



Time-course biofilm formation and presence of antibiotic resistance genes on everyday plastic items deployed in river waters

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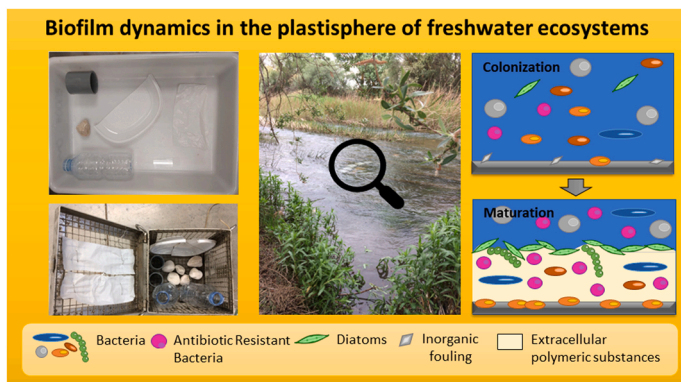
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HIGHLIGHTS

- The evolution of microbial communities in the plastisphere was studied for one year.
- Site, substrate, and colonization time were relevant in shaping the plastisphere.
- Core microbiome/biomes could be identified in each plastic item along time.
- Everyday plastic items can be considered reservoirs of antibiotic resistance genes.

GRAPHICAL ABSTRACT



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ABSTRACT

The plastisphere has been widely studied in the oceans; however, there is little information on how living organisms interact with the plastisphere in freshwater ecosystems, and particularly on how this interaction changes over time. We have characterized, over one year, the evolution of the eukaryotic and bacterial communities colonizing four everyday plastic items deployed in two sites of the same river with different anthropogenic impact. α -diversity analyses showed that site had a significant role in bacterial and eukaryotic diversity, with the most impacted site having higher values of the Shannon diversity index. β -diversity analyses showed that site explained most of the sample variation followed by substrate type (i.e., plastic item) and time since first colonization. In this regard, core microbiomes/biomes in each plastic at 1, 3, 6 and 12 months could be identified at genus level, giving a global overview of the evolution of the plastisphere over time. The measured concentration of antibiotics in the river water positively correlated with the abundance of antibiotic resistance genes (ARGs) on the plastics. These results provide relevant information on the temporal dynamics of the plastisphere in

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freshwater ecosystems and emphasize the potential contribution of plastic items to the global spread of antibiotic resistance.

1. Introduction

The unique properties of plastics, such as durability, low density, versatility, and malleability, have made it one of the most widely manufactured material since its invention in the mid-19th century (P. Li et al., 2021; Thompson et al., 2009). This is reflected in the global plastic production, which reached its maximum in 2019 with 368 million tonnes (excluding polyethylene terephthalate (PET)-fibers, polyamide (PA)-fibers, and polyacryl-fibers) and stabilized in 2020 (PlasticsEurope, 2021). In Europe (referred to EU27 plus Norway, Switzerland and the United Kingdom. Among the most commonly used polymers are low and high-density polyethylene (LDPE and HDPE), polypropylene (PP), polyvinyl chloride (PVC), PET, polyurethane (PUR) and polystyrene (PS) (PlasticsEurope, 2021). Subsequently, its poor management means that at least 60% of the plastics produced end up in landfills or in the environment (Chamas et al., 2020; Wang et al., 2021). In the environment, plastics can be transported from the soil and wastewater treatment plants (WWTPs) to rivers and consequently to oceans (Jambeck et al., 2015; Martínez-Campos et al., 2022). In fact, the major point-source of plastic to the oceans is known to be by river discharge (Meijer et al., 2021).

Currently, scientific studies have shown concerns regarding the potential impact that plastics may have on aquatic ecosystems (Chae and An, 2017; Thushari and Senevirathna, 2020; Vighi et al., 2021). However, the effects of plastics on river ecosystems, despite their key role in the plastics life cycle, are poorly understood in comparison to the marine environment (Azevedo-Santos et al., 2021). Recent studies have reported evidence of plastic ingestion by freshwater organisms (Azevedo-Santos et al., 2021) and their potential risk to carry potentially hazardous substances, such as toxic additives (Bolívar-Subirats et al., 2021) or legacy and emerging contaminants (Puckowski et al., 2021; Schell et al., 2022). Ingestion of larger plastics can cause wounds or tears in the digestive tract, malnutrition problems or even death in animals, in addition to possible bioaccumulation and biomagnification processes; in the case of the smallest fragments (nanoplastics), these may be able to cross the cellular barriers and affect cells mainly by triggering oxidative stress (Huang et al., 2021). However, most studies have been performed in marine organisms, the few studies on freshwater ecosystems limits the knowledge on the possible impact and consequences, both ecologically and to human health (Barros and Seena, 2021).

Plastics form a novel biotope, quoted as the *plastisphere*, in which organisms use plastics to support their growth or to find some shelter (Barros and Seena, 2021; Zettler et al., 2013). Most of the research analysing the *plastisphere* has been conducted in marine environments (Agostini et al., 2021; Keswani et al., 2016; Zhang et al., 2022). These studies proved that microbial communities attached to plastic are remarkably different from those that are found in the surrounding environment (Xu et al., 2019; Zettler et al., 2013). Moreover, organisms that are considered pathogens (Kirstein et al., 2016), invasive (Barnes, 2002), or carriers of antibiotic resistance genes (ARGs), may be part of the *plastisphere*, implying plastics can enhance risks to ecosystems and to human health (Yang et al., 2019).

Some studies show that the structure of microbial communities forming the *plastisphere* differs according to the type of plastic or substrate (Kirstein et al., 2019; Oberbeckmann et al., 2018), and that such associations are dependent on the environmental conditions and the geography of the sampled locations (Wright et al., 2021b). However, to date, there is limited scientific knowledge on the microbial communities forming the *plastisphere* of river ecosystems (Barros and Seena, 2021; Kettner et al., 2019; Martínez-Campos et al., 2021; McCormick et al., 2014, 2016; Oberbeckmann et al., 2018). Plastics can remain in the

same stretch of a river for months to years (Newbould et al., 2021). The communities attached to these plastics could evolve and be significantly influenced by the environment in which it remains, as in marine ecosystems (Vannini et al., 2021). Furthermore, the proximity to WWTPs, considered to be one of the main pathways for antibiotics into the environment (Guo et al., 2017), could facilitate the attachment and/or proliferation of antibiotic resistant bacteria (ARB) carrying cognate ARGs, which could be exported along the river with plastics. Another factor that influences the communities that constitute the *plastisphere* is the time since colonization. The community attached to the *plastisphere* exhibits a clear ecological succession during the early stages of colonization (Galloway et al., 2017; Rummel et al., 2021; Wright et al., 2020), as these communities gradually adapt to the new environment over time and tend to form more established communities (Chen et al., 2020; Du et al., 2022; Lorite et al., 2011). A similar phenomenon occurs with the plastic resistome. Yang et al. (2020) analysed the temporal evolution of ARGs in plastics exposed to urban waters for one month, detecting changes in ARGs abundance and determining the presence of pioneer, intermediate and persistent bacteria carrying ARGs. Furthermore, Yang et al. (2020) highlighted the necessity to conduct long term investigations to comprehend the ecological dynamics of the *plastisphere* and its potential risk for the environment and human health.

In this study, we characterized the colonization and long-term dynamics of the bacterial and eukaryotic communities attached to four types of everyday plastic items: a LDPE bag, a PET bottle, a PS dish and a PVC pipe. These everyday plastics were incubated for one year in two different river sites with different levels of anthropogenic impact. Site 1 was located in an area characterized by natural land use, while site 2 was located downstream of a WWTP. We hypothesized that the biofilm formed in each of these everyday plastic items will be influenced by the type of polymer and will be notably different to the microbial communities in non-plastic substrates or those living in the water column. Furthermore, we expected that sampling time and environmental conditions will have a profound effect on the *plastisphere* complexity. We also evaluated the aging of these everyday plastic items and their capacity to host bacteria carrying ARGs in relation to antibiotic contamination in the study area. Antibiotic-mediated changes in biofilm structure and dynamics have been observed before (R. Li et al., 2021; Yang et al., 2019). The specific antibiotics analysed in this study were selected based on previous studies (Arenas-Sánchez et al., 2019; Rico et al., 2019) that report antibiotic occurrences at relatively large concentrations at site 2.

2. Material and methods

2.1. Study area

This study was performed in the Henares River, located in the upper part of the Tagus River Basin (Spain). Two sampling sites (Fig. S1 in Supplementary Material 1) were selected covering two levels of anthropogenic impact. Site 1 was located in the upper reach of the Henares River (40°50'10.94"N 3° 7'14.23" W) and was mainly surrounded by natural areas. Site 2 was located approximately 50 m downstream of the discharge point of the west WWTP of Alcala de Henares (40° 27' 58.15"N, 3° 24' 55.12" W). It was mainly characterized by urban impact and a moderate influence of semi-intensive agriculture (Arenas-Sánchez et al., 2019; Rico et al., 2019).

2.2. Plastic substrates and non-plastic substrates used for microbial colonization

Four types of everyday plastic items were acquired from local supermarkets (Madrid, Spain): a LDPE bag, a PET drinking water bottle, a PS dish, and a PVC pipe. The selection of these materials was made based on the polymers most demanded in Europe, according to the report made by [PlasticsEurope \(2021\)](#). These items have already shown significant abundances in rivers in the period between 2016 and 2020 ([Cordova et al., 2021](#)). Glass microscope slides and limestone rocks were used as chemically inert, non-plastic substrate controls as already reported by previous studies ([Erni-Cassola et al., 2020](#); [Martínez-Campos et al., 2021](#); [Oberbeckmann et al., 2016](#); [Wu et al., 2020](#)). More details on the substrates used in this study are shown in [Table S1](#) of the [Supplementary Material 1](#).

2.3. Design of the colonization experiment and sampling methods

The LDPE bags, the PET bottles and the PS dishes were pre-treated prior to the experiment: the LDPE bags were cut with sterilized scissors to produce 8×25 cm plastic sheets (not including coloured areas); the PET bottle (height of 33 cm and diameter of 8 cm) bases were punctured to avoid the accumulation of sediments inside the plastic container and labels were discarded; the PS dishes were divided into two parts using sterilized scissors producing 10.5×10.5 cm sheets. The PVC pipes, with a diameter of 5 cm and a length of 8 cm, did not receive previous treatment.

Two units of each substrate (6 units in the case of rocks) were properly attached inside a stainless-steel cage with flanges and submerged in the middle section of the river. More details about the deployment of the substrates inside the cage are shown in [Fig. S2](#) in the [Supplementary Material 1](#). Four cages were deployed at each sampling site. The cages were fixed in the river using chains and ropes to avoid being dragged away by the river. One cage was recovered from each sampling site after one month (20/06/2018; T1), three months (04/09/2018; T3), six months (21/11/2018; T6) and twelve months (21/05/2019; T12) since the start of the incubation period (22/05/2018; T0). Immediately after sampling, all samples were transported to the laboratory, where half of the substrates were kept frozen at -20°C until DNA extraction. The rest of samples were stored at 4°C to be used for other analyses as explained below.

In order to obtain a representative sample of the microbial community in the surrounding water, 3 L of water were sampled in wide-mouthed polyethylene bottles and kept cool in the dark. 1 L water was filtered through a $2.7\ \mu\text{m}$ glass Millipore filter to collect the particulate material in suspension. Subsequently, 250 mL of the filtered water was further filtered through a $0.22\ \mu\text{m}$ membrane Millipore filter to collect the free-living microbial community. Filters were frozen in liquid nitrogen and stored at -20°C until DNA extraction.

2.4. Nutrients and physicochemical parameter analyses

Sampling site water parameters were characterized at the beginning of the incubation period (T0) and at the moment of collecting each cage (T1, T3, T6 and T12). Water temperature, pH, dissolved oxygen (DO, expressed in % and mg/L) and conductivity were measured *in situ* using a portable multimeter probe (HANNA Instruments, Woonsocket, RI, USA, model HI98194). Basic hydrological parameters (water depth and water flow) were measured using a flowmeter. During each sampling, 1 L of water was taken in the middle section of each sampling site for analysis of nutrients and total organic carbon (TOC). Ammonium (NH_4^+), nitrate (NO_3^-), Total Kjeldahl Nitrogen (TKN), orthophosphate (PO_4^{3-}) and total phosphorus were also measured according to the methods described in the Standard Methods for the Examination of Water and Wastewater ([Chambers, 2019](#)). TOC concentration was measured on a Shimadzu TOC-VCSH/CSN coupled to an ASI-V autosampler (Shimadzu

Corporation, Kyoto, Japan).

2.5. Antibiotic concentrations measurements

1 L of water was taken at T0, T1, T3, T6 and T12 in the middle section of each sampling site in amber glass bottles and kept frozen at -20°C until further analysis. In total, 10 antibiotics were analysed: amoxicillin, azithromycin, ciprofloxacin, clarithromycin, erythromycin, lincomycin, metronidazole, sulfamethoxazole, ofloxacin and trimethoprim. Antibiotic selection was based on the pharmaceuticals detected in the same river by [Rico et al. \(2019\)](#). Antibiotic concentration was quantified by liquid chromatography using an HPLC system (Agilent 1200 Series, Agilent Technologies) coupled to an Agilent 6495 triple quadrupole (QQQ) mass spectrometer (LC-MS/MS). Further details of the analytical procedure are provided in [Rico et al. \(2019\)](#).

2.6. Scanning electron microscopy (SEM) analysis

For qualitative assessment of biofilm structure, a random collection of three areas per substrate was chosen for scanning electron microscopy (SEM) analysis. Virgin non-exposed substrates were used as controls. Rocks were not considered in the analysis. The selected areas were cut out, preventing damage of the biofilm. Afterwards, the fragments were fixed with a solution of glutaraldehyde 5% (v/v) in sodic cacodylate 0.2 M (pH 7.2) for 1 h and then washed two times with sodium cacodylate 0.2 M (pH 7.2). Subsequently, samples were dehydrated in a stepwise increasing ethanol series of 10 min immersion in 25% ethanol, 50% ethanol, 75% ethanol, 90% ethanol and absolute ethanol. Then, samples were dried at 50°C for 24 h. The dry samples were metalized with a chromium layer of 15 nm using a sputter Quórum model Q150T-S. Then, the substrate surfaces were analysed using a Scanning Electron Microscope Hitachi S-3000 N.

2.7. Microbial diversity analysis

2.7.1. DNA extraction

DNA was extracted from the microbial community attached to the exposed plastics, rocks, BS glass and surrounding water filters. For that, samples were divided into three fragments by cutting them with sterilized scissors (in the case of rocks, each rock was considered as one replicate). After that, all the sample surfaces, except filters, were scratched using a sterilized scalpel, separating the biofilm from the substrate, which was transferred and divided into various 2 mL tubes according to the biomass volume. Water filters were cut into small fragments and transferred to 2 mL tubes. DNA extraction was performed using phenol/chloroform method extraction followed by absolute ethanol precipitation according to [Martínez-Campos et al. \(2021\)](#).

2.7.2. DNA metabarcoding sequencing

PCR amplification and Miseq Illumina sequencing of the regions V3-V4 of the 16S rRNA and the region V4-V5 of the 18S rRNA of each of the three replicates of each sample (192 sequenced samples) were carried out by the Genomics Service of the Parque Científico de Madrid (Madrid, Spain). The used primers are shown in [Table S2](#) of the [Supplementary Material 1](#). DNA libraries and amplicon sequencing were performed as previously described in [Martínez-Campos et al., \(2018\)](#).

2.7.3. Microbial data analysis

16S rRNA and 18S rRNA profiling was performed using Quantitative Insights into Microbial Ecology 2 (QIIME 2) v.2020.8 using a modified pipeline described in [Martínez-Campos, et al. \(2021\)](#).

Quality filtering of reads (the quality was previously checked using the q2-demux plugin), trimming paired ends and denoising process were performed using DADA2 ([Callahan et al., 2016](#)) via q2-dada2 plugin. All Amplicon Sequence Variants (ASVs) were aligned using MAFFT ([Katoh et al., 2002](#)) and used to construct a phylogeny with FastTree2 (using

q2-phylogeny) (Price et al., 2010).

For α -diversity analysis, Shannon-Wiener diversity Index (Shannon, 1948) was calculated via q2-diversity after samples were rarefied (subsampling without replacement) to 46242 sequences per sample. The Kruskal Wallis test was used to determine if Shannon diversity indexes were significantly different between samples (pairwise comparison) and between the different treatments (sampling site, time, and substrates). Taxonomy was assigned to ASVs via q2-feature classifier plugin (Bokulich et al., 2018) classify-sklearn naïve Bayes taxonomy classifier against the SILVA 132, 99% OTUs database (Quast et al., 2013) previously trained via q2-feature plugin (Bokulich et al., 2018) using the region of the target sequences that were sequenced for 16S rRNA and 18S rRNA.

For β -diversity analysis, an unweighted-pair group method with arithmetic mean (UPGMA) dendrogram was performed based on Bray-Curtis dissimilarity matrix (Sorensen, 1948) using ASV abundance. The UPGMA dendrogram was obtained via “hclust” function of the stats package (Team, 2013) in R Studio (RStudio, 2020). A PERMANOVA (permutational multivariate analysis of variance) test (Anderson, 2001) performed with 999 Monte Carlo permutations was applied to assess significant differences between samples.

To determine the influence of the sampling site, time and substrate (comparison between surrounding water and tested substrates) on the microbial communities, a distance-based redundancy analysis (dbRDA) (Legendre and Anderson, 1999) was performed based on the Bray-Curtis dissimilarity matrix (Sorensen, 1948). In the 16S rRNA samples, the analysis also included the antibiotics detected with the highest concentration (macrolides, sulphonamides, quinolones and trimethoprim). The dbRDA was performed using the “dbRDA” function from the vegan package (Dixon, 2003). The “anova.cca” function of the vegan package (Dixon, 2003) with 999 permutations was used to perform the significance test of the dbRDA. All regression coefficients (R²) were adjusted for multiple testing. Db-RDA graph was performed using the Statistica 13 Software.

To identify differences among substrates for specific microbial taxa, the linear discriminant analysis effect size method (LEfSe) (Segata et al., 2011) was used. This was performed with the LEfSe tool v. 1.1.2 available through Bioconda (Grünig et al., 2018), using all default settings for data formatting and LDA (Linear Discriminant Analysis) effect size. The input data included non-transformed relative abundance genera and the strategy for multi-class analysis “one-against-all” was performed.

2.7.4. Accession number

Sequence data obtained in this study were submitted to the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>) under the Bioproject accession number PRJNA783293 for 16S rRNA sequences and PRJNA783563 for 18S rRNA sequences.

2.8. Analysis of plastic surface alterations

One-year colonized plastics samples were softly brushed and washed with deionized water to eliminate as much adhered material as possible, dried at 35 °C for 24 h in an oven and stored in a desiccator. Attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) was applied to assess the potential alteration of the plastic surface on five randomly selected places in each plastic. Spectra were collected in absorbance mode using a Thermo Nicolet IS10 spectrometer with a Smart iTR-Diamond ATR module using the OMNIC software version 9.1.26. The spectral range was at wavenumber 3500–650 cm⁻¹ and for each measurement, 16 scans were accumulated. The spectral resolution was 4 cm⁻¹, window aperture was at medium resolution, gain was two and optical velocity 0.4747. These parameters allowed obtaining good quality spectra with low spectral noise.

The hydroxyl index was calculated for each polymer as a measure of the hydroxyl groups formed during their environmental oxidation (Brandon et al., 2016). The index was obtained by dividing the maximum absorption in the 3300–3400 cm⁻¹ region by the absorption

of a reference peak. The reference taken was the stretching vibration of C-H bonds, which has been shown as relatively insensitive to the transformations due to polymer ageing (Brandon et al., 2016). The following equations summarize the calculations performed for each plastic:

- Hydroxyl index (LDPE bag) =
$$\frac{\text{Absorption corresponding to the hydroxyl group (3300–3400 cm}^{-1}\text{)}}{\text{Reference peak in the main stretching vibration of } -\text{CH}_2 \text{ (2920 cm}^{-1}\text{)}}$$
- Hydroxyl index (PET bottle) =
$$\frac{\text{Absorption corresponding to the hydroxyl group (3300–3400 cm}^{-1}\text{)}}{\text{Reference peak in the C–H stretching (2970 cm}^{-1}\text{)}}$$
- Hydroxyl index (PS dish) =
$$\frac{\text{Absorption corresponding to the hydroxyl group (3300–3400 cm}^{-1}\text{)}}{\text{Reference peak in the C–H aliphatic stretching of } -\text{CH}_2 \text{ (2900 cm}^{-1}\text{)}}$$
- Hydroxyl index (PVC pipe) =
$$\frac{\text{Absorption corresponding to the hydroxyl group (3300–3400 cm}^{-1}\text{)}}{\text{Reference peak in the C–H stretching (2900 cm}^{-1}\text{)}}$$

2.9. Relative abundance of ARGs

The relative abundance of four ARGs (*ermF*, responsible for erythromycin resistance *sul1*, for sulphonamide resistance; *dfrA1*, for trimethoprim resistance and *qnrSrtF11A*, for quinolone resistance) was compared between the plastics substrates, the non-plastic substrates (BS glass and rock) and the free-living bacterial community using quantitative PCR (qPCR). The selection of ARGs was based on the most abundant antibiotics detected in the two sampling sites (see below) and their wide distribution and high abundance in European WWTPs (Pärnänen et al., 2019). qPCR experiments were carried out by the Genomics Service of the Parque Científico de Madrid (Madrid, Spain). qPCR assays were performed using 1 ng of template DNA and LightCycler® 480 SYBR Green I Master (Roche; USA) in a LightCycler® 480 system (Roche; USA). The primers for amplification of the genes are detailed in Table S3 of the Supplementary Material 1. Thermal cycling details were as described in Pärnänen et al. (2019). Two technical replicates were run for each gene and each sample obtaining in each one a detectable cycle threshold (Ct) value. Both positive and negative controls were included in every run.

The 2^{-ΔCt} method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the 16S gene of the same sample. The different values of 2^{-ΔCt} between samples indicate different gene abundance between samples/conditions (Silver et al., 2006). Student-Newman-Keuls tests were used to see if there were significant differences between times and substrates in the relative abundance of each of the genes. Spearman correlations were developed to test whether there was a relationship between the antibiotic concentration at each of the sampling sites and the 2^{-ΔCt} values obtained for each substrate.

3. Results

3.1. Environmental parameters

The interpretation of the environmental data was divided into physicochemical parameters (Table S4 of the Supplementary Material 1), nutrients (Table S5 of the Supplementary Material 1) and antibiotics (Table S6 of the Supplementary Material 1). The statistical analysis of these parameters between both sampling sites is reported in Table S7 of the Supplementary Material 1.

3.1.1. Physicochemical characterization of the sampling sites along the time course of the colonization experiment

The main physicochemical parameters of water are shown in Table S4 of the Supplementary Material 1. Although samples were taken at regular intervals for one year, seasonality was clearly observed in water temperature and flow rate, with higher temperature and lower

water flow rate in spring and summer. The reduced conductivity detected mostly in June in site 2 is probably related to the increase in the treated/natural water ratio during the summer period. The percent saturation of DO was in the 70–100% range in both sampling sites, meaning that no remarkable oxygen depletion occurred during the sampling period as established in Arenas-Sánchez et al. (2019). However, DO levels were slightly but significantly lower at site 2 compared to site 1 (p -value < 0.05; Table S7 of the Supplementary Material 1) due to the influence of the WWTP, which is located 50 m upstream from the sampling site. pH values were in the range of 7.1 – 8.3, which is considered a regular range for freshwater ecosystems (Bundschuh et al., 2016). Water depth was significantly higher at site 2 with respect to site 1 (p -value < 0.05; Table S7 of the Supplementary Material 1).

3.1.2. Nutrients

The influence of the WWTP effluent discharge on inorganic nutrient concentrations and TOC (shown in Table S5 of the Supplementary Material 1) was significant, showing the highest values at the more anthropogenically impacted site 2 (p -value < 0.05; Table S7 of the Supplementary Material 1). In fact, the concentration of inorganic nutrients at site 2 corresponds to a moderately impacted site (Poikane et al., 2019). N-nitrate and, particularly, N-ammonium levels were higher at the more impacted site 2 (Table S5 of the Supplementary Material 1). The difference between the two sampling sites was even more striking concerning the phosphate concentration. Phosphate concentration was two orders of magnitude higher at sampling site 2 than at sampling site 1 (Table S5 of the Supplementary Material 1), exceeding the local threshold for poor ecological status (Poikane et al., 2019).

3.1.3. Occurrence of antibiotics

Ten antibiotics were measured and detected at site 2 (Table S6 of the Supplementary Material 1), with concentrations ranging from 1.2 ng L⁻¹ (lincomycin) to 7282 ng L⁻¹ (azithromycin), whereas at site 1, only seven antibiotics were found (azithromycin, ciprofloxacin and ofloxacin were below quantification limits) with concentrations ranging from 0.20 ng L⁻¹ (erythromycin) to 211 ng L⁻¹ (metronidazole). The total antibiotic concentration at site 1 was 306 ng L⁻¹, while at site 2 was 24,438 ng L⁻¹, indicating a higher antibiotic pressure at site 2. Seasonality did not have any clear influence on the measured antibiotic concentrations except for azithromycin and ofloxacin at site 2, which fluctuated widely over time. Antibiotics, as well as other pharmaceuticals, are considered point source contaminants; the significantly higher levels of individual antibiotics at site 2, located downstream of a WWTP, namely ciprofloxacin, clarithromycin, erythromycin, sulfamethoxazole, azithromycin, ofloxacin and trimethoprim (p -value < 0.05; Table S7 of the Supplementary Material 1), confirmed the role of wastewater discharge as a major pathway of antibiotics to rivers.

3.2. Microbial colonization of plastics

The visual inspection of the plastics after river incubation (Fig. S3) showed that their surface was covered by microorganisms. To assess microbial colonization, the surface of plastics and BS glass was inspected using SEM microscopy (Fig. 1).

A detailed analysis showed that the surface of non-incubated substrates (T0) was smooth, and no depressions or cracks could be observed, except for the LDPE bag, which presented an irregular surface in some small areas. After the first month of colonization, large substrate areas covered with biofouling were observed, and mostly diatoms were seen; some inorganic fouling (crystalline and inorganic particles) was also observed particularly in substrates incubated in the anthropogenically impacted site 2. After 3 months of incubation, the formation of a thick biofilm on all plastic surfaces was confirmed. Furthermore, in some areas, no clear individual cells could be observed, which might imply that extracellular polymeric substances (EPS) secreted by microorganisms enabled microbes and suspended particles in water to clump

together, an indication of biofilm maturity. In the last phases of incubation (6 and 12 months), the biofouling layer on the plastic surface increased its thickness, showing a clear multilayer biofilm with diverse types of microorganisms, such as diatoms or bacteria clumped between inorganic particles.

3.3. Taxonomical annotation

In total, 12175631 reads (6426961 reads corresponding to 16S rRNA gene and 5748670 reads corresponding to 18S rRNA gene) were obtained using Illumina sequencing. After quality filtration, reads merging and chimera removal using DADA2, 9334841 sequences remained (4470467 reads of 16S rRNA gene and 4864374 reads of 18S rRNA gene). Based on 99% sequence similarity, these reads were clustered into 16943 ASVs for bacteria and 11129 ASVs for eukaryotes. The rarefaction curves for all samples (Fig. S4 for 16S rRNA and Fig. S5 for 18S rRNA in Supplementary Material 1) approached the saturation plateau, indicating that the libraries were adequately sampled. To validate the statistics results, the sequencing depth used to evaluate the α - and β -diversity was 14953 reads per sample for 16S rRNA and 10263 reads per sample for 18S rRNA.

3.4. α -Diversity analysis

Microbial α -diversity was estimated using the Shannon Index. Diversity plots for the different substrates, incubation times and sampling sites for 16S rRNA and 18S rRNA are shown in Fig. 2. The diversity of bacterial and eukaryotic communities differed according to site, but also according to incubation time and substrate (Global p -value < 0.05; Tables S8 and S9 of the Supplementary Material 1). The sampling site had a significant role in bacterial and eukaryotic α -diversity. Sampling site 1 samples had significantly lower values of eukaryotic and bacterial α -diversity (according to the Shannon Index) than samples from sampling site 2 (p -value < 0.05).

Shannon diversity index average values of the bacterial communities at both sampling sites on LDPE bag, PET bottle, PS dish, PVC pipe, Rock, BS glass and water were 7.62 ± 0.74 , 7.98 ± 1.05 , 7.61 ± 1.05 , 7.62 ± 1.05 , 7.93 ± 0.71 , 7.35 ± 0.75 and 7.13 ± 1.32 respectively. As shown in the figure, a fluctuating pattern of α -diversity was observed along the one year of sampling. Remarkably, after three months of incubation, bacterial α -diversity increased significantly in all plastic substrates (p -value < 0.05) except site 2 PVC pipe, where they slightly decreased (Table S8 of the Supplementary Material 1); also, water free-living bacteria community diversity at site 1 significantly decreased (p -value < 0.05) after 3 months and more markedly after 6 months (p -value < 0.05), probably due to the heavy rain that preceded the sampling. Similarly, water free-living bacterial diversity also decreased after 6 months in site 2 (p -value < 0.05; Table S8 of the Supplementary Material 1). In general, after 12 months of incubation, there was an increase in diversity in all tested substrates at site 1 except in the PVC pipe (Fig. 2); this could be due to the fact that PVC pipes release various chlorinated compounds, organotin compounds and aldehydes and these compounds may be more toxic to bacteria than substances released by other polymers (Rožej et al., 2015). This trend was not so evident in the more impacted site 2, where diversity, except for the LDPE bag, decreased in all tested samples.

The eukaryotic mean Shannon diversity average index values at both sampling sites on the LDPE bag, PET bottle, PS dish and PVC pipe, Rock, BS glass and water were 4.43 ± 1.00 , 4.65 ± 1.65 , 4.14 ± 0.96 , 4.08 ± 1.49 , 4.08 ± 1.56 , 4.43 ± 1.03 and 6.24 ± 0.7 respectively. A fluctuating pattern of α -diversity was also found along time (Fig. 2). In general, Shannon diversity was lower in site 1 samples as compared with that in site 2 samples. After 12 months of incubation, the eukaryotic community α -diversity in all plastics, except the LDPE bag, significantly increased in site 1 (p -value < 0.05; Table S9 in Supplementary Material 1). In the more impacted site 2, similar to what was found for bacterial

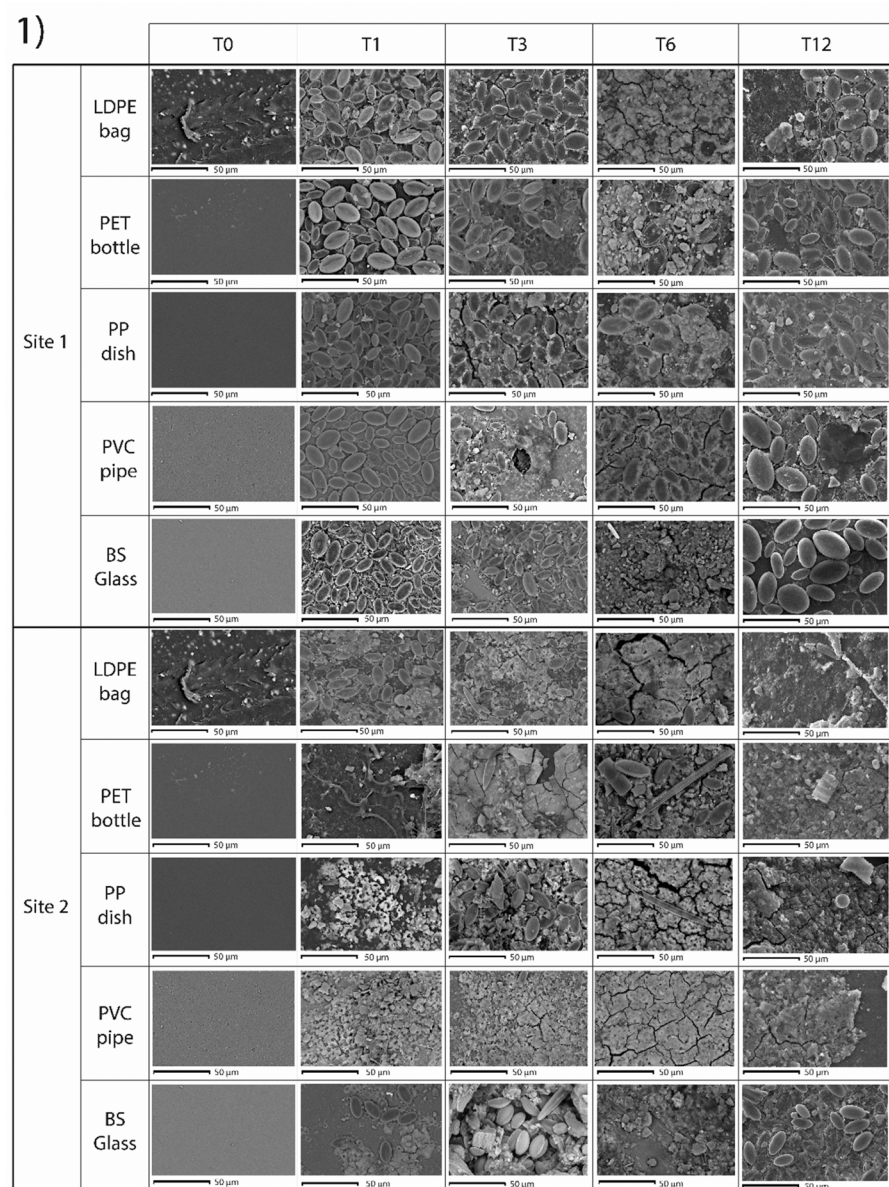
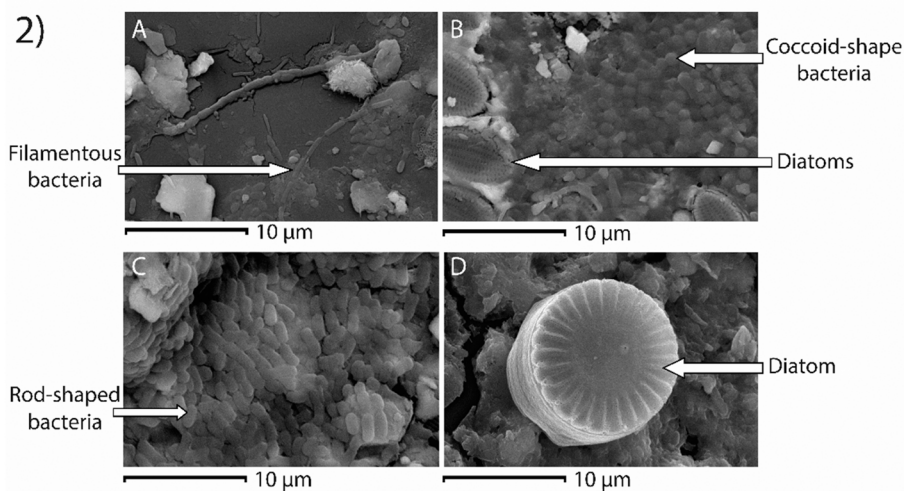


Fig. 1. (1) SEM image showing microbial colonization on the different substrates in both sites along incubation time [T0 (start of the incubation period); T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization)]. (2) detailed SEM images showing the different microorganism morphologies found colonizing the plastic surface along the incubation time: A) filamentous bacteria detected on the PET bottle after 1 month of colonization; B) coccoid-shape bacteria and pennate diatoms identified on the PS dish after 3 months of incubation; C) rod-shaped bacteria over the PVC pipe surface after 6 months of colonization; D) a centric diatom located on the LDPE bag after 12 months of incubation.



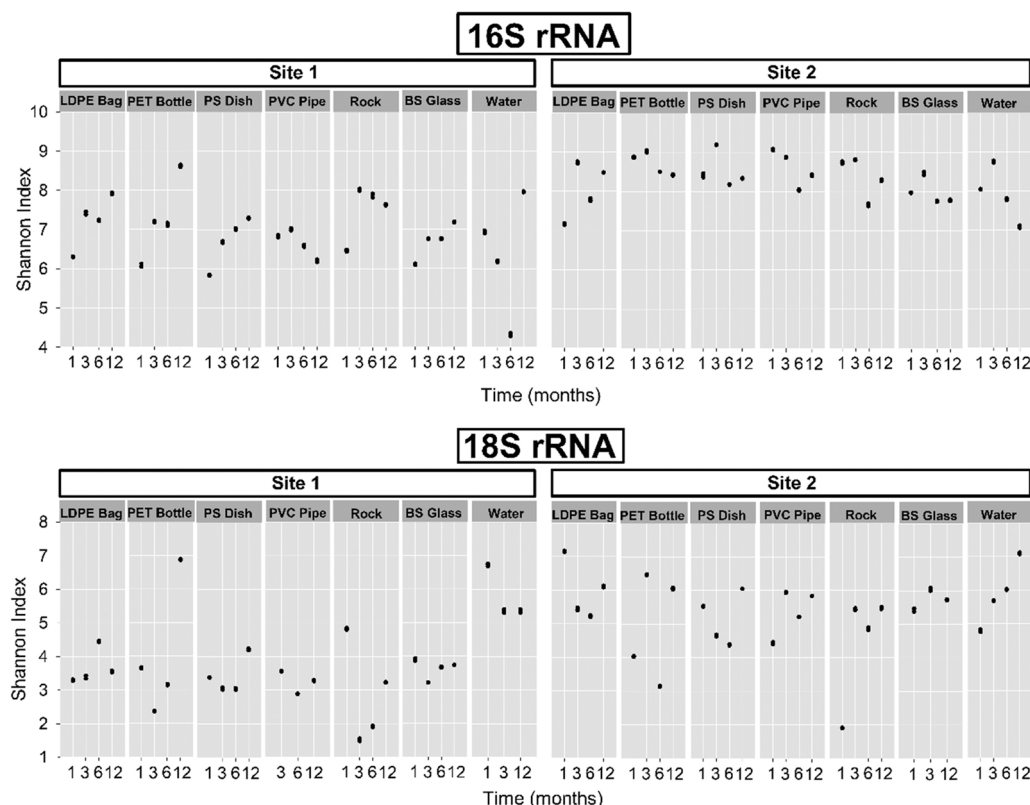


Fig. 2. Shannon Index was used as an estimator of α -diversity of bacteria (16S rRNA) and eukaryotes (18S rRNA) in plastics (LDPE bag, PET bottle, PS dish and PVC pipe), BS glass, rock, and surrounding water (2.7–0.22 μ m) at the two sampling sites after 1, 3, 6 and 12 months of plastic, BS glass and rock deployment in the river. The results of the Kruskal-Wallis test are shown in [Tables S8 and S9](#) of the [Supplementary Material 1](#).

diversity, some fluctuations were found, but a clear increase in diversity was not found in any plastic substrate ([Fig. 2](#)).

In conclusion, there were significant changes in the diversity of bacteria and eukaryotes colonizing plastic substrates over time that differed between the two sampling sites and differed from the diversity of those attached to rocks and BS glass and that of the free-living.

3.5. Composition of bacterial communities on plastics

Fifty-two bacterial phyla containing 150 classes were identified in all the samples ([Supplementary Material 2](#)). Five archaeal phyla were also identified, including 8 classes. The relative abundance of the two domains was markedly unequal, with bacteria representing more than 99.9% of the relative abundance of the samples. Therefore, the following analysis focuses on the most abundant taxa in the bacterial community.

Taxonomic analyses showed a bacterial community dominated by the phylum Proteobacteria followed by phyla Bacteroidetes and Cyanobacteria, independently of the collected substrate/environment (plastic, BS glass, rock, or water), sampling site and month of collection.

At the class level, the analyses confirmed the specificity of the plastisphere compared to the bacterial communities on BS glass, rock and freshwater, significantly influenced by the sampling site ([Supplementary Material 2](#)). In sampling site 1, the plastisphere was dominated by the classes Alphaproteobacteria, Oxyphotobacteria and Gammaproteobacteria, similarly to the bacterial community associated with rocks. BS glass-attached bacterial communities were dominated by Alphaproteobacteria, Gammaproteobacteria and Bacteroidia. The greatest change in the bacterial community was detected in the water, highlighting the abundance of the classes Bacteroidia, Gammaproteobacteria and Actinobacteria. In contrast, at sampling site 2 the more abundant attached bacterial classes were Gammaproteobacteria, Alphaproteobacteria and Bacteroidia, independently of tested substrates or free-living bacteria in

the water column.

At lower taxonomic levels, such as order ([Fig. 3](#)) and family, the temporal evolution of the bacterial community associated with plastic in both sites was followed. Early colonizers (after 1 month of incubation), intermediate colonizers (after 3 months of incubation) and late colonizers (after 6–12 months of colonization) were recognized as the bacterial community stabilizes over time at each of the sampling sites. At both sites, early colonizers of the plastisphere were Betaproteobacteriales (mostly represented by the family Burkholderiaceae), Rhodobacteriales (family Rhodobacteraceae), Rhizobiales and Sphingomonadales (family Sphingomonadaceae), independently of the selected sampling site. These orders were followed in abundance by the orders Methanomassiliicoccales (family Methylophilaceae) and Chitinophagales (family Saprospiraceae) at sampling site 1, and the orders Betaproteobacteriales (family Rhodocyclaceae) and Methylococcales (family Methylomonaceae) at sampling site 2, denoting some variability in the bacterial community according to site.

After 3 months of incubation, a considerable change took place in the most abundant bacteria. The overall relative abundance of all identified taxa decreased ([Fig. 3](#); [Supplementary Material 2](#)). However, the orders Betaproteobacteriales (mostly represented by the family Burkholderiaceae) and Rhizobiales are still the dominant orders at both sites. In addition to these, the order Chitinophagales (mostly represented by the family Saprospiraceae at site 1 and by the family Chitinophagaceae at site 2) was another abundant order in both sampling sites. At each site, the plastisphere at sampling site 1 showed high abundance of the orders Sphingomonadales (family Sphingomonadaceae) and Pirellulales (family Pirellulaceae). On the other hand, at site 2, the orders Betaproteobacteriales (family Rhodocyclaceae) and Methylococcales (family Methylomonaceae) were still dominant.

After 6 months of incubation, the bacterial community attached to the plastisphere seemed to be sufficiently established, with no further

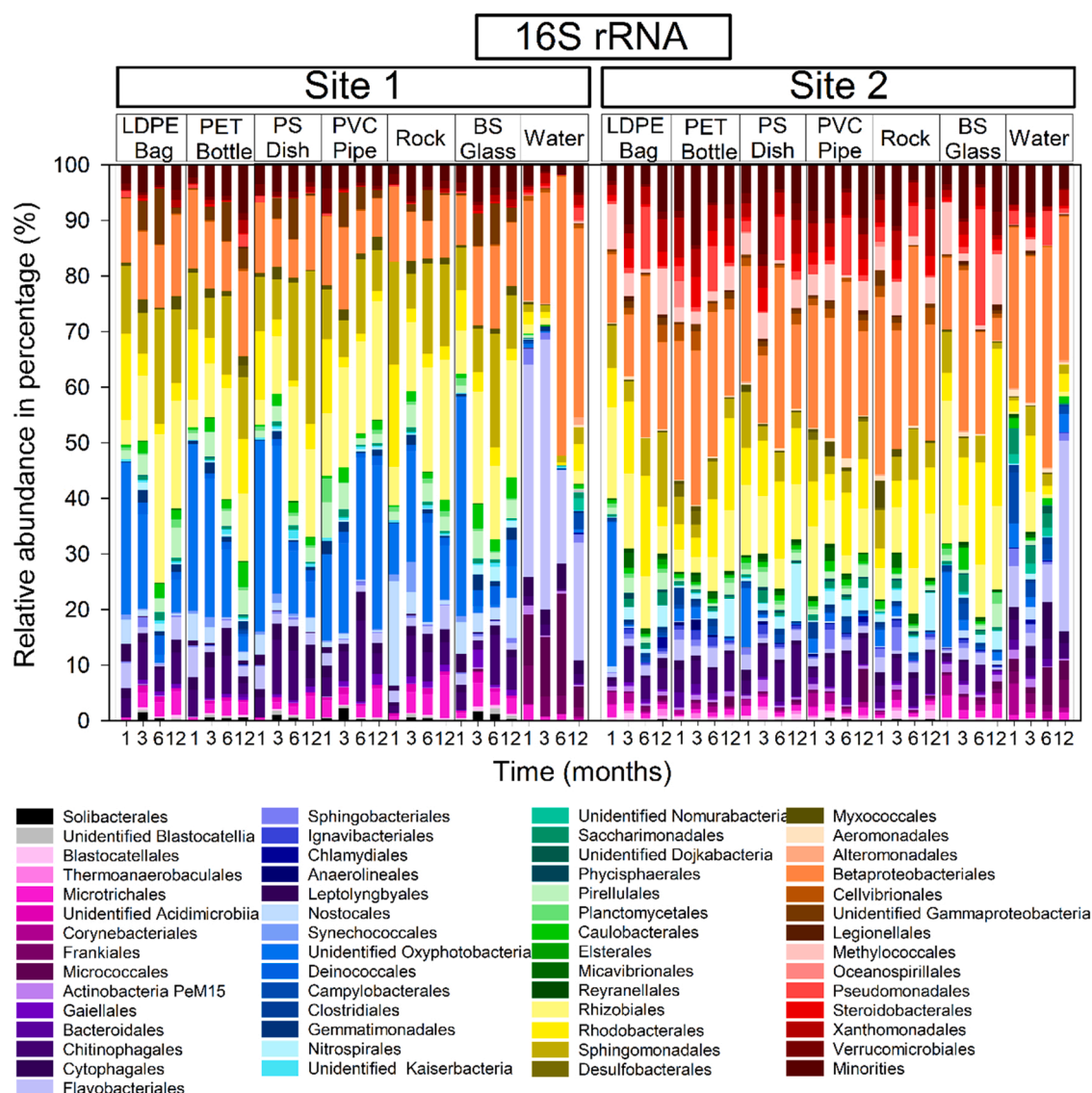


Fig. 3. Relative abundance of bacterial orders in the plastic substrates (LDPE bag, PET bottle, PS dish and PVC pipe), BS glass, rock, and surrounding water at both sampling sites with increasing incubation periods (1, 3, 6 and 12 months). Minorities include orders whose representation was lower than 1%.

significant changes (Fig. 3; Supplementary Material 2). Therefore, the orders Rhizobiales (represented mostly by the family Hyphomicrobiaceae in the plastisphere of site 1 as well as by the family Rhizobiaceae in site 2) and Betaproteobacteriales (represented mostly by the family Burkholderiaceae) and Chitinophagales (represented by the family Chitinophagaceae at site 1 and by the family Saprospiraceae in site 2) were the most abundant ones. The order Sphingomonadales (mostly represented by the family Sphingomonadaceae) was again dominant at both sites. Some of the most abundant orders at this time of colonization were only relevant in each site, with the order Microtrichales, represented mainly by the family Microtrichaceae, at site 1. At site 2, the most abundant bacterial order was Rhodobacterales, mainly represented by the family Rhodobacteraceae.

After 1 year, there were no further significant changes in the bacterial community at the order level (Fig. 3; Supplementary Material 2). The orders with the highest abundance at both sampling sites include Rhizobiales (family Rhizobiaceae), Sphingomonadales (family Sphingomonadaceae), Betaproteobacteriales (family Burkholderiaceae) and Chitinophagales (represented mostly by the family Saprospiraceae at site 1 and by the family Chitinophagaceae at site 2). Moreover, the abundance of Rhodobacterales (family Rhodobacteraceae) increased in

both sites. However, there were some orders whose relative abundance was higher according to the site and incubation time, such as the order Cytophagales (family Hymenobacteraceae) at site 1 and Nitrospirales (family Nitrospiraceae) at site 2.

3.6. Composition of eukaryotic communities on plastics

Full taxonomic assignment obtained using SILVA 132 database can be found in Supplementary Material 3. All 18S rRNA sequences were identified as eukaryotes. The eukaryotic organisms identified do not exclusively consist of microorganisms but include multicellular organisms that can also colonize the plastisphere. Most of the sequences collected from the sample set were identified as being part of the clades Opisthokonta, SAR or Harosa (represented mainly by the group Stramenopiles), and Archaeplastida (constituted primarily by Chloroplastida). 6.3% of the sequences were identified only as eukaryotic, with no further assignment.

At lower taxonomic levels, the effect of the sampling site on the sample set was more prominent. Samples collected at sampling site 1 showed the dominance of the phylum Ochrophyta, specifically of the class Diatomea. Another frequent phylum was Mollusca, represented

mainly by the class Gastropoda, and Platyhelminthes, represented by the class Rhabditophora. In the case of sampling site 2, a higher diversity of taxa was found. The dominant phyla were Bryozoa (highlighting the presence of the class Phylactolaemata), Annelida (mainly the class Clitellata) and Platyhelminthes (represented mainly by the class Gastropoda).

The taxonomic analysis detected changes in relative abundance in all samples at the order (Fig. 4) and family level. As with bacteria, an ecological succession of the eukaryotic community attached to the plastisphere could be observed at both sites. As an exception, the order Achnanthes (site 1), specifically, most of these sequences were as the genus *Cocconeis* of the family Cocconeidae was found on all substrates (plastics, BS glass, and rock), representing approximately 50% of the relative abundance of taxa found in these samples, regardless of colonization time.

Regarding temporal succession, potential early eukaryotic colonizers of the plastisphere could be identified after the first month of incubation (Fig. 4; Supplementary Material 3). In this first phase, the eukaryotic orders with the highest relative abundance differed widely between both sites. Only the superorder Heterobranchia (unassigned family and order)

showed a high relative abundance at both sites. At site 1, the orders with the highest relative abundance in the plastic assemblage were Tricladida (family Planariidae) and the orders of photosynthetic organisms Chaetophorales (mostly represented by the family Chaetophoraceae), Cymbellales (family Gomphonemataceae) and Ulvales (family Monostromataceae). Instead, at site 2, several types of multicellular organisms of the order Haptotaxida (such as the family Naididae), Diptera, Catenulida (family Stenostomidae) as well as the protist order Tectofilosida dominated.

After 3 months of incubation, the eukaryotic taxa with the highest relative abundances were clearly different to those found after 1 month of colonization and could be considered as intermediate colonizers. (Fig. 4; Supplementary Material 3). Superorder Heterobranchia, the order Tricladida (family Planariidae) and Diptera are the most abundant. At site 1, photosynthetic organisms still play a major role in the community, with the algae of the order Chaetophorales (represented mainly by the family Chaetophoraceae) again prominent. The most abundant novel taxa at this time of colonization included the order Bubarida (whose most abundant family is Scopalinidae) and Caenogastropoda (represented mainly by the family Caecidae). At sampling

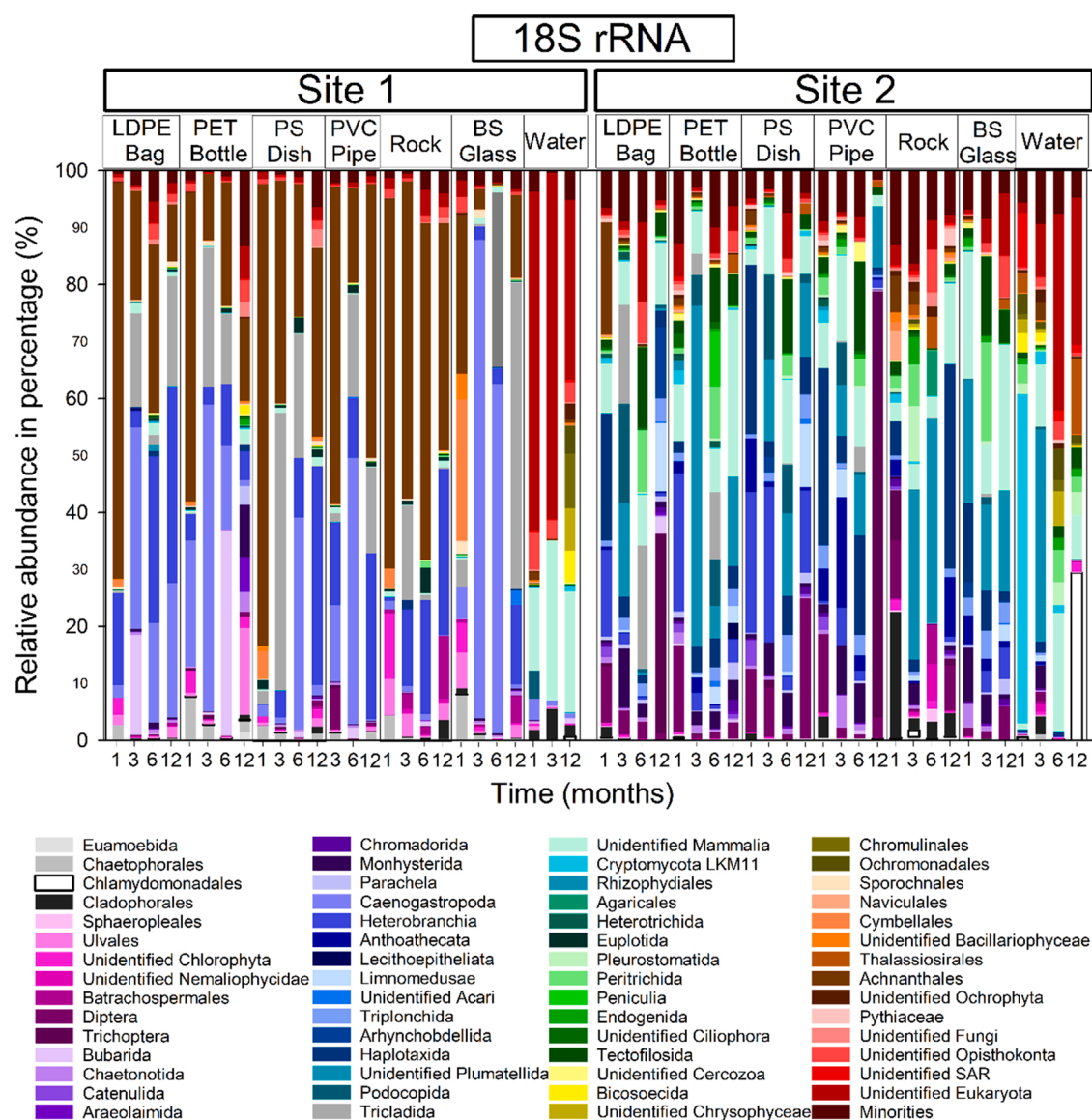


Fig. 4. Relative abundance of the eukaryotic community at the order level associated with the different plastic substrates (LDPE bag, PET bottle, PS dish and PVC pipe), BS glass, rock, and surrounding water incubated at both sampling sites with increasing incubation times (1 month, 3 months, 6 months, and 12 months). Minorities include orders whose representation is less than 1%.

site 2, the order Haplotaxida (family Naididae) was quite abundant. Other relevant taxa were the ostracod order Podocopa (family Cypriidae), the bryozoan order Plumatellida (family unidentified) and the nematode order Monhysterida.

After six months of incubation, as already found for the bacterial community, the eukaryotic community in the platisphere at the order level was already settled since previously detected taxa remained (Fig. 4; Supplementary Material 3). At site 1, the superorder order Bubarida (whose most abundant family is Scopalinidae) and Caenogastropoda (represented mainly by the family Caecidae) were still well established. In addition, the relative abundance of the order Euplotidae (represented mainly by the family Aspidiscidae) increased markedly. At sampling site 2, the orders Plumatellida, Monhysterida and Podocopa (family Cypriidae) were still very abundant. The orders Haplotaxida and Tectofilosida, which were already prominent in the early stage of colonization, increased their relative abundance after 6 months of incubation. However, some new orders with high relative abundances appeared, such as the order Peritrichida (family Opisthionectidae) and Triplonchida. Specifically, the order Tricladida (represented mainly by the family Planariidae) showed a high relative abundance at both sites.

After one year, there were no further significant changes in the eukaryotic community (Fig. 4; Supplementary Material 3). The abundance of the superorder Heterobranchia remained high at both sites. At site 1, the order Tricladida (family Planariidae) and the order Caenogastropoda (family Caecidae) have been retained from the mid-stage of colonization. The orders Ulvales (family Monostromataceae) and Chaetophorales increased their abundance at this stage. In addition, the nematode order Monhysterida increased in relative abundance to a considerable degree in this late phase of colonization. At site 2, the orders Tectofilosida, Plumatellida, Haplotaxida, and Triplonchida were retained from the intermediate stage of colonization. The order Diptera was also found at this stage and several taxa increased their abundance at this stage, such as Trichoptera and Arhynchobdellida (family Erpobdellidae).

3.7. β -diversity

The db-RDA analysis (based on the Bray-Curtis distance matrix using ASVs) revealed a similar clustering structure for bacterial and eukaryotic communities (Fig. 5). The distribution of the samples is mainly based on their site (sampling site), finding a very clear differentiation in the distance on the X-axis (which explained the 26.42% of the difference between clusters in bacteria and 22.63% of that difference in eukaryotes). The samples from site 1 are mostly distributed around the Y-axis in a homogenous way according to the substrate and to a lesser extent to colonization time. Samples from site 2 showed a more dispersed distribution, although there was a certain homogenous pattern of distribution along the Y-axis (which explained the 10.26% difference between clusters in bacteria and 7.77% of that difference in eukaryotes) according to the type of the substrate and the time of the colonization. The bacterial and eukaryotic community in water was distinctly different from those substrates at both sites, which is illustrated in the clustering of these samples far apart on the X-axis from the rest of the samples.

The distribution of the samples hierarchized by UPGMA dendrograms (Fig. S6 for bacteria and Fig. S7 for eukaryotes in Supplementary Material 1) confirms these results. The clusters were ordered according to the factors considered in our study (location, incubation time and type of substrate). In this regard, the dendrograms clearly show that the clusters are ordered first by sampling site, then by substrate type and lastly by the time of colonization.

A Monte-Carlo permutation test (999 unrestricted permutations) was performed to better explain the potential influence of the parameters considered in this study regarding bacterial and eukaryotic communities; the parameters were: sampling site, colonization time, substrate and within substrates only plastic; in the case of bacterial communities, the concentration of antibiotics was also added (Table 1). The analysis

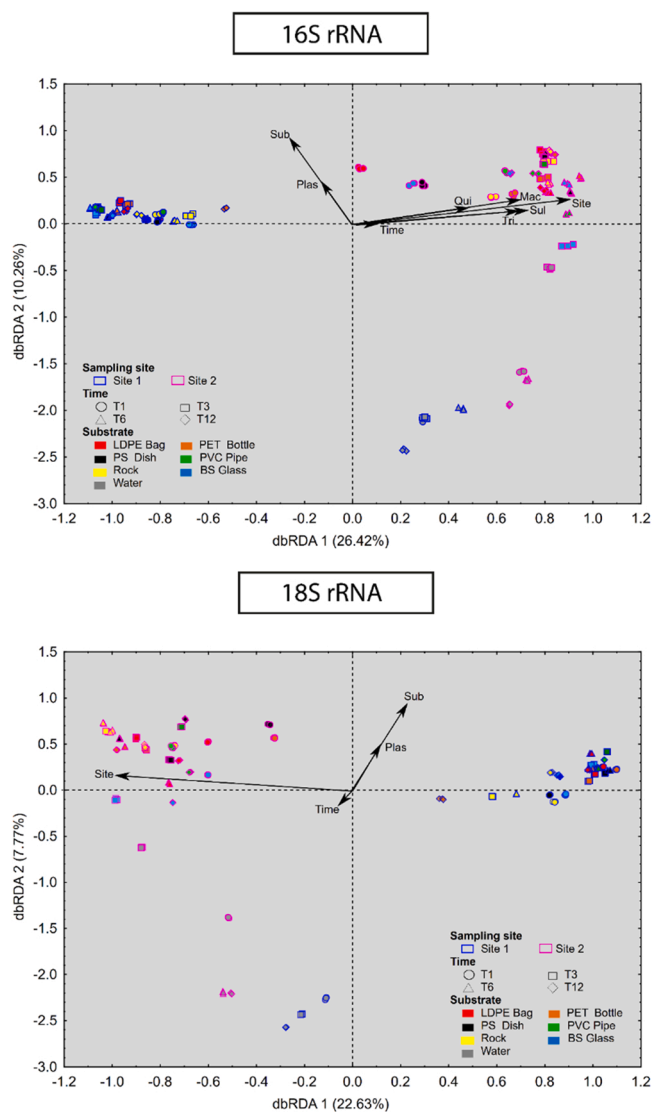


Fig. 5. Distance-based redundancy analysis (dbRDA) of bacterial (16S rRNA) and eukaryotic (18S rRNA) communities. Each point in the ordination plot represents the community in a given sample. The factor abbreviations are Sub (Substrate); Plas (plastic); Qui (Quinolones); Mac (Macrolides); Tri (Trimethoprim); Sul (sulphonamide). T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization).

confirmed a significant influence of the sampling site, type of substrate, plastic and colonization time in eukaryotes and concentration of the antibiotics (p -value < 0.05) in the case of bacteria. This analysis confirmed the previous ones as the factor that explained most of the variation in the microbial communities was the sampling site (24.6% for bacteria and 22.1% for eukaryotes). In the bacterial communities, the second factor explaining most of the variation was the concentration of antibiotics, namely, sulphonamides (17.6%), trimethoprim (16.4%), macrolides (16.8%); the third factor was the type of substrate (11.4%), followed by the concentration of quinolones (9.2%), whether the material was plastic or not (3.63%) and the sampling period (3.62%). In eukaryotes, the order of the factors explaining the variation was similar, with type of substrate accounting for 8.4% of the differences, plastic type 3.1%, and the sampling period 3.14%. This model explained 56.2% of the differences between samples in the bacterial communities, and 32.7% in the eukaryotic communities showing that several factors are already relevant, as found in the db-RDA model.

The differences in the microbial community between samples were

Table 1

Results of the Monte-Carlo permutation tests (999 unrestricted permutations) and percent variation explained for variables considered in the db-RDA analysis.

Gene	Factor	Sum of Squares	F	P-value	Proportion of explained variation (%)
16S rRNA	Site	13.99	93.99	0.001	24.64
	Time	2.05	13.82	0.001	3.62
	Substrate	6.51	43.69	0.001	11.45
	Plastic	0.55	3.71	0.004	3.63
	Quinolones	2.17	14.57	0.001	9.19
	Sulphonamides	2.49	16.76	0.001	17.65
	Trimethoprim	2.98	20.00	0.001	16.45
	Macrolides	2.37	15.88	0.001	16.82
	Residual	23.68			
	Model	33.13	27.80	0.001	56.22
18S rRNA	Site	13.48	52.02	0.001	22.15
	Time	1.91	7.38	0.001	3.14
	Substrate	4.94	19.08	0.001	8.37
	Plastic	0.66	2.55	0.007	3.19
	Residual	39.92			
	Total	21.00	20.26	0.001	32.77

confirmed using global and category-based PERMANOVA in this study: sampling site, colonization time and substrate (p -value < 0.05; Table S10 and Table S11 of the Supplementary Material 1). In contrast, pairwise comparisons were not significant in either bacterial or eukaryotic communities regarding sampling site, sampling time or substrate (p -value > 0.05; Table S10 and Table S11 of the Supplementary Material 1). As pairwise PERMANOVA tests did not detect significant differences among microbial communities, linear discriminant analyses (LEfSe) were subsequently used to confirm further whether certain taxa (mostly at the genus level) were significantly more abundant in each substrate considering sampling sites and colonization time (Table S12 of the Supplementary Material 1 and Table S13 of the Supplementary Material 1); A summary of the taxa identified in each of the plastics is reflected in Tables 2 and 3 (differential prokaryotic taxa) and Tables 4 and 5 (differential eukaryotic taxa).

The presence of these taxa in both the bacterial and eukaryotic communities of each plastic at each sampling point at different times at each sampling point allows for defining a core microbiome (biome in the case of eukaryotic taxa). An ecological succession of the community attached to the plastisphere could be observed at both sites as the core microbiome/biome could be categorized in each plastic according to early colonizers (after one month of colonization), intermediate colonizers (after 3 months of colonization), and late colonizers (after 6–12 months of colonization, although LEfSe analyses allowed to identify specific core microbiomes for both time periods) (Tables 2, 3, 4 and 5).

3.8. Plastic polymer alterations

Plastic samples incubated at the two sampling sites were characterized by ATR-FTIR analysis at the end of the experiment (12 months) and compared with virgin, non-incubated plastics, as shown in Fig. 6. There were clear changes in chemical structure with time, as evidenced by the formation of new functional groups as a result of environmental aging of plastics in comparison with non-incubated plastics (Fig. 6). Some differences in the spectra of PS dish were observed between sampling sites, but no significant changes were noticed between the spectra of LDPE bags, PET bottles and PVC pipes deployed at the two different sampling sites (Fig. 6).

Most of the aged plastic samples were characterized by the appearance of new absorption bands in the regions of 3300–3305 cm^{-1} and 1745–1635 cm^{-1} , corresponding to the formation of hydroxyl and carbonyl groups, respectively (Fig. 6). In LDPE bags, two significant peaks appeared in the 1000–1200 cm^{-1} region, which could be attributed to the formation of carbon-oxygen bonds (Fig. 6). Furthermore, the presence of a new absorption band around 1640 cm^{-1} may be assigned to unconjugated C=C, previously described and considered characteristic of the degradation process of LDPE (Otake et al., 1995).

The results for the evolution of hydroxyl indices (Table S14 of the Supplementary Material 1) revealed that all deployed plastics underwent certain degradation after 1 year of incubation in both sites. The degradation process showed some differences depending on the type of plastic and the sampling site (Table S14 of the Supplementary Material 1). Hydroxyl index was higher in the LDPE bag and PET bottle from sampling site 1 in comparison with sampling site 2 (Table S14 of the Supplementary Material 1). In contrast, hydroxyl index was higher in the PS dish and PVC pipe from sampling site 2 in comparison with sampling site 1 (Table S14 of the Supplementary Material 1).

3.9. Antibiotic resistance genes (ARGs) detected on plastics

Site 1 was characterized by quite low or even undetectable antibiotic concentrations in water as compared to site 2 (Table S6 of the Supplementary Material 1). ARGs abundance in the substrates (plastics, glass, or rocks) was negligible, although low abundance of the tested ARGs was found at some sampling times in the free-living bacteria in the surrounding water (Fig. 7).

The sampling site 2 was characterized by higher antibiotic concentrations in water (Table S6 of the Supplementary Material 1) and had a higher abundance of ARGs not only in the free-living bacteria but also in plastics, glass, and rocks (Fig. 7) as compared to site 1. In general, neither of the tested ARGs were more abundant in the substrates compared to tested ARGs in the free-living bacteria, implying that the tested substrates (plastic, BS glass or rock) did not concentrate them. *sul1* was relatively more abundant in the free-living bacteria throughout all colonization times (p -value < 0.05, Table S15 of the Supplementary Material 1), except for the 3-month time. The relative abundance of the *sul1* gene in the plastics was always lower than in the free-living bacteria but significantly higher than in the rock and glass after 6 and 12 months of incubation (p -value < 0.05; Table S15 of the Supplementary Material 1).

Regarding the *ermF* gene, its relative abundance was only higher in free-living bacteria after the first month of colonization ($2^{-\Delta\text{ct}}$ value of 0.006, Fig. 7; p -value < 0.05; Table S16 of the Supplementary Material 1). After the first 3 months of colonization, the abundance of this gene in BS glass surpassed by far the abundance in the rest of the substrates ($2^{-\Delta\text{ct}}$ value of 0.006, Fig. 7; p -value < 0.05; Table S16 of the Supplementary Material 1). In contrast, in the later incubation periods (6 and 12 months of incubation) the relative abundance of this gene in the plastic was the highest among substrates ($2^{-\Delta\text{ct}}$ value of 9.5×10^{-3} and 1.8×10^{-3} respectively),

Concerning *dfrA*, the relative abundance of this gene in the free-living bacteria was higher than in any of the substrates used, regardless of incubation time (p -value < 0.05; Table S16 of the Supplementary Material 1).

Table 2

Summary of the differential bacterial taxa in sampling site 1 identified by comparing plastic items between sampling sites over time using linear discriminant analyses (LEfSe). Taxa with the highest Log LDA (Linear Discriminant Analysis) score in each group are listed. The complete dataset (with all substrates) is shown in Table S12 of the Supplementary Material 1.

Time	Substrate	Taxa	Log LDA score
1 month	LDPE bag	<i>Pseudorhodobacter</i>	4.41
		<i>Calothrix</i>	4.32
		Flavobacteriaceae	4.02
		<i>Porphyrobacter</i>	3.88
		<i>Lacihabitans</i>	3.27
	PET bottle	<i>Silvanigrella</i>	3.18
		<i>Streptococcus</i>	3.57
		<i>Pseudorhodobacter</i>	3.45
		<i>Stigeoclonium</i>	2.05
		<i>Rhodopirellula</i>	4.21
	PS dish	<i>Gemella</i>	2.91
		<i>Haemophilus</i>	2.62
		<i>Rothia</i>	2.24
	PVC pipe	<i>Gemmatimonas</i>	3.89
		<i>Pirellula</i>	3.76
		<i>Fluviicola</i>	3.56
		<i>Limnobacter</i>	3.26
		<i>Rhizobacter</i>	3.98
3 months	LDPE bag	<i>Maribacter</i>	3.49
		<i>Blastopirellula</i>	3.27
		<i>Imbrioglobus</i>	2.91
		<i>Sandaracinus</i>	2.39
		<i>Pleurocladia</i>	3.43
	PET bottle	<i>Rhodopirellula</i>	3.26
		<i>Nannocystis</i>	2.89
		<i>Silvanigrella</i>	2.82
		<i>Neochloris</i>	2.56
		<i>Oligoflexus</i>	2.37
	PS dish	<i>Ferrovibrio</i>	2.16
		Methylophilaceae	3.84
		Rhodocyclaceae	2.80
	PVC pipe	<i>Snodgrassella</i>	2.34
		<i>Schizothrix</i>	4.27
		<i>Paludibaculum</i>	3.80
		<i>Bryobacter</i>	3.42
		<i>Rhodopirellula</i>	2.97
6 months	LDPE bag	<i>Hyphomicrobium</i>	3.98
		<i>Amoebophilus</i>	3.84
		<i>Luteolibacter</i>	2.53
	PET bottle	<i>Gallionella</i>	2.40
		<i>Lacihabitans</i>	4.09
		<i>Steroidobacter</i>	2.57
	PS dish	<i>Schleiferia</i>	2.21
		<i>Roseibacillus</i>	2.79
	PVC pipe	<i>Taeseokella</i>	3.65
		<i>Pajaroellobacter</i>	2.78
		<i>Polyangium</i>	2.69
	LDPE bag	<i>Cytophaga</i>	2.05
		<i>Pirellula</i>	3.66
		<i>Fimbrioglobus</i>	2.98
		<i>Massilia</i>	2.72
		<i>Bdellovibrio</i>	2.65
12 months	LDPE bag	<i>Lacibacter</i>	2.56
		<i>Peredibacter</i>	2.52
		<i>Hymenobacter</i>	4.30
		<i>Hyphomonas</i>	3.83
		<i>Hirschia</i>	3.69
	PET bottle	<i>Acidibacter</i>	3.59
		<i>Leptothrix</i>	3.37
		<i>Dongia</i>	3.30
		<i>Rhodobacter</i>	3.12
		<i>Pleurocapsa</i>	3.83
	PS dish	<i>Sphingorhabdus</i>	3.83
		<i>Haliangium</i>	3.76
		<i>Rickettsia</i>	3.52
		<i>Deinococcus</i>	3.48
		<i>Hymenobacter</i>	3.46
	PVC pipe	<i>Ilumatobacter</i>	4.29

Table 3

Summary of the differential bacterial taxa in sampling site 2 identified by comparing plastic items between sampling sites over time using linear discriminant analyses (LEfSe). Taxa with the highest Log LDA (Linear Discriminant Analysis) score in each group are listed. The complete dataset (with all substrates) is shown in Table S12 of the Supplementary Material 1.

Time	Substrate	Taxa	Log LDA score
1 month	LDPE bag	<i>Tychonema</i>	3.63
		<i>Amoebophilus</i>	3.21
		Desulfatitalea	2.15
		<i>Arenimonas</i>	4.16
		<i>Pseudomonas</i>	3.82
	PET bottle	<i>Pseudohongiella</i>	3.58
		<i>Desulfomicrobium</i>	3.23
		<i>Accumulibacter</i>	2.90
		<i>Inhella</i>	3.35
		<i>Verrucomicrobium</i>	2.97
	PS dish	<i>Lacunisphaera</i>	2.77
		<i>Cellvibrio</i>	2.76
		<i>Bdellovibrio</i>	2.68
		<i>Sphingomonas</i>	3.51
		<i>Altererythrobacter</i>	3.08
3 months	LDPE bag	<i>Competibacter</i>	3.05
		<i>Propionivibrio</i>	2.70
		<i>Rhizobacter</i>	2.66
		<i>Defluviimonas</i>	3.80
		<i>Chryseobacterium</i>	3.34
	PET bottle	<i>Aeromonas</i>	3.12
		<i>Blastopirellula</i>	3.09
		<i>Peredibacter</i>	2.98
		<i>Nitratireductor</i>	2.89
		<i>Thiobacillus</i>	3.91
	PS dish	<i>Dechloromonas</i>	3.65
		<i>Roseomonas</i>	3.39
		<i>Desulfobacter</i>	3.39
		<i>Competibacter</i>	3.17
		<i>Crenothrix</i>	3.01
6 months	LDPE bag	<i>Reyranella</i>	3.55
		<i>Dinghuibacter</i>	3.37
		<i>Luteitalea</i>	3.19
		<i>Rickettsia</i>	2.76
		<i>Planctopirus</i>	2.53
	PET bottle	<i>Competibacter</i>	2.67
		<i>Permianibacter</i>	2.66
		<i>Nitrosomonas</i>	2.62
		<i>Chloroflexi</i>	2.34
		<i>Gemmobacter</i>	3.13
	PS dish	<i>Paracoccus</i>	2.96
		<i>Thiothrix</i>	2.90
		<i>Acetobacterium</i>	2.53
		<i>Brachymonas</i>	2.35
		<i>Dialister</i>	2.34
12 months	LDPE bag	<i>Arenimonas</i>	3.13
		<i>Acetoanaerobium</i>	2.96
		<i>Acinetobacter</i>	2.78
		<i>Rhodoferrax</i>	2.54
		<i>Tolomonas</i>	2.48
	PET bottle	<i>Accumulibacter</i>	2.18
		<i>Lautropia</i>	3.30
		<i>Staphylococcus</i>	3.09
		<i>Lawsonella</i>	2.91
		<i>Comamonas</i>	2.80
	PS dish	<i>Pirellula</i>	2.51
		<i>Pedobacter</i>	2.47
		<i>Zoogloea</i>	4.01
		<i>Thauera</i>	4.00
		<i>Terrimonas</i>	3.81
12 months	LDPE bag	<i>Aeromonas</i>	3.75
		<i>Acidovorax</i>	3.73
		<i>Cloacibacterium</i>	3.68
		<i>Methyloparacoccus</i>	3.22
		<i>Terrimicrobium</i>	2.73
	PET bottle	<i>Finegoldia</i>	2.70
		<i>Pirellula</i>	2.69
		<i>Paracaedibacter</i>	2.62
		<i>Anaerococcus</i>	2.60
		<i>Nitroga</i>	3.51

(continued on next page)

Table 3 (continued)

Time	Substrate	Taxa	Log LDA score
	PS dish	<i>Tahibacter</i>	3.21
		<i>Lautropia</i>	2.90
		<i>Vogesella</i>	2.33
		<i>Schlesneria</i>	2.24
		<i>Nitrospira</i>	4.42
		<i>Chthoniobacter</i>	3.10
		<i>Pseudoduganella</i>	2.88
		<i>Chromobacterium</i>	2.75
	PVC pipe	<i>Alysiosphaera</i>	2.55
		<i>Corynebacterium</i>	3.36
		<i>Chthoniobacter</i>	3.03
		<i>Luteolibacter</i>	2.87
		<i>Rhodovastum</i>	2.83
		<i>Atopostipes</i>	2.76
		<i>Leeia</i>	2.71

Table 4

Summary of the differential eukaryotic taxa in sampling site 1 identified by comparing plastic items between sampling sites over time using linear discriminant analyses (LEfSe). Taxa with the highest Log LDA (Linear Discriminant Analysis) score in each group are listed. The complete dataset (with all substrates) is shown in Table S13 of the Supplementary Material 1.

Time	Substrate	Taxa	Log LDA score
1 month	PET bottle	<i>Aphanochaete</i>	3.92
		<i>Chaetopeltis</i>	3.22
3 months	PS dish	<i>Cocconeis</i>	5.55
		<i>Poales</i>	2.35
	LDPE bag	<i>Pseudourostyla</i>	3.19
		<i>Daptonema</i>	2.65
		<i>Catenula</i>	2.36
		<i>Marsilea</i>	2.28
	PET bottle	<i>Schmidtea</i>	3.18
		<i>Continenticola</i>	2.74
	PVC pipe	<i>Mermithida</i>	3.85
	LDPE bag	<i>Oenothera</i>	2.55
6 months	LDPE bag	<i>Rheum</i>	2.41
		<i>Synchaeta</i>	2.30
		<i>Haptoria</i>	2.08
		<i>Heteromita</i>	2.00
	PET bottle	<i>Bubarida</i>	5.17
	PS dish	<i>Sialis</i>	2.41
	PVC pipe	<i>Vorticella</i>	3.60
		<i>Eucapnopsis</i>	3.04
	LDPE bag	<i>Taphrina</i>	2.50
		<i>Paulinella</i>	2.54
		<i>Copromyxa</i>	3.69
		<i>Dictyamoeba</i>	2.69
		<i>Filamoeba</i>	2.60
		<i>Angulamoeba</i>	2.47
	PS dish	<i>Rhizamoeba</i>	2.07
		<i>Erynia</i>	3.02
		<i>Fabales</i>	2.29
		<i>Navicula</i>	2.25
12 months	PVC pipe	<i>Plantago</i>	2.20
		<i>Eimeriidae</i>	2.33

The *qnrSrtF11A* gene was relatively more abundant in the free-living bacteria in comparison with the rest of substrates at most incubation times, except for the three-month incubation where this gene was more abundant both in plastic ($2^{-\Delta ct}$ value of 8.12×10^{-4}) and BS glass ($2^{-\Delta ct}$ value of 7.85×10^{-4}) than in rock and free-living bacteria (p -value < 0.05; Table S18 of the Supplementary Material 1).

After 3 months of incubation, the Spearman's correlation analysis (Table S19 of the Supplementary Material 1) confirmed a significant correlation between antibiotic concentration and the abundance of the corresponding ARG at both sampling sites and sampling time (p -value < 0.05) independently of the substrate. However, most correlations were not significant if only site 1 was considered because there was little change in the antibiotic concentration in the water (Table S19 of the

Table 5

Summary of the differential eukaryotic taxa in sampling site 2 identified by comparing plastic items between sampling sites over time using linear discriminant analyses (LEfSe). Taxa with the highest Log LDA (Linear Discriminant Analysis) score in each group are listed. The complete dataset (with all substrates) is shown in Table S13 of the Supplementary Material 1.

Time	Substrate	Taxa	Log LDA score
1 month	LDPE bag	<i>Poales</i>	3.07
		<i>Caryophyllales</i>	2.72
		<i>Stentor</i>	2.34
		<i>Chaetomium</i>	2.33
		<i>Tetraselmis</i>	2.21
		<i>Nematostelium</i>	2.00
		<i>Pelagotherix</i>	3.14
		<i>Plagiopyla</i>	2.71
	PET bottle	<i>Clevelandella</i>	2.43
		<i>Epaxella</i>	2.42
		<i>Stenostomum</i>	4.15
		<i>Stenostomum</i>	3.68
		<i>Entamoeba</i>	2.50
		<i>Angulamoeba</i>	2.17
		<i>Leptomyxida</i>	2.14
		<i>Adineta</i>	3.30
3 months	LDPE bag	<i>Scotinospaera</i>	2.53
		<i>Chlorellales</i>	2.24
		<i>Ephemeroptera</i>	2.22
		<i>Haltidites</i>	2.72
		<i>Rhizoclonium</i>	2.55
		<i>Euplotia</i>	2.41
		<i>Bullera</i>	2.16
		<i>Cypridopsis</i>	4.02
	PET bottle	<i>Dorylaimida</i>	3.24
		<i>Cryptosporidium</i>	2.46
		<i>Pterocystis</i>	2.26
		<i>Hydra</i>	4.89
		<i>Radix</i>	3.51
		<i>Stentor</i>	3.49
		<i>Placorhynchus</i>	3.24
		<i>Saccamoeba</i>	2.25
6 months	LDPE bag	<i>Girardia</i>	5.00
		<i>Pelodera</i>	3.54
		<i>Geotrichum</i>	2.74
		<i>Rhabditis</i>	2.49
		<i>Flabellula</i>	2.46
		<i>Candona</i>	4.36
		<i>Actinidia</i>	2.53
		<i>Schistonchus</i>	2.51
	PET bottle	<i>Ichthyospora</i>	2.48
		<i>Tripyrella</i>	4.38
		<i>Caenorhabditis</i>	2.77
		<i>Candida</i>	2.39
		<i>Mononchoidea</i>	2.37
		<i>Haplotaxida</i>	4.90
		<i>Epistylis</i>	3.57
		<i>Cyclopoida</i>	3.53
12 months	PS dish	<i>Apodibius</i>	2.40
		<i>Ptolemba</i>	2.13
		<i>Erpobdella</i>	4.77
		<i>Hypsibius</i>	3.57
		<i>Trinema</i>	2.74
		<i>Paraphanolaimus</i>	2.68
		<i>Aphelenchoides</i>	2.54
		<i>Parachela</i>	4.07
	PVC pipe	<i>Chromadorida</i>	3.98
		<i>Caenorhabditis</i>	3.07
		<i>Pinustaeda</i>	2.94
		<i>Pinophyta</i>	2.62
		<i>Limnolacarus</i>	3.51
		<i>Macrostromida</i>	3.26
		<i>Rhabditida</i>	2.62
		<i>Geotrichum</i>	2.11

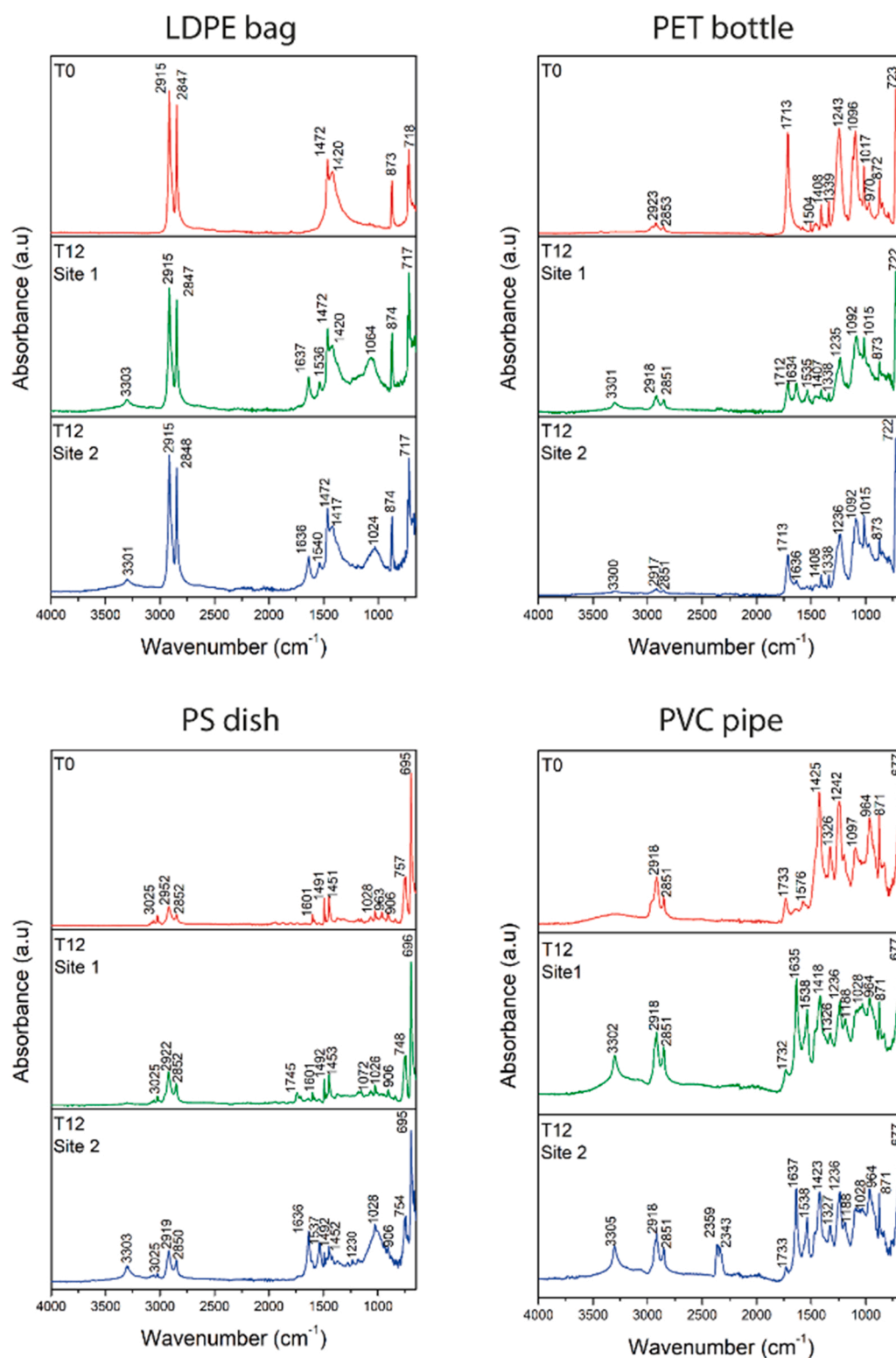


Fig. 6. ATR—FTIR comparative spectra of each plastic surface substrate after 1 year of colonization (T12) at the two sites compared with the virgin, non-incubated plastic (T0) treated following the same cleaning protocol.

Supplementary Material 1). The correlations obtained in all cases were positive (particularly in site 2 which was the site with the higher anthropogenic impact and higher antibiotic concentrations, Table S6), although their strength varied depending on the substrate and each particular ARG (Table S19 of the Supplementary Material 1). The strongest correlations of *sul1* (0.89) and *dfrA* genes (0.97) were with

plastic, the *ermF* gene with rock (0.78), and the *qnrSrtF11A* gene with BS glass (0.83; Table S19 of the Supplementary Material 1). In general, the weakest correlations were in the water where the *sul1* (0.5) and *qnrSrtF11A* (0.54) genes showed the strongest correlation (Table S19 of the Supplementary Material 1).

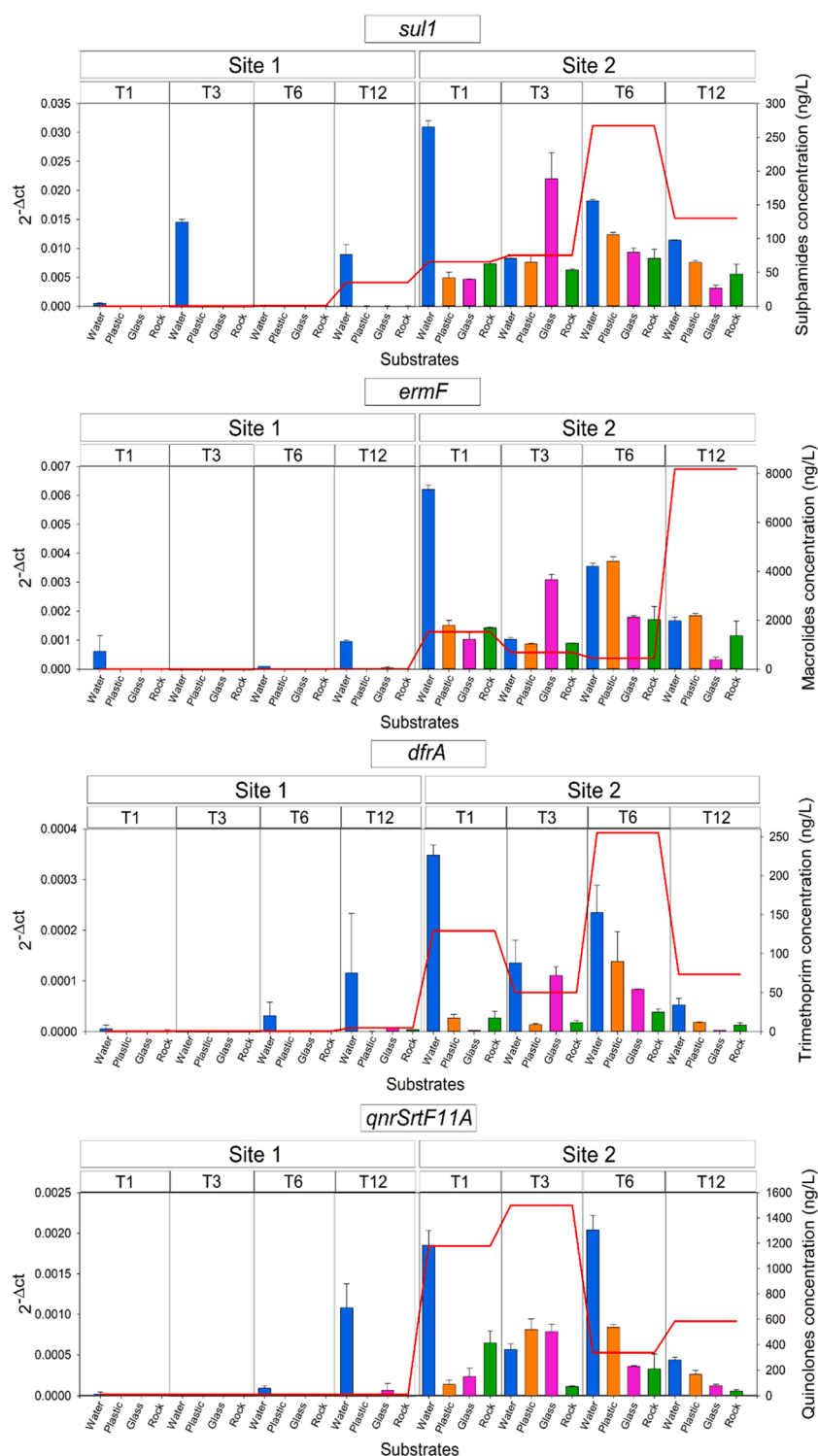


Fig. 7. Relative abundance ($2^{-\Delta ct}$) of *sul1*, *ermF*, *dfrA* and *qnrSrtF11A* genes in comparison with the concentration of sulphonamides, macrolides, trimethoprim, and quinolones, respectively, in both sampling sites at the different sampling times [T1 (1 month of colonization); T3 (3 months of colonization); T6 (6 months of colonization); T12 (12 months of colonization)]. The colour of the graph bar corresponds to the type of substrate: blue: water; orange: plastic; pink: BS glass; green: rock. The red line represents the variation in the measured antibiotic concentration.

4. Discussion

This study represents a time-course evaluation of the dynamics of the eukaryotic and bacterial communities developed on everyday plastic items over a year in two sites with different levels of anthropogenic impact. The evaluation of these three factors (site, type of substrate, and incubation time) is essential to understand the plastisphere dynamics. The results show that sampling site is the main factor influencing the microbial diversity of the different substrates used. Previous studies in freshwater and marine ecosystems, at different times of plastic

colonization and with different types of plastics, have reported site as the main factor determining the structure of bacterial communities in the plastisphere (Barros and Seena, 2021; Di Pippo et al., 2020; Wright et al., 2021b, Martínez-Campos et al., 2021). The most comprehensive report to date was performed by Wright et al. (2021b) and included meta-analysis of 16S rRNA sequencing results from more than 30 studies developed in a variety of environments (terrestrial, freshwater and marine water) as well as different plastics, including those used in this study (LDPE, PET, PS and PVC). Wright et al. (2021b) concluded that site is the decisive factor in the constitution of the bacterial community.

In our analysis, the two sampling sites selected showed different environmental conditions: sampling site 1, located in a natural area, was characterized by a low concentration of both, nutrients, and antibiotics, as well as good oxygenation, close to saturation. At sampling site 2, the high concentration of nutrients and antibiotics influenced by the WWTP discharge was found to be a relevant factor for bacterial communities in the plastisphere. Previous studies have also shown that WWTPs affect the biodiversity of receiving rivers, in some cases increasing it (Bondarczuk and Piotrowska-Seget, 2019; Price et al., 2018). This could explain why α -diversity values were significantly higher on all substrates at site 2. Consequently, our assay confirms that the site is the factor that mostly affects the development of plastisphere regarding both bacterial as well as in eukaryotic communities.

In this research, substrate type was the second most influential factor shaping microbial diversity. In addition, within substrate types (plastic, BS glass, rock) and surrounding water, plastic explains most of the variation regarding microbial diversity. Most of the current studies comparing different substrates (wood, glass, or rock) with plastic have found no significant differences between substrates, although there are clear differences with the surrounding water (Dussud et al., 2018; Oberbeckmann et al., 2016). On the contrary, other studies found differences between the tested substrates as well as a distinctive microbiome core in each plastic, either between different types of plastics (Martínez-Campos et al., 2021; Xu et al., 2019) or concerning other artificial surfaces (Mieczan, 2020). The differences in the morphology of the plastics used in the study could also explain the changes in the eukaryotic and attached bacterial community as Cheng et al. (2021) suggested, indicating that the morphology of the plastics could promote the development of certain specific taxa as well as the characteristics of the polymer it is made of.

The colonization time was the least significant factor influencing the development of the eukaryotic and bacterial community attached to the plastics in our experiment. Over time, the surface of the plastic begins to suffer a certain degree of degradation, as indicated by the hydroxyl index values obtained at both sites. Subsequently, the plastisphere matures as time progresses and the plastic-associated community tends to converge and become more similar over time, reducing the differences between microbial communities in different substrates (Mincer et al., 2019). This explains the decreasing difference between the substrates in the db-RDA analysis. Secondly, the season of the year promotes the growth and development of certain organisms in the environment, which is relevant for the constitution of the plastisphere, as it has been previously evidenced in marine ecosystems (Amaral-Zettler et al., 2020) and freshwater ecosystems in shorter time windows than the one evaluated here (Mieczan, 2020). In this report, and concerning the differential taxa identified throughout the 12 months of the colonization experiment, we could identify an early stage (1 month of incubation), an intermediate stage (corresponding to 3 months of incubation) and a late stage of colonization (corresponding to 6–12 months of incubation).

In the early stage of development of the plastisphere, the pioneer organisms that attached to the plastic generate EPS, decreasing the hydrophobicity and roughness of the material (Yang et al., 2020). In our study, the families Rhodobacteraceae and Sphingomonadaceae probably played these roles. These families have previously shown their ability to attach to different plastic substrates without showing any type of preference, producing exopolysaccharides and surface-adhesion proteins (Balkwill et al., 2006; Di Pippo et al., 2020; Kviatkovski and Minz, 2015). In addition, the family Sphingomonadaceae is characterized by its high capacity to form biofilms in aquatic environments and its ability to degrade a wide range of organic compounds (Di Gregorio et al., 2017). Another family found in the early phase was Burkholderiaceae characterized by several generalist genera with the ability to degrade different organic compounds, as well as to develop under different nutrient concentrations and be widely distributed in different aquatic environments (Balkwill et al., 2006). Regarding the most abundant eukaryotic taxa in the early phase, the order Achmannthales, specifically

the genus *Cocconeis* (family Cocconeidaceae) was found in all substrates and was maintained throughout the entire year of colonization. The dominance of this order of diatoms could explain the vast abundance of diatoms observed by SEM analysis, particularly in plastics deployed at site 1 (where they were found covering the surface of all materials). The presence of the genus *Cocconeis* has been previously reported in the marine plastisphere (Dudek et al., 2020; Oberbeckmann et al., 2014). Khan et al. (2020) showed the ability of diatoms to colonize different plastic surfaces. Initially, the roughness of the material is an important factor, but later, exopolymers generated by previous pioneer microorganisms influence colonization. Although diatoms were not the only primary producers attached to plastics, the abundance of the families Chaetophoraceae, Gomphonemataceae and Monostromataceae was also remarkable in the early stage of colonization, confirming the importance of photosynthetic organisms in the early shaping of the community that constitutes the plastisphere (Yokota et al., 2017).

In the mid-phase of the plastisphere colonization, which includes the colonization phase after 3 months of incubation, the presence of biofilm-forming organisms is still prominent, although bacteria with defined roles within the microbial community develop. A family that became important during this phase, although it had already appeared in an early phase, is the Burkholderiaceae family. The family Burkholderiaceae is also frequently found as part of the plastisphere in different aquatic environments (Nguyen et al., 2021; Wen et al., 2020). The importance of this family lies in its great metabolic capacity being able to degrade polymers such as polyhydroxyalkanoate (PHA) (Ma et al., 2022), or different organic complex substances (van der Zaan et al., 2012). This could explain the formation of hydroxyl and carbonyl groups associated with all the plastics used in this study. Another important family at this phase is the family Saprospiraceae. The family Saprospiraceae, such as the family Sphingomonadaceae, is also capable of producing exopolysaccharides and can utilize products generated in the biofilm as a source of carbon and energy (Yun et al., 2008). The family Microtrichaceae had been previously detected as an intermediate colonizer (4 months of incubation) in marine environments (Tu et al., 2020). This family is generalist, so it can also metabolize plastic carbon, using different types of plastics as substrates in oligotrophic environments (Agostini et al., 2021). Regarding eukaryotic organisms, the presence of certain families of multicellular organisms, such as Caecidae, Planariidae, Cyprididae, or Diptera, was remarkable. These organisms play roles as primary consumers or predators, and when they are consolidated in the plastisphere, a complex food web is developed (Amaral-Zettler et al., 2020). These findings are in line with other previous reports and indicate that many multicellular organisms can use plastics as safe refuges. This has already been demonstrated in plastic litter in the ocean (De-la-Torre et al., 2021). Furthermore, De-la-Torre et al. (2021) reported the presence of various organisms that have so far been considered invasive and others that, although not invasive, could become invasive if the plastics drift through the ecosystems.

In the late stages of the plastisphere formation (6–12 months of colonization), many of the previously described families are already consolidated, so there were no substantial changes in the families with the highest relative abundances, although there were some exceptions. This is the case for the family Hymenobacteraceae, which has been previously described in association with greenhouse plastics in rivers (Martínez-Campos et al., 2022). The Nitrospirales family, characterized by its participation in the nitrogen cycle, also formed part of the plastisphere of site 2, which may be an adaptation of the community attached to the plastisphere to the nitrogen compounds (Baskaran et al., 2020) released by the WWTP effluent. In the case of the family Hiphomicrobiaceae, its abundance increased after 6 months of colonization; some members of the family, such as the genus *Hyphomicrobium*, are restricted facultative methylotrophs, growing on C1 components, such as methanol but not compounds with three or more carbon atoms (Liu et al., 2014). These bacteria could therefore take up these compounds from other organisms already developed in the biofilm. With respect to

eukaryotes, the changes in the community were also minor. The case of the order Ulvales is particularly remarkable as it appeared again in great abundance in this phase. It has been previously recognized as a colonizer of different artificial substrates such as plastic and may colonize the inner side of packaging items, in our study it developed inside the PET bottle (Bravo et al., 2011). The order Trichoptera is also relevant at this stage. This is noteworthy, considering that Gallitelli et al. (2021) showed that certain freshwater macroinvertebrates, such as Trichoptera larvae, have a slight preference for microplastics to build their refuges as compared to natural substrates.

LEfSe analyses allowed the identification of differential genera colonizing each of the tested plastics in the different colonization times, this allowed the identification of plastic core microbiomes (biomes in the case of eukaryotic taxa) in each plastic substrate at the different stages of colonization. Some of the genera found in the core microbiome/biome of each of the plastics have relevant ecological implications or could pose a risk to human health or the environment. Specifically, in the LDPE bag microbiome core, several bacterial genera had already been reported in previous studies. *Lacihabitans* was previously found attached to plastics and was characterized by their ability to degrade compounds such as cellulose (Szabó et al., 2021). *Nitratireductor*, which appeared at site 2, is a nitrate-reducing bacteria, indicating that plastics and the associated biofilms might influence nitrogen cycling in the aquatic environment (Ashar et al., 2020). *Calothrix* is notable for its ability to produce toxins, which are dangerous to humans (Shardlow, 2021). *Aeromonas*, a potential pathogen for humans and fish, also was relatively abundant in this plastic. (Amaral-Zettler et al., 2020). Other bacterial genera previously detected as attached to LDPE plastics in river water include *Pseudorhodobacter* and *Porphyrobacter* (Martínez-Campos et al., 2022). Regarding eukaryotes, the presence of different types of plants such as *Marsilea* or *Pinophyta* could result in the input of organic matter and compounds such as cellulose on the plastics, which can be used by certain bacteria such as *Lacihabitans* (Szabó et al., 2021). *Daptonema* showed a tendency to colonize artificial surfaces after a few days in a water column (Fonséca-Genevois et al., 2006). *Nematostelium* was reported to develop in aquatic biofilms, feeding on bacteria attached to the biofilm (Lindley et al., 2007).

In the PET bottle, some of the bacteria found in the associated microbiome core had been previously described as part of the community associated with the plastisphere in aquatic environments, such as *Streptococcus* (Oberbeckmann et al., 2014), *Ferrovibrio* (Zhu et al., 2022), *Hymenobacter* (Martínez-Campos et al., 2022) and *Hyphomonas* (Zettler et al., 2013). *Pseudomonas*, found in the core microbiome of the PET bottle at site 2 after 3 months of incubation, is widely known for its ability to produce exopolymers that aid in the formation of biofilms (Chien et al., 2013). In addition, this genus has a high metabolic capacity, which enables it to degrade highly complex substances such as plastics like PET (Vague et al., 2019). *Roseomonas*, which is significantly abundant at different incubation times, is known to have members that are opportunistic pathogens for humans (Rihs et al., 1993). As far as the eukaryotic core biome is concerned, a significant abundance of *Aphanochaete* has already been reported in other types of plastics in aquatic environments (Chia et al., 2020). Several species of the genus *Rhizoclonium* have shown a tendency to colonize artificial substrates such as glass rather than natural substrates (Danilov and Ekelund, 2001).

In the PS dish core microbiome, the genus *Pirellula* was found previously colonizing PS in different ecosystems (Purohit et al., 2020). Other associated genera identified in the PS dish which have been found in the plastisphere in previous studies were *Pleurocapsa* (Rogers et al., 2020), *Sphingorhabdus* (Di Pippo et al., 2020) and *Hymenobacter* (Martínez-Campos et al., 2022). Also noteworthy is the presence of the genus *Rhodopirellula*, a genus with the ability to degrade hydrocarbons (de Araujo et al., 2021). *Rickettsia* is known to cause waterborne infectious diseases (Walker et al., 2003). The genus *Staphylococcus* could resist various antibiotics such as β -lactams. (Fuda et al., 2005). Among eukaryotic biome taxa in PS dish, the genus *Ploimidia* appeared attached

to plastic litter in different aquatic ecosystems (Kettner et al., 2019). *Cryptosporidium* is a parasite that requires removal from drinking water, so its attachment to plastic could pose a risk to human health (Gómez-Couso et al., 2010). The genus *Candida* is characterized as a potential multi-drug resistant pathogen (Spivak and Hanson, 2018) and some species of this genus also have the potential to degrade polymeric substances (Zahari et al., 2021).

The most abundant bacterial genera in the PVC pipe included *Fluviicola* and *Chthoniobacter*, previously described as plastic colonizers (Cappello et al., 2021; Rummel et al., 2021). *Sphingomonas*, which is already present during the first month of colonization, is characterized as a pioneer species in biofilm formation (Bereschenko et al., 2010). This genus has been reported as a dominant colonizer on PVC surfaces since it could participate in the degradation of PVC (Z. Wang et al., 2021; Wright et al., 2021a). The genus *Bryobacter*, also very abundant, has been reported as having members which are multi-resistant bacteria to several antibiotics in wastewater (Zhao et al., 2021). Regarding the eukaryotic biome core, only the genus *Radix* (snail) has been detected associated to the plastisphere; some members of this genus prefer to attach to plastics in comparison with other natural substrates (Vossage et al., 2018).

The increase of antibiotic-resistant bacteria (ARBs) and subsequent implications for human health is one of the major concerns of modern societies. Plastics may have an important role in this problem because they can function as a reservoir of ARBs and cognate ARGs in marine ecosystems (Liu et al., 2021; Moore et al., 2020; Yang et al., 2019). WWTPs are considered to be one of the major pathways for ARGs and microplastics into the environment, and may favour their interaction (Syranidou and Kalogerakis, 2022). Martínez-Campos et al. (2021) showed an enrichment of microorganisms carrying the *sul1* gene in different types of plastics after 48 h of incubation in the effluent of a WWTP. Yang et al. (2020) studied the temporal dynamics of 64 ARGs over one month in urban waters showing an increase in ARGs over time. Our study found a higher concentration of ARGs in the plastics colonized at sampling site 2, downstream of the WWTP, than at site 1 (located in a natural area). However, the surrounding water showed the highest relative abundance of all tested ARGs in both sampling sites. There was one exception: the gene *ermF*, which was more abundant in plastic than in water after 6 months of incubation. Wang et al. (2020) found similar results regarding this gene in different environments (river and estuary), suggesting the possibility that the integrase gene, *intI1*, could play an important role in the transmission of the *ermF* gene from bacteria in the surrounding water to bacteria attached to plastics, which would explain its increasing abundance over time on the plastisphere.

In this context, some of the bacterial taxa found in the plastisphere in the present study have been found to carry ARGs, such as the Burkholderiaceae family, which is a primary carrier of ARGs in situations of high antibiotic concentrations (Cao et al., 2021), such as those occurring in site 2. The genus *Acinetobacter*, which is part of the core microbiome detected in the PET bottle after 6 months of colonization in site 2, is responsible for the persistence of macrolide resistance ARGs in WWTP effluents, which would also explain the higher relative abundance of *ermF* in this sites and colonization phase (April et al., 2022).

Our results reveal that the concentration of antibiotics in the environment is a factor to be considered since there is a positive correlation with the presence of ARGs on plastics. This correlation is stronger for ARGs on plastics than for the other substrates analysed in this study, especially in the case of both *sul1* and *dfrA* genes. This study shows that the antibiotics released by WWTPs may facilitate the selection of ARBs on the plastisphere of nearby plastics and these could, therefore, function as a reservoir for ARGs. On the contrary, in river sites characterized by trace levels of antibiotics, this correlation does not appear. The correlation between antibiotics and bacteria-associated ARGs had been previously analysed in freshwater environments (Luo et al., 2010). Our findings are in line with the results obtained by Wang et al. (2020), who proposed that the concentration of ARGs on the surface of microplastics increased through the interaction with the surrounding environment.

5. Conclusions

This study evaluates, for the first time, the long-term dynamics of the plastsphere in four commonly used plastics deployed in areas of the same river with different anthropogenic impact. It shows that sampling site, type of substrate and sampling time are key determinants for these dynamics. The LEfSe analyses allowed the identification of core microbiomes/biomes related to three evolutionary stages of the plastsphere: early or initial (1 month of incubation), intermediate (3 months) and late colonizers (6–12 months). Some of the identified taxa attached to the plastics could be potential pathogens and pose a risk to human health and the environment. Others could be potential plastic degraders. Different types of higher organisms were also identified which could use the plastics for shelter and could be transported to other habitats in drifting plastics. The presence of certain bacteria and eukaryotes suggest the possibility of complex interactions, such as food webs or the involvement of plastics in biogeochemical cycles. Moreover, positive correlations were observed between the concentration of antibiotics in the surrounding environment and cognate ARGs on plastics, which emphasizes the potential role of plastic items for the environmental spread of antibiotic resistance.

CRediT authorship contribution statement

Sergio Martínez-Campos: Conceptualization, Methodology, Investigation, Writing – original draft. **Miguel González-Pleiter:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Andreu Rico:** Conceptualization, Methodology, Investigation, Writing – review & editing. **Theresa Schell:** Methodology, Investigation, Writing – review & editing. **Marco Vighi:** Conceptualization, Writing – review & editing. **Francisca Fernández-Piñas:** Supervision, Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Roberto Rosal:** Supervision, Conceptualization, Methodology, Writing – original draft, Writing – review & editing. **Francisco Leganés:** Supervision, Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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.

Data availability

Data will be made available on request.

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Novelty statement

There is little information on how living organisms interact with the plastsphere (new habitat where organisms colonize plastics) in

freshwaters, and particularly on how this interaction changes over time. Our study reports for the first time on temporal dynamics of the plastsphere (up to one year), identifies core microbiomes/biomes for each plastic at increasing colonization times and, as a novel result, finds a positive correlation between the concentration of antibiotics in the surrounding environment and the abundance of cognate antibiotic resistance genes (ARGs) on plastics, which emphasizes the potential role of plastic items for the environmental spread of antibiotic resistance.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2022.130271.

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