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The dangerous transporters: A study of microplastic-associated bacteria passing through municipal wastewater treatment

Antonina Kruglova [a,*], Barbara Muñoz-Palazón [b,c], Alejandro Gonzalez-Martinez [b], Anna Mikola [a], Riku Vahala [a], Julia Talvitie [d]

[a] Department of Built Environment, Aalto University, PO Box 15200, FI-00076, AALTO, Finland
[b] Department of Microbiology, University of Granada, Campus Universitario de la Cartuja C.P., 18071, Granada, Spain
[c] Dipartimento di Ecologia e Biologia, Università degli Studi della Tuscia, Largo Università snc, 01100, Viterbo, Italy
[d] Marine Research Center, Finnish Environment Institute (SYKE), P.O.Box 140, FI-00251, Helsinki, Finland

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ABSTRACT

Microplastics (MPs) provide a stable and protective habitat for diverse wastewater bacteria, including pathogenic and antibiotic-resistant species. Therefore, MPs may potentially transport these bacteria through wastewater treatment steps to the environment and far distances. This study investigated bacterial communities of MP-associated bacteria from different stages of municipal wastewater treatment processes to evaluate the potential negative effect of these biofilms on the environment. The results showed a high diversity of bacteria that were strongly attached to MPs. After all treatment steps, the core bacterial groups remained attached to MPs and escaped from the wastewater treatment plant with effluent water. Several pathogenic bacteria were identified in MP samples from all treatment steps, and most of them were found in effluent water. These data provide new insights into the possible impacts of wastewater-derived MPs on the environment. MP-associated biofilms were proved to be important sources of pathogens and antibiotic-resistant genes in natural waters.

1. Introduction

During the past decades, plastic production has been continuously increasing (Plastic Europe, 2021). This increase brings along with it an accumulation of plastic waste in the environment and an escalation of environmental pollution by microplastics (MPs), a smaller fraction of plastics (1 μm–5mm in diameter) that are harder to stop from spreading due to their small size and persistence (Thompson et al., 2004; Hartmann et al., 2019; Hale et al., 2020). MPs are entering the environment either as primary MPs or as secondary microplastics that are breaking down from larger plastic items due to biological, chemical, or biological processes (GESAMP, 2016). Consequently, MPs are everywhere in our environment and society: in soils, aquatic environments, wildlife, food products, drinking water, and in the air we breathe (Xu et al., 2020; Zhou et al., 2020; Susanti et al., 2020; Kwon et al., 2020; Zhang et al., 2020).

MPs have been suspected to have detrimental impacts on wildlife and human health (Lo and Chan, 2018; Xia et al., 2020; Vethaak and Legler, 2021; Ragusa et al., 2021). Due to their tiny size, MPs can be ingested by a variety of species (Gouin, 2020; Wang et al., 2021; Yin et al., 2022), and although field-based evidence is still relatively scarce, many laboratory studies have demonstrated that, in aquatic environments, MPs can be transferred in food webs from lower to higher trophic levels and potentially to humans (Nelms et al., 2018). MPs may also include organic and inorganic micropollutants, originally present or adsorbed to their surfaces, which may cause harmful effects on organisms ingesting them (Rochman et al., 2019). In addition, plastic litter and microplastics provide a durable substrate for the growth of microbial biofilms, which can include non-indigenous and harmful species. The microbial community in the plastics can be very different from the surrounding environments forming a unique ecosystem called the plastisphere (Zettler et al., 2013; Amaral-Zettler et al., 2020).

Municipal wastewater treatment plants (WWTPs) are one of the main recipients of MPs originating from our daily activities like washing synthetic clothes and using MP-containing personal care products (Browne et al., 2011; Carr et al., 2016; Talvitie et al., 2017a, 2017b; Liu et al., 2021). Although WWTPs act as a barrier for MPs to the aquatic environment, removing microplastics from wastewater (up to 99%),

* Corresponding author.
E-mail address: antonina.kruglova@aalto.fi (A. Kruglova).

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studies show that no wastewater treatment technique leads to complete retention of microplastics, and hence WWTPs are viewed as pathways for microplastics to the aquatic environment (Liu et al., 2021). Therefore, despite their high reduction ability, conventional WWTPs are a still significant source of MPs in the aquatic environment due to the large volumes of discharged effluents (Murphy et al., 2016; Talvitie et al., 2017a, 2017b; Lares et al., 2018; Salmi et al., 2021).

The majority of municipal WWTPs include biological treatment based on the activated sludge (AS) process. Wastewater entering WWTPs contains a wide range of human microbiota, including pathogenic bacteria. In the biological treatment step, these bacteria meet with AS microorganisms (Saunders et al., 2016). In the AS step, the treated water is separated via sedimentation, and most of the bacteria are returned to the process. However, due to their durability and small size associated with low density, some of the MPs pass through these wastewater treatment steps while being colonized by dense bacterial biofilm (McCormick et al., 2014; Auta et al., 2017; Talvitie et al., 2017a, 2017b).

Few studies have evaluated the role of wastewater-derived MPs as transporters of harmful or pathogenic microbiota to the aquatic environment and how wastewater treatments, particularly disinfection, may modify the bacterial community in the plastisphere. Boni et al., 2021 and Shen et al. (2021) showed in their laboratory studies how MPs can act as a protective habitat for pathogenic bacteria in wastewater, allowing them to survive disinfection treatments (UV, chlorine disinfection, and disinfection by peracetic acid), which is usually effective for free-living bacteria. A recent study of MP biofilms at two different WWTPs demonstrated an increase in bacterial species richness and an abundance of taxa during the wastewater treatment processes, suggesting that WWTPs can have a significant role in modifying the plastisphere (Kelly et al., 2021). Additionally, studies of MP biofilms and free-living bacteria before and after the disinfection treatment (ozonation) at a municipal WWTP demonstrated no effect of the treatment on the total bacterial communities, the composition of the potentially pathogenic bacteria, or the antibiotic resistance gene (ARG) abundances in MP biofilms (Galafassi et al., 2021). This is especially concerning since Imran et al. (2018) showed that MPs can act not only as transporters but also as hotspots for the spread of antibiotic resistance between bacteria, including phylogenetically distinct species.

Our study aimed to evaluate the possible effects of wastewater-derived MP biofilms on the performance of current municipal wastewater treatment in terms of hygienic risks and environmental water quality. For this, we followed the MP bacterial biofilm persistence and dynamics through the main treatment steps of a large, advanced WWTP, comparing plastispheres of influent, before and after biological treatment and in the effluent released into the aquatic environment.

2. Materials and methods

2.1. Description of the WWTP and removal of microplastics

The sampling of MPs took place during the winter and spring of 2017. Samples were taken from the Viikinmäki WWTP, a large municipal WWTP in Helsinki, Finland, (~300,000 m^3 d^{-1}, population equivalent to ~800,000). The wastewater treatment process at the Viikinmäki WWTP consists of pre-treatment (including screening, grit removal, preaeration, and primary sedimentation), conventional AS process (secondary treatment), secondary sedimentation, and a tertiary biologically active filtration (BAF) for denitrification. The treated effluents are discharged into an open sea area ~4 km away from the shoreline in the Gulf of Finland, Baltic Sea. The Principle diagram of the WWTP process with sampling points is presented in Fig. S1 in the Supplementary materials. According to previous studies, Viikinmäki WWTP efficiently removes MP, 20 μm–5mm in size (Talvitie et al., 2017a, 2017b). Most of the influent MP (~98%) is already removed during the pre-treatment (from 686.7 to 10.9 MP L^{-1}). The AS process further removes about 88% of the microplastics from the primary effluent (from 10.9 to 1.3 MP L^{-1}). Only <0.5% of the incoming microplastics are left in the final effluent and discharged into the Baltic Sea (Talvitie et al., 2017a). The most common microplastics in the wastewater at the Viikinmäki WWTP consist of polyester fibers, and polyethylene (PE) and polypropylene fragments (Talvitie et al., 2017a, 2017b).

2.2. Sample collection and processing

The irregular-shaped PE fragments from personal care products commonly detected MPs in the wastewater of Viikinmäki WWTP (Talvitie et al., 2017a, 2017b), were chosen to represent wastewater-based MPs in our study (hereafter PE). In addition to that, we collected a mixture of different kinds of secondary microplastics (hereafter MIX), including polyethylene and polypropylene fragments, to see the selectivity of the MP surface. Polyester fibers were not included in the examination, although they are a very common MP type in wastewater, as it is not possible to distinguish synthetic fibers from natural fibers prior to material analyses. To compare synthetic and natural (organic) microparticles as selective surfaces for bacterial colonization, the microscopic (<5 mm) film-like organic microparticles (hereafter OP) were also collected from the effluent.

The samples were collected from four sampling points along the wastewater treatment process: influent, after pre-treatment, after AS, and final effluent (Table 1). Two or three replicates were taken from each sampling site except influent, where the collection of MPs was challenging due to the large amount of suspended solids and large variation in microplastic concentration. Sampling was performed with a simultaneous pump and filtering technique, where the wastewater is pumped from the surface of the wastewater stream into the filter device with an electric pump (Biltema art.17–953). The filter device consists of transparent acryl tubes (60 mm in diameter) and connectors attaching the tubes. The 300 μm mesh-sized filter was placed between the connectors (Talvitie et al., 2015). The sample volumes varied from ~1 L (influent) to >1 m^3 (effluent). After the sampling, the filters were collected in a Petri dish with forceps and kept moist at a temperature of ~4 °C and transported to laboratory facilities for further processing.

MPs were picked from the filters with sterile forceps under a stereomicroscope (Fiberoptic – Heim LQ 1100) rinsed with sterile saline solution (0.9%) and collected in Eppendorf tube filled also with sterile saline solution (0.9%). To compare the fate of wastewater-derived MP-associated bacteria for free-living bacteria, influent water and activated sludge bacteria were also collected. The samples were centrifuged in 50 ml Falcon tubes for 10 min at 5000 rpm, and pelleted biomass was collected. The PE, MIX, natural biofilm microparticles as well as influent and activated sludge free-living bacteria were collected in separate Eppendorf tubes, immediately frozen, and kept at −20 °C before the DNA extraction.

2.3. Scanning Electron Microscopy (SEM)

The visualization of biomass attached to MPs was done by Scanning Electron Microscopy (SEM). For SEM, samples of MPs were fixated in the glutaraldehyde solution according to Gonzalez-Martinez et al. (2017). After that, samples were transported to the Center of Scientific Instrumentation at the University of Granada for visualization by a Carl Zeiss LEO 906E scanning electron microscope. The detailed protocol of sample preparation is described in Gonzalez-Martinez et al. (2017).

2.4. FTIR analyses

To ensure that the PE fragments and OP microparticles were what they were suspected to be, part of those particle types were selected for chemical identification with an imaging Fourier Transform Infrared Spectrometer (FTIRi) (Spectrum Spotlight 300, PerkinElmer, Waltham, Massachusetts, USA). The particles chosen for material analyses were picked from the filters, rinsed with distilled water and placed onto ZnSe

Table 1
The types of samples successfully obtained on the six sampling attempts.

Sampling days	Influent			After pre-treatment		Activated sludge (secondary treatment)	After secondary treatment		Effluent		
Bacterial community	free-living	PE	MIX	PE	MIX	free-living	PE	MIX	PE	MIX	OP
1	■					■			■	■	
2				■		■		■			
3				■	■	■			■		
4				■	■		■	■			
5		■	■				■	■			
6		■							■	■	■

PE = polyethylene microbeads, MIX = mixture of MPs, OP = organic microparticles
■ = *successful sampling*

windows, and left to dry for approximately 1 h, after which each window was photographed and analyzed with the FTIRi. The FTIR spectra were recorded in transmittance mode, in the wavelength region of 700–4000 cm^{-1} at a resolution of 4 cm^{-1} with 15 scans. The Thermo Scientific™ Hummel Polymer and Additives FT-IR Spectral Library were used to compare the microbead spectra to reference spectra from plastics of known composition.

2.5. Microbial analyses

For bacterial analyses, MPs were first rinsed with a sterile saline solution, then the total DNA was extracted from the surfaces of the MPs using the FastDNA SPIN Kit for Soil (MP Biomedicals, Solon, Ohio, USA).

A high-throughput sequencing procedure of DNA samples was conducted at Research & Testing Laboratory (Lubbock, Texas, USA) using Illumina MiSeq equipment and the Illumina MiSeq Reagent Kit v3. The taxonomical affiliation was done using the 16S rRNA gene taxonomy from the NCBI database, as described by Kruglova et al. (2017).

2.6. Bioinformatics pipeline

The raw data treatment from massive parallel sequencing was done using the software mothur v.1.45.3. First, the forward and reverse paired ends were merged into contigs. The contigs were subjected to quality analysis in order to remove any ambiguous bases and sequences with more than eight polymers. The unique sequences were identified and aligned against the SiLVA SEED using Needleman alignment conditions. The aligned sequences that were not in the right forward and reverse positions were eliminated. Then the chimerical sequences were removed using the VSEARCH algorithm as a template. Then any sequence belonging to any lineage other than *Bacteria* was removed. The remaining sequences were clustered into OTUs with a similarity up to 97% and a cut-off of 0.03. The cluster was calculated employing the algorithm based on an abundance-based greedy algorithm to construct the operational taxonomic unit (OTUs). The sequences were classified using the k-nearest-neighbor algorithm with the k-mer search method using a k-mer size of 8 bp with the SiLVA SEED v132. Finally, the singleton OTUs were removed, and the remaining sequences were used to create a taxonomic consensus.

2.7. Statistical analyses

Heat maps of the samples were represented using the most dominant OTUs, with more than 1% of total relative abundance in at least one sample.

Estimates for α-diversity and β-diversity were calculated using Past v3.14 software. The α-diversity calculation for species richness, diversity, and evenness was estimated through the Chao index, Simpson, Shannon-Wiener, Pielou's evenness, and Berger Parker indices. The β-diversity was calculated for a pair of samples using Whitaker and Williams indices. The diversity indices were calculated whenever possible according to success in obtaining the replicates for the sample. The principal coordinated analysis (PCoA) was calculated using the Bray-Curtis algorithm under 999 bootstrap using PAST software.

Similarity Percentages analysis (SIMPER) was used to observe the contribution of dominant bacterial taxa (OTUs with >1.5% of total relative abundance) to dissimilarities between samples in pairs. This was done to estimate which taxa in the community contributed to MPs' colonization the most and how they affected communities of other MP types (PE or Mix) at all stages of the treatment (Influent, Before AS, After AS, and Effluent).

The compositional statistics captured all the diversity of the biological samples, including rare phylotypes. Thus, the OTU table was corrected to avoid zero values and the log-ratio was transformed using R-project and CoDaPack software, respectively. The OTU distributions were employed to calculate the expected effect size (EES).

3. Results

During our six sampling campaigns, ten successful (with at least 20 microparticles found in the sample) PE samples and 8 MIX samples were collected and analyzed from four sampling points at the WWTP. AS was sampled to see the effect of the biological process step on MP biofilm. One sample of the influent microbial community and one sample of biofilm attached to OP were also included in the study as references. However, due to the lack of replicates, these samples were excluded from statistical analyses.

3.1. Microplastic colonization

Examples of PE and MIX samples are shown in Fig. 1 (A). SEM pictures of bacteria attached to effluent MPs after rinsing are shown in Fig. 1 (B), demonstrating a persistent bacterial biofilm attached to MPs.

3.2. Statistical comparison: α-diversity, principle component analysis, and β-diversity analysis

The Simpson, Shannon, and Chao indices varied considerably among the samples as well as the replicates (Table S1), showing great differences in communities. These differences could be driven by the morphology of the MPs such as size, roughness, form, etc. (Yang et al., 2020). There were no conclusive results about differences in species diversity between the PE and Mix, although previously, some authors described materials such as polystyrene and PE as having lower species richness than micropieces of natural materials (Kettner et al., 2019), while in general, the species richness is greater than in free-living

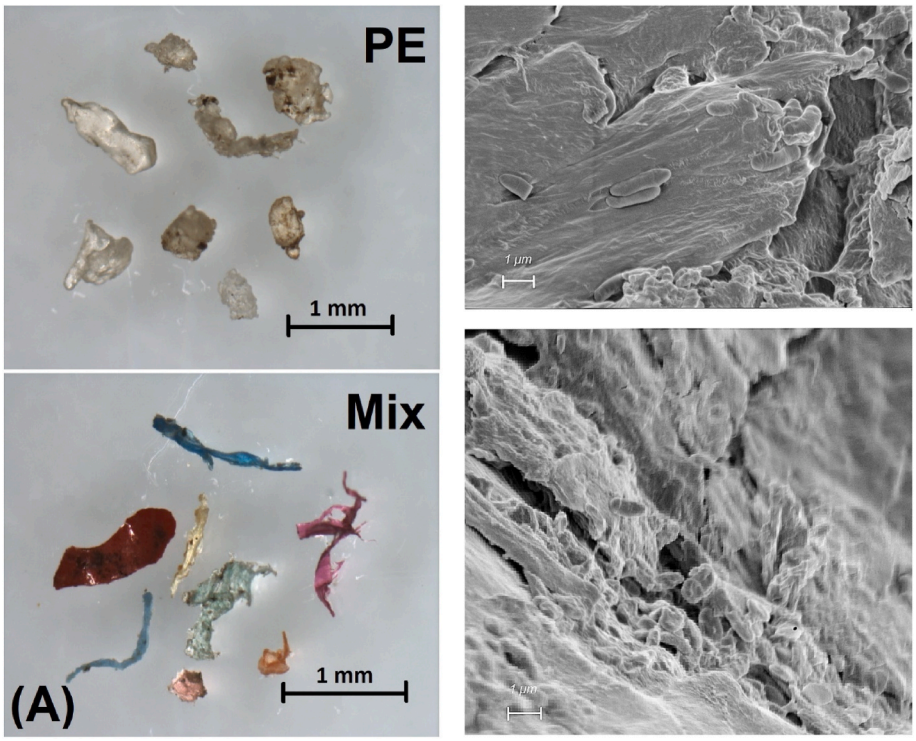

Fig. 1. **(A)** Examples of PE (polyethylene) and MIX (mixture) samples of MPs. **(B)** SEM images showing biofilm bacteria on the effluent microplastic surface.

bacterial niches (Dussud et al., 2018).

In the evenness study, the results similarly presented great variances. The highest evenness was observed in influent MIX and effluent MIX samples, showing the selective growth of bacteria on MP passing through wastewater treatment compared to influent MPs and especially on PE.

The Bray-Curtis PCoA plot (presented in Fig. 2) demonstrated a clear clustering of AS communities, influent MP biofilm, and MP biofilms before and after the AS process (secondary treatment). In addition, MIX communities were separated from PE communities, especially after the AS process.

These results suggest that the AS bacteria, despite their high diversity and population density, have only a limited effect on the MP biofilm bacteria of PE. However, the AS step noticeably altered the MIX biofilm communities. This shows the selective properties of PE for bacterial attachment compared to the variety of surface materials in the MIX samples.

β-diversity analyses (Fig. S2 in the Supplementary material) have demonstrated remarkable differences between the free-living bacterial populations found in influent and AS compared to all MP biofilms. For MP biofilms, the larger differences among pairs of samples were found before and after the AS for both types of MPs but to a greater extent for PE, which is consistent with other analyses. In contrast, the samples taken after the biological treatment (after AS and from effluent) were highly similar to each other, demonstrating no effect of the tertiary treatment. However, it is noteworthy to mention that the Whittaker index revealed a great standard deviation in the data. Altogether, the analyses showed that the composition of AS influenced biofilm colonization. But it is important to mention that the nature of MPs also reflected differences in the dominant OTUs (Section 3.4), as described by Kelly et al. (2021).

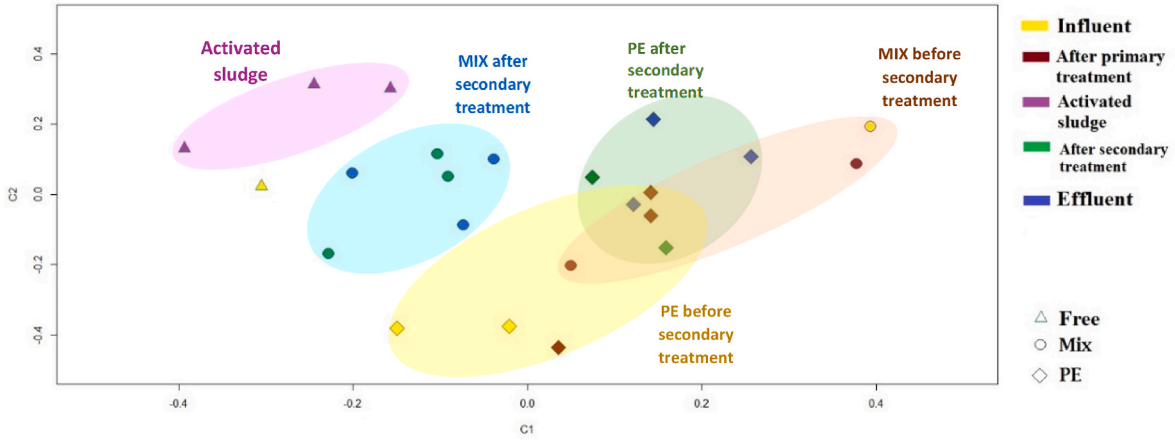

Fig. 2. Principal Coordinate Analysis (PCoA) analysis of the studied samples based on Bray-Curtis dissimilarity.

3.3. Contribution to dissimilarities

The contribution of OTUs to dissimilarities between samples was calculated by SIMPER analysis, which showed that 52 OTUs contribute to dissimilarities with more than 1.00% in at least one sample (Fig. S3 in Supplementary materials). The most striking contribution to dissimilarities between sampling points was given by *Blastocatella* (OTU007). Being dominant in AS and almost absent in all other samples, *Blastocatella* contributed more than 20% of the relative abundance to differentiate the bacterial communities found in the AS compared to other samples. Next, *Corynebacterium* (OTU004) had the heaviest impact on dissimilarities of MP biofilm samples after AS since its phylotypes proliferated after the biological treatment and almost completely disappeared in the biofilm of effluent, regardless of the nature of the MP pieces. Furthermore, *Methylotenera* (OTU001) and *Aquabacterium* (OTU002) contributed to wide dissimilarities between MP biofilm samples before AS and the rest, with values of dissimilarity promotion ranging from 5 to 10% of relative abundance. The same pattern in the OTU001 and OTU002 SIMPER analyses was shown between effluent and the rest of the samples, which demonstrates selectivity for colonization of these two phylotypes to the MPs during the wastewater treatment steps.

The expected effect size analyses determined the greatest number of OTUs differences between the influent MIX and effluent MIX samples (Fig. S4 E in Supplementary materials) as well as before and after AS (Fig. S4 F in Supplementary materials). In contrast, the PE samples had minor effects on the OTU populations observed between influent and effluent, and even lower before and after AS (Fig. S3 C in Supplementary materials). Furthermore, the dominant OTUs in MIX had a stronger effect on the dissimilarities, while the dominant and rare phylotypes of PE bacteria had similar importance. Finally, for all types of MPs, the effect of spatial disturbance in the number of OTUs showed statistically more significant differences before and after AS compared to influent and effluent MP biofilms.

3.4. Bacterial community composition

The results showed a high diversity of bacteria attached to MPs of both PE and MIX sample types in all of the successfully processed samples. The taxonomic richness of bacterial communities (presented in Fig. 3) was similar between all MP biofilm samples as well as free-living bacteria of influent and AS samples.

Noticeably lower taxonomic richness was observed in MIX biofilm from influent and OP biofilm from effluent. Given that influent sampling was the most challenging and that only one sample was collected, this may have affected this result. Similarly, for OP biofilm, more sample replicates would be needed to support these results.

The relative abundance of bacterial phyla and bacterial classes is presented in Fig. 4. Consistent with most of the published MP biofilm data (Yang et al., 2020), all studied MPs were mostly colonized by *Proteobacteria* (up to 60% of the community). Additionally, *Firmicutes, Acidobacteria, Bacteroidetes, Actinobacteria, Chloroflexi, and Fusobacteria* were among the dominant phyla with a great variety between the samples and even the replicates.

In contrast to MPs, influent free-living bacteria were mostly represented by *Firmicutes* (30%) and *Fusobacteria* (17%), followed by *Proteobacteria* (only 9%). Among AS free-living bacteria, the most abundant dominant phylum was *Acidobacteria* (up to 50%), mostly represented by the family *Microtrichaceae,* followed by *Proteobacteria* (up to 27%). OP biofilm had a very different structure of the community, with four dominant phyla: *Proteobacteria* (35%), *Firmicutes* (20%), *Bacteroidetes* (12%), and *Actinobacteria* (7%).

Within the phylum *Proteobacteria,* the dominant bacteria of all PE biofilms were from the class *Betaproteobacteria* (up to 55% of the total community), followed by *Gammaproteobacteria* (up to 18%) and *Epsilonproteobacteria* (up to 6%).

By contrast, MIX biofilm in influent was colonized mostly by *Gammaproteobacteria* (29% of the total community), followed by *Alphaproteobacteria* (6.5%). However, in the next treatment steps, *Betaproteobacteria* become the most abundant class in MIX biofilm (up to 51% of the total community after primary treatment and up to 29% in the effluent). This change could be explained by the different composition of MIX microparticles due to the effect of the primary wastewater treatment.

Altogether, these results demonstrated that MP-associated bacterial communities have a different structure and dominant groups than free-living or OP-associated bacteria. In addition, during secondary wastewater treatment (AS process), some of the AS free-living bacteria attached to MPs develop a new bacterial composition of MP biofilms. Both wastewater-derived and AS-derived bacterial populations were detected in effluent MPs and OPs.

3.5. Microplastic biofilm dynamics through the wastewater treatment process

MP biofilm analysis on a genera level revealed that bacterial communities shifted greatly after primary, secondary, and tertiary treatment. The most abundant genera of MP biofilms at different steps of the

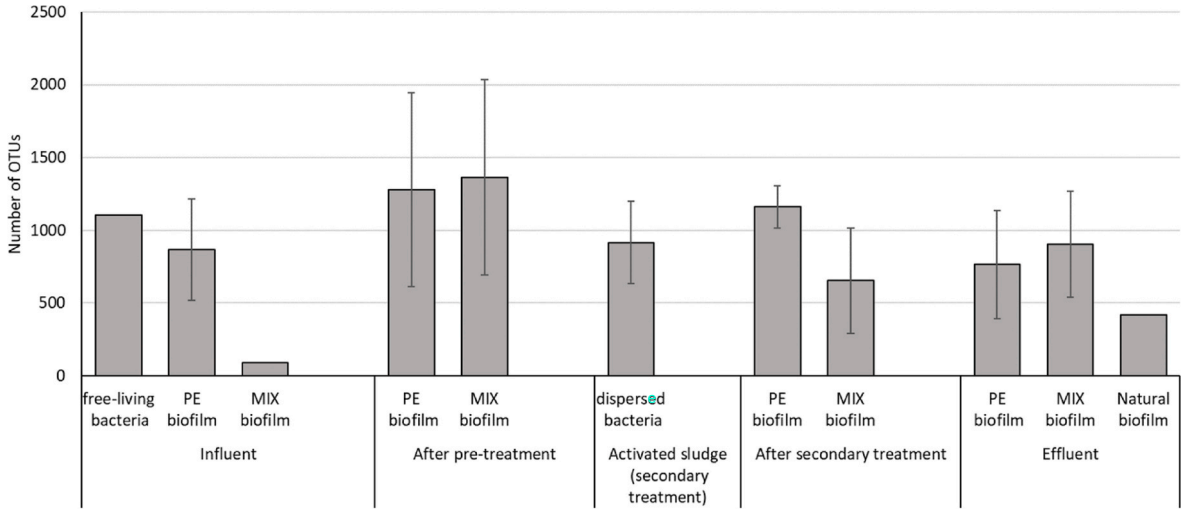

Fig. 3. Number of operational taxonomic units observed within the studied bacterial communities. Bars represent the mean values. The error bars represent the standard deviation.

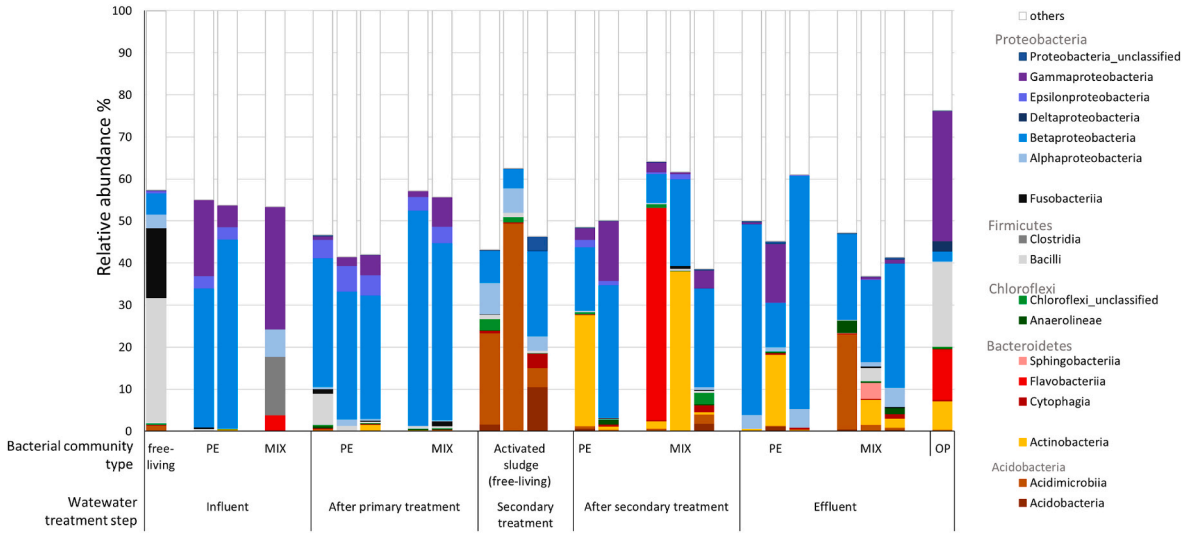

Fig. 4. Relative abundance of bacteria at the class level associated with mixture (MIX) microplastics, polyethylene microbeads (PE), and free-living bacteria at different steps in the municipal wastewater treatment process. Others are classes with less than 2.5% abundance.

municipal wastewater treatment process are presented in Fig. 5.

Influent PE biofilm was mostly represented by *Aquabacterium* (up to 32%) and *Pseudomonas* (up to 18%) genera, followed by unclassified *Betaproteobacteria* (up to 17%). These taxa continued to dominate the PE biofilm communities through the whole wastewater treatment process. Both *Aquabacterium* and *Pseudomonas* were previously reported as dominant bacterial genera of wastewater MP, and our data support the suggestions on the selective advantage of these bacterial groups for PE colonization. Additionally, both genera presumably include species with plastic-degrading capabilities (Kelly et al., 2021; McCormick et al., 2016).

Similarly to the results obtained by Kelly et al. (2021), in this study, the abundance of *Arcobacter* significantly decreased during the treatment steps (from 2% to 6% before secondary treatment to below 0.2% in effluent). Previously, Kristensen et al. (2020) reported a high abundance of *Arcobacter* in many wastewater treatment plant effluents in Denmark.

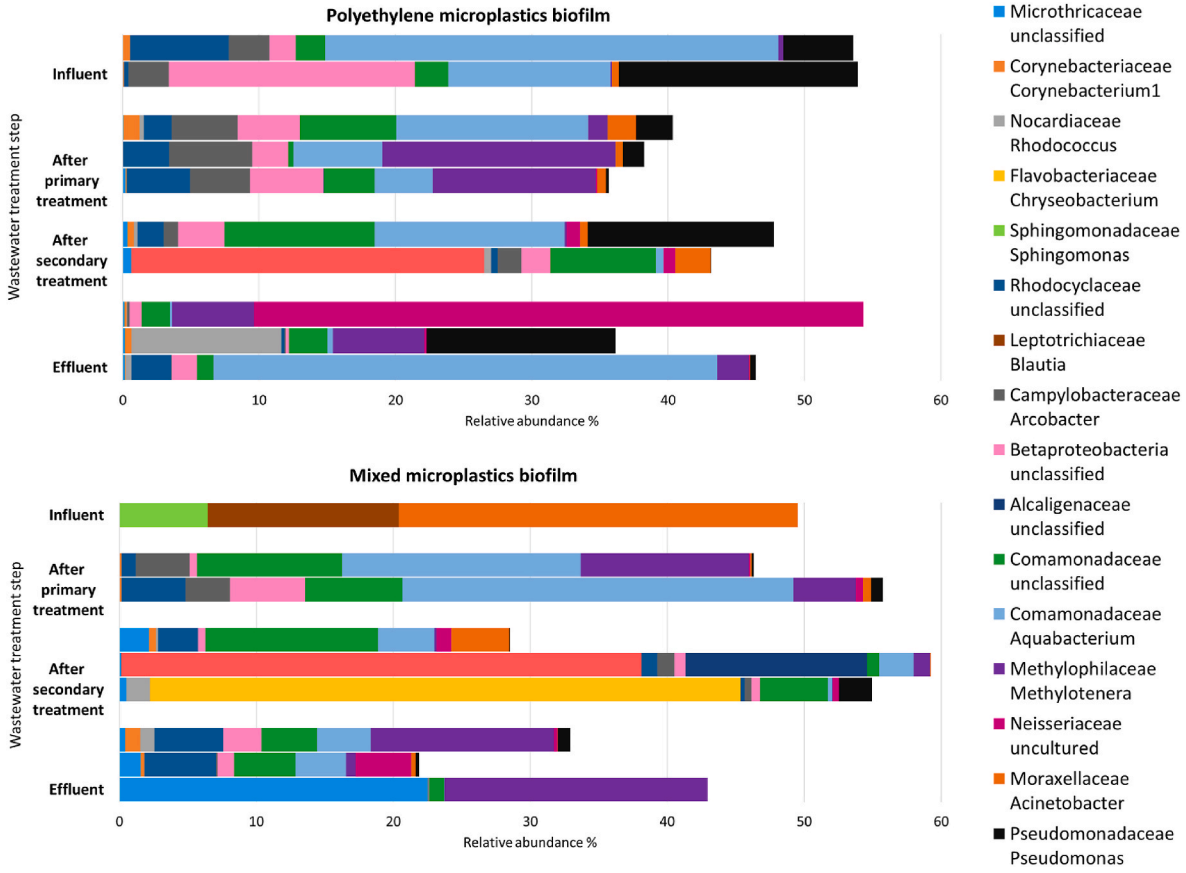

Fig. 5. Relative abundance of the most abundant bacterial genera associated with polyethylene microbeads (PE) and mixture (MIX) microplastics at different steps in the municipal wastewater treatment process.

The suggested reason for the low removal of these bacteria was their loose attachment to flocs, which could also be a reason for them to be washed out from MP biofilms. In contrast, all other dominant bacterial taxa showed no decrease in abundance after primary, secondary, and tertiary treatment.

The composition of MIX biofilms showed similarities to MP biofilms previously reported by Galafassi et al. (2021), mostly after secondary treatment. In particular, similarly to this study, MIX MPs after AS had high an abundance of *Flavobacteriaceae* (~50% after AS), Comamonadaceae (up to 13%), and *Rhodocyclaceae* (up to ~5%). Additionally, *Microtrichaceae* had a clear increase in abundance after secondary treatment (up to 23% in effluent MIX biofilm) due to the effect of the dominant bacteria from AS. *Methylophilaceae* abundance is also most likely related to the effect of wastewater treatment that is typical in a bacteria community (Hultman et al., 2018).

3.6. Human-associated and potentially pathogenic bacteria colonizing microplastics

Almost 35% of influent free-living bacteria and up to 48% of influent MP biofilm bacteria could be associated with the human gastrointestinal system and potentially pathogenic species. The abundances of identified families and genera of enteric bacteria are presented in Fig. 6.

Leptotrichiaceae represent 16% of influent free-living organisms and from 0.5 to 1% of MP communities in both MIX and PE in all the treatment steps, including 0.1% of PE biofilm and 0.4% of MIX biofilm in the effluent. *Leptotrichiaceae* are human-associated taxa that include dangerous pathogens (Eisenberg et al., 2016). Another dominant group of influent bacteria was represented by the *Streptococcus* genus, which was about 15% of free-living bacteria and up to 1.2% of MP biofilm bacteria in influent. After the secondary treatment, no *Streptococcus* was detected on PE, but up to 2.1% of the effluent MIX community still belonged to this genus. Furthermore, members of the previously mentioned abundant genus *Arcobacter* include several dangerous pathogens for humans and animals (Collado and Figueras, 2011).

Lachnospiraceae are common human and animal enteric bacteria (McLellan et al., 2013). Though they were abundant on influent MIX biofilm (18% of the total community), these bacteria seem to be quickly removed from MPs and the total wastewater bacteria composition already after the primary treatment steps. Similarly, the genus *Lactobacillus* is a known group of human enteric bacteria that appeared to be present on PE biofilm (about 7% of the bacterial community) but have not been detected in any MP samples after secondary treatment.

In addition to the groups presented in Fig. 6, two important genera—*Diezia* and the previously discussed *Pseudomonas*—were observed in high abundances on MP biofilms. Bacteria from the *Diezia* genus represented up to 2.5% of MP biofilm after secondary treatment. Species of these genera may have various habitats and also include human pathogens (Gharibzahedi et al., 2014).

Almost 45% of one PE biofilm sample was represented by the *Neisseriaceae* family. In other effluent PE samples, it was present in 0.1%, and in MIX samples, up to 4.1%. Although no *Neisseriaceae* were found in the influent, after pre-treatment, it represented 0.1% of PE biofilm and up to 0.5% of MIX biofilm. Interestingly, after secondary treatment, a higher abundance was indicated (up to 1% in both types of MP biofilm samples).

Finally, *Nocardiaceae* were detected in AS (up to 1%) after secondary treatment in MIX (up to 1.7%) and in effluent samples in both PE and MIX (~2%). *Nocardiaceae* include multiple species commonly detected in wastewater, some of which are the clinically significant and pathogenic species *Nocardia* spp. (Bafghi and Yousefi, 2016; Jia and Zhang, 2020).

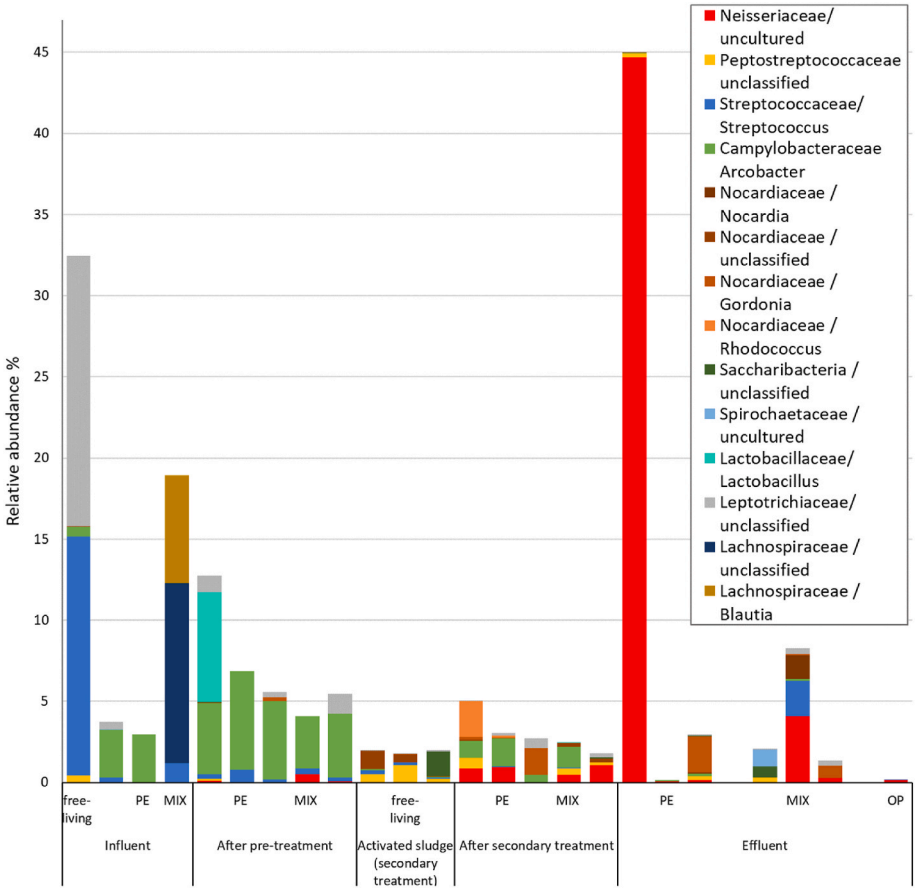

Fig. 6. Abundance of human-associated and potentially pathogenic bacteria among studied microbial communities.

Interestingly, OP biofilm in effluent had almost none of the discussed bacterial taxa except *Neisseriaceae* in 0.1% of the community and a ten times lower abundance of *Streptococcus*.

4. Discussion

Overall, in this study, several factors showed a strong effect on the MP biofilm structure. First, the diversity of MP materials increased the diversity of bacteria attached to MP, showing that the polymer structure can be selective for attaching bacteria. Secondly, despite the abundance and density of AS bacterial populations and different water processing steps, MP biofilms were able to keep a similar abundance of human gut bacteria. Once the MP particle was colonized by certain groups of bacteria, the porous surface structure of MPs with pore sizes similar to bacterial sizes (Fig. 1B) could accommodate bacterial cells as well as act as a shield against different environmental factors. Finally, AS bacteria colonized MP biofilms, increasing the diversity of particle-attached bacterial communities. AS is a diverse population of environmental bacteria mixed with wastewaters. In wastewater, activated sludge is constantly receiving different types of human-associated bacteria as well as trace amounts of multiple antibiotics. Close and long-term contact with environmental and human gut bacteria may potentially increase the spread of antibiotic resistance among environmental bacterial species through interspecies horizontal gene transfer.

The above-mentioned factors could also be attributed to OP biofilm formation. However, according to our results, biofilm from organic particles accommodated much less human gut bacteria, which can be attributed to the different origins of these microparticles. Additionally, the effect of OP on the environment would generally be secondary, considering the rather short surface material lifetime due to faster decomposition and higher biodegradability compared to plastic polymers.

Earlier, Basili et al., 2020 demonstrated on marine plastic biofilm that a microbial community depends on both environmental features and the biofilm formation process. In the study by Martínez-Campos et al. (2021) on wastewater MP biofilm, a bacteria community was mostly defined by the environment rather than the polymer structure. Yang et al. (2020) reported that colonization and distribution of microorganisms in MPs biofilm depend on the interaction between exopolysaccharides segregated by bacteria with factors not yet known (Amaral-Zettler et al., 2020). If the biofilm formation process could be controlled by operational conditions at a WWTP, this knowledge would help limit the amount of dangerous MP-associated bacteria escaping the process. Therefore, more studies are needed on the effect of engineering design and conditions of WWTPs on the microbial communities' development and bacterial metabolic functions in MPs biofilms.

The analyses on the diversity and richness of bacterial-associated biofilms have indicated no effect of treatment processes and wastewater treatment process conditions on multiple bacterial groups, including potentially pathogenic and antibiotic-resistant bacteria. Therefore, the source and amount of microplastics passing through WWTPs are important to control in order to secure environmental water quality. Similarities between the samples before and after tertiary treatment demonstrated the importance of additional advanced polishing steps, although in this case, the purpose of the tertiary treatment was only to denitrify solids (not remove them). Previous studies reported that effluent MP biofilms have higher microbial diversity compared to effluent free-living bacteria (Martínez-Campos et al., 2021; McCormick et al., 2014). This could be explained by the beneficial conditions of the MP surface for bacterial survival and growth. It also suggests the importance of MP-associated biofilm analysis in evaluating water safety. The microbiological safety of wastewater effluents is traditionally evaluated through coliform indication methods, which give very limited information compared to high-throughput sequencing (Lu et al., 2015). Our data suggest that targeted cultivation tests may not reflect the diversity of dangerous bacteria escaping WWTPs hidden in MPs.

Pathogenic microorganisms are considered to be efficiently removed during the municipal wastewater treatment process. However, this study shows that while some pathogenic groups of bacteria are effectively removed, for other species, microplastics can provide a protective habitat. Not only do these bacteria survive all wastewater treatment steps, including water-sludge separation, but the retention of microplastics in sludge could increase their abundance. Selective growth of these bacteria on MPs forced by wastewater treatment operational conditions could be one possible explanation for these results. Most of MPs together with bacteria are returned to the AS process after secondary sedimentation (Fig. S1 of Supplementary material). Therefore, these MPs may spend a long time returning to the AS before they reach effluent, while process conditions could favor the growth of certain bacterial groups. For instance, in our study, a noticeable increase in *Neisseriaceae* was observed on MPs between primary and secondary and after tertiary treatment, suggesting preferable growth conditions for this group of bacteria. Earlier, Hultman et al. (2018) reported *Neisseriaceae* among the dominant bacteria of effluents from two Finnish WWTPs, including Viikinmäki WWTP hosting tetracycline resistant genes (Hultman et al., 2018). Additionally, antibiotic resistance genes associated with *Neisseriaceae* were found in effluents of several WWTPs by Chu Binh et al. (2018). Therefore, the presence of certain pathogenic bacteria in WWTP effluents could be region-specific and attributed to a combination of local factors such as wastewater sources, wastewater composition, climate, and operational conditions. Furthermore, the diversity of region-specific human-associated bacteria released into natural waters can be linked to the spread of certain antibiotic resistance genes in this region.

Additionally, presence of *Streptococcus, Pseudomonas, Lactobacillus* and *Acinetobacter* on MP biofilms in our study raises concerns about the possibility for transferring antibiotic resistance. For decades, antibiotic treatment of *Streptococcus* diseases has been challenged due to the increase in resistance to penicillin and non-β-lactam antibiotics in its species worldwide. The same resistance mechanisms have emerged in other gram-positive pathogens (Ambrose Karita et al., 2005). The strains of *Pseudomonas, Lactobacillus,* and *Acinetobacter* have well-known multidrug-resistant isolates and are responsible for growing healthcare-related infections (Anisimova and Yarullina, 2019; Santajit and Indrawattana, 2016). Also, *Pseudomonas* was previously reported among major antibiotic resistance carriers at WWTPs (Sun et al., 2016). The high abundance of the above-mentioned bacteria on effluent MPs demonstrates the potential risks of antibiotic resistance spread through wastewater-derived MPs and the need to optimize the wastewater treatment process for better MP removal.

According to our results, MPs provide a durable substrate for biofilm-forming micro-organisms in municipal wastewater, potentially including pathogens and antibiotic resistance genes. WWTPs can efficiently remove MPs from the wastewater, but due to their small size and light weight, part of the MPs escape the wastewater treatment processes and end up in the aquatic environments with attached biofilm. To understand what kinds of harm, if any, can wastewater-derived biofilms cause in aquatic environments, more information is needed on the effect of MP-associated biofilm on the spread of antibiotic-resistant genes, particularly among AS bacteria. In addition, the effect of advanced treatment technologies such as advanced oxidation and membrane technologies on biofilm formation and MP removal should be examined. Finally, studies on the fate of microplastic-associated bacteria in environmental water near WWTP discharge are of high importance.

5. Conclusions

MPs provide a long-lasting favorable and protective habitat for the high diversity of bacteria, including several known human gastrointestinal and pathogenic species. MPs' surface composition affects bacterial colonization, selecting certain groups of bacteria, after which environmental factors have a smaller effect on the bacterial community. Both,

primary, and tertiary treatment had a minor effect on the MP biofilm structure.

The AS community and MP-associated bacteria may have a mutual effect due to the circulation of MPs reproducing pathogenic bacteria from influents leading to higher abundances of clinically significant bacterial groups in effluent MP biofilms. These interactions also provide favorable conditions for the spread of antibiotic-resistant genes in AS, as well as in the environmental waters. Since the WWTP process conditions did not efficiently reduce the diversity of MP-associated clinically significant bacteria, it can be suggested that MP will as well protect and maintain these bacteria in the environment, increasing the risks related to these species.

CRediT author statement

Antonina Kruglova: Conceptualization, Methodology, Investigation, Visualization, Writing- Original draft preparation. **Barbara Muñoz-Palazón:** Validation, Software, Visualization, Writing- Reviewing and Editing. **Alejandro Gonzalez-Martinez:** Methodology, Software, Project administration. **Anna Mikola:** Supervision, Project administration, Writing- Reviewing and Editing. **Riku Vahala:** Supervision, Funding acquisition. **Julia Talvitie:** Conceptualization, Methodology, Investigation, Writing- Reviewing and Editing, Project administration.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Antonina Kruglova reports financial support was provided by Finnish Water Utilities Association.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.120316.

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