % of the dissimilarity between MPs in seawater and freshwater fractions (**Fig. 4**). Interestingly, except for one ASV identified as *Acinetobacter* sp., the ASV that contributed to the difference between the two fractions were abundant in one fraction (average abundance from 226 to 1,628 of the total ASVs for each fraction) but not detected in the other, thus reinforcing the difference between

marine and riverine plastispheres. Seawater MPs exhibited 12 ASVs significantly contributing to the difference between the two fractions, with ASVs identified as Rhodobacterales, *Lewinella* sp., *Sulfitobacter* sp., *Maribacter* sp. *Acinetobacter* sp. and one uncultured bacterium contributing between 1 and 5 % of the difference between the seawater and freshwater samples (other ASV contributing to <1 % were Phenylobacterium, Flavobacteriaceae, *Polaribacter* sp., *Winogradskyella* sp., and *Alteromonas* sp.). Freshwater MPs exhibited 7 ASVs with *Deinococcus* sp. that contributed to more than 5%, *Aeromonas* sp. and *Acinetobacter* sp. contributing between 1 to 5%, and *Hymenobacter* sp., *Hydrogenophaga* sp., *Chitinibacter* sp. and *Qipengyuania* sp. contributing below 1% of the difference between the seawater and freshwater samples.

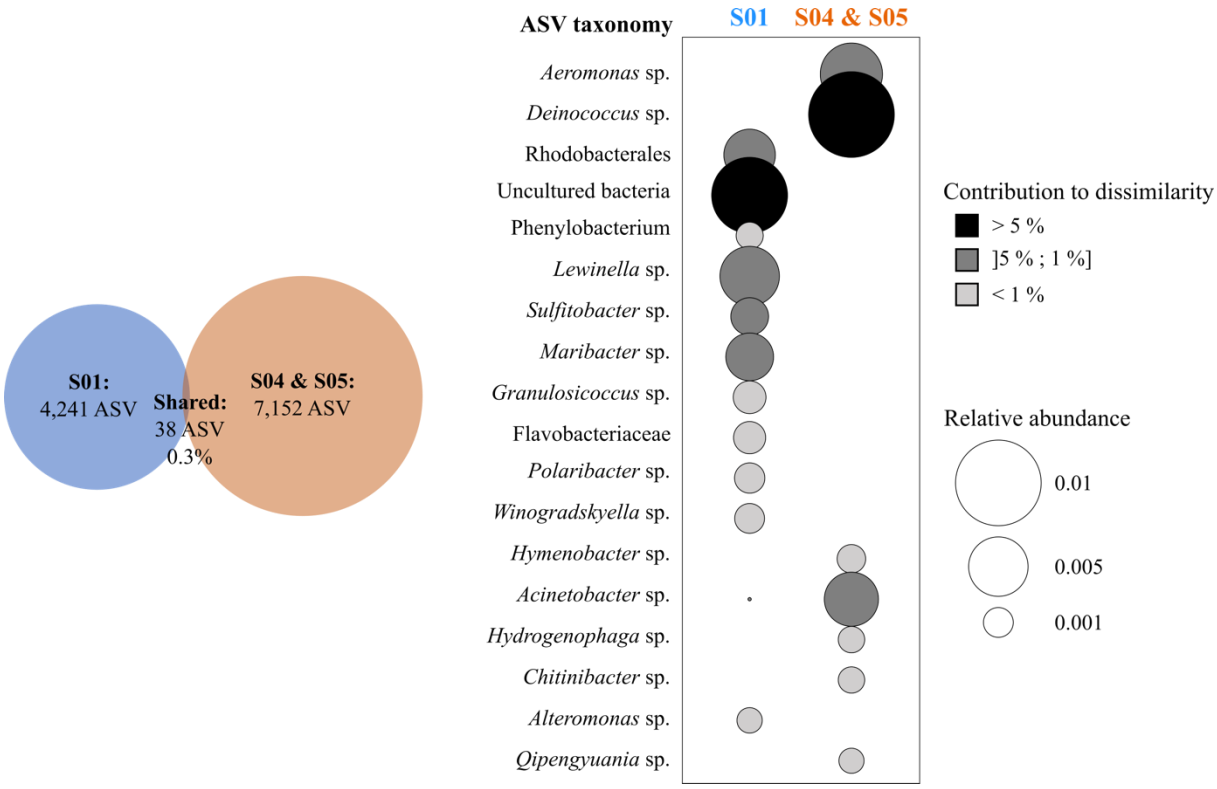


Figure 4. Comparison between communities associated to MPs at seawater station (1) and riverine stations (4 and 5) in the Seine River. Left: Venn diagram identifying the shared and unique ASV for the considered stations. Right: Bubble plot showing the relative abundance and taxonomy of the ASVs contributing up to 30% of the difference between the stations, based on a SIMPER analysis. Bubbles are sized according to the relative abundance and colored according to their contribution to the global dissimilarity.

3.5. Focus on bacterial pathogens

3.5.1. Putative pathogens based on 16S rRNA sequencing data

We identified 30 bacterial genera with known pathogenic effects in the aquatic environment or for human health, with 10 times higher ASV abundances on microplastics (MPs and *p*MPs) compared to the surrounding water (FL and PA) (see data and references in **Suppl. Table 2**). The presence of putative pathogens was observed at all sites on microplastics, with lower abundances at the sampling station S3 and S5 (**Fig. 5**). The 30 bacterial genera represented 6750 ASVs, with only 1% shared across sampling sites along the river, and even when considering MPs and *p*MPs only (**Fig. 5 and Suppl. Fig. 3**).

A focus on each river highlighted the absence of putative pathogen transfer across stations from the freshwater to seawater. For both MPs or *p*MPs, we observed a clear shift of the dominant putative pathogenic bacteria between the freshwater and seawater (**Suppl. Fig. 3**). Most of the identified putative pathogens exhibited decreased in relative abundance in the river-to-sea continuum (i.e., *Vibrio*, *Aquibacter*, *Sulfitobacter*, *Glaciecola*, *Erythrobacter*, *Lactobacillus*, *Winogradskyella*, *Pseudoalteromonas*, *Psychrobacter*, *Aquimarina*), with some appearing only upstream of the major city (i.e., *Desulfovibrio*, *Aeromonas*, *Arcobacter*, *Acinetobacter*, *Sphingomonas*), while others almost disappeared in the seawater stations (i.e., *Staphylococcus*, *Corynebacterium*, *Ruminococcus*). On the opposite, several putative pathogens increased in relative abundance in the river-to-sea continuum (i.e., *Stenotrophomonas*, *Acidovorax*, *Massilia*, *Paracoccus*, *Limnothrix*), whereas some do not exhibit any pattern (i.e., *Streptococcus*, *Fusobacterium*, *Aliivibrio*, *Peptostreptococcus*, *Shewanella*, *Lacinutrix*).

Only 10% of the putative pathogen ASVs were shared between MPs and *p*MPs (**Suppl. Fig. 4**). The MPs contain higher bacterial diversity and relative abundance of putative pathogenic ASVs compared to the *p*MPs, with some exclusively present on the MPs (*Prevotella*, *Corynebacterium*, *Lactobacillus*, *Acidovorax*, *Ruminococcus*, and *Arcobacter*).

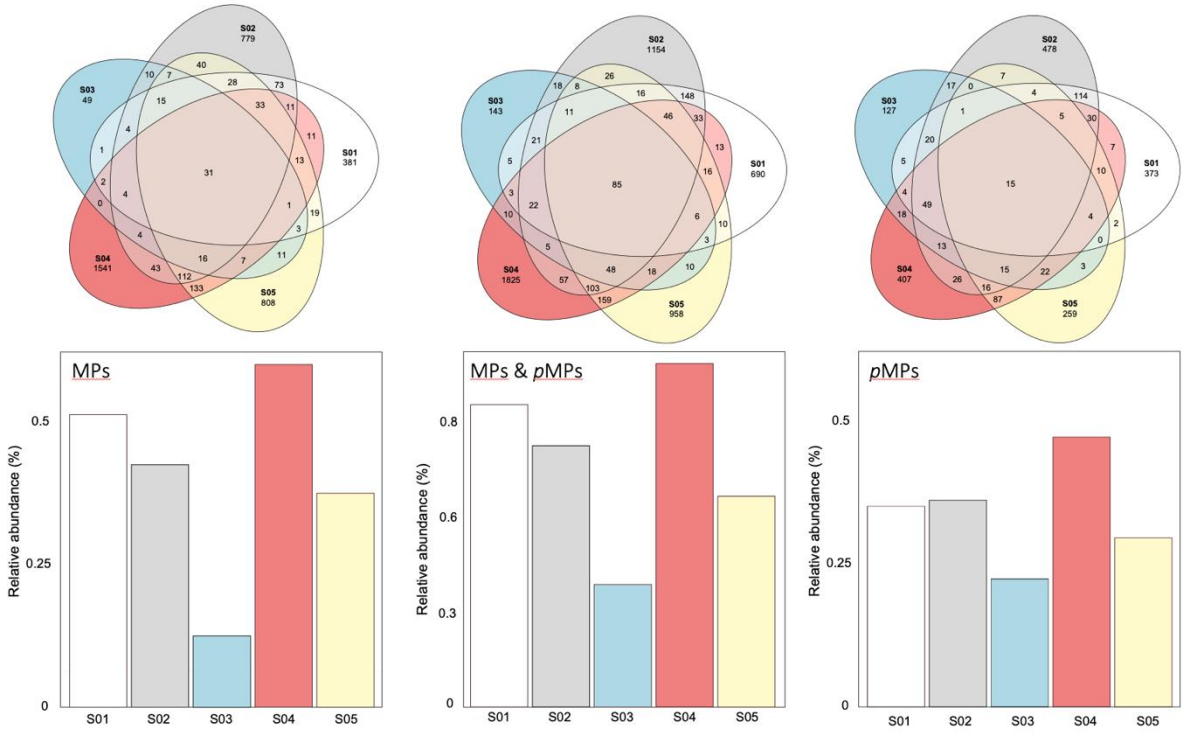


Figure 5: Venn diagrams and histogram bars showing the relative abundances of putative pathogens across sampling stations (S01 to S05) in the river-to-sea continuum for MPs only (left), *p*MPs only (right) and both MPs and *p*MPs (middle).

3.5.2. Identification of human bacterial pathogens in MPs

16S rRNA genes sequencing revealed that most of the cultured bacteria associated with MPs sampled in the Rhine and Loire river-to-sea continuum belonged to Pseudomonas (*P. zhaodongensis*), Pseudoalteromonas (*P. mariniglutinosa, P. nigrigaciens, P. profundi*), Cellulophaga (*C. baltica*), Neptunomonas (*N. acidivorans*), Paracoccus (*P. yeei*) in the marine station and to Flavobacterium (*F. compostarboris, F. cupreum*), Pseudomonas (*P. atacamensis*) and Shewanella (*S. putrefaciens*) in the freshwater station. Among the isolated colonies, only one strain Y651 affiliated to *Shewanella putrefaciens* presented positive results to the virulence and biofilm formation tests. First, we found similar ability of *Shewanella putrefaciens* Y651 to attach and invade/internalize the human carcinoma (Caco-2) cells when compared to the positive human pathogen *S. putrefaciens* control strain previously isolated from the animal feces (**Fig. 6**). Interestingly, *S. putrefaciens* Y651 showed higher capability to form biofilms on polystyrene (PS) than the control *S. putrefaciens* previously isolated from animal gut. None of these strains formed biofilm on polypropylene (PP) under our experimental conditions.

The 16S rRNA database generated in this study on the nine European rivers showed that ASVs affiliated with the *Shewanella* sp. genera were detected at the same sampling station but with very low abundance (6 ASV, eq. 0.04% of the total ASV per sample at station 5 in the Loire river). *Shewanella* sp. represented >0.1% of the total ASV per sample in individual MPs and *p*MPs but not in the surrounding waters collected in most rivers (Ebro, Elbe, Loire, Rhine, Seine, Thames) and exceptionally >1% in MPs and *p*MPs collected in Loire and Thames rivers. It is noteworthy that ASV affiliated to the species *Shewanella putrefaciens* was only detected in our 16S rRNA database for one MP collected in the Thames river (station 4), but not detectable at the original location of the cultured Y651 strain (station 5 of the Loire river).

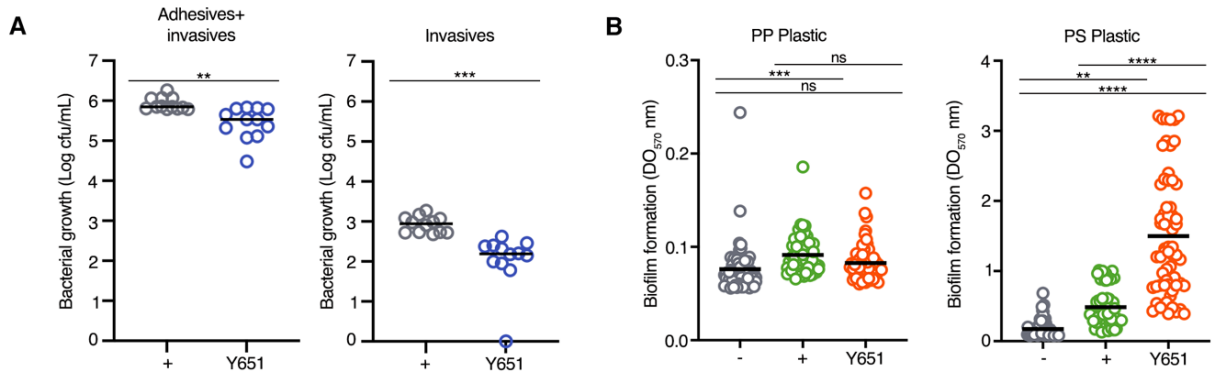


Figure 6. Bacterial virulence (A) and biofilm formation (B) of *Shewanella putrefaciens* Y651 isolated from MPs in the freshwater of Loire river. Another *S. putrefaciens* pathogenic bacteria previously isolated from animal faeces served as positive control (Dias et al. 2019). CFU for colony forming unit, DO for optical density, PP for polypropylene and PS for polystyrene. Statistical significance was assessed by comparing the mean values (black bar) using Fisher T test on (A) and one-way ANOVA analysis test on (B) with ns: p-value>0.05; **: p-value<0.01; ***: p-value<0.001.

4. Discussion

4.1. Plastisphere niche partitioning is a common feature in the river-to-sea continuum

Plastisphere has been extensively studied in the marine environment since the last decade (Zettler et al., 2013), whereas plastisphere in freshwater is an emerging concept (Barros and Seena, 2021). The “plastic life cycle” is based on the fact that plastics mainly originate from terrestrial sources and are primarily transported via rivers to reach the ocean

(Jambeck and Walker-Franklin, 2023; Sonke et al., 2022). It is therefore crucial to study the plastisphere sampled across a transect that includes rivers, estuary and inshore seawater from the same geographical zone, but also across different river types as it was done during the Mission *Tara* Microplastics. Alpha-diversity (richness, evenness and diversity) within the plastisphere followed the same order as the surrounding seawater, with the highest diversity indexes observed in the PA and the lowest in the FL. The significant differences in beta-diversity between the plastisphere (MPs and *p*MPs) and the surrounding communities in waters (FL and PA) observed here was consistently found in various marine ecosystems (Jacquin et al., 2019) and more recently in the highly urban river in Chicago, USA (McCormick et al., 2014). This aligns with a study that compared bacterial communities living on microplastics and their surrounding water samples in freshwater and seawater ecosystems within the Shandong Province, China (Li et al., 2021). Similar niche partitioning was also found in several lakes (Di Pippo et al., 2022). Thus, the extension to a pan-European approach given by our study reinforces the general feature of microplastics providing a unique habitat for microorganisms in all aquatic ecosystems.

It has been previously shown that PA generally differ from FL in all aquatic ecosystems, including marine (Dussud et al., 2018b), lakes (Zhao et al., 2017), and riverine environments (Zhao et al., 2021). Here, we found that dissimilarities between PA and FL community structures were smoothed when compiled together with the plastisphere from 43 sampling stations (within and between stations) along the river-to-sea continuum of nine of the major European rivers. This is in line with the conclusion by Li et al. (2021), which showed that niche-based processes (deterministic) govern the structure of the anthropogenic plastisphere community, while neutral-based processes (stochastic) dominate the planktonic community structure, extending beyond the difference between PA and FL communities. These authors speculated that such findings resulted from the high heterogeneity as well as the fragmented and disconnected nature of the plastics as a habitat.

We could have expected that the difference in composition and load of organic particles between rivers and seawaters may affect the plastisphere communities afterwards. Plastics are constantly submitted to sorption/desorption of hydrophobic and hydrophilic organic materials (Liu et al., 2019) together with the particle-attached bacteria that may have taken the opportunity to colonize plastics. Clear dissimilarities were already observed between plastisphere and organic particle-attached bacterial communities in the marine environment (Dussud et al., 2018b), but no equivalent study was made in freshwater. Despite encountering heterogeneous environments along the river-to-sea continuum, the same conclusion was

reached, thus indicating minimal interaction between bacteria living in the two co-existing particle types (organic matter and plastics) regardless of the sampling zones. This observation underscores the notion that plastics represent unique habitats along this continuum.

4.2. Salinity is an important driver of the plastisphere bacterial communities

Several studies conducted in seawater demonstrated the importance of environmental factors in shaping the plastisphere diversity and community structure (Basili et al., 2020). Geographical location and seasons (Amaral-Zettler et al., 2015; Coons et al., 2021; Oberbeckmann et al., 2014) as well as chemical polymer composition, plastic shape, size (Cheng et al., 2021; Delacuvellerie et al., 2022) or even colors (Wen et al., 2020) were shown to influence the bacterial community structure living on *p*MPs in the marine environment. Other factors were shown to play a substantial role in shaping the early plastic colonizers, such as hydrophobicity, topography, roughness, crystallinity, and surface charge (Rummel et al., 2017), whereas these factors may play a limited role when the biofilm become mature (Cheng et al., 2021; Dussud et al., 2018a).

Interestingly, the chemical composition of the polymers was not shown as driving forces of the plastisphere community structure in this work. The dominance of PE, PP and PS within the sorted 115 MPs across the nine rivers observed here is in accordance with MPs characterization in seawater (Auta et al., 2017) and in freshwater (Li et al., 2020), thus rending our dataset representative of the large microplastics (LMP, from 500 µm to 5mm) generally encountered in the environment. Our study provides further evidence that environmental conditions rather than polymer properties determine the plastisphere at the global (Amaral-Zettler et al., 2015), oceanic basin (Dussud et al., 2018b) or regional scales (Basili et al., 2020). This result is in contradiction with other studies showing clear differences between biofilms grown on different polymers (Dussud et al., 2018a; Oberbeckmann et al., 2018; Pinto et al., 2019). Such discrepancy could be explained by the fact that later studies focused on the long-term colonization of *p*MPs, which is different from sampling MPs directly into the environment.

Based on our nine European river-sea gradient sampling strategy, estuaries were associated with a plastisphere that differed from freshwater and seawater communities, highlighting that salinity appears as a key factor determining the bacterial plastisphere assemblages. We are aware that the salinity is not the unique factor determining changes in plastisphere community composition and especially in the estuarine zone that is the places where salt and fresh water meet. These regions are greatly influenced by river floods but also

585 tidal movements, storm surges which strand plastic on intertidal or wrack zones (Eerkes-
586 Medrano et al., 2015).
587
588 **4.3. Strong segregation between seawater and freshwater plastisphere**
589 Because rivers are major sources of plastics to the sea, their associated plastisphere has
590 been thought to be a vector of bacterial species from freshwater to the ocean, including
591 potentially harmful microorganisms to human and marine animal health (Barros and Seena,
592 2021). Freshwater and marine habitats share a number of features, but there are also strong
593 differences between them that affect the plastisphere consortia.
594 All along the nine European rivers and for all plastic types (MPs or *p*MPs) and
595 characteristics (chemical composition, shape, size, color), we found a strong selective
596 pressure exerted between freshwater and the marine environments, with very few examples of
597 resilience. We noted that the richness, evenness and diversity on marine MPs were
598 significantly lower than from the riverine stations. We found only 0.3% of common ASV
599 between MPs originated from freshwater and seawater across the Seine River, with only five
600 of them belonging to genera that comprises pathogenic taxa but with very low relative
601 abundance. All the other ASVs were unique to one or the other compartments (freshwater *vs.*
602 seawater), thus suggesting that common ASVs between freshwater and seawater (including
603 putative pathogens) were rather an exception than a rule. Deinococcales and Burkholderiales
604 dominated the freshwater plastisphere but had a minor contribution in the seawater
605 plastisphere, which is consistent with a recent study in a small river that flows into the
606 Mediterranean Sea (Var, France) (Delacuvellerie et al., 2022). Conversely, Rhodobacterales,
607 Flavobacteriales and Chitinophagales that dominated coastal seawater but were minor in the
608 freshwater have been classically found on plastics in previous studies in marine environments
609 (Dussud et al., 2018b; Oberbeckmann et al., 2016). In particular, the SIMPER analysis
610 highlighted an ASV affiliated to *Lewinella* sp. as a major contributor to the difference
611 between the marine and riverine bacterial communities across the Seine River, abundant on
612 samples from seawater (station S01) and absent in freshwater (stations S04 and S05). This
613 taxon was previously found on plastic debris in the North Pacific Subtropical Gyre (Li et al.,
614 2021) and on PET bottles incubated for 6 weeks in the North Sea (Oberbeckmann et al.,
615 2016). Interestingly, two taxa highlighted by the SIMPER analysis comprise strains
616 previously identified as plastic degraders, isolated from insect larvae. In particular, two
617 isolates affiliated to the genus Acinetobacter were identified as PE (Kim et al., 2023) and PS
618 (Wang et al., 2020) degraders.

Finally, some isolates affiliated to the genus *Aeromonas* sp., which was identified as a main contributor to the difference between marine and riverine communities across the Seine River, were previously identified as putative pathogens. In particular, *Aeromonas salmonicida* has been identified on floating MPs in the North Adriatic and described as a fish pathogen (Viršek et al., 2017b). It is noteworthy that in our study, *Aeromonas* were abundant in riverine samples but absent from sea samples, as well as other putative pathogens such as *Acidovorax*, *Arcobacter*, and *Prevotella*. On the other hand, *Vibrio* sp. was in our study one of the most abundant putative pathogen in the marine plastispheres. This genus has been depicted as an early MP colonizer that helps attachment of other bacteria, with several putative pathogen species found as member of plastisphere communities all around the world (Pedrotti et al. 2022). Interestingly, we observed a higher relative abundance of the putative pathogenic ASVs downstream the first heavily populated city with a decrease heading towards the estuarine waters, thus suggesting that the impact of human activities was not retained along the river. Overall, we could not find a transfer of specific putative pathogen ASVs in the river-to-sea continuum, which is consistent with the results discussed above.

However, the analysis conducted here (16S rDNA amplicon sequencing with short reads) does not allow to precisely identify the species, nor to highlight effective pathogenicity. We therefore used a culture approach in order to test adhesion and invasion of human intestinal epithelial cells by alive plastisphere bacterial isolates. Positive response was found with the Gram-negative bacterium *Shewanella putrefaciens* Y651 living on PP debris in the freshwater of the Loire river. We also demonstrated higher capability of the Y651 strain to form biofilms on plastic than another *S. putrefaciens* strain previously isolated from animal tissues (Dias et al., 2019). To our knowledge, this is to date the first *in situ* demonstration of the presence of a virulent human pathogen on MPs collected in aquatic environment. *S. putrefaciens* has been previously found in the marine waters in moderate and warm climates (Yu et al., 2022), but also in protein-rich refrigerated foods as a spoilage agent (Vogel et al., 1997). It is considered as an emerging opportunistic human pathogen associated mainly with intra-abdominal, skin and soft tissue infections (Vignier et al., 2013) as well as pneumonia induction in ventilated patients (Huynh and Abdeen, 2023). *S. putrefaciens* infection may also lead to bacteraemia, sepsis and even death (Müller et al., 2023). Nevertheless, the presence of human pathogen on MPs should be considered as an exception rather than a rule. It is noteworthy that very few ASV assigned to *Shewanella putrefaciens* were found in the 16S rRNA database generated in this study on the nine European rivers. This species was found in the so-called “rare biosphere” (0.08 % of the total number of ASVs) of one MP collected in

653 the freshwater of the Thames river, and even not detectable by environmental 16S rRNA
654 sequencing at the original location of the cultured Y651 strain (station 5 of the Loire river).
655 Moreover, the absence of putative pathogens in the shared ASV between marine and riverine
656 plastisphere across the Seine River suggests that the transfer of pathogens from the rivers to
657 the sea by rafting on floating plastics is strongly limited.
658 Overall, our results are in accordance with the only other study compiling the
659 plastisphere in a river-to-sea continuum, focusing on the Shandong Province, China (Li et al.,
660 2021). We confirm the strong dispersal limitation for the plastisphere microorganisms in the
661 river-to-sea continuum at the European level, with freshwater plastispheres being almost
662 completely reshaped when entering the sea, suggesting that very few bacteria are able to adapt
663 to these fragmented habitats, and reinforcing the notion of an extremely low resilience of
664 bacteria in the plastisphere along the river-to-sea continuum.
665
666 **4.4. Similarities between MPs and *p*MPs**
667 Most of the studies on the plastisphere were based on incubation experiments under
668 controlled conditions with plastics of known composition, but only a few explored the plastic
669 debris sampled in the aquatic environment. Most of them considering marine waters and very
670 few conducted in freshwaters (Amaral-Zettler et al., 2020). Only one example of companion
671 studies compared the marine plastisphere associated to MPs (Dussud et al., 2018b) and *p*MPs
672 incubated during one month in the same geographic region (Dussud et al., 2018a). The same
673 colonization period of one month was chosen in this study, with various polymer types (PE,
674 POM and NYL) immersed all along the river-to-sea continuum of the nine European rivers.
675 We observed that MPs and pMPs plastispheres presented similar diversity index values and
676 presented the same clear niche partitioning when compared to the surrounding water bacteria
677 (PA and FL). They also followed the same geographical gradient, all along the river-sea
678 continuums. Such similarities between MPs and *p*MPs suggest that the heterogeneous
679 environmental conditions of the river-to-sea continuums were more important than the plastic
680 history, chemical composition, shape or size in driving changes in plastisphere diversity.
681 Likewise, *p*MPs composition (PE, POM or NYL) had no statistical effect on the plastisphere
682 diversity and community structure after 1-month incubation at each sampling site. These
683 results are in accordance with other studies showing no influence of the polymer composition
684 on the diversity of bacteria colonizing conventional plastics, even for the primo-colonizers
685 and for several months of incubation (Dussud et al., 2018; Odobel et al., 2021). However, a
686 more subtle distinction could be noticed between the MPs and *p*MPs. UPGMA analysis

showed that MPs and *p*MPs plastisphere community structures slightly differed within each station, with less dissimilarity dispersion in *p*MPs samples that always grouped together. This is likely the signature of the different histories in the MPs, that was not a driving factor at the river-to-sea continuum scale, but exerted a selective pressure at the local scale. This is in line with a recent study on a small river (Var, France), which found that the most important drivers of the plastisphere structure along the river-to-sea continuum were mainly the sampling site, and in a lesser extent the polymer chemical composition (Delacuvellerie et al., 2022).

5. Conclusion

Our pan-European study confirms that the niche partitioning between the plastisphere and the surrounding aquatic bacteria is a common feature all along the river-to-sea continuum. We also demonstrated the strong segregation between seawater and freshwater plastisphere, with the exception of few common ASVs. In the river-to-sea continuum, the drastic changes in salinity were shown here as a major barrier for the freshwater plastispheres to cope with transitioning conditions and thus survive in the coastal seawaters. Such findings suggest that the transfer of bacterial pathogens by microplastics from the rivers to the sea is unlikely. This is of outmost importance as we demonstrated that microplastics collected in freshwater harboured a pathogenic bacterium (*Shewanella putrefaciens*) that remained its virulence towards human intestinal epithelial cells.

Our results present the first pan-European set of data associated with the plastisphere along the river-to-sea continuum. To date, the biodiversity of microbial assemblages on the freshwater plastisphere was mainly limited to few geographic locations. Further studies are needed to evaluate the temporal changes of a plastisphere that can be greatly challenged between the low water period and flood events. There is also a substantial need for studies on plastisphere eukaryotes, and especially fungal communities, as recent studies have highlighted this microbial component as non-trivial contributors to the ecosystem.

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Declarations

-Ethical Approval: This article follows the Committee on Publication Ethics (COPE) guidelines. including the ethical responsibilities of the authors. The authors declare that they obtained study-specific approval from the appropriate ethics committee for the research content of this article.

-Consent to Participate: All the authors agreed to participate in coauthorship. The authors have no competing interests to declare that they are relevant to the content of this article.

-Consent to Publish: All the coauthors agreed with the content of this article, and they all provided explicit consent for submission. The authors obtained consent from the responsible authorities at the institute where the work was carried out before the work was submitted.

Author Contributions (CRediT taxonomy)

Léna Philip: Formal analysis, Methodology, Visualization, Writing original draft, review & editing; **Leila Chapron:** Formal analysis, Visualization, Writing review & editing; **Valérie Barbe**: Investigation, Methodology, Supervision, Writing review & editing; **Gaëtan Burgaud:** Investigation, Methodology, Writing review & editing; **Isabelle Calvès**: Methodology, Visualization; **Ika Paul-Pont:** Methodology, Visualization, Writing review & editing; **Odon Thiébeauld:** Methodology, Visualization, Writing review & editing; **Brice Sperandio:** Methodology; **Lionel Navarro:** Investigation, Writing review & editing; **Alexandra ter Halle:** Conceptualization, Investigation, Methodology, Writing review & editing; **Boris Eyheraguibel:** Conceptualization, Investigation, Methodology, Writing review & editing; **Wolfgang Ludwig:** Conceptualization, Investigation, Methodology, Writing review & editing; **Maialen Palazot:** Investigation, Methodology, Writing review & editing; **Mikael Kedzierski:** Investigation, Methodology, Writing review & editing; **Anne-Leila Meistertzheim:** Conceptualization, Investigation, Supervision, Methodology, Writing review & editing; **Jean-François Ghiglione:** Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Visualization, Writing original draft, review & editing.

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Supplemental Tables and Figures

Supplemental Table 1: Location and environmental parameters measured at the four to five sampling stations in each of the nine European river. *ND*: not determined.

Country	Latitude	Longitude	Sampling date	Temp (°C)	Salinity	SPM (mg/L)	POC (%)	Si(OH)4 (mM)	PO4 (mM)	NO
Spain	N 40°41.00	E 00°56.00	8/14/2019	25.81	37.59	5.50	6.8	1.46	0.01	
Spain	N 40°42.00	E 00°51.00	8/15/2019	25.85	3.85	4.90	19.8	104.03	0.67	
Spain	N 40°42.52	E 00°43.15	8/15/2019	26.08	2.34	6.40	11.3	77.73	0.57	
Spain	N 40°42.57	E 00°35.02	8/16/2019	26.16	1.15	4.30	10.3	76.28	0.75	
Spain	N 40°48.64	E 00°31.14	8/16/2019	26.16	1.15	2.50	20.9	83.88	1.11	
Germany	N 54°07.254	E 07°57.391	6/15/2019	14.20	32.8	3.10	13.32	1.28	0.05	
Germany	N 53°51.278	E 08°56.705	6/16/2019	18.20	12,0	27.80	4.59	10.90	1.06	
Germany	N 53°46.900	E 09°22.706	6/16/2019	21.30	1,0	101.50	3.74	34.03	1.74	
Germany	N 53°32.003	E 09°49.354	6/17/2019	21.27	0.42	42.90	5.24	7.04	1.54	
Germany	N 53°26.455	E 10°05.928	6/18/2019	23.37	0.46	41.40	15.57	4.30	0.05	
France	N 45°33.802	W01°26.139	11/15/2019	14.13	32.93	18.51	1.59	*ND*	*ND*	
France	N 45°24.95	W00°45.75	11/14/2019	12.73	28.92	89.59	1.27	*ND*	*ND*	
France	N 45°07.55	W00°40.05	11/13/2019	13.20	2,0	262.87	1.58	*ND*	*ND*	
France	N 44°52.732	W00°32.076	11/6/2019	12.50	0.8	14.20	2.24	*ND*	*ND*	
France	N 44°48.013	W00°31.544	11/7/2019	12.80	0.8	76.51	2.15	*ND*	*ND*	
France	N 47°08.4	W02°23.5	11/18/2019	12.47	32.52	5.29	3.12	*ND*	*ND*	
France	N 47°15.56	W02°11.132	11/17/2019	11.81	25.24	21.47	3.08	*ND*	*ND*	
France	N 47°18.042	W02°03.497	11/20/2019	8.84	4.48	15.15	3.69	*ND*	*ND*	
France	N 47°11.865	W01°40.667	11/18/2019	9.21	0.13	16.64	6.28	*ND*	*ND*	
France	N 47°14.4531	W01°27.677	11/19/2019	9.07	0.12	12.71	7.96	*ND*	*ND*	
Netherlands	N 52°07.824	E03°42.718	7/10/2019	22.20	30.00	3.90	13.40	0.12	0.01	
Netherlands	N 51°58.517	E04°06.573	7/11/2019	21.60	14.00	10.00	8.20	11.26	0.60	
Netherlands	N 51°53.755	E04°21.055	7/11/2019	21.20	2.00	20.50	6.90	18.89	0.40	
Netherlands	N 51°54.20	E04°34.7	7/11/2019	21.40	2.00	37.90	5.80	5.70	0.16	
France	N 43°09.30	E04°58.11	8/20/2019	21.76	37.09	3.40	6.30	0.69	0.01	
France	N 43°18.68	E04°51.15	8/21/2019	21.67	20.28	6.70	7.20	1.22	0.02	
France	N 43°23.37	E04°47.46	8/21/2019	23.19	2.92	9.60	5.70	19.92	0.53	
France	N 43°39.16	E04°36.20	8/22/2019	23.22	0.19	12.90	3.90	16.94	0.82	
France	N 43°42.68	E04°36.50	8/23/2019	22.92	0.20	35.50	2.70	37.28	0.69	
France	N 49°28.738	E00°07.097	7/22/2019	20.40	34.00	6.30	14.00	0.35	0.02	
France	N 49°25.820	E00°09.266	7/18/2019	21.40	27.00	55.90	3.40	13.06	0.75	
France	N 49° 25.921	E00°18.873	7/20/2019	21.80	15.00	83.00	3.30	19.32	1.07	
France	N 49° 21.053	E00°56.557	7/19/2019	21.40	0.71	40.20	5.80	105.98	2.18	
France	N 49° 20.116	E01°05.223	7/19/2019	21.70	0.71	17.10	7.30	136.68	4.15	
England	N 51°27.218	E01°27.65	6/10/2019	14.92	34.66	19.60	3.40	1.73	0.13	
England	N 51°30.225	E00°41.625	6/10/2019	16.16	32.61	19.40	4.50	1.45	2.48	
England	N 51°27.763	E00°15.56	6/11/2019	17.03	19.32	32.60	4.80	11.74	16.63	
England	N 51°27.6033	W00°18.872	6/12/2019	16.25	0.32	28.90	11.90	164.98	8.62	
Italy	N 41°38.55	E12°00.78	9/11/2019	22.91	38.25	2.99	4.50	*ND*	*ND*	
Italy	N 41°44.74	E12°15.23	9/12/2019	22.03	3.98	5.40	8.40	*ND*	*ND*	
Italy	N 41°45.44	E12°17.07	9/12/2019	21.46	6.19	5.92	10.70	*ND*	*ND*	
Italy	N 41°48.21	E12°25.09	9/13/2019	21.01	0.61	9.98	8.50	*ND*	*ND*	
Italy	N 42°01.02	E12°31.15	9/14/2019	20.50	0.62	15.88	4.40	*ND*	*ND*	

Supplemental Table 2: List of putative bacterial pathogens (genus level) with the number of associated ASVs across microplastic types (MPs, *p*MPs and shared) in the nine European river-to-sea continuum. Literature references are indicated for each putative pathogen.

Bacterial genus	References	Tot ASVs	MPs specific	*p*MPs specific	Shared
Vibrio	Pedrotti et al., 2022	171	42	17	46
Staphylococcus	Cheng et al., 2023; Rupp and Archer, 1992	32	15	2	1
Aquibacter	Kopprio et al., 2021	151	17	24	89
Sulfitobacter	Jacquin, 2020	103	14	35	45
Glacieola	Rendueles et al., 2017	83	30	6	38
Erythrobacter	Zheng et al., 2016	615	186	162	224
Prevotella	Ruan et al., 2015	168	91	5	3
Desulfovibrio	Fournier, 2022	595	304	6	126
Corynebacterium	Riebel et al., 1986	96	28	12	7

Streptococcus	Marques et al., 2023; Zadjelovic et al., 2023	115	65	9	7
Lactobacillus	Fournier, 2022; Harty et al., 1994	59	26	6	7
Fusobacterium	Epaulard et al., 2006; Muchova et al., 2022	45	18	1	13
Aeromonas	Pessoa et al., 2022; Viršek et al., 2017	110	68	8	17
Stenotrophomonas	Pham et al., 2017	79	47	10	5
Acidovorax	Javaid et al., 2021; Tavelli et al., 2022	48	13	6	3
Massilia	Ali et al., 2022; Ran et al., 2024	91	35	14	25
Paracoccus	Lasek et al., 2018	138	57	40	20
Aliivibrio	Kelly et al., 2022	34	14	2	11
Ruminococcus	Zhai et al., 2023	276	146	9	14
Peptostreptococcus	Van Dalen et al., 1998	14	12	1	1
Winogradskyella	Canada et al., 2020	223	60	29	115
Arcobacter	Ramees et al., 2017; Zhong et al., 2023	137	75	4	4
Limnothrix	Nguyen et al., 2022	87	54	5	28
Pseudoalteromonas	Delacuvellerie et al., 2022; Pujalte et al., 2007	387	196	28	93
Psychrobacter	Bonwitt et al., 2018; Koh et al., 2023	237	165	9	45
Aquimarina	Koh et al., 2023; Silva et al., 2022	112	21	10	73
Acinetobacter	Almasaudi, 2018; Tavelli et al., 2022	1706	1226	50	160
Shewanella	Tavelli et al., 2022	144	17	9	4
Lacinutrix	Cheng et al., 2021; López et al., 2017	29	9	6	11
Sphingomonas	Tavelli et al., 2022	1004	461	124	206

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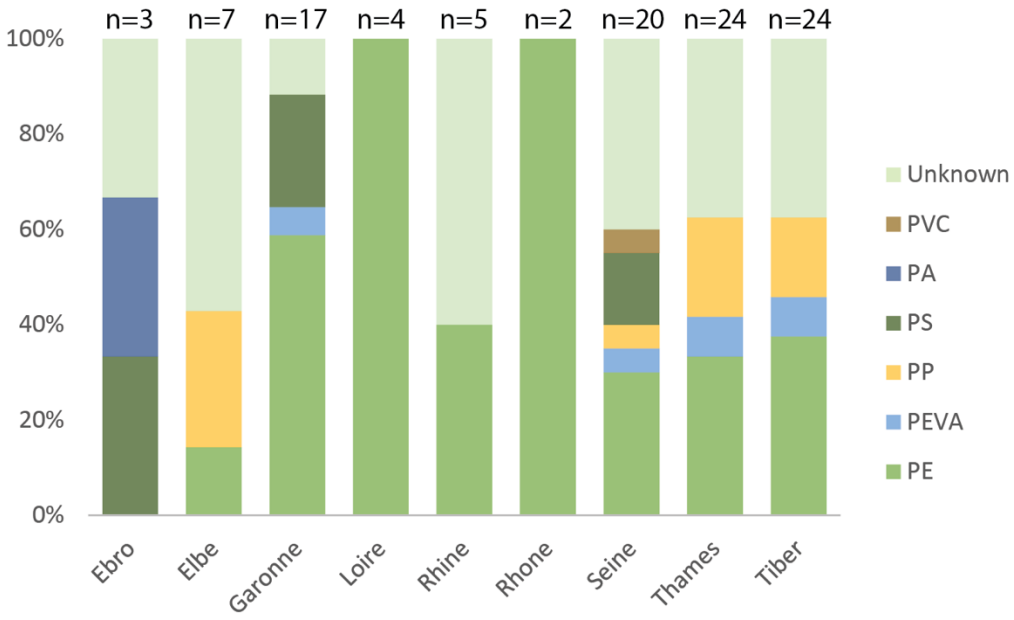
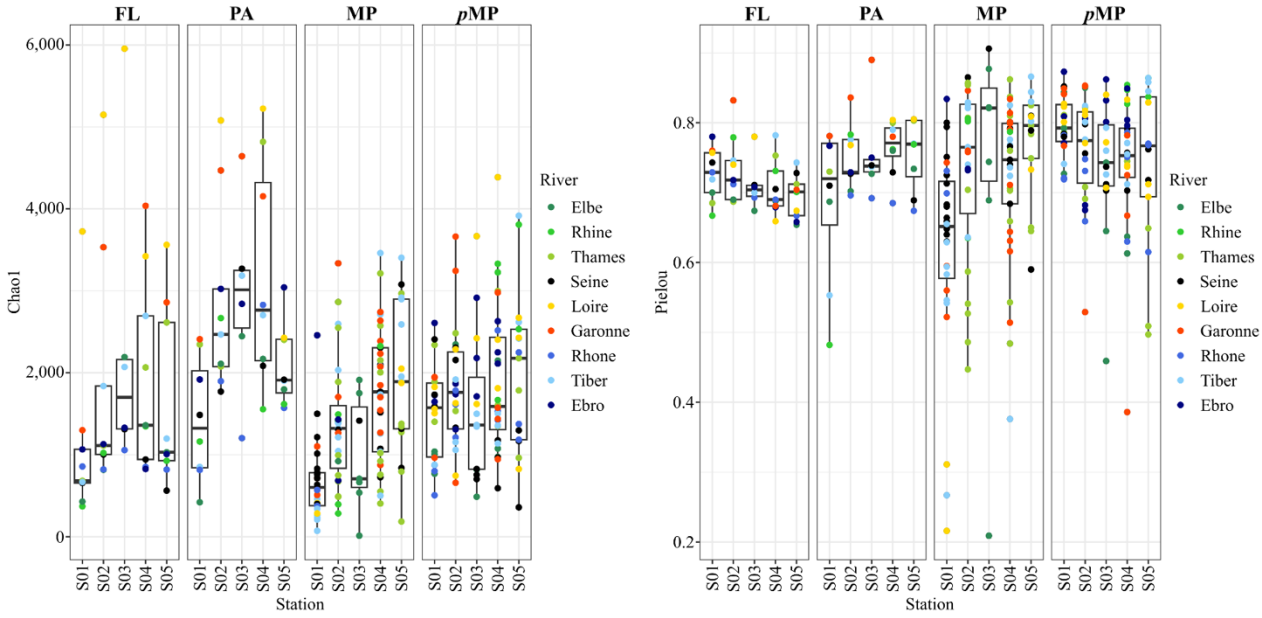
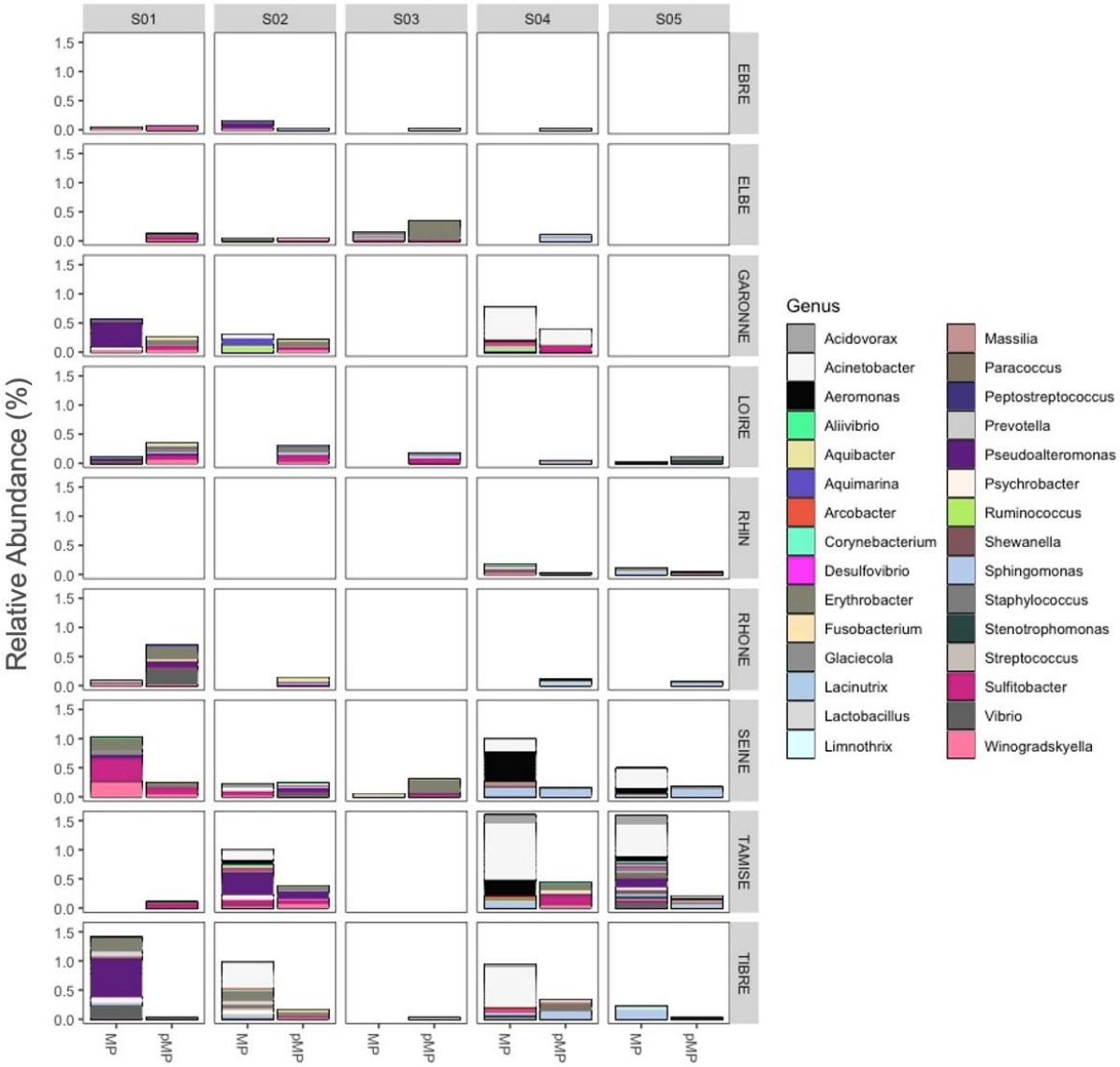
Supplemental Figure 1. Composition of polymers (in %) and number of MPs (n) whose plastisphere was analyzed from the nine European rivers.



Supplemental Figure 2. Chao1 and Pielou eveness indexes at each station, all rivers considered. PA refers to the particle-attached bacteria, FL to free-living bacteria, MP to floating microplastics and *p*MP to one-month plastispheres growing on pristine plastics. Colors indicate the sampling river.



Supplemental Figure 3. Relative abundance of putative pathogen bacterial genus across the sampling stations (S01 to S05), rivers and plastic types (MPs and *p*MPs).



Supplemental Figure 4. Venn diagrams and histogram bars highlining the shared ASVs and the relative abundances of putative pathogens across MPs and *p*MPs for all stations (top), for the seawater only (station S01, bottom left), and for the freshwater only (stations S04 and S05, bottom right).