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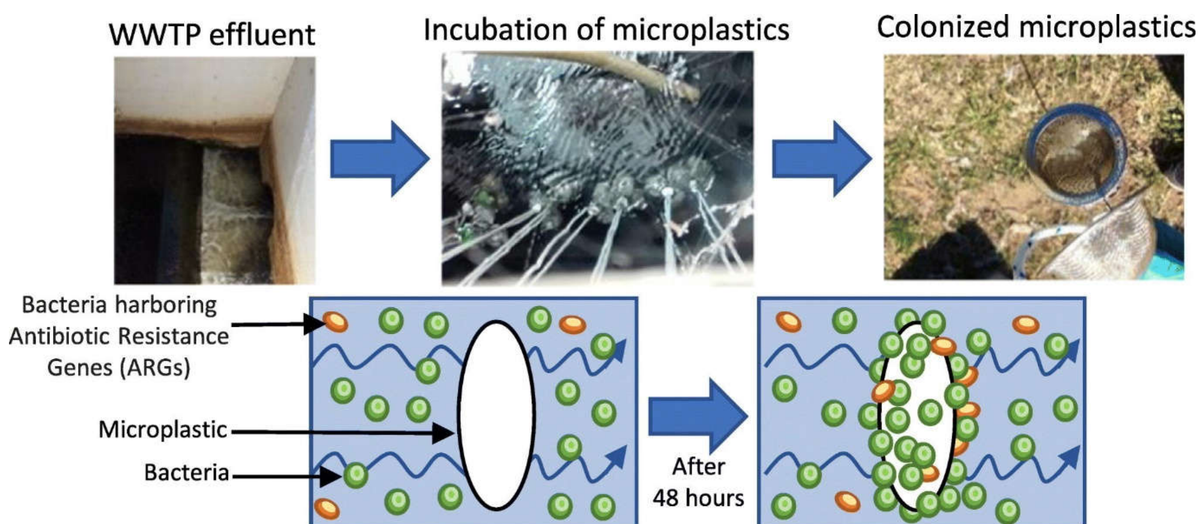

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Early and differential bacterial colonization on microplastics deployed into the effluents of wastewater treatment plants

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Abstract

Microbial colonization of microplastics (MPs) in aquatic ecosystems is a well-known phenomenon; however, there is insufficient knowledge of the early colonization phase. Wastewater treatment plant (WWTP) effluents have been proposed as important pathways for MPs entry and transport in aquatic environments and are hotspots of bacterial pathogens and antibiotic resistance genes (ARGs). This study aimed at characterizing bacterial communities in the early stage of biofilm formation on seven different types of MPs deployed in two different WWTPs effluents as well as measuring the relative abundance of two ARGs (*sulI* and *tetM*) on the tested MPs. Illumina Miseq sequencing of the 16S rRNA showed significant higher diversity of bacteria on MPs in comparison with free-living bacteria in the WWTP effluents. β-diversity analysis showed that the in situ environment (sampling site) and hydrophobicity, to a lesser extent, had a role in the early bacterial colonization phase. An early colonization phase MPs-core microbiome could be identified. Furthermore, specific core microbiomes for each type of polymer suggested that each type might select early attachment of bacteria. Although the tested WWTP effluent waters contained antibiotic resistant bacteria (ARBs) harboring the *sulI* and *tetM* ARGs, MPs concentrated ARBs harboring the *sulI* gene but not *tetM*. These results highlight the relevance of the early attachment phase in the development of bacterial biofilms on different types of MP polymers and the role that different types of polymers might have facilitating the attachment of specific bacteria, some of which might carry ARGs.

Keywords: Microplastics; Early colonization; Wastewater treatment plant effluents; Bacterial communities; Antibiotic resistance genes

1. Introduction

Plastics have been widely used since 1950 and their use is increasing (Drzyzga and Prieto, 2019). These plastics usually have a short product lifetime and because of their persistence, accumulate in the environment, especially in aquatic ecosystems (Duis and Coors, 2016; Ivleva et al., 2017).

Plastics interact with co-occurring organisms (from mammals to microorganisms) in aquatic ecosystems in different ways (Kettner et al., 2019; Macreadie et al., 2017). One important impact of this pollution is that plastics provide an artificial, hard and persistent surface for microbial colonization (Miao et al., 2019; Rummel et al., 2017). The attached microbial communities on plastic surface are termed as "plastisphere" (Amaral-Zettler et al., 2020; Zettler et al., 2013). Thus, plastics have emerged as novel ecological habitats, that are usually constituted by microbial communities significantly different to those living in the surrounding environment (De Tender et al., 2015).

According to NOAA's definition, plastics fragments below 5 mm are considered microplastics (Gago et al., 2016). They are easily transported between environmental compartments (Law and Thompson, 2014), including freshwaters, oceans, polar environments and pristine mountain lakes (Free et al., 2014; Kettner et al., 2017; Oberbeckmann et al., 2018; Waller et al., 2017), staying in the environment for long periods of time serving as a vector for the dispersal of invasive species, including pathogens but also antibiotic resistance bacteria (ARBs) carrying antibiotic resistance genes (ARGs) (Arias-Andres et al., 2018; Kirstein et al., 2016;

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Laganà et al., 2019; McCormick et al., 2014; Oberbeckmann et al., 2018). Furthermore, MPs provide a large surface area that increases the available space for microbial colonization (Hidalgo-Ruz et al., 2012).

Microbial colonization of MPs in freshwater environments is poorly known in comparison with marine environments (Jacquin et al., 2019). Recent studies reported that wastewater treatment plants (WWTPs) is one of the principal pathways of MPs entering into freshwater and marine ecosystems (Edo et al., 2020; McCormick et al., 2014). The MPs that end up in the WWTPs not only come from the degradation of macroplastics, but many are a common formulation in cosmetics and other personal care products (Carr et al., 2016). Although WWTPs usually have the capacity to remove 99 % of the MPs, a small but significant fraction of MPs ends up in the effluent with the potential to interact with the river biota (Murphy et al., 2016). In this context, recent studies have performed colonization experiments in rivers and in locations close to the discharge of WWTP (Kettner et al., 2019; Kettner et al., 2017; Oberbeckmann et al., 2018). However, these studies analyzed microbial communities established on the MPs after two weeks of in situ incubation. Peng et al. (2018) studied early (24–48 h) biofilm colonization on polypropylene (P)P large bio-cords deployed downstream of a WWTP outlet but, specifically on MPs, early colonization studies seem to be lacking although the first hours or days of biofilm formation affects the subsequent maturation of the biofilm (Goecke et al., 2010; Peng et al., 2018).

Furthermore, numerous previous studies have recognized that WWTPs are one of the most important hotspots for propagation of pathogens and ARBs and their cognate ARGs in the environment (Bouki et al., 2013; Guo et al., 2017; Hendriksen et al., 2019; Pärnänen et al., 2019). So far, only few studies have addressed the potential of MPs as vectors of pathogens and ARGs mostly in marine systems (Wang et al., 2020; Yang et al., 2019); regarding freshwaters, Oberbeckmann et al. (2018) detected certain bacteria commonly associated with antibiotic resistance downstream of a WWTP. Arias-Andres et al. (2018) stablished the capacity of MPs to be “hot-spots” of horizontal gene transfer (HGT).

In this study, we characterized, for the first time, early bacterial colonization on seven types of MPs [three biodegradable plastics, namely polylactic acid (PLA), poly-3-hydroxybutyrate (PHB), polycaprolactone (PCL), and four non-biodegradable plastics, namely polyethylene terephthalate (PET), low-density polyethylene (LDPE), polystyrene (PS) and polyoxymethylene (POM)]. These MPs were deployed during 48 h into the effluents of two WWTPs with different water treatments, different water sources and located in different towns. We hypothesized that early MP-biofilm forming bacteria might be different among the tested MPs and different to free-living water bacteria and to those colonizing another artificial substrate (borosilicate spheres). Furthermore, we hypothesized that MPs-colonizing bacteria might act as vectors of ARGs and contribute to their spread.

2. Material and methods

2.1. Study site

Two full-scale activated sludge WWTPs in Spain were selected for this study. Cantoblanco (Universidad Autónoma de Madrid) wastewater plant, denoted as WWTP1, processes approximately 931 m^3 per day from the university facilities, various research institutes located in the campus, a hospital and an elderly nursing home. The Guadalajara wastewater treatment plant, denoted as WWTP2, processes approximately 45,000 m^3 per day. It treats domestic and industrial water from the city of Guadalajara (medium-size city with about 86,000 inhabitants). The operational variables and treatments performed in each WWTP is depicted in Table S1 (Supplementary Material 1). The location of the WWTPs is shown in Fig. S1a (Supplementary Material 1).

2.2. Plastic substrates used for microbial colonization and characterization of their surface properties

Seven types of polymers were considered; the biodegradable polylactic acid (PLA), poly-3-hydroxybutyrate (PHB) and polycaprolactone (PCL) and the non-biodegradable but in widespread use, polyethylene terephthalate (PET), polyoxymethylene (POM), polystyrene (PS) and low-density polyethylene (LDPE); the size range of all tested MPs was 3–5 mm. Borosilicate spheres (BS) were used as non-plastic substrate control (size range between 2 and 8 mm). All substrates were commercial and additive-free. The most important information of

these substrates is detailed in Table S2 (Supplementary Material 1).

The surface properties of the materials used as substrates for microbial colonization were studied by contact angle measurements. Contact angles were determined with an optical contact angle meter (Krüss DSA25 Drop Shape Analysis System) at room temperature using the sessile drop technique. Contact angles were measured using drops of MilliQ water, glycerol and diiodomethane delivered by the built-in syringe. Contact angle measurements were taken at least at three different positions for each solvent and material and analyzed using the software Drop Shape Analysis (DSA4) release 2.1. Surface tension was calculated using the procedure by Van Oss (2007). The procedure allowed obtaining the free energy of interaction between two identical surfaces immersed in a liquid, ΔG_{SWS}, which is a measure of the hydrophobicity or hydrophilicity of the surface. If $\Delta G_{SWS} > 0$, the surface is hydrophilic, whereas if $\Delta G_{SLS} < 0$, it is hydrophobic. The different calculated parameters are shown in Table S3.

The microtexture of all substrate materials was evaluated using a high-resolution 3D microscope with interferometry and profilometry model Leica DCM 8 with the analysis mode in confocal mode (green LED). The software used to process the result is Leica Scan version 6.5. The areas considered were 649 μm × 488 μm using three measurements per particle and three different particles. The measured parameters were the developed interfacial area ratio (Sdr) and kurtosis value (Sku). The Sdr parameter is expressed as the percentage of additional surface area contributed by the texture as compared to the planar definition area, the Sdr of a completely level surface is 0, but when a surface has any slope, its Sdr value becomes larger. The Sku value is a parameter of the sharpness of the surface height: height normal distribution has a value of 3; a value of Sku less than 3 indicates that height distribution is skewed above the mean plane; on the contrary, Sku values higher than 3 indicates that its height distribution is spiked. (high Sku values indicated a spiky surface, low Sku values indicates a bumpy surface) (Blunt and Jiang, 2003).

2.3. Design of the colonization experiment

The substrates were sterilized according to their properties: PLA, PHB, POM, PET and BS were sterilized by autoclave (120 °C, 20 min); PCL, LDPE and PS, because of their low melting temperature, were sterilized using 10% hydrochloric acid 1 min and cleaning with sterilized Milli-Q water. Approximately, 5 g of each polymer type pellet and BS were introduced into sterilized metallic cages with 1 mm holes by triplicate. These cages were deployed during 48 h at a depth of 20 cm at the exit of the WWTP secondary clarifiers, separated from each other by 15 cm. (see Fig. S1b–f for details on the colonization experiment). WWTP1 incubation was carried out on October 19th-21st 2017, WWTP2 on March 14th-16th, 2018.

After the incubation, all MP pellets and BS were carefully extracted from the metallic cages to avoid the destruction of the biofilm and the residual water of the sample dried with sterilized filter paper. Dried MP pellets and BS were put into sterile tubes, frozen in liquid nitrogen and finally stored at −20 °C until DNA extraction.

In order to obtain a representative sample of the bacterial community in surrounding water, 1 L of water was sampled in wide mouthed polyethylene bottles and kept cool in the dark. Water was filtered by 0.22-μm membrane Millipore filter. Filters were frozen in liquid nitrogen and stored at −20 °C until DNA extraction.

Environmental properties of WWTP effluent waters were analyzed at the beginning of the experiment (0 h) and at the end of the incubation time (48 h) (Table S4; Supplementary Information 1). Dissolved oxygen, temperature, pH and conductivity were measured in situ using an oxygen portable meter ProfiLine Oxi 3310 (WTW), an electrical conductivity meter CDTM 523 and a microprocessor pH Meter pH 96 (WTW), respectively. Nutrient (nitrate, nitrite, ammonium and phosphate) concentrations were determined by duplicate using colorimetric methods as previously described (Perona et al., 1999). The chemical oxygen demand (COD) was measured using the COD cell Test kit (Merck Millipore).

2.4. Microbial diversity analysis

2.4.1. DNA extraction

Phenol:chloroform method was essentially carried out as previously described (Debeljak et al., 2017). Total DNA was extracted from all frozen MP pellets and frozen BS and water filters in triplicate. Pellets of each substrate were

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distributed in three 2 ml Eppendorf tubes. Water filters were cut into small fragments with sterilized scissors and distributed in three 1.5 mL Eppendorf tubes. The procedure started with the addition of Tris-HCL 10 mM, EDTA 0.1 mM pH 7.5, 0.05 % SDS (*W*/*V*) and 0.01% of silica pellets (W/V). After that, 0.5 volumes of hot phenol ultrapure pH 7.9 (65 °C) was added, and the samples were vortexed and warmed to 65 °C for 1 min three times to fully release the DNA from the biofilms developed in the samples. After that, 0.5 volume of chloroform was added, and the samples were vortexed and frozen again six times. Finally, samples were centrifuged at 13,000 rpm at 4 °C for 20 min. The supernatant of the samples was transferred to a new Eppendorf tubes and 1 volume of hot phenol pH 7.9 (65 °C) was added to wash the sample which was subsequently centrifuged at 13,000 rpm at 4 °C for 20 min. The process was repeated twice. Finally, all supernatants that belonged to the same sample were pooled and 2 volumes of absolute ethanol was added, the sample was mixed and frozen at −20 °C overnight to precipitate the DNA. Samples were subsequently centrifuged at 13000 rpm at 4 °C for 20 min. The supernatant was discarded, and the pellet was washed with 1 volume of ethanol 70% to remove the salts. Samples were further centrifuged at 13000 rpm at 4 °C for 2 min. Finally, samples were dried, and the DNA was resuspended in 40 μL of Milli-Q water. All samples were stored at −20 °C.

2.4.2. DNA sequencing

PCR amplifications of the regions V3-V4 of the 16S rRNA of each of the three replicates of each microplastic plus three replicates of BS and water effluent filters (54 sequenced samples) were carried out by the Genomics service of the Parque Científico de Madrid (Madrid, Spain). The primers used are shown in Table S5 (Supplementary Material 1). DNA libraries and amplicon sequencing were performed as previously described (Martínez-Campos et al., 2018).

2.4.3. Data analysis

16S rRNA profiling was determined using Quantitative Insights Into Microbial Ecology 2 (QIIME 2) v. 2019.4 (Bolyen et al., 2019) (https://docs.qiime2.org/2019.1). The complete pipeline of the process can be found in Supplementary Material 1.

Briefly, the quality of the reads (fastq format) was evaluated with FastQC 0.11.18 (Bioinformatics, 2011) and with the q2-demux plugin. The reads, cleaned and trimmed paired ends, were filtered and denoised using DADA2 (Callahan et al., 2016) via q2-dada2. Identified amplicon sequence variants (ASVs) were aligned using MAFFT (Katoh et al., 2002) via q2-alignment, and used to construct a phylogeny with FastTree2 (Price et al., 2010). Rarefaction curves were estimated via q2-diversity to 71,940 lectures depth per sample. α-diversity methods, that includes Shannon index (Shannon and Weaver, 1949) Chao1 index (Chao and Lee, 1992) and Pielou's evenness (Pielou, 1966), were estimated via q2-diversity and the differences between samples were evaluated using Kruskal Wallis statistics method (Kruskal and Wallis, 1952).

ASVs were taxonomically assigned using the q2-feature classifier plugin (Bokulich et al., 2018) based on classify-sklearn naïve Bayes taxonomy classifier using Silva 128, 99 % OTUs database (Quast et al., 2012). A specific classifier for the amplified 16S region was trained using the primers specified above and a maximum fragment size of 300 nts.

For β–diversity analysis, two type of analysis were performed. Between-treatment variability was analyzed with principal coordinate Analysis (PCoA) based in ASV abundance (Bray- Curtis dissimilarity) (Sorenson, 1948) and visualized using EMPeror (Vázquez-Baeza et al., 2013). Permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was applied to test significant differences between sites and substrates considering 999 permutations.

Redundancy analysis (RDA) was performed to establish a correlation between environmental and intrinsic plastic factors (site, roughness and hydrophobicity) and the bacterial community established in each substrate. The relative abundance of the microbial groups at genus level in each sample was used as “species data”, filtering out genera with a relative abundance less than 0.5 %. Environmental variables were transformed using log (x + 1) to avoid the differences in scale (binary data were not transformed, and hydrophobicity was transformed to positive values). A Monte Carlo permutation test with 999 permutations was carried out to test the significance of the environmental parameters in relation to distribution pattern of samples. The

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analysis was performed using vegan package in Rstudio.

To identify differentially attached taxa among the different substrates and water at both WWTPs, the linear discriminant analysis effect size method (LEfSe) (Segata et al., 2011) was used. This was performed with the LEfSe online tool in the Galaxy framework, using all default settings for data formatting and LDA (Linear Discriminant Analysis) effect size. The factors "substrate" and "location" were set as classes. Non-transformed relative abundance was used and the strategy for multi-class analysis "one-against-all" was performed.

2.4.4. Accession numbers

Sequences used in this study were submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) under the Bioproject accession number: PRJNA543601.

2.5. Relative abundance of ARGs

The relative abundance of two ARGs (*sulI* and *tetM*) in the bacterial community attached to the tested substrates was compared to the relative abundance of the two genes in free-living water bacteria using quantitative PCR (qPCR). *sulI* confers resistance to sulfonamides which are a class of antibiotics for which resistance is a worldwide problem and has been documented in wastewater impacted environments (Garner et al., 2018). *tetM* provides a high level resistance to tetracycline (Morse et al., 1986), a class of antibiotics used to treat a number of human infections such as cholera, brucellosis, plague, malaria, and syphilis; the *tetM* gene has also been documented in urban sewage (Hendriksen et al., 2019; Pärnänen et al., 2019).

qPCR assays were carried out in a LightCycler® 480 (Roche; USA) system using 2.5 ng of template DNA and using. LightCycler® 480 SYBR Green I Master (Roche; USA). The primers for amplification of the *sulI* and *tetM* genes are depicted in Table S6 (Supplementary Material 1). Three 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 $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001) was used to normalize and calibrate transcript values relative to the 16S gene of the same sample.

3. Results

3.1. Characterization of substrates

Surface properties were shown in Table 1. The Gibbs free energy of interaction, ΔG_{SWS}, gives a measure of surface hydrophobicity, which was, in increasing order:
PHB < PS < PCL < POM < LDPE < PET < BS < PLA. Surface topography was visualized using 3D microscopy.

Fig. S2 (Supplementary Material 1) revealed substrate-dependent differences. LDPE displayed the highest roughness (expressed as Sdr, see Table 1) mostly with ridge-and-valley appearance. PHB, PCL and POM displayed intermediate roughness and uneven surfaces. PS and PET had the flattest surface roughness with Sdr values close to BS. Besides, PET, PLA, PHB, POM, LDPE and PCL with kurtosis values (Sku > 3; Table 1) showed spiked surfaces, while BS and PS were softer.

Table 1. Surface properties of the materials.

	ΔG_{SWS} (mJ/m^2)[a]	Sdr (%)[b]	Sku[c]
PLA	-54.5 ± 8.1	24.1 ± 13.4	6.9 ± 2.8
PHB	-20.4 ± 4.5	41.5 ± 3.9	4.9 ± 2.2
PCL	-34.6 ± 2.1	37.7 ± 8.7	3.6 ± 0.8
PET	-45.5 ± 3.9	8.2 ± 1.7	7.4 ± 7.1
LDPE	-42.4 ± 2.3	84.6 ± 30.7	3.8 ± 0.4
POM	-41.5 ± 5.2	22.4 ± 41.0	4.1 ± 1.7
PS	-29.0 ± 3.9	8.2 ± 3.4	2.9 ± 0.4
BS	-45.6 ± 5.8	3.8 ± 1.1	2.9 ± 0.3

[a] ΔG_{SWS} is the Gibbs free energy of interaction. The more negative, the more hydrophobic is the surface.
[b] Sdr is the developed interfacial area ratio defined as the percentage of additional area due to texture if compared to planar area (zero represents a flat surface).
[c] Sku: kurtosis of roughness profile; Sku > 3: spiked distribution with numerous high peaks and low valleys; Sku < 3: means few peaks and low valleys.

3.2. Taxonomical annotation

About 7,111,208 reads were obtained using Illumina sequencing. After quality filtration, reads merging and chimera removal using DADA2, 5,620,437 sequences remained (79.0% of the total reads) which were assigned to 9075 ASVs. 3970 ASVs were identified In WWTP1 while 6293 ASVs were identified in WWTP2. Rarefaction plot (Fig. S3; Supplementary Material 1) reached the plateau with the current sampling effort in all samples, pointing out that the bacterial libraries were adequately sampled. In order to validate the statistics results, the sequence depth used to

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evaluate the α- and β-diversity was 70,940 reads per sample.

3.3. α-Diversity analysis

Bacteria diversity was estimated using the alpha components, namely diversity (Shannon Index), evenness (Pielou's evenness) and bacterial richness (Chao1 Index), These indexes are represented in Fig. 1 according to location (WWTP1 or 2) and substrates. The WWTP1 samples had significant lower values of Shannon index (Global Kruskal Wallis p value: $2.9 * 10^{-10}$) (Fig. 1a), Pielou evenness (Global Kruskal Wallis p value: $2.8 * 10^{-10}$) (Fig. 1b) and Chao1 (global Kruskal Wallis p value: 0.0004) (Fig. 1c) than WWTP2 samples. This indicated a higher bacterial diversity in WWTP2 than in WWTP1, underpinning an important difference in species richness between both locations that could be related both to the operational conditions of both WWTPs (Table S1) as well as nutrient loads which are higher in WWTP1 (Table S4).

The comparison among the studied substrates revealed that effluent water, independently of the WWTP, presented significant lower Shannon Index value (pairwise Kruskal Wallis p value <0.05) (Fig. 1a), and Pielou evenness value (pairwise Kruskal Wallis p value <0.05) (Fig. 1b) than all the other tested substrates, revealing a less diverse bacterial community than those present in MPs and BS.

The sampling site had a significant role on bacterial diversity on the different tested substrates: PHB and PCL presented significant higher diversity and evenness in WWTP1 (pairwise Kruskal Wallis p value < 0.05) while PLA and BS presented a slightly higher diversity in WWTP2 (pairwise Kruskal p value < 0.05) (Fig. 1a, b). These results might suggest that, in general, bacterial assemblages attached to biodegradable MPs were more diverse on these than on the rest of substrates (except for BS in WWTP2).

3.4. Bacterial community composition

Fifty-one bacterial phyla divided in 188 classes and 2 Archaea phyla divided in 6 classes were identified in the whole sample set (Supplementary Material 2).

Taxonomic analysis showed that the majority of the reads in the sample set were associated with the phyla Proteobacteria with 59.9% relative abundance followed by Bacteroidetes (14.7 %), Actinobacteria (6.6 %), Chloroflexi (5.1 %), Firmicutes (4.2 %), Saccharibacteria (1.4 %) and Planctomycetes (1.3 %) (Supplementary Material 2). Proteobacteria (60.7 %), Bacteirodetes (16.2 %), Actinobacteria (6.6 %) were the most abundant phyla in MPs biofilms¸ Proteobacteria (55.75%), Bacteroidetes (11.6 %), Antinobacteria (10.5 %) in BS biofilms and Proteobacteria (58.3 %), Parcubacteria (11.0 %), Firmicutes (7.1 %) in free-living bacteria in water.

The most abundant classes were Betaproteobacteria (23.1 %), Alphaproteobacteria (21.6 %) and Gammaproteobacteria (11.8 %) in the phylum Proteobacteria, Sphingobacteria (8.5 %) in the phylum Bacteroidetes, Acidimicrobia (3.9 %) in the phylum Actinobacteria and Clostridia (2.5 %) in the phylum Firmicutes (Supplementary Material 2). Betaproteobacteria (24.2 %), Alphaproteobacteria (21.4 %), Gammaproteobacteria (12.0 %) were the most abundant classes in MPs biofilms; Alphaproteobacteria (26.2 %), Betaproteobacteria (18.5 %), Shingobacteriia (8.3 %) in BS biofilms and Betaproteobacteria (19.4 %), Alphaproteobacteria (18.1 %), Gammaproteobacteria (14.1 %) in free-living bacteria in water.

The bar chart represented in Fig. 2 shows the bacterial distribution at the order level associated to the tested substrates and WWTPs. Firstly, microbial community composition at his level was clearly different between the two WWTPs. WWTP1 was characterized by a high abundance of the orders Rhizobiales (22.3 %), Rhodocyclales (17.8 %), Burkholderiales (9.8 %), Pseudomonadales (6.7 %) and Flavobacteriales (5.5 %). In addition to these shared orders, Neisseriales (16.9 %) was dominant in the free-living bacteria in water samples, while Sphingobacteriales (4.7 %) dominated in the MPs-attached biofilms. In contrast, Anaerolineales (7.5 %) and Clostridiales (5.0 %) were more abundant in BS.

In contrast, Sphingobacteriales (12.6 %), Burkholderiales (12.8 %), Pseudomonadales (8.7 %), Acidimicrobiales (7.4 %) and Rhodobacterales (6.3 %) characterized the distribution of bacterial order abundance in WWTP2. The abundance of the orders Campylobacterales (9.2 %) Legionallales (7.1 %) and Rickettsiales (4.0 %) was higher in in the free-living bacteria in comparison with BS- and

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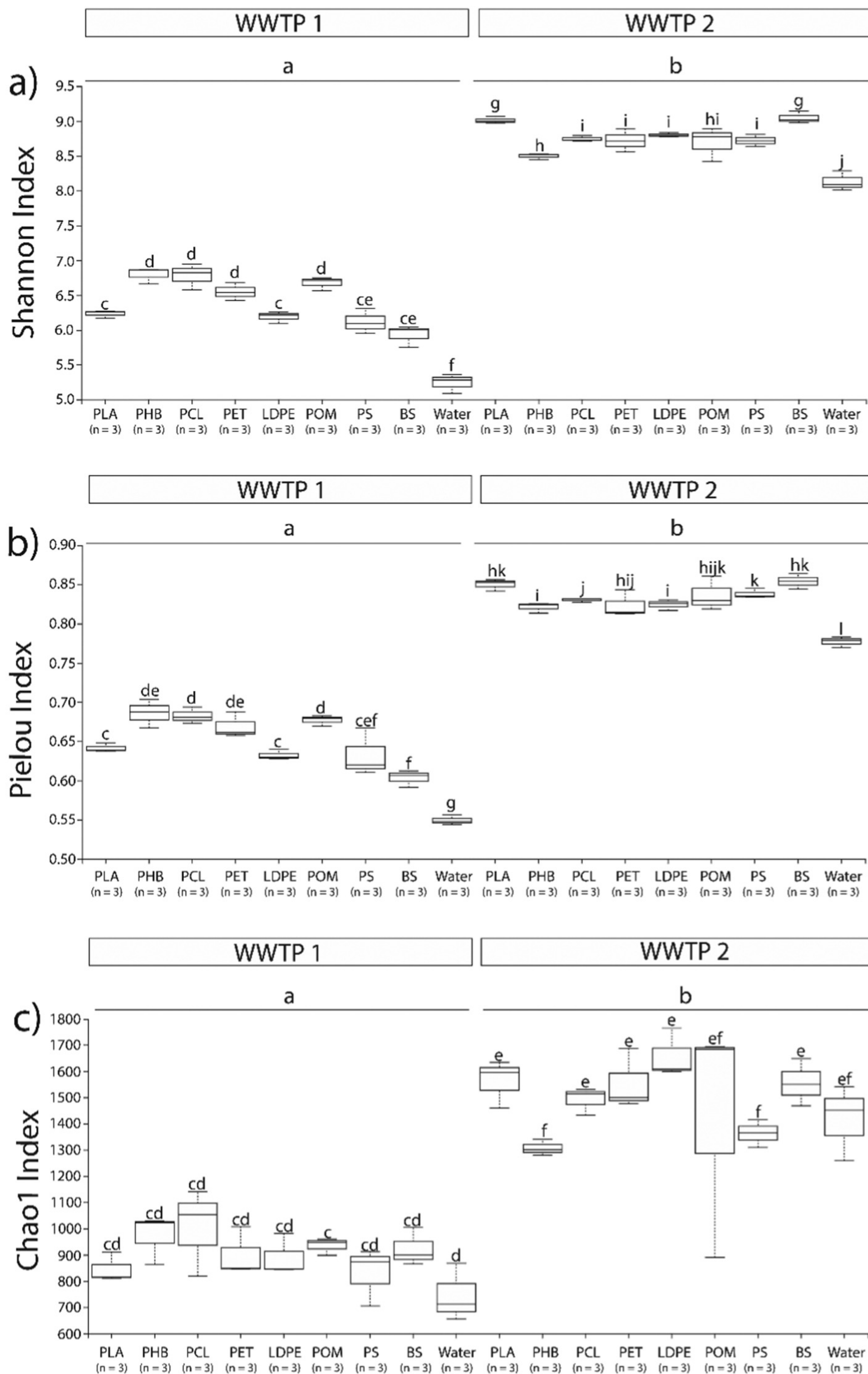

Figure 1. Boxplots of ASVs representing α-diversity using (a) the Shannon-Wienner index, (b) Pielou Evenness index and (c) Chao 1 index in the different substrates in WWTP1 and WWTP2. Lowercase letters indicated significant differences in the Kruskal-Wallis analysis. Statistical significance was a *p*-value <0.05. Polylactic acid (PLA), poly-3-hidroxybutirate (PHB), polycaprolactone (PCL), polyethylene terephthalate (PET), low-density polyethylene (LDPE), polystyrene (PS) and polyoxymethylene (POM), borosilicate spheres (BS).

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MPs-attached biofilms. Conversely, the order Rizhobiales dominated both BS (5.2 %) and MP (5.8%) biofilms. There were not clear differences at the order level between the different tested substrates (MPs and BS).

However, at family-level resolution (Supplementary Material 2), there were differences in the relative abundance with respect to the tested substrates. Comamonadaceae (11.9 %), Rhodocyclaceae (10.1 %), Moraxellaceae (7.5 %), Hyphomicrobiaceae (4.8 %) and Rhodobacteraceae (4.3 %). displayed a higher relative abundance on MPs compared to BS and water samples, independently of the location. Considering the location, family Campylobacteraceae (7.1 %), specifically the genus *Arcobacter*, was found as predominant in PHB (6.0 %) and PCL (8.1 %) in WWTP1. In comparison, the unassigned family JG35-K1-AG5 (23.3 %) dominated in BS samples and Neisseriaceae (17.0 %) dominated in WWTP1 effluent water. Regarding WWTP2, Saprospiraceae (10.4 %) predominated in MPs and BS assemblages in comparison with free-living bacteria in the effluent water; families Campylobacteraceae (9.1 %) and Legionellaceae (7.1 %) were more abundant in the effluent water.

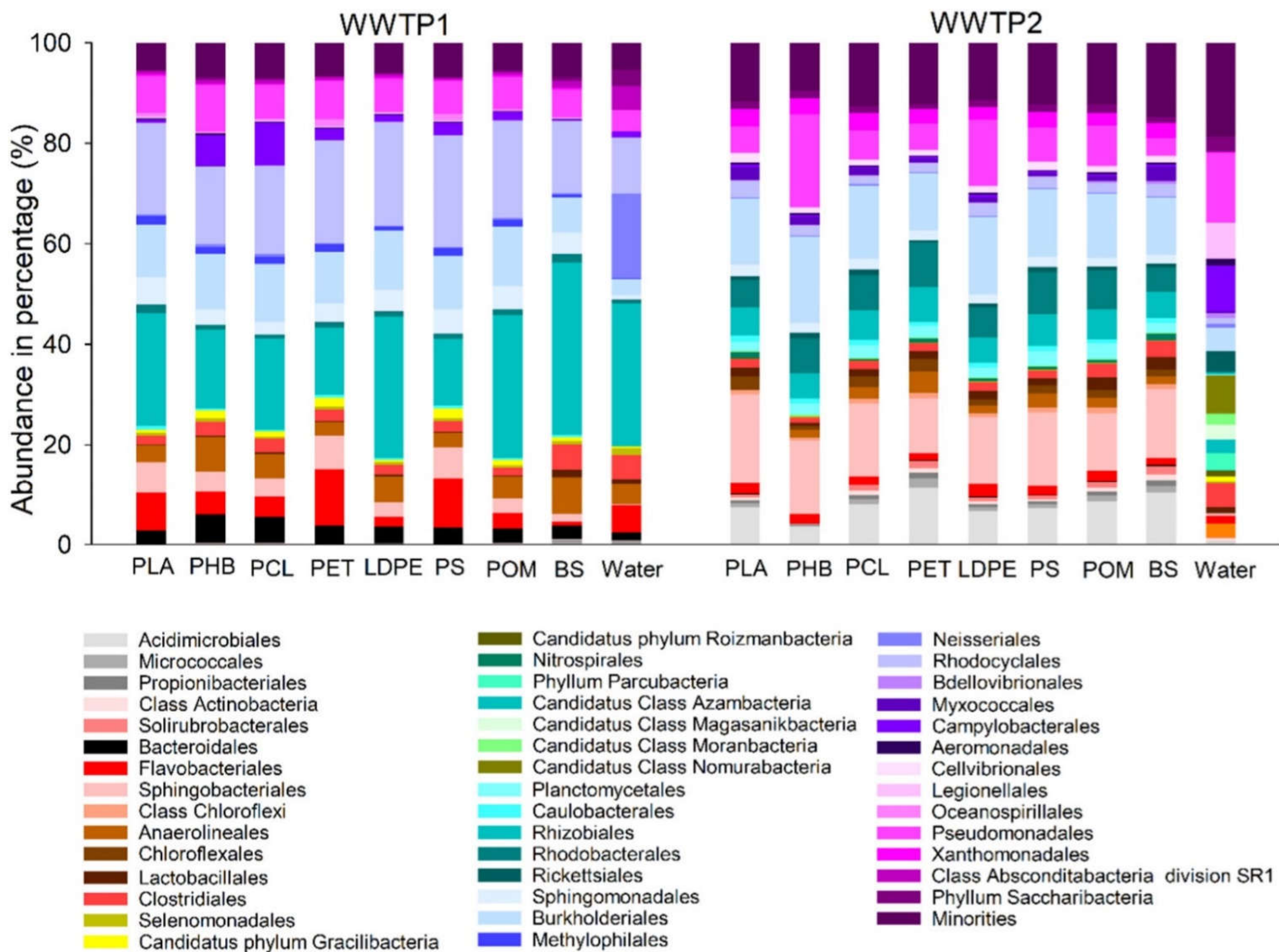

Figure 2. Relative abundance of bacteria communities at the order level associated to the different substrates in WWTP1 and WWTP2. Minority are orders whose representation is less than 1 %.

3.5. β- diversity

The data suggest that there are significant differences in bacterial composition between the two WWTPs and between MPs and BS and water; to further, explore this, β-diversity metrics was used. A Principal Coordinate Analysis (PCoA) (Fig. 3) was performed to determine the relevance of the site factor (WWTP1 or WWTP2) or tested substrate (MP, BS or effluent water).

The statistical relevance of factors was analyzed by PERMANOVA tests (Table 2). The LEfSe analysis was subsequently used to confirm which taxa, if any, were significantly more abundant in each group.

Site (in situ environment) (Fig. 3) had a highly significant effect on the bacterial community (PERMANOVA; p value <0.05). The Bray-Curtis PCoA plot revealed an important pattern of

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clustering structure according to the sampling location, finding a very clear differentiation in the distance on the first axis, which explained the 65.2 % of the difference between clusters. It should be noticed that water samples were clearly separated from BS and MPs according to the second axis coordinate, which explained only 10.5 % of the difference between clusters. The significant differences among the two WWTPs was confirmed by PERMANOVA tests (PERMANOVA; p value < 0.05, Table 2). In addition; LEfSe analysis revealed significant differences in the abundance of different bacterial taxa among WWTP1 and WWTP2 (Table 3) highlighting the taxa Rhodocyclaceae, Hyphomicrobiaceae, Rhizobiales JG35 K1 AG5 *Fluviicola*, Sphingomonadaceae, *Arcobacter*, *Aquabacterium*, *Zoogloea*, *Paludibacter*,

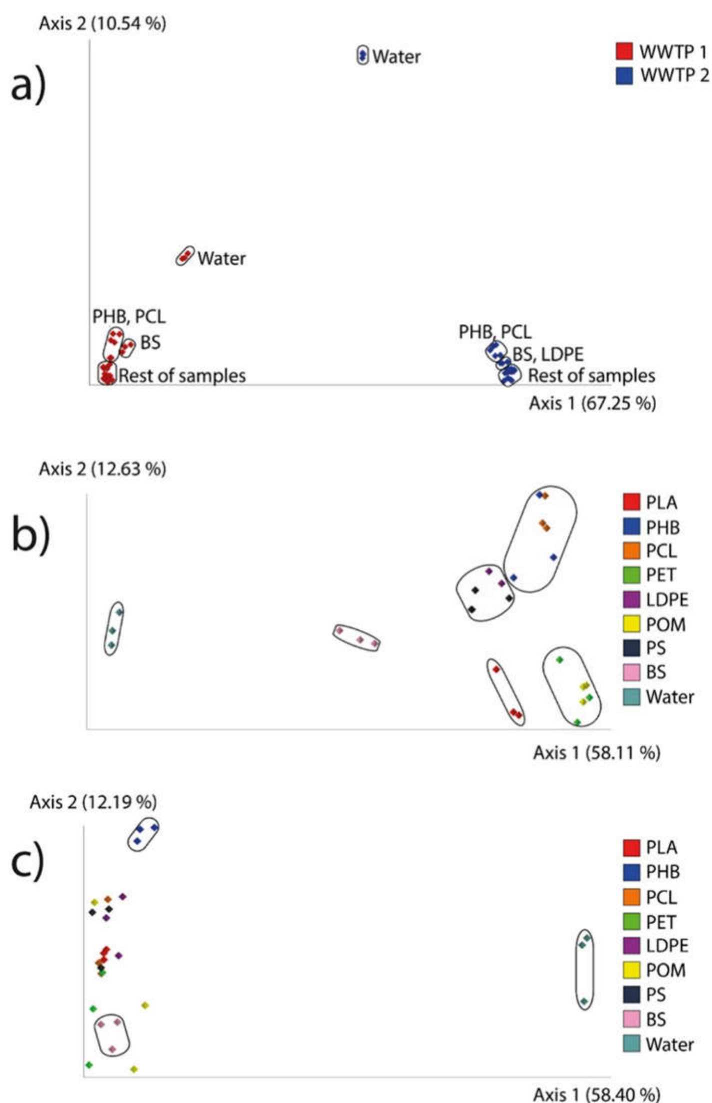

Figure 3. PCoA analysis of the microbial composition in samples based on Bray-Curtis dissimilarity. (a) Global analysis of all samples, (b) analysis of WWTP1 samples, (c) analysis of WWTP2 samples. Percentage in axes represent % of variation explained by that axis.

Table 2. Global and pairwise PERMANOVA analysis.

	Groups	PERMANOVA	
		Pseudo-F	p value
Global		36.34	< 0.01
Pairwise	WWTP1- WWTP2	97.37	< 0.01
	Water-MP	5.67	< 0.01
	Water- BS	2.75	0.07
	MP- BS	0.95	0.20
	Water WWTP1-water WWTP2	96.70	0.10
	BS WWTP1- BS WWTP2	59.67	0.08
	MP WWTP1-MP WWTP2	195.34	< 0.01
	MP WWTP1-water WWTP1	30.67	< 0.01
	MP WWTP1- BS WWTP1	8.17	< 0.01
	Water WWTP1-BS WWTP1	39.81	0.10
	MP WWTP2-water WWTP2	36.72	< 0.01
	MP WWTP2-BS WWTP2	38.84	0.07
	Water WWTP2-BS WWTP2	39.43	0.10
	PLA-PHB	0.51	0.58
	PLA-PCL	0.42	0.58
	PLA-PET	0.28	0.57
	PLA-LDPE	0.31	0.57
	PLA-POM	0.24	0.57
	PLA-PS	0.26	0.58
	PLA-BS	0.45	0.57
	PLA-Water	3.22	0.08
	PHB-PCL	0.26	0.57
	PHB-PET	0.57	0.57
	PHB-LDPE	0.38	0.56
	PHB-POM	0.41	0.57
	PHB-PS	0.42	0.57
	PHB-BS	0.95	0.56
	PHB-water	3.05	0.08
	PCL-PET	0.33	0.58
	PCL-LDPE	0.27	0.57
	PCL-POM	0.29	0.58
	PCL-PS	0.24	0.58
	PCL-BS	3.04	0.06
	PCL-Water	3.04	0.06
	PET-LDPE	0.43	0.57
	PET-POM	0.14	0.64
	PET-PS	0.29	0.60
	PET-BS	0.66	0.58
	PET-water	3.38	0.01
	LDPE-POM	0.38	0.57
	LDPE-PS	0.16	0.67
	LDPE-BS	0.54	0.57
	LDPE-water	3.15	0.09
	POM-PS	0.32	0.59
	POM-BS	23.69	0.10
	POM-Water	3.43	0.00
	PS-BS	76.46	0.09
	PS-Water	48.27	0.11
	PS-BS	2.78	0.08

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Table 3. Differential bacterial taxa abundance comparing WWTP1 and WWTP2 samples by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed.

Sampling point	Taxa	Log LDA score
WWTP1	Rhodocyclaceae	4.79
	Hyphomicrobiaceae	4.56
	Rhizobiales JG35 K1 AG5	4.43
	Fluviicola	4.24
	Sphingomonadaceae	4.06
	Arcobacter	4.01
	Aquabacterium	3.92
	Zoogloea	3.84
	Paludibacter	3.82
	Uncultured Anaerolineaceae	3.80
	Uncultured Sphingobacteriales	3.78
	Acidovorax	3.74
	Uncultured Gracilibacteria	3.68
	Rhizobiales	3.65
	Pseudomonas	3.55
WWTP2	Uncultured Saprospiraceae	4.50
	Acinetobacter	4.14
	Rhodobacteraceae	4.11
	Comamonadaceae	4.10
	Microthrix	4.08
	Leeia	4.01
	Rhodocyclaceae 12up	3.86
	Acidimicrobiaceae	3.78
	Roseiflexus	3.73
	Saccharibacteria	3.71
	Variovorax	3.66
	Terrimonas	3.62
	Dokdonella	3.57
	Chloroflexi ambiguous taxa	3.50
	Iamia	3.48

Uncultured Anaerolineaceae, Uncultured Sphingobacteriales, *Acidovorax* and *Pseudomonas* in WWTP1 and Uncultured Saprospiraceae *Acinetobacter*, Rhodobacteraceae, Comamonadaceae, *Microthrix*, *Leeia*, Rhodocyclaceae 12up, Acidimicrobiaceae, *Roseiflexus*, Saccharibacteria, *Variovorax, Terrimonas*, *Dokdonella*, Chloroflexi ambiguous taxa, *Iamia* and *Mycobacterium* in WWTP2.

RDA analysis (Fig. 4) further confirmed a significant influence of the in situ environment in the community diversity, factor that was strongly correlated with the first RDA axis that explained 70.1 % of the variation. The physicochemical substrate properties hydrophobicity and roughness were highly correlated with the second RDA axis that only explained 2.89 %. Results of Monte-Carlo test showed that the influence of site (*p* value: 0.001) and hydrophobicity (*p* value: 0.015) was significant although roughness was not (*p* value: 0.094).

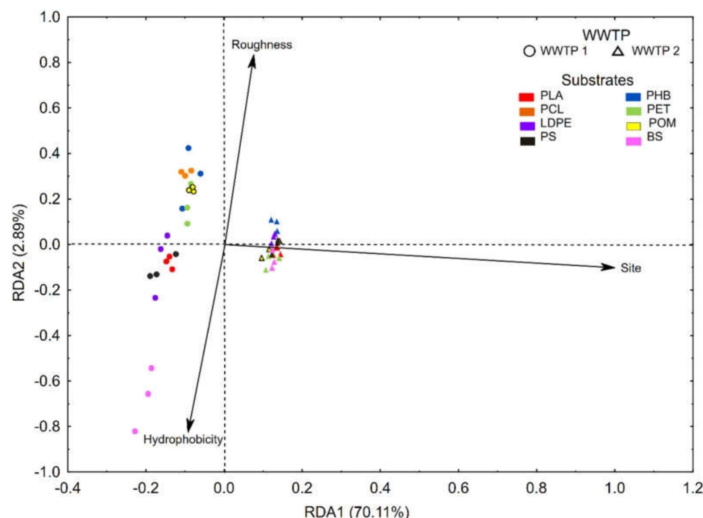

Figure 4. Redundancy analysis plot (RDA) of bacterial diversity in relation to site (in situ environment) and substrate surface proprietaries (roughness and hydrophobicity).

Although pairwise PERMANOVA tests did not detect significant differences among bacterial communities when comparing, as a whole, water-MPs, water-BS and MPs-BS (PERMANOVA; *p* value < 0.05) (Table 2), when the samples were globally analyzed by LEfSe analysis, certain taxa were significantly more abundant in MPs compared to water and BS (Table 4). In this context, uncultured Saprospiraceae, Comamonadaceae, Rhodobacteraceae, *Aquabacterium*, *Zoogloea*, *Acidovorax*, *Sphaerotilus*, Uncultured Sphingobacteriales, Acidimicrobiaceae, *Variovorax*, *Roseiflexus*, *Terrimonas*, *Dodonella, Pseudomonas* and *Perludibaca* might constitute the MP "core microbiome" in the studied WWTP effluents. BS selected for quite different taxa, including Rhizobiales, Sphingomonadaceae and photosynthetic ones like cyanobacteria. Effluent water free-living bacteria included, among others, Enterobacteriaceae which were not found in MPs. Nevertheless, when considering each WWTP separately, LEfSe analysis revealed some differences in the MPs core microbiome which might be due to the clear differences in the performance and characteristics of each WWTP (Tables S7 and S8; Supplementary Material 1).

LEfSe analyses also reported differential abundance of certain taxa in each specific MP, regardless of the WWTP as shown in Table 5. Of

Table 4. Differential bacterial taxa abundance comparing microplastic-associated assemblages to borosilicate-associated assemblages and water sample bacterial communities by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed.

Substrate	Taxa	Log LDA score
MPs	Uncultured Saprospiraceae	4.35
	Comamonadaceae	4.33
	Rhodobacteraceae	4.04
	Aquabacterium	3.80
	Zoogloea	3.77
	Acidovorax	3.69
	Sphaerotilus	3.65
	Uncultured Sphingobacteriales	3.63
	Acidimicrobiaceae	3.56
	Variovorax	3.53
	Roseiflexus	3.47
	Terrimonas	3.41
	Dokdonella	3.38
	Pseudomonas	3.37
	Perludibaca	3.32
BS	Uncultured Anaerolineaceae	4.17
	Rhizobiales	3.95
	Sphingomonadaceae	3.85
	Chistensenellaceae 7 group	3.65
	Uncultured Aeroccaceae	3.20
	Leucobacter	3.16
	Paucibacter	3.14
	Chlorella sp. CC Bw 9	3.03
	Ignatzschineria	3.01
	Proteiniclasticum	3.88
	Holdemania	3.87
	Caldisericum	3.87
	Paucisalibacillus	3.82
	Dermacoccus	3.80
	Cyanobacteria subsection IV Family I	3.78
Water	Rhizobiales JG35 K1 AG5	4.95
	Leeia	4.85
	Rhodocyclaceae	4.65
	Flavobacterium	4.38
	Unculture candidate division SR1	4.28
	Saccharibacteria	3.93
	Alcaligenaceae GK98 fresh water group	3.53
	Methylocystaceae	3.50
	Uncultured Veillonaceae	3.33
	Enterobacteriaceae	3.28
	Dialister	3.23
	Saccharofermentans	3.22
	Uncultured compost bacterium Saccharibacteria	3.11
	Alistipes	3.08
	Bifidobacterium	3.03

the tested MPs, PLA showed the higher diversity with fifteen taxa with the highest scores, followed by PET with ten taxa; PS MPs showed the lowest diversity. In general, the tested MPs did not share taxa suggesting that each MP might select different attached bacteria.

Table 5. Differential bacterial taxa abundance comparing the different microplastic-associated assemblages by linear discriminant analyses (using LEfSe). Taxa with the highest Log LDA score in each group are listed

Plastic	Taxa	Log LDA score
PLA	Uncultured Saprospiraceae	4.46
	Uncultured Sphingobacteriales	3.84
	Dokdonella	3.56
	Spongiibacteraceae BD1 7clade	3.47
	Comamonas	3.38
	Aeromonas	3.24
	Flavobacteriales NS9 marine group	3.18
	Xanthomonadaceae uncultured	3.16
	Bacteroidetes	3.15
	Sphingomonadales	3.12
	Thauera	3.03
	Dechloromonas	3.02
	Sphingobacteriales	3.02
	Chitinophagaceae	2.97
	Sorangium	2.95
PHB	*Acinetobacter*	4.59
	Aquabacterium	4.12
	Pseudomonas	3.65
	Lautropia	3.35
	Ferruginibacter	3.32
	Vibrio	2.84
	Gracilibacteria	2.60
PCL	*Sphaerotilus*	3.88
	Variovorax	3.77
	Terrimonas	3.60
	Simplicispira	3.24
	Sphingobium	3.01
	Cyanobacteria	2.73
PET	Rhodobacteraceae	4.15
	Thermomonas	3.25
	Xantomonadales Incertae Sedis	3.16
	Agitococcus lubricus group	3.09
	Betaproteobacteria SC I 84	3.02
	Ferribacterium	2.97
	Uncultured Rhizobiales A08329	2.91
	Uncultured Sphingobacteriales	2.79
	Acetobacteraceae	2.67
	Reyranella	2.62

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LDPE	Comamonadaceae	4.40
	Zooglea	3.98
	Ernhydrobacter	3.24
	Betaproteobacteria	3.18
	Candidatus Competibacter	2.60
POM	Sphingobacteriales OPS17	3.11
	Uncultured Fimbrimonadaceae	3.01
	Uncultured Verrucomicrobiaceae	3.00
	Gammaproteobacteria	2.74
	Bdellovibrio	2.65
	Deltaproteobacteria SAR324 glade marine group B	2.59
	Verrucomicrobiaceae	2.55
	Prosthecobacter	2.50
PS	*Acidovorax*	3.81
	Hydrogenophaga	3.15

3.6. Relative abundance of the ARGs *tetM* and *sulI*

Fig. 5 shows the relative abundance of ARGs *tetM* and *sulI* on the tested substrates and WWTP water effluents. Pairwise Kruskal Wallis test for significant differences among substrates and water in the two WWTPs is shown in Table S9 (Supplementary Material 1).

The relative abundance of the *sulI* gene changed significantly depending on the WWTP. In WWTP1 no significant differences were found among MPs and water; however, BS had a significant lower relative abundance of the *sulI* gene compared to water (p value < 0.05). Regarding WWTP2, the *sulI* gene was detected in a significantly higher relative abundance attached to POM and PS MPs as well as on BS than in water (p value < 0.05). In general, significantly less *tetM* was detected in MPs and BS than in water in both WWTPs.

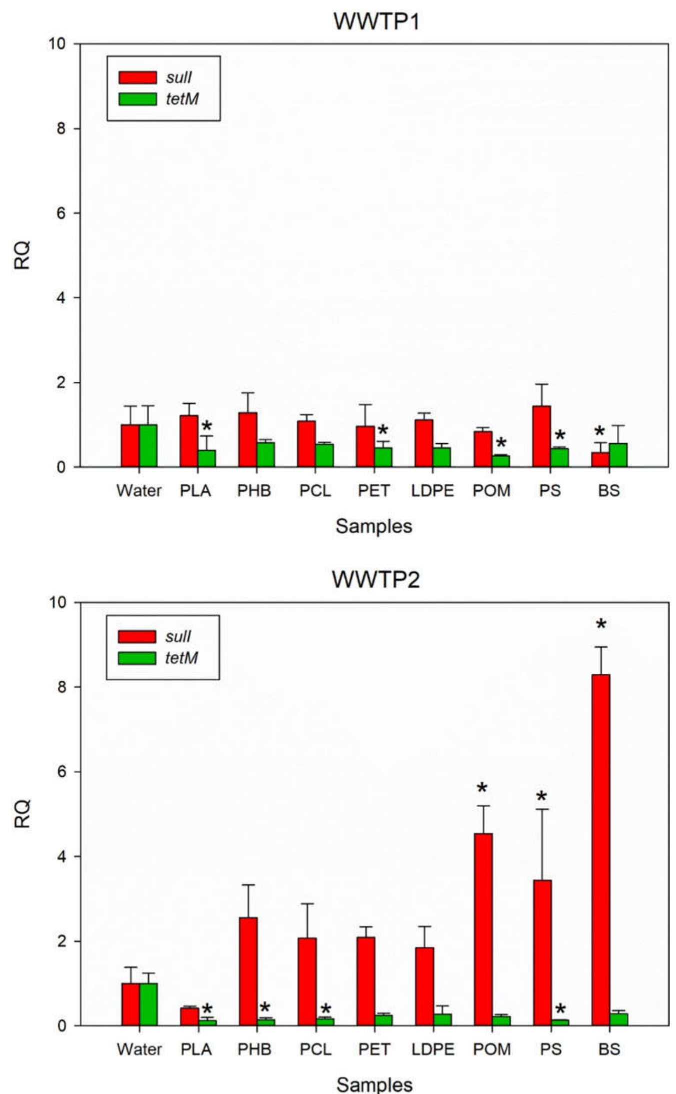

Figure 5. Relative abundance of *sulI* and *tetM* genes measured in the different substrates and effluent water in WWTP1 and WWTP2. Error bars indicate standard deviations of triplicates. Asterisk (*) denotes a statistically significant difference between the relative abundance of *sulI* and *tetM* genes in substrates and effluent water in each WWTP (Kruskal Wallis test; p value < 0.05).

4. Discussion

The present study provides relevant information about bacterial community assemblages in different MPs exposed for a short time (48 h) to WWTP effluents in situ. To our knowledge, this is the first study to do so in seven different types of polymers including biodegradable (PLA, PHB, PCL) as well as non-biodegradable (PET, LDPE, PS, POM) MPs. Marine plastic debris is mainly composed of PE, PP and PS; in this context, most marine studies have focused on the use of these polymers for the colonization studies as well as PET or polycarbonate (PC) (De Tender et al., 2017; Dussud et al., 2018; Oberbeckmann et al., 2016; Ogonowski et al., 2018). Dussud et al. (2018) besides virgin PE, have used artificially aged PE as well as the biodegradable polyester PHBV. Marques et al. (1997) also used PHBV. Lee et al. (2014) used PS and PVC plates deployed on a cold seep in the Red Sea. Regarding freshwater systems, Hoellein et al. (2014) compared hard and soft substrata including plastics deployed on a river, a pond and recirculating laboratory streams. Oberbeckmann et al. (2018) analyzed the colonization of HDPE and PS pellets incubated for 14 days at sampling stations in the estuary of the river Warnow (including WWTP discharge) and in the Baltic Sea. McCormick et al., 2014, McCormick et al., 2016 did not perform colonization experiments

but collected plastics from surface river waters and WWTPs effluent and analyzed the microbial assemblages on the collected MPs.

WWTPs have been revealed as one of the main hotspots for the release of MPs in freshwater (Edo et al., 2020; Magnusson and Norén, 2014; McCormick et al., 2014), as well as pathogens and ARBs (Pazda et al., 2019). MPs can interact with sewage-related microorganisms, including pathogens and ARBs, and transport them downstream, ending up in the oceans (McCormick et al., 2014; Oberbeckmann et al., 2018; Oberbeckmann et al., 2014). In this context, Hoellein et al. (2014) suggested that plastic biofilms might be more stable and remain intact longer and transport biofilms further compared to natural surfaces like wood or other natural particles.

The signs of the existence of the plastisphere were denoted for the high diversity in the MPs in comparison with the water, independently of the WWTP. These results were novel in comparison with previously studies that evaluated the bacterial biofilm formation in WWTP effluent. Peng et al. (2018) analyzed the early biofilm formation (24–48 h) in a WWTP effluent using a bio-cord of PP fine fiber as substrate and reported a diversity bacterial richness much lower in the water than in the biofilm. McCormick et al. (2014) recollected MPs from the WWTPs effluent and showed a higher diversity in MPs than in WWTP effluent water. Our results reported that MPs had a higher bacterial diversity than WWTP effluent water free-living bacteria, that could be explained because the early colonization that occurs on bare substrates implies active adhesion capacities for pioneer bacterial species, and these pioneers facilitate the adhesion of new species from water column in the first hours (Lyautey et al., 2005).

The factor in situ environment sampling site was the most significant explaining the bacterial diversity in the different tested MPs. Oberbeckmann et al. (2018) demonstrated that the degree of specificity of the marine microbiome on MPs depended on the environmental conditions and they only observed significant differences between MP microbiomes in areas with lower nutrients; they refer the term plastisphere, proposed by Zettler et al. (2013), to be used in certain environmental conditions such as “lower nutrients, high salinity”. In this context, the two WWTPs of this study showed significant operational differences (Table S1): In WWTP1, the treatment is based on a contact-stabilization process, unable to remove nutrients efficiently presenting difficulty in generating a stable effluent of good quality. On the contrary, in WWTP2, the secondary treatment was based in the A2O method, which removed nutrients efficiently using two anaerobic ponds and an anoxic pond as well as an oxic pond allowing a high-quality effluent; also A/A/O (A/O) systems, as compared to other systems such as membrane bioreactors (MBRs), usually show higher Simpson's diversity index and evenness index meaning also a higher bacterial diversity (Hu et al., 2012) as also found in this study. This could be related to the chemical parameters of each WWTP effluents, with WWTP1 effluent showing a higher nutrient load than WWTP2 particularly regarding PO_4^{3-}, NH_4^+ and COD (Table S4). Previous studies confirmed that the microbial communities adhered to the MPs depended mainly on the location (Amaral-Zettler et al., 2015; McCormick et al., 2014; Oberbeckmann et al., 2018; Oberbeckmann et al., 2014).

Rummel et al. (2017) defined hydrophobicity and roughness as the two principal superficial parameters of polymers that can affect the colonization of MPs. This information suggests that the first phases of colonization might be dependent on the MP surface properties.

In this study, MP superficial parameters were secondary to the factor in situ environment, nevertheless, hydrophobicity had a significant role although smaller and roughness did not play any significant role. Oberbeckmann et al. (2018) found that in situ environment was the major factor in their two-week experiment. Ogonowski et al. (2018), in a two-week study also, found that substrate hydrophobicity strongly correlated with bacterial composition across all tested substrate. Clearly, more studies on how changes in surface properties of the same material over time affect colonization process are needed before reaching significant conclusions in this matter.

Illumina sequencing data highlighted significant differences among bacterial assemblages on MPs, BS and bacterial communities in WWTP effluent water samples after 48 h of colonization. However, most studies have shown that the microbial community in plastics is similar to that in other substrates (glass, metal, organic

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particulate matter) although clearly different to that of free living microorganisms in the water column or marine sediment (Bryant et al., 2016; Dussud et al., 2018; Harrison et al., 2014; Hoellein et al., 2014; McCormick et al., 2014; McCormick et al., 2016). A few studies, however, found significant changes in microbial diversity depending on polymer type (De Tender et al., 2017; Ogonowski et al., 2018; Webb et al., 2008). Most of these studies considered colonization data over a week. In this context, Hoellein et al. (2014) and Oberbeckmann et al., 2016, Oberbeckmann et al., 2018 suggested that future experiments on MP biofilms should include the colonization phase of the first few hours to days because difference in microbial diversity between substrate types might be stronger during early stages of biofilm formation on MPs. Biofilms are envisaged as an effective strategy for microbes to survive in unfavorable environments. The formation of a biofilm is a dynamic sequence of events, which, for better understanding, has been divided into distinct developmental stages: it is initiated by planktonic bacteria that first attach to each other (cell-to-cell attachment, termed as cohesion). Then, they attach themselves reversibly to a surface usually through physical forces and in real time, a number of the reversibly adsorbed cells remain immobilized and become irreversibly adsorbed onto the surface (physical appendages of bacteria such as fimbriae or pili as well as adhesins have a predominant role in this phase). Once adsorbed, they form microcolonies and produce the extracellular polymeric substance (EPS), the glue that holds the microbial community together and acts as a barrier to chemicals (containing exopolysaccharides, proteins, nucleic acids and other bacterial detritus). In the final stage, the biofilm disperses, and the free microbes look for new niches to be established (Hall-Stoodley et al., 2004). During biofilm initiation, nutrients and dissolved organic matter (DOM, which may facilitate the formation of a surface organic layer on the substrate) and bacterial input form the surrounding water will affect the microbial communities and their interaction.

Phyla Proteobacteria [Betaproteobacteria (24.24%), Alphaproteobacteria (21.39 %), Gammaproteobacteria (12.04 %)], Bacteirodetes and Actinobacteria dominated MPs biofilms in this study. Members of alpha and gammaproteobacteria as well as Bacteroidetes and Firmicutes are characteristic of early biofilm colonization and are known to produce the EPS (Dang and Lovell, 2000). Peng et al. (2018) in their study on early biofilm formation on a PP bio-cord deployed downstream of a WWTP outlet found that Alphaproteobacteria dominated the biofilm and that this class showed “biofilm-specific” property, suggesting that the ability of colonization was more relevant in the very early stage of biofilm formation; also Actinobacteria may contribute significantly to organic matter processing. Some members of Bacteroidetes are reported to have a role in initial biofilm formation as they can degrade biopolymers to low molecular weight DOM that helps in biofilm conditioning (Kirchman, 2002).

At the family level, Comamonadaceae, Rhodocyclaceae, Moraxellaceae, Hyphomicrobiaceae and Rhodobacteraceae predominated on MPs compared to BS and water samples. Comamonadaceae, has been found as dominant in MPs collected from urban rivers and associated WWTP effluents (McCormick et al., 2014; McCormick et al., 2016). Family Rhodobacteraceae and Flavobacteriaceae were found as dominant in MPs colonized in marine waters (Bryant et al., 2016; De Tender et al., 2017; Oberbeckmann et al., 2018; Zettler et al., 2013).

An interesting question is whether a MPs-core microbiome can be identified. De Tender et al. (2017) identified 25 bacterial core OTUs on both plastic sheets and dolly ropes deployed in a harbor in Belgian part of the North Sea. Oberbeckmann et al. (2018) reported a marine MPs-microbiome core where Hyphomonadaceae and Erythrobacteraceae were dominant. Ogonowski et al. (2018) in their colonization experiment using PE, PP and PS in the Baltic Sea (brackish system) reported that Alphaprotrobacteria, Bacteroidetes and Plantomycetes predominated in plastics compared to non-plastic substrates. Regarding freshwaters, McCormick et al. (2014) identified 46 OTUs that accounted for more than 60 % variation between plastic and non-plastic substrates, the most common taxa on plastics were Pseudomonadaceae, Proteobacteria and Campylobacteraceae, other relevant taxa were *Arcobacter* and *Aeromonas*. In a similar but more recent study, McCormick et al. (2016) identified Pseudomonadaceae, Gammaroteobacteria and Comamonadaceae in MPs collected also from

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urban rivers; other relevant taxa more abundant on collected MPs were *Pseudomonas* and *Aquabacterium*. Peng et al. (2018) identified 44 OTUs as dominant in the plastic biofilms deployed in the effluent of a WWTP; these OTUS corresponded to members of the Alphaproteobacteria, Gammaproteobacteria, Firmicutes, and Bacteroidetes. In this study, we have been able to identify a core microbiome of fifteen taxa that have colonized MPs deployed into the effluent of two quite different WWTP effluents; it is noteworthy that there were some coincidences with those taxa described by McCormick et al., 2014, McCormick et al., 2016 like Comamonadaceae, *Aquabacterium* or *Pseudomonas* and also with some taxa described by Peng et al. (2018) such as Rhodobacteraceae or *Pseudomonas*. Despite the coincidences, there are many differences that might suggest that the specific environment (site) is the parameter that might select the indicator species. More studies in a range of different environments are necessary before reaching a conclusion about MPs-core microbiomes.

It is noteworthy that some of the genera found as dominant in MPs such as *Pseudomonas*, *Variovorax*, *Aquabacterium* or *Acidovorax* have species with the capacity to metabolize recalcitrant substances, including plastics. *Pseudomonas* has already been previously described as one of the first colonizers of the plastisphere (McCormick et al., 2014; Wu et al., 2019), it is one of the main producers of exopolysaccharide (EPS), that facilitates the adhesion of new bacteria (Chien et al., 2013) and also provides protection against harmful substances, such as heavy metals (Pal and Paul, 2008). In addition, it can metabolize plastics such as PE, PET and PS to some extent as a source of carbon and energy under laboratory conditions (O'Leary et al., 2005; Ronkvist et al., 2009; Yoon et al., 2012). Likewise, some species of the genus *Acidovorax* can accumulate PHB inside (Schulze et al., 1999). Morohoshi et al. (2018) detected the presence of this genus associated to biofilms that degraded PHB. Some species of the genus *Aquabacterium* are able to metabolize plasticizers used in PVC (Kalmbach et al., 1999); this genus has been identified as dominant in biofilms attached to plastics in drinking water plants (Kalmbach et al., 2000). The isolation of these strains could be very important to establish new metabolic pathways that favour the biodegradation of plastics. The genus *Variovorax* is able to degrade several aquatic pollutants such as trichloroethylene, linuron and arsenite (Satola et al., 2013).

The high relative abundance of the genus *Roseiflexus*, on MPs, whose only representative species is the photosynthetic *Roseiflexus castenholzii* (Hanada et al., 2002), indicates the importance of microbial primary producers other than cyanobacteria associated with MPs (Yokota et al., 2017).

An issue with MPs colonization is the presence of pathogenic bacteria. Genus *Pseudomonas* include species that are opportunistic pathogens to humans such as *Pseudomonas aeruginosa*, which has already been found in WWTPs with multiple resistance to antibiotics (McCormick et al., 2014; Slekovec et al., 2012). Within family *Campylobacteraceae*, genus *Arcobacter*, which also contains some opportunistic pathogenic members which are known to cause human gastrointestinal infections, has also been found attached to MPs and remarkably, it has been found in both freshwaters and marine habitats (Harrison et al., 2014; McCormick et al., 2014). In this study, *Arcobacter* was found in MPs biofilms specifically in WWTP1, which has a higher organic load than WWTP2. Interestingly, also in this study, the well-known human pathogenic genus *Mycobacterium* (belonging to the *Mycobacteriaceae* family, Actinobacteria phylum) was also found in bacterial assemblages on MPs in WWTP2. Other studies have found *Vibrio* spp. on MPs which also has some pathogenic species for man and aquatic fauna (Kirstein et al., 2016; Zettler et al., 2013) or fish pathogens such as *Aeromonas* (McCormick et al., 2014). The fact that some of these pathogens may be early MP colonizers and could be transported from WWTPs to rivers and even oceans may raise some concerns on potential risk to human health. However, at present, the role of plastics in general as vectors of pathogenic microorganisms is unknown. Future studies should examine the survival rates of the bacteria adhered to the MPs as they drift along the river to the sea.

In this study LEfSe Analysis allowed the identification of early bacterial colonizers on each of the seven tested MPS; this implies that the type of polymer might select for such early colonizers. This finding is not reported in most studies because it might be possible that this is mostly

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evident only in early colonization studies (Oberbeckmann et al., 2018). However, Ogonowski et al. (2018) found differences in bacterial colonization of PE, PP and PS in their two-week study of colonization in brackish waters from the Baltic Sea, with PS being the substrate with a higher diversity.

It is noteworthy that the biodegradable MPs used in this study, PLA, PHB and PCL, showed a significant abundance of genera with potential pathogenic members: *Pseudomonas*, *Comamonas*, *Aeromonas* and *Vibrio*. Does this mean that biodegradable MPs might be vectors of pathogenic bacteria in aquatic environments? This is an issue to be further investigated and clarified. Biodegradable MPs also were enriched on potential degrading taxa such as *Aquabacterium* and *Pseudomonas* in PHB and *Variovorax* in PCL.

Regarding non-biodegradable plastics, (PET, LDPE, POM and PS), genus *Ferribacterium* was selected in PET, this taxon has previously been reported as a characteristic microorganism in sewage sludge (Luo et al., 2020) and as an early colonizer attached to PP bio-cords deployed in a WWTP outlet (Peng et al., 2018). Genus *Zooglea*, very abundant in LDPE, has a crucial role in aerobic wastewater treatments due to its ability to degrade organic carbon and promote floc formation (Dris et al., 2015). Although it is usually more frequent in wastewater effluent water, it has already been found in MP assemblages in freshwater environment (McCormick et al., 2014) and related to the early formation of biofilm at PP bio-cords in WWTP effluents (Peng et al., 2018). In PS, the most abundant genus was the potential plastic *Acidovorax*, which is very frequent in activated sludge (Heylen et al., 2008).

There is also a growing concern that MPs, in general, may be reservoirs of ARBs and cognate ARGs. ARBs may survive in the presence of one or more antibiotics and that might be a potential threat for human health (Proia et al., 2016). Most ARGs are located on broad-host range conjugative plasmids or other mobile elements that can be transferred to nearby receptors leading to global spread of resistance (Sultan et al., 2018; Wang et al., 2020). The main source of ARBs/ARGs is to be found in urban sewage as has been proved by global monitoring of antibiotic resistance (Hendriksen et al., 2019; Pärnänen et al., 2019).

The role of plastic biofilm as ARG reservoirs has been seldom studied. Yang et al. (2019) in an in situ study found 64 ARG subtypes of 11 ARG types and 47 MRG subtypes in microbes on plastic particles in the North Pacific Gyre and Wang et al. (2020) under laboratory conditions using river water collected from the pristine headwater zone of the Taihu Lake, China, and sea water collected from the East sea of China found that PE MPs concentrated most ARGs from the surrounding water including *sulI*, *tetA*, *tetC*, *tetX*, *ermE* and *ermF*. Our study showed that the *sulI* gene was already present in WWTP effluents and that it was present in MPs at the same level than in effluent water in MPs deployed in WWTP1 and at higher abundances in POM, PS and BS than in effluent water in MPs in WWTP2. Proia et al. (2016) found a significant abundance of *sulI* in biofilms situated after a WWTP effluent. However, Yang et al. (2019) that did not detect the presence of sulfonamide resistance genes in marine MPs, stating that sulfonamide resistance is associated with anthropic environments and not with relatively pristine environments such as marine sediments or lakes. On the contrary, *tetM*, abundance was significantly lower in MPs than in effluent water, meaning that MPs do not seem to concentrate ARBs, which harbor this gene in particular. *SulI* genes are part of the 3′ conserved segments of Class 1 integrons. In this context, the *sulI* gene is usually considered as a marker of the presence of this class of integrons associated with resistance to sulfonamides and quaternary compounds. Class 1 integrons is the one most frequently detected in *Enterobacteriaceae*, including *Campylobacter* spp., *Escherichia coli*, and *Salmonella enterica* serotype Typhimurium (Lucey et al., 2000; Carattoli, 2001; Zhao et al., 2001). The environmental relevance of this class of integrons is that it as a primary source of resistance genes and is suspected to serve as reservoirs of antimicrobial resistance genes within microbial populations (Carattoli, 2001). Regarding plastics, Wang et al. (2020) found a significant correlation between ARGs and class 1 integron integrase gene (*intI1*) suggesting that *intI1* might facilitate the transmission of *sulI*, *tetX*, *ermE* and *ermF* between water and MPs through horizontal gene transfer which might underpin the role of MPs as conveyors of microbial resistance in aquatic environments.

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This study is the first to evaluate seven different types of MPs as potential vectors of *sulI* and t*etM* finding that they could be conveyors of *sulI* but not *tetM*. High throughput studies should analyze more globally the ability of MPs to accumulate ARBs and cognate ARGs and the possible impact on the environment and human health.

5. Conclusions

This study addresses for the first time the early bacterial colonization phase of seven different types of MPs including biodegradable and non-biodegradable ones deployed in WWTP effluent water.

In situ environment (sampling site) along with hydrophobicity to a lesser extent were the factors explaining bacterial diversity in the tested MPs.

The MPs clearly showed a different bacterial diversity when compared to that of WWTP effluent water or borosilicate glass. An early colonization phase MPs-core microbiome was identified. Furthermore, LEfSe analysis allowed identifying core microbiomes specific for each type of polymer suggesting that each type might select early attachment of bacteria.

It is of concern that some of the taxa identified on MPs could have pathogenic members and be a threat to human health. The fact that these taxa are found in biodegradable MPs suggests that the capacity of the MPs to act as vector of potentially pathogenic taxa may be facilitated by their biodegradability.

The tested WWTP effluent waters contained ARBs harboring the *sulI* and *tetM* ARGs, MPs concentrated the ARBs harboring the *sulI* gene, particularly those deployed in WWTP2, but not *tetM*. This might have to do with the specific sites and/or the ARG-carrying bacteria present in the site and their ability to attach to different MP polymers. This merits further study before claiming that MPs may act as global vectors of ARGs.

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SUPPLEMENTARY MATERIAL

Early and differential bacterial colonization on microplastics deployed into the effluents of wastewater treatment plants

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Contents:

Table S1. Operational variables of the two wastewater treatment plants (WWTP) evaluated in this study.

Table S2. Principal characteristics of the different substrates used in this study.

Table S3. Contact angle measurements and surface free energy components.

Table S4. Physical and chemical parameters in the two WWTP effluents.

Table S5. Description of the primers for 16S rRNA Illimina sequencing. The amplified region and the sequences of the primers are indicated. The primer tail is shown in bold.

Table S6. qPCR primers for specific detection and quantification of ARGs.

Table S7. Differential bacterial taxa abundance comparing microplastic-associated assemblages to borosilicate-associated assemblages and water sample bacterial communities in WWTP1 by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed.

Table S8. Differential bacterial taxa abundance comparing microplastic-associated assemblages and water sample bacterial communities in WWTP2 by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed. BS is not included as LEfSe analysis did not find any taxa clearly more abundant in BS with respect to MPs and water.

Table S9. Pairwise Kruskal Wallis test.

Figure S1. Sampling sites and details of the colonization experiments. a) Spain map showing location of WWTP1 and WWTP2, b) Virgin MPs before the colonization experiment c) metal cage with MPs inside, d) deployment of metal cages with MPs into WWTP effluent, e) cages after 48 h of colonization f) drying of the colonized MPs onto sterilized filter paper.

Figure S2. Images of the surface of each substrate obtained by 3D microscopy. Red color represents roughness crests and blue color represents the sunken areas.

Figure S3. Rarefaction curve that compares the observed ASVs index in comparison with number of reads for each sample (sequencing depth).

Metagenomics pipeline.

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Table S1. Operational variables of the two wastewater treatment plants (WWTP) evaluated in this study.

Operation variable	**WWTP1**	**WWTP2**
Location	Cantoblanco (Madrid, Spain)	Guadalajara (Castilla La Mancha, Spain)
Coordinate (DG)	Longitude: 40.5442 Latitude: -3.6845	Longitude: 40.6211 Latitude: -3.1909
Type of sewage	Domestic and hospital	Industrial and domestic
Discharge (m^3/d)	931	45000
Population equivalent	5927	91600
Total suspended solids (mg/L)	265	300
BOD_5 (mg/L)	382	350
TKN (mg/L)	54.1	50
TP (mg/L)	12.7	12
Pretreatment	Bar screens, grit removal	Bar screens, grit removal and fat and grease removal
Primary treatment	No	Three primary tanks sedimentation
Secondary treatment	Aerobic system by contact	A2O

Table S2. Principal characteristics of the different substrates used in this study.

Name	Abbr.	Biodegradable	Manufacturer	Shape	Size (mm)	Density (g/cm^2)
Polylactic acid	PLA	Yes	Goodfellow	pellet	3	1.24
Polyhydroxybutyrate	PHB	Yes	Goodfellow	pellet	5	1.25
Polycaprolactone	PCL	Yes	Sigma Aldrich	pellet	3 - 5	1.15
Polyethylene terephthalate	PET	No	Goodfellow	pellet	4	1.39
Low-density polyethylene	LDPE	No	Goodfellow	pellet	3.5	0.92
Polyoxymethylene	POM	No	Goodfellow	pellet	5	1.41
Polystyrene	PS	No	Goodfellow	pellet	3	1.05
Borosilicate glass pearls	SS	No		sphere	2	2.23
Borosilicate glass pearls	MS	No		sphere	5	2.23
Borosilicate glass pearls	BS	No		sphere	8	2.23

Table S3. Contact angle measurements and surface free energy components.

	Contact angle (°)			Surface free energy components (mJ/m^2)					
Material	Water	Glycerol	Diiodomethane	gSLW	gS(+)	gS(-)	gSAB	gS	ΔG_{SWS}
PLA	86.7 ± 7.0	64.5 ± 9.4	62.5 ± 5.1	27.13	3.22	0.82	3.25	30.38	-54.5 ± 8.1
PHB	69.3 ± 7.1	46.8 ± 8.7	76.5 ± 7.6	19.34	9.52	6.10	15.24	34.58	-20.4 ± 4.6
PCL	74.6 ± 2.1	49.8 ± 4.1	63.1 ± 4.9	26.80	6.39	2.80	8.47	35.26	-34.6 ± 2.1
PET	84.1 ± 2.9	64.3 ± 4.8	68.6 ± 7.4	23.65	3.81	1.92	5.42	29.06	-45.5 ± 3.9
LDPE	95.0 ± 3.4	54.5 ± 3.1	74.3 ± 2.9	20.09	8.73	0.00	0.00	20.09	-42.4 ± 2.3
POM	81.7 ± 3.3	65.6 ± 2.2	68.9 ± 7.0	23.47	3.03	3.68	6.67	30.15	-41.5 ± 5.2
PS	77.1 ± 3.5	59.3 ± 2.9	78.3 ± 7.0	18.37	6.24	4.99	11.16	29.53	-29.0 ± 3.9
BS	74.0 ± 2.4	62.3 ± 3.7	41.0 ± 4.47	39.11	0.43	7.51	3.60	42.70	-45.6 ± 5.8

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Table S4. Physical and chemical parameters in the two WWTP effluents.

Location	**WWTP1**		**WWTP2**	
Time	**0 h**	**48 h**	**0 h**	**48 h**
Temperature (°C)	13.7	12.4	19.3	19.6
pH	7.40	7.52	7.27	7.06
Oxygen (mg/L)	1.52	2.09	4.62	4.06
Oxygen (%)	13.9	20.6	53.5	47.6
Salinity (µs/cm)	622	649	903	970
PO_4^{3-} (mg/L)	6.95	9.20	4.80	5.70
NO_2^- (mg/L)	0.04	0.05	0.02	0.03
NO_3^- (mg/L)	0.25	0.20	35.7	21.8
NH_4^+ (mg/L)	69.9	55.2	0.35	0.35
COD (mg/L)	85.0	50.0	8.5	14.0

Table S5. Description of the primers for 16S rRNA Illumina sequencing. The amplified region and the sequences of the primers are indicated. The primer tail is shown in bold.

Region	Reference number	Sequence
16S	16SV3-V4-CS1	**ACACTGACGACATGGTTCTACA**CCTACGGGNGGCWGCAG
	16SV3-V4-CS2	**TACGGTAGCAGAGACTTGGTCT**GACTACHVGGGTATCTAATCC

Table S6. qPCR primers for specific detection and quantification of ARGs.

Target gene	Primer	Sequence (5' - 3')	References
16S rRNA	F1048	GTGSTGCAYGGYTGTCGTCA	[1]
	R1194	ACGTCRTCCMCACCTTCCTC	
sulI	sul(I)-FX	CGCACCGGAAACATCGCTGCAC	[1]
	sul(I)-RX	TGAAGTTCCGCCGCAAGGCTCG	
t*etM*	tetM-FW	ACAGAAAGCTTATTATATAAC	[2]
	tetM-RV	TGGCGTGTCTATGATGTTCAC	

[1] Pei R, Kim SC, Carlson KH, Pruden A. Effect of river landscape on the sediment concentrations of antibiotics and corresponding antibiotic resistance genes (ARG). Water research 2006; 40(12): 2427-2435.

[2] Mao, D., Yu, S., Rysz, M., Luo, Y., Yang, F., Li, F., Hou, J., Mu, Q. and Alvarez, P. J. J. Prevalence and proliferation of antibiotic resistance genes in two municipal wastewater treatment plants. Water research 2015; 85: 458-466.

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Table S7. Differential bacterial taxa abundance comparing microplastic-associated assemblages to borosilicate-associated assemblages and water sample bacterial communities in WWTP1 by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed.

Substrate	Taxa	Log LDA score
MPs	Rhodocyclaceae	4.82
	Hyphomicrobiaceae	4.58
	Fluviicola	4.33
	Arcobacter	4.17
	Comamonadaceae	4.08
	Aquabacterium	4.08
	Zooglea	4.03
	Uncultured Sphingobacteriales	3.92
	Acidovorax	3.91
	Sphaerotilus	3.77
	Paludibacter	3.74
	Pseudomonas	3.70
	Uncultured Gracilibacteria	3.64
	Perludibaca	3.61
	Comamonas	3.54
BS	Uncultured Anaerolineaceae	4.19
	Sphingomonadaceae	4.17
	Rhizobiales	4.10
	Christensenellaceae 7 group	3.66
	Rhodobacteraceae	3.62
	Trichococcus	3.58
	Ottowia	3.53
	Gammaproteobacteria WN HWB 116	3.53
	Peptostreptococcaceae	3.52
	Alkanindiges	3.42
	Uncultured Verrumicrobia LD1 PB3	3.26
	Methylotenera	3.25
	Clostridium	3.24
	Leucobacter	3.20
	Cyanobacteria Subsection IV family I	3.18
Water	*Leeia*	4.92
	Rhizobiales JG35 K1 AG5	4.89
	Rhodocyclaceae 12 up	4.62
	Flavobacterium	4.38
	Unculture candidate division SR1	4.32
	Saccharibacteria	4.13
	Alcaligenaceae GK98 freshwater group	3.59
	Methylocystaceae	3.51
	Uncultured Veillonellaceae	3.32
	Dialister	3.25
	Enterobacteriaceae	3.23
	Uncultured compost bacterium *Saccharibacteria*	3.22
	Bifidobacterium	3.16
	Streptococcus	3.16
	Ruminococcaceae UCG 014	3.15

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Table S8. Differential bacterial taxa abundance comparing microplastic-associated assemblages and water sample bacterial communities in WWTP2 by linear discriminant analyses (using LEfSe). Fifteen taxa with the highest Log LDA score in each group are listed. BS is not included as LEfSe analysis did not find any taxa clearly more abundant in BS with respect to MPs and water

Material	Taxa	LDA effect score
MPs	Uncultured Saprospiraceae	4.61
	Comamonadaceae	4.46
	Rhodobacteraceae	4.27
	Candidatus *Microthrix*	4.18
	Acidimicrobiaceae	3.90
	Variovorax	3.79
	Roseiflexus	3.78
	Terrimonas	3.74
	Dokdonella	3.68
	Chloroflexi	3.62
	Iamia	3.57
	Rhodobacter	3.54
	Lautropia	3.49
	Sphaerotilus	3.46
	Pirellula	3.45
	Mycobacterium	2.85
Water	Rhizobiales JG35 K1 AG5	5.05
	Leeia	4.84
	Rhodocyclaceae 12up	4.65
	Flavobacterium	4.39
	Unculture candidate division SR1	4.24
	Uncultured Anaerolineaceae	4.21
	Rhodocyclaceae	3.94
	Rhizobiales	3.80
	Christensenellaceae 7 group	3.65
	Saccharibacteria	3.51
	Methylocystaceae	3.51
	Alcaligenaceae GK98 fresh water group	3.46
	Arcobacter	3.45
	Uncultured Veillonellaceae	3.45
	Hyphomicrobiaceae	3.43

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Table S9. Pairwise Kruskal Wallis test.

WWTP	Gene	Comparison	Difference of Means	Test statistic	*p* value
WWTP1	*sulI*	PLA vs. Water	0.154	0.048	0.827
		PHB vs. Water	0.225	0.429	0.513
		PCL vs. Water	0.024	0.048	0.827
		PET vs. Water	-0.099	0.429	0.513
		LDPE vs. Water	0.051	0.196	0.658
		POM vs. Water	-0.218	0.429	0.513
		PS vs. Water	0.382	1.190	0.275
		BS vs. Water	-0.719	3.857	0.050
	tetM	PLA vs. Water	-0.811	-20.000	0.020
		PHB vs. Water	-0.476	-4.333	0.504
		PCL vs. Water	-0.516	-6.000	0.335
		PET vs. Water	-0.777	-20.000	0.002
		LDPE vs. Water	-0.604	-9.667	0.136
		POM vs. Water	-0.790	-21.000	0.001
		PS vs. Water	-0.624	-11.333	0.080
		BS vs. Water	-0.495	-9.667	0.136
WWTP2	*sulI*	PLA vs. Water	-0.636	-3.333	0.564
		PHB vs. Water	1.506	10.000	0.083
		PCL vs. Water	1.015	6.000	0.299
		PET vs. Water	1.036	6.667	0.248
		LDPE vs. Water	0.793	5.167	0.423
		POM vs. Water	3.492	15.167	0.019
		PS vs. Water	2.384	12.333	0.033
		BS vs. Water	7.240	18.167	0.005
	tetM	PLA vs. Water	-0.901	-19.833	0.002
		PHB vs. Water	-0.877	-18.000	0.050
		PCL vs. Water	-0.858	-15.833	0.015
		PET vs. Water	-0.858	-8.677	0.181
		LDPE vs. Water	-0.744	-10.333	0.111
		POM vs. Water	-0.802	-10.000	0.123
		PS vs. Water	-0.889	-19.000	0.003
		BS vs. Water	-0.736	-6.333	0.328

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Figure S1. Sampling sites and details of the colonization experiments. a) Spain map showing location of WWTP1 and WWTP2, b) Virgin MPs before the colonization experiment c) metal cage with MPs inside, d) deployment of metal cages with MPs into WWTP effluent, e) cages after 48 h of colonization f) drying of the colonized MPs onto sterilized filter paper.

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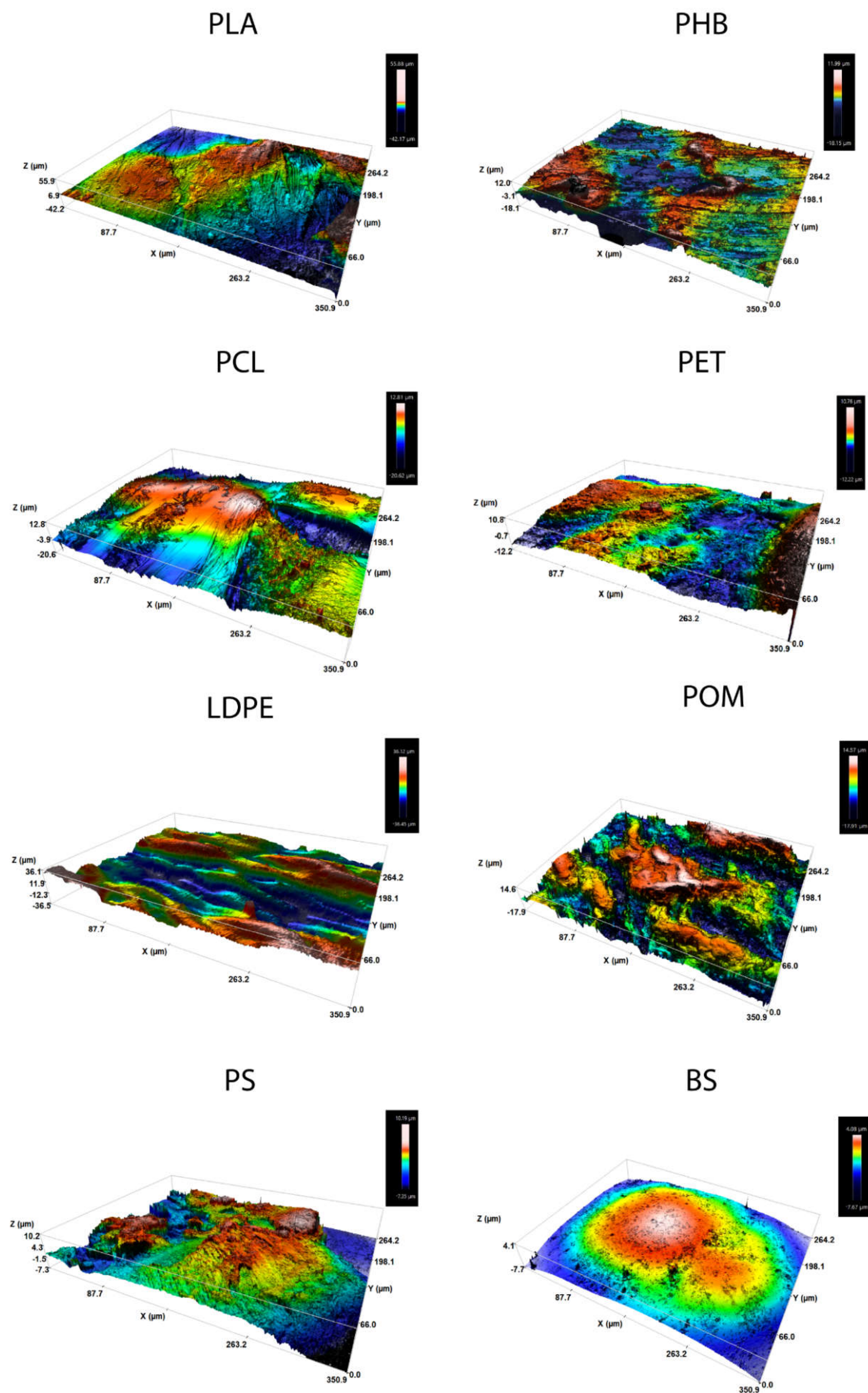

Figure S2. Images of the surface of each substrate obtained by 3D microscopy. Red color represents roughness crests and blue color represents the sunken areas.

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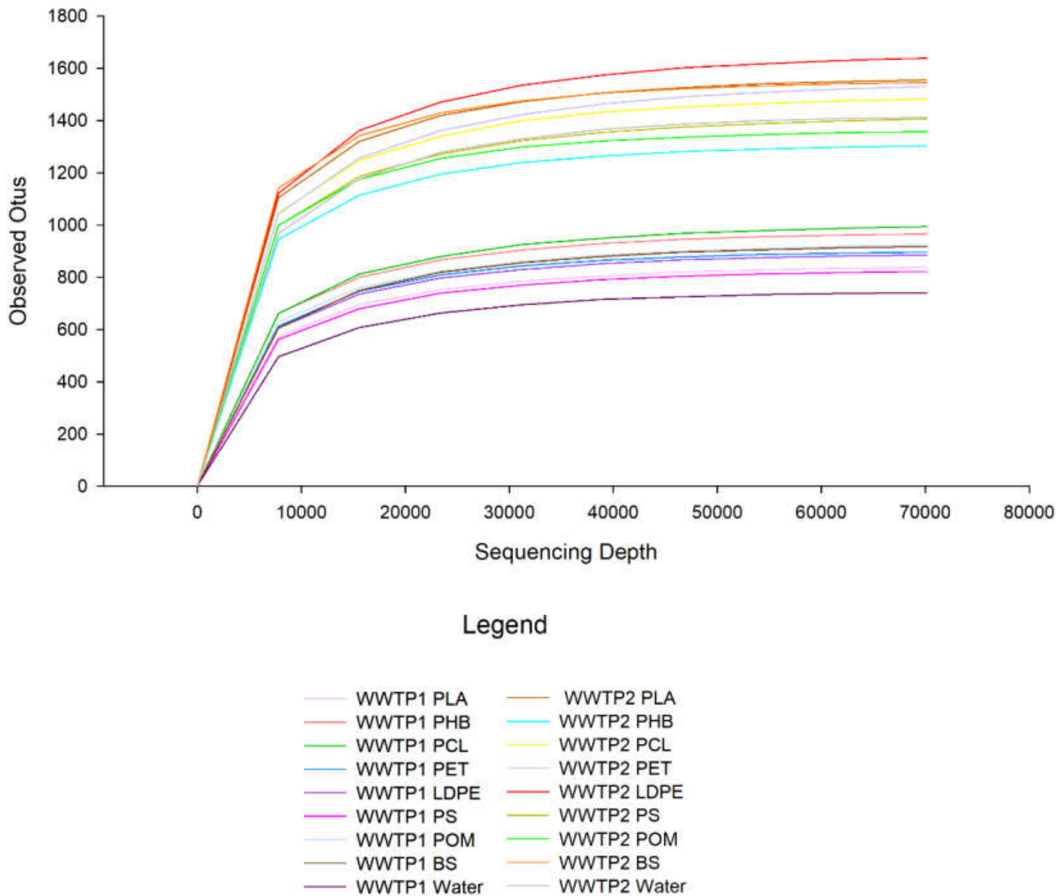

Figure S3. Rarefaction curve that compares the observed ASVs index in comparison with number of reads for each sample (sequencing depth).

Metagenomics pipeline. The guide of this information can be found in the QIIME 2 user documentation (https://docs.qiime2.org/2019.10/)

#1 Group the files within the same folder

#2 Import data to QIIME2
qiime tools import --type 'SampleData[PairedEndSequencesWithQuality]'
--input-path lecturas
--input-format CasavaOneEightSingleLanePerSampleDirFmt
--output-path GuadaUAMjunto.qza

#3 Check the quality of the samples according to QIIME2
qiime demux summarize
--i-data GuadaUAMjunto.qza
--o-visualization calidadsecuenciasmicroplastics.qzv

#4 Use of dada2 to denoises single-end sequences, dereplicates them, and filters chimeras. According the quality obtained before, the lectures are trimmed and truncate
qiime dada2 denoise-single
--i-demultiplexed-seqs GuadaUAMjunto.qza
--p-trim-left 20

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--p-trunc-len 240
--o-representative-sequences microplasticsdada2.qza
--o-table microplasticstable-dada2.qza
--o-denoising-stats microplasticstats-dada2.qza

#5 Create metadata file and validate with Keemei

#6 Generate a summarise table of the content
qiime feature-table summarize
--i-table microplasticstable-dada2.qza
--o-visualization microplasticstable.qzv
--m-sample-metadata-file microplasticosrevision.tsv

#7 Generate tabular view of feature identifier to sequence mapping, including links to BLAST each sequence against the NCBI nt database
qiime feature-table tabulate-seqs
--i-data microplasticsdada2.qza
--o-visualization microplasticsrep-seqs.qzv

#8 Create a sequence alignment using MAFFT. The result is used to infer a phylogenetic tree
qiime phylogeny align-to-tree-mafft-fasttree
--i-sequences microplasticsdada2.qza
--o-alignment microplasticsaligned-rep-seqs.qza
--o-masked-alignment microplasticsmasked-aligned-rep-seqs.qza
--o-tree microplasticsunrooted-tree.qza
--o-rooted-tree microplasticsrooted-tree.qza

#9 Generate interactive alpha rarefaction curves considerating the "min_depth" and the "max_depth"
qiime diversity alpha-rarefaction
--i-table microplasticstable-dada2.qza
--i-phylogeny microplasticsrooted-tree.qza
--p-max-depth 70139
--m-metadata-file microplasticosrevision.tsv
--o-visualization microplasticsalpha-rarefaction.qzv

#10 Applies a collection of diversity metrics (including Shannon Index and Bray-Curtis matrix)
qiime diversity core-metrics-phylogenetic
--i-phylogeny microplasticsrooted-tree.qza
--i-table microplasticstable-dada2.qza
--p-sampling-depth 70139
--m-metadata-file microplasticosrevision.tsv
--output-dir core-metrics-results

#11 Compare visually and statistic the alpha diversity by Shannon index, Pielou evenness and Chao 1 index
mkdir alpha
qiime diversity alpha-group-significance
--i-alpha-diversity core-metrics-results/shannon_vector.qza
--m-metadata-file microplasticosrevision.tsv
--o-visualization alpha/GuadalajaraUAMshannongroup.qzv
qiime diversity alpha
--i-table microplasticstable-dada2.qza
--p-metric pielou_e
--o-alpha-diversity alpha/microplasticsallpielou.qza

```
qiime diversity alpha-group-significance
--i-alpha-diversity alpha/microplasticsallpielou.qza
--m-metadata-file microplasticosrevision.tsv
--o-visualization alpha/microplasticsallpielou.qzv
qiime diversity alpha
--i-table microplasticstable-dada2.qza
 --p-metric chao1
 --o-alpha-diversity alpha/microplasticsallchao1.qza
qiime diversity alpha-group-significance
--i-alpha-diversity alpha/microplasticsallchao1.qza
--m-metadata-file microplasticosrevision.tsv
--o-visualization alpha/microplasticsallchao1.qzv
```

#12 Grouping of samples and comparison of statistics PERMANOVA and PERMDISP using Bray Curtis distance matrix

```
mkdir beta
qiime diversity beta-group-significance
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Materialplace
 --p-method permanova
  --p-pairwise
 --p-permutations 999
 --o-visualization beta/permanovaMaterialplace
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column WWTP
 --p-method permanova  --p-pairwise
 --p-permutations 999
 --o-visualization beta/permanovaWWTP
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Paper
 --p-method permanova  --p-pairwise
 --p-permutations 999
 --o-visualization beta/permanovaPaper
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Material
 --p-method permanova
  --p-pairwise --p-permutations 999
 --o-visualization beta/permanovaMaterial
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
 --m-metadata-file microplasticosrevision.tsv
 --m-metadata-column Materialplace
 --p-method permdisp  --p-pairwise
 --p-permutations 999
 --o-visualization beta/permdispMaterialplace
qiime diversity beta-group-significance
 --i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
```

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```
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column WWTP
--p-method permdisp
--p-pairwise
--p-permutations 999
--o-visualization beta/permdispWWTP
qiime diversity beta-group-significance
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column Paper
--p-method permdisp
--p-pairwise
--p-permutations 999
--o-visualization beta/permdispPaper
qiime diversity beta-group-significance
--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column Material
--p-method permdisp
--p-pairwise
--p-permutations 999
--o-visualization beta/permdispMaterial
```

#13 Train the classifier Silva 128 at 99 % similitude with the primers.

```
qiime tools import
--type 'FeatureData[Sequence]'
 --input-path 99_otus_16S.fasta
 --output-path 99_otus_16S.qza
qiime tools import
--type 'FeatureData[Taxonomy]'
--input-format HeaderlessTSVTaxonomyFormat
--input-path consensus_taxonomy_7_levels.txt
--output-path consensus_taxonomy_7_levels.qza
qiime feature-classifier extract-reads
--i-sequences 99_otus_16S.qza
--p-f-primer CCTACGGGNGGCWGCAG
--p-r-primer GACTACHVGGGTATCTAATCC
--o-reads consensus_taxonomy_7_levelsref-seqs.qza
qiime feature-classifier fit-classifier-naive-bayes
--i-reference-reads consensus_taxonomy_7_levelsref-seqs.qza
 --i-reference-taxonomy consensus_taxonomy_7_levels.qza
 --o-classifier SILVA_128_99_classifier.qza
```

#14 Assign taxonomy using the classifier Silva 128 at 99 % similitude. After that, generate a taxa bar plot interactive

```
mkdir taxonomy
qiime feature-classifier classify-sklearn
--i-classifier SILVA_128_99_classifier.qza
--i-reads  microplasticsdada2.qza
--o-classification microplasticsalltaxonomysilva.qza
qiime metadata tabulate
--m-input-file microplasticsalltaxonomysilva.qza
--o-visualization microplasticsalltaxonomysilvavisualizationtaxonomysilva.qza
qiime taxa barplot
```

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```
--i-table microplasticstable-dada2.qza
--i-taxonomy microplasticsalltaxonomysilva.qza
--m-metadata-file  microplasticosrevision.tsv
--o-visualization microplasticosrevisiontaxa-bar-plotsSilva.qzv
```

#15 Group the replicates and create a taxa bar plot using a new metadata file

```
qiime feature-table group
--i-table microplasticstable-dada2.qza
--p-axis sample
--p-mode sum
--m-metadata-file microplasticosrevision.tsv
--m-metadata-column Paper
--o-grouped-table Papergrouptable.qza
qiime taxa barplot
--i-table Papergrouptable.qza
--i-taxonomy microplasticsalltaxonomysilva.qza
--m-metadata-file microplasticosrevisionPaper.tsv
--o-visualization taxonomy/microplasticsPaper-bar-plotsSilvasimple.qzv
```

#16 Convert the archives in txt to use in Lefse. For this process, if it is necessary to collapse the taxa results at speciess level, export the data and convert to txt format.

```
qiime taxa collapse
--i-table microplasticstable-dada2.qza
--i-taxonomy microplasticsalltaxonomysilva.qza
--p-level 7
--o-collapsed-table microplastics_collapsedspecies.qza

qiime tools export
--input-path microplastics_collapsedspecies.qza
--output-path speciesslefsetable

biom convert
--i speciesslefsetable/feature-table.biom
--o feature-tablespecies.txt
--header-key “taxonomy” --to-tsv
```

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