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# Growth and prevalence of antibiotic-resistant bacteria in microplastic biofilm from wastewater treatment plant effluents

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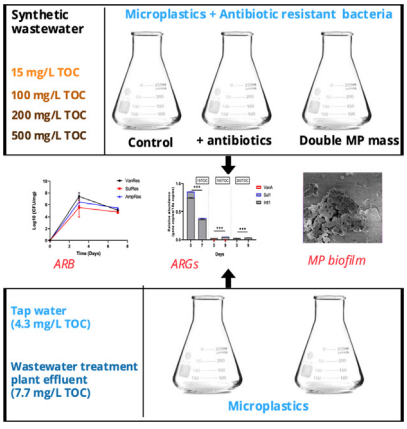
## HIGHLIGHTS

- ARB belonging to the genus Aeromonas dominated in WWTP effluent.
- Viable ARB numbers increased significantly in MP biofilm on day 3.
- ARB in MP biofilm differed as a function of time and TOC.
- ARGs and intl1 distribution in MP biofilm differed significantly across varying conditions.
- MP biofilm in treated water contained *van*A, *sul1* and *intl1* on day 30.

## GRAPHICAL ABSTRACT

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## ABSTRACT

It is accepted that Microplastic (MP) biofilms accumulates antibiotic-resistant bacteria (ARB) and antibiotic-resistant genes (ARGs) in water. ARB/ARGs and MPs are emerging pollutants of concern due to various associated health risks. The objective of this study was to 1) investigate the ARB community in a pilot-scale wastewater treatment plant (WWTP) effluent, 2) to study and visualize the ARB/ARGs in MP biofilm grown in WWTP effluent and tap water, and 3) to analyze microplastic adherent ARB/ARGs in the biofilm and planktonic ARB/ARGs in the filtrate under controlled conditions. Results indicated the dominance of *Pseudomonas*, *Aeromonas*, and *Bacillus* among isolated ARB in WWTP effluent. Representative resistance strains were incubated in 300 mL water containing commercial polystyrene beads of 300550 μm diameter (MP) in a series of batch experiments. Microbiological, molecular, and microscopic analyses were performed by enumeration, 16srRNA, real-time polymerase chain reaction (qPCR), and Field Emission-Scanning Electron Microscopy (FEG-SEM) techniques. The analyzed viable ARB indicated an increasing trend in MP biofilms between days 3 and 5. It further decreased on days 7 and 9. The prevalence of ARB in the filtrate and MP biofilm varied as a function of time and TOC level, while no significant impacts were observed for minor temperature variation, low antibiotic pressure, and increased MP mass with few exceptions. Relative abundance of ARGs (*van*A, *sul1*) and integron integrase gene (*intl1*) in MP biofilm were significantly different across different TOC levels, time, and antibiotic pressure. ARGs and *intl1* were detected in the MP biofilm in tap water and WWTP effluent on day 30.

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## 1. Introduction

The spread of antibiotic resistance has been identified as one of the 21st century's most serious threats to public health (WHO, 2014). Moreover, in the European Union, the Marine Strategy Framework Directive (MSFD, 2008/56/EC) included microplastics (MPs) pollution in a legislative proposal (Gago et al., 2016). However, no stipulated minimal reductions for MP, antibiotics, antibiotic-resistant bacteria (ARB), and antibiotic-resistant genes (ARGs) are found in water and wastewater treatment guidelines (Hong et al., 2018). MPs, ARB, and ARGs continue to remain global contaminants in natural and engineered water systems.

One of the main concerns associated with MP pollution is the MP-associated microorganisms. The MP particles harboring bacterial strains in drinking water sources potentially pose a water disinfection challenge. Bacterial species embedded in the biofilm matrix are more resistant to disinfection techniques due to the physical protection barrier composed of extracellular polymeric substances (EPS) and dead cells (M. Zhang et al., 2019; X. Zhang et al., 2019; Rodrigues et al., 2019; Boni et al., 2020). The functional composition and contribution of the MP biofilm towards the spreading of antibiotic resistance is less understood.

Antibiotic resistance is spread through the ARB, ARGs, and mobile genetic elements (MGEs), including integrated conjugative elements, integrons, plasmids, insertion sequences, and transposable elements that carry genetic traits encoding antimicrobial resistance function. Horizontal gene transfer (HGT) is mediated by processes of transformation, conjugation, transduction, and fusion of outer membrane vesicles. Some of the mechanisms through which ARGs facilitate antimicrobial resistance are reduced permeability, active efflux of antibiotics, antibiotic target alteration, post-translational modification, chemical modification, or hydrolysis of antibiotics (Bag et al., 2019; Das et al., 2020). The colonizing microbial communities on the MP surface, the ARB/ARGs in effluent water, and the HGT phenomena provide a thriving environment for the spread of antibiotic resistance. Furthermore, the MPs are a hypothesized carrier of ARB, ARGs, and other antibiotic resistance elements in the drinking water sources (Costerton et al., 1999; Arias-Andres et al., 2018a,b; Wu et al., 2019).

Various studies indicated the presence of ARB and ARGs in WWTP effluent, drinking water sources, and tap water (Armstrong et al., 1981; Bai et al., 2019; Chen et al., 2019; Hao et al., 2019; Khan et al., 2016). Garner et al. (2018) reported the abundance of 590 different types of ARGs in potable water in the range of 3.93–6.83 log gene copies per mL and 4.33–5.32 log gene copies per swab in the biofilm of the water distribution network. Hunter et al. (2008) reported the antimicrobial resistance genes transfer rate in the interval of $10^{-2}$ to $10^{-9}$. Therefore, the microbial colonization on plastic surfaces becomes a reservoir for ARB and ARGs (Yang et al., 2019). Meanwhile, a full understanding of antimicrobial resistance at a microbial community level, the genome level, and the transmission of resistance in the biofilm are absent.

Conventional WWTP design does not allow for the complete removal of MPs, ARB, and antibiotic resistance elements, especially ARGs and integrons in water resources. Although WWTPs remove a high proportion of the MPs, $4 \times 10^9$ MP particles and fibers are expected in the annual WWTP effluent discharge (Mintenig et al., 2017). Therefore, the situation calls for further research on MP-associated microorganisms for their potential role in antibiotic resistance proliferation.

The influence of biofilms on the fate, effects, and characteristics of MPs remains less understood (Rummel et al., 2017). More research is required via controlled laboratory conditions to evaluate the relative contribution of various factors, including the microbial community structure, antibiotics, other compounds, HGT, and the water parameters in shaping the resistome of treated water (Garner et al., 2018). Furthermore, previous research studies on microplastic biofilm are focused on field observations, whereas replicable studies under controlled exposure conditions are lacking (Ogonowski et al., 2018).

This research aims to contribute to an understanding of the ARB colonization on MP surfaces as a function of TOC, time, antibiotic pressure, and available MP in the water matrix. To achieve this goal, ARB and ARGs were quantified across varying conditions. The study provides an insight into the selectivity and abundance of ARB, ARGs, and MGE in the MP biofilm. It highlights the potential of MPs as a hub for ARB, ARGs, and MGE. It further highlights the potential role of MP biofilm as a carrier of antibiotic-resistant elements in water.

## 2. Experimental

### 2.1. Bacterial strains and antibiotics

Indigenous ARB were isolated from the effluent of a pilot-scale WWTP, located at the Universidad Rey Juan Carlos Mostóles, Madrid, Spain. The WWTP, with a total treatment capacity of 150 $m^3$/day receives wastewater influent from the campus. The water line of the WWTP consists of a physicochemical treatment of coagulation-flocculation followed by dissolved air flotation, a secondary treatment in rotating biological contactors followed by a lamellar decanter and a tertiary treatment with a sand-carbon filter before disinfection. 50 mL grab samples of the effluent were collected in sterile bottles. Bacterial strains were isolated thrice during the summer, winter, and spring seasons to have representative bacterial species throughout the year. The bacterial colony morphology was analyzed to attain diverse species. In total, 19 morphologically different colonies were selected for identification by 16srRNA partial sequencing.

Trypticase soy agar (TSA) plates were supplemented with vancomycin (70 μg/mL), ciprofloxacin (0.25 μg/mL), sulfamethoxazole (20 μg/mL) and ampicillin (25 μg/mL) (Sigma-Aldrich) for the isolation of the respective ARB. The effluent samples were plated on the antibiotic-supplemented TSA agar plates within 10 min of WWTP effluent sample collection. The plated cultures were incubated at 37 °C for 24 ± 2 h. Pure colonies were grown in antibiotic-supplemented Luria-Bertani (LB) broth, and the glycerol stocks were stored at −20 °C.

### 2.2. Bench studies for the MP biofilm analysis

Batch flasks of 300 mL working volume were used in four batch experiments conducted in replicate (Table 1). The synthetic wastewater recipe was adopted from literature (OECD, 1999) with the following chemical composition in mg/L (TOC 500 mg/L): peptone (400), meat extract (275), urea (75), $K_2HPO_4$ (70), NaCl (17.5), $CaCl_2.2H_2O$ (10) and $Mg_2SO_4.7H_2O$ (5). Subsequent dilutions were prepared for the batch experiments to simulate WWTP effluent of 200, 100, and 15 mg/L TOC levels. Literature reported TOC level of settled influent determined for 11 WWTPs appeared below 170 mg/L, and the final effluent TOC reported was between 5 and 16 mg/L (Dubber and Gray, 2010). In the present study, the TOC values were determined at an interval of three days by the TOC analyzer, TOC-VCSH, Shimadzu (Standards Methods 2540-C).

The fourth batch was conducted with tap water (TOC = 4.3 mg/L) and real WWTP effluent (TOC = 7.7 mg/L). Grab effluent samples of volume 300 mL were collected at the end of July from a pilot-scale WWTP located in URJC, Móstoles Campus, Madrid, Spain. Tap water was collected from the same location and incubated with MPs without any supplemented carbon sources at 26–30 °C with continuous stirring (60 rpm).

The MP used in the batch experiments was polystyrene (PS) (Goodwills) of between 300 and 550 μm diameter. PS is among the most common MP found in freshwater and marine environments. It has been used in similar studies (Pinto et al., 2019; Parrish and Fahrenfeld, 2019; Eckert et al., 2018).

*Salmonella enterica* (ATTC BAA-190) containing integrase integron gene class 1 (*intl1*) was added in the study to represent MGEs. *Intl1* is used as a marker for MGE, a proxy for ARG contribution by anthropogenic sources. The proximity of microbes in the biofilm matrix may trigger MGEs exchange (Gillings et al., 2015; Eckert et al., 2018).

Three indigenous ARB species were selected out of the 19 ARB isolates (Fig. 1). The selected ARB were *Aeromonas hydrophila* AmpRes.I (MT882264), *Bacillus cereus* SulRes.II (MT882275) and *Pseudomonas aeruginosa* VanRes.SM (MT882279), spiked in the synthetic wastewater matrix batch experiments (Table 1). A higher proportion of ARB species

**Table 1**
MP biofilm batch experiment design and conditions.

| Batch # | Water matrix (replicates) | Initial TOC (mg/L) | Temperature (°C) | MP[a] mass (mg) | Sampling period (days) | Antibiotic mixture (μg/L) | Added bacterial strains |
|---|---|---|---|---|---|---|---|
| 1 | Synthetic wastewater | 15 | 30 | 600 | 0, 3, 7 | 0 | *Salmonella enterica* (ATTC BAA-190), *Aeromonas hydrophila* AmpRes.I |
| | Synthetic wastewater | 15 | 30 | 1200 | 0, 3, 7 | 0 | *Bacillus cereus* SulRes.II, *Pseudomonas aeruginosa* VanRes.SM |
| | Synthetic wastewater | 15 | 30 | 600 | 0, 3, 7 | 80 | |
| 2 | Synthetic wastewater | 100 | 26 | 600 | 0, 3, 7, 9 | 0 | |
| | Synthetic wastewater | 200 | 26 | 600 | 0, 3, 5, 7, 9 | 0 | |
| | Synthetic wastewater | 200 | 30 | 600 | 0, 3, 5, 7, 9 | 0 | |
| 3 | Synthetic wastewater | 500 | 26 | 600 | 0, 3, 5, 7 | 0 | |
| 4 | WWTP effluent | 7.7 | 26 | 600 | 0, 30 | 0 | Non |
| | Tap water | 4.3 | 26 | 600 | 0, 30 | 0 | Non |

[a] PS MP size range = 300–550 μm.

isolated in the present study belonged to three genera, *Aeromonas* (5), *Pseudomonas* (3), and *Bacillus* (2) (Fig. 1). The selection was made on abundance and representation of ARB resistance to three different antibiotics: vancomycin, sulfamethoxazole, and ampicillin.

Combinations of varying conditions were designed for the batch experimental set-up (Table 1). The total incubation period was 7 to 9 days in three batch experiments. The time interval was selected to acquire mature MP biofilm for subsequent analysis. In the fourth batch, conducted with tap water and real WWTP effluent, the incubation period was 30 days. MPs in water bodies and engineered systems might exist for much longer periods, 30 days in treated waters was selected to represent a longer time period. Continuous shaking was maintained throughout the experiments (60 rpm) to maintain the shear stress that exists due to water flow in water channels. The temperature range (26–30 °C) was adopted from a past similar study (Parrish and Fahrenfeld, 2019). The MP concentration (2 mg/mL & 4 mg/mL), and antibiotic mixture of total concentration (0 & 80 μg/L) in equal proportion of vancomycin, ciprofloxacin, sulfamethoxazole, and ampicillin was used. The antibiotics and MP concentrations chosen in the batch experiments do not simulate the reported concentrations in real treated waters. Higher MP masses were used in the experiments to acquire higher MP biofilm volume for ARB and ARGs quantification. While in the natural environment, the MP mass varies greatly across time and location, and could appear between <0.001 to >1000 MPs/L (Li et al., 2018). Similarly, an antibiotic mixture with a total concentration of 2.7 μg/L was reported in river water (Diwan et al., 2018). Similarly, the four antibiotics were selected to represent four different classes of antibiotics namely glycopeptides, fluoroquinolones, sulfonamides, and beta-lactams. Antibiotics belonging to these classes were reported in treated and/or freshwater bodies (Gu et al., 2021; Voigt et al., 2020; Diwan et al., 2018). Preliminary results for ARB resistant to fluoroquinolones group of antibiotics (ciprofloxacin) appeared below detection level in the batches and was therefore excluded from the experiment.

### 2.3. Sample preparation for ARB quantification

Bacterial strains were grown in tryptic soy broth (TSB) supplemented with vancomycin (70 μg/mL), sulfamethoxazole (20 μg/mL) and ampicillin (25 μg/mL) for the respective ARB in initial concentration of log10 6–7 CFU/mL. Samples were collected from the flasks at time intervals of 0, 3, 5, 7, and 9 days. Flasks were gently mixed to attain a uniform distribution of MP across the flask. 50 mL filtrate was filtered through a stainless-steel (63 μm diameters mesh-size) filter. 5 mL of deionized water was run twice through the MP retained on the filter to remove the free bacterial cells.

The filter retaining the MP biofilm was transferred to a 20 mL sterile tube with 5 mL sterile salt solution (0.85 % NaCl), briefly sonicated thrice for 30 s, and vortexed for 5 min. Phenotypic antibiotic resistance was assessed by culture-based methods. The filtrate and MP biofilm samples were plated in triplets on TSA plates supplemented with vancomycin (70 μg/mL), sulfamethoxazole (20 μg/mL), and ampicillin (25 μg/mL). Colonies forming units (CFU) were counted after 24 ± 2 h of incubation at 37 °C.

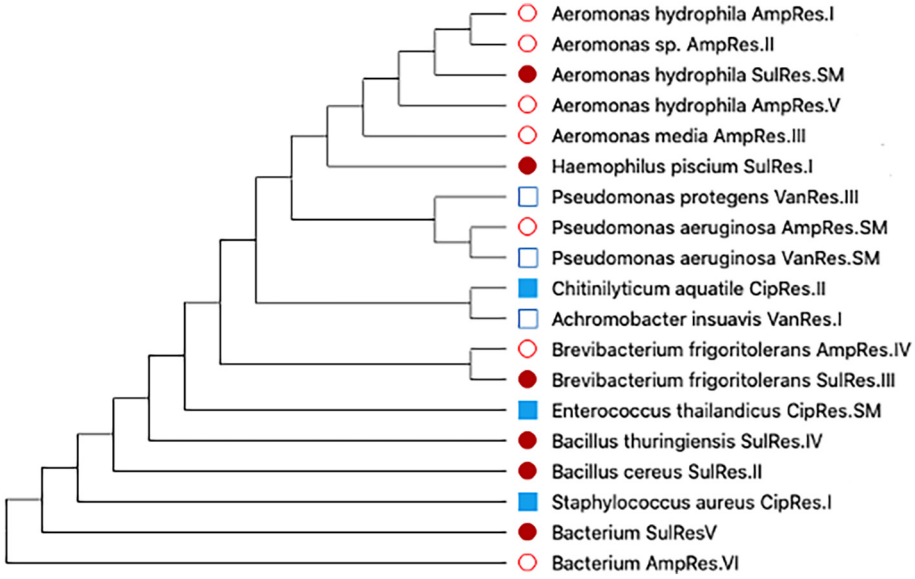


**Fig. 1.** Evolutionary relationships of taxa computed by the Neighbor-Joining method. The optimal tree with the sum of branch length is 3.01. Evolutionary analyses were conducted in MEGA X.

### 2.4. Sequencing and molecular analysis

Partial 16srRNA sequences were carried out for the ARB identification by Sanger sequencing technology (Macrogen, South Korea). Each strain was amplified using universal primers, 785F/907R, and 27F/1492R. 16srDNA sequences of the ARB were acquired from the forward and reverse strain sequences of size range 1350–1400 base pairs (bp).

50 mL filtrate from the batch flask was filtered through a 63 μm dia stainless-steel filter to retain MP and washed thrice with deionized water. The filter was directly introduced into the lysing tube for DNA extraction. The filtrate was filtered through a membrane filter of 0.22 μm (Polycarbonate Track-Etched Filters, Sartorius Germany) for DNA extraction. Total DNA was extracted using a commercial DNA extraction kit (Qiagen Power-Kit).

Universal primers were used for the quantification of 16srRNA, 2 ARGs, and 1 MGE, contributed by the spiked bacterial strains in the batch experiments (Table 1), quantified by real-time quantitative polymerase chain reaction (qPCR). The 3 target genes in this study, *van*A, *sul1*, and *intl1* were reported in drinking water sources in previous studies (Hao et al., 2019; Destiani and Templeton, 2019). For vancomycin-resistant gene detection, the *van*A gene was quantified by qPCR. Primers for *van*A amplification were (5′-AA TGT GCG AAA AAC CTT GC-3′/5′-AAC AAC TAA CGC GGC ACT-3′) (Volkmann et al., 2004). Integron-integrase gene class 1 *int1* was quantified by previously reported primers for *intl1* (5′-GCC TTG ATG TTA CCC GAG AG-3′/5′-GAT CGG TCG AAT GCG TGT-3′) (Goldstein et al., 2001). Primers for *sul1* gene detection were *sul1*-FW (5′-CGCACC GGAAACATCGCTGCAC-3′/5′-TGAAGTTCCGCCGCAAGGCTCG-3′) (Pei et al., 2006). Amplification conditions were optimized following the literature (Volkmann et al., 2004; Goldstein et al., 2001; Pei et al., 2006) (Table A - Supplementary materials) and qPCR was conducted in qPCR system ABI 7500 Fast and ABI 7000 SDS (Applied Biosystems).

### 2.5. FEG-SEM analysis

MP samples were collected from the batch flasks and washed thrice with deionized water for microscopic analysis. The MP samples were prepared by previously reported methods (Pinto et al., 2019). Briefly, the MPs were fixed in 2.5 % glutaraldehyde and stored overnight at 4 °C. Ethanol dehydration were carried out in 30 %, 50 %, 70 %, and 80 % ethanol in PBS solution for 10 min each. Lastly, 3 cycles of 10 min dehydration was carried out in absolute ethanol. The samples were dried and stored in a desiccator. The samples were coated with 6 nm of gold. The images were analyzed under Nova Nano SEM230 (FEG-SEM).

### 2.6. Data analysis

Quality control and bioinformatic analysis were conducted using online platforms and open-source software resources. The raw sequence data were processed, and contigs were acquired in BioEdit version 7.2.5 (Hall, 1999). After classification, accession numbers were acquired from the GenBank portal of the National Center for Biotechnology Information (NCBI). MEGA X software was used for the phylogenetic analysis (Stecher et al., 2020).

The data analysis and statistics were performed using Prism 8 and Microsoft Excel 2010. The mean comparison by ANOVA ($p < 0.05$) and standard deviations of replicates were determined in OriginPro 6.1.

## 3. Results and discussion

### 3.1. Ecology and phylogenetic analysis of ARB in the WWTP effluent

Partial 16srDNA sequences of bacterial species isolated from a pilot-scale WWTP were classified into genus and species. The bacteria were grown on four different types of antibiotics (vancomycin, ciprofloxacin, sulfamethoxazole, and ampicillin) supplemented in TSA agar plates. The classification indicated that most species belong to the genus *Aeromonas*, followed by *Bacillus* and *Pseudomonas*. Around 65 % of the total species were Gram-negative. The species identities are available under the accession numbers MT882263–MT882280 (NCBI) (Table B - Supplementary materials).

The phylogenetic study was conducted to analyze the evolutionary relationship among the classes of isolated ARB in WWTP effluent.

Most of the ampicillin-resistant bacterial species indicated a close evolutionary relationship among themselves, affiliated with the same genus. Ampicillin-resistant species also indicated a shorter evolutionary distance relationship with the sulfamethoxazole and vancomycin-resistant bacterial species (Fig. 1). Moreover, among the isolated ARB, few ARB belonging to the same genus indicated resistance to more than one antibiotic. *Aeromonas* and *Pseudomonas* genus showed multiple antibiotic resistance.

The abundance of *Aeromonas* among ARB was in agreement with previous studies. *Aeromonadaceae* had been indicated as a possible marker of ARB and ARG pollution in an aquatic environment due to its association with high ARG numbers (Stalder et al., 2019). In our study, *Aeromonas* species were resistant to sulfamethoxazole and ampicillin. Moreover, over 26 % of the ARB belonged to the genus *Aeromonas*.

Biofilm formed by Gram-positive bacteria is considered more resistant to antimicrobial agents (Oliva et al., 2021). *Staphylococcus aureus* was found among the antimicrobial-resistant species in biofilm as compared to planktonic bacteria (Olson et al., 2002). Among the isolates, 35 % were Gram-positive bacteria. Likewise, *Staphylococcus aureus* indicated resistance to ciprofloxacin at the concentration of 0.25 μg/mL.

The second most abundant species, *Pseudomonas* was resistant to vancomycin and ampicillin. *Pseudomonas aeruginosa*, carbapenem-resistant is listed among the priority-1 pathogenic ARB (Critical). Furthermore, *Staphylococcus aureus*, methicillin-resistant, vancomycin-intermediate, and resistant are among priority-2 (High) (WHO, 2017). WWTP effluent majorly flows into freshwater sources, is reused for multiple purposes, or even utilized for drinking purposes after disinfection. The prevalence of these species in WWTP effluent raises a potential public health concern.

### 3.2. Factors affecting the ARB colonization on MP surface

#### 3.2.1. Time and TOC level

The colonization of three ARB: *Aeromonas hydrophila* AmpRes, *Bacillus cereus* SulRes, and *Pseudomonas aeruginosa* VanRes were quantified in MP biofilm incubated in synthetic wastewater of low and higher TOC levels, as a function of time, simulating WWTPs influent and effluent.

In the synthetic wastewater of TOC 15 mg/L, all three ARB increased in the initial three days, both in the surrounding water and in the MP biofilm (Fig. 2a, b). The growth and survival of the ARB in the surrounding water may be attributed to the available organic supplements and enabling growth environment. The TOC decreased from 15 mg/L to 5.9 mg/L on day three. It further decreased to 3 mg/L on day 7 (Table C - Supplementary materials). The decreasing TOC level might have impacted the viable ARB survival in the surrounding water and the MP biofilm. In this study, there was a decrease in the viable cell numbers with a decrease in the TOC level. Moreover, the increase by day 3 and the decrease by day 7 were observed both in the MP biofilm and the surrounding water. It suggested a similar impact on viable ARB numbers across time with decreasing TOC levels in low-strength synthetic wastewater.

The depleted TOC with time in the water might create competition over available nutrients for bacterial growth and proliferation. But the ARB indicated persistence in the low nutrient environment on day 7. Although the growth showed a decrease after day 3, the number of dead cells, especially in the MP biofilm, may remain high and contribute to the number of genetic materials including ARGs. During the growth period of day 3, the percent distribution analysis showed that the MP surface (600 mg) was constituted by 53 % VanRes, 8 % SulRes, and 3 % AmpRes ARB among the total ARB in the flask. The majority of viable ARB were present in the surrounding water (300 mL) throughout the study period of 7 days (Fig. 3a, b, c). The results suggested that although MP particles harbored ARB in water bodies, a high proportion of the total bacterial cells existed in the water matrix. The distribution might be explained since the number of MP particles per

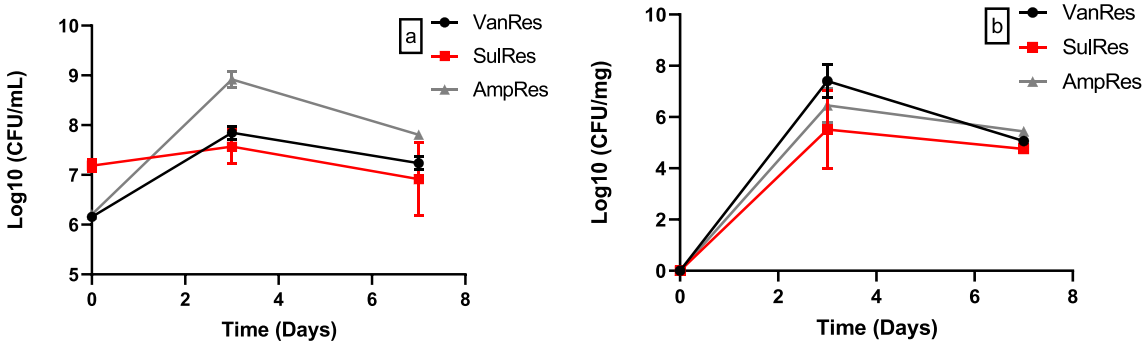


**Fig. 2.** Viable ARB across time in the synthetic wastewater of initial TOC of 15 mg/L; a) in the surrounding water and, b) in the MP biofilm.

water volume is expected to be low. These results do not discard the potential of MP particles in accumulating ARB in water bodies because of the potential protection provided by MP biofilm to the embedded bacterial cells. While the surrounding water would remain the major target of disinfection techniques for the removal of ARB, the removal of MP particles would result in complete removal of ARB in water.

To verify the evolution of biofilm formation on the MP surface, the MPs were analyzed under SEM. On day 3, a smooth surface with visible depositions, assumed to be extracellular substances (EPS), and embedded cells in the matrix were observed. By day 7, the surface was characterized by rough deposits (Fig. 4). Previous studies indicated that the biofilm was strong enough by 8–9 h, and after 24 h the biofilm mass started stabilizing, and dead cells were among the biofilm community (Govaert et al., 2018; Parrish and Fahrenfeld, 2019). While a comparison with previous studies on surface biofilm formation would be unrealistic because previous studies on biofilm development were conducted in culture media rich in available nutrients for bacterial growth (Govaert et al., 2018). Moreover, there are differences in experimental conditions, bacterial strains, and research methods. In the present study, the visual observation suggested bacterial attachment and microcolony formation on day 3 (Fig. 4b). It further indicated biofilm maturation and detachment on day 7 (Fig. 4c). Therefore, the MP biofilm at a 15 mg/L TOC level might contribute to the dispersion of ARB at maturation. The dispersed ARB might further colonize newly available areas. The results suggested an active biofilm formation process on MP biofilm surface involving ARB attachment and detachment in the low strength water matrix.

Similarly, in the synthetic wastewater of TOC 200 mg/L, no significant increase in ARB colony numbers in the surrounding water was found by day 3. ARB accumulation on MP surface was significantly high on day 3. The ARB CFUs indicated variability across time, VanRes slightly decreased on day 5. Moreover, the variability was observed only in the surrounding water. The ARB growth in the MP biofilm followed a similar increasing trend in the initial period and a decrease afterward (Fig. A - Supplementary materials). The percent distribution in the 300 mL flask indicated 14 % VanRes, 10 % SulRes, and 9 % AmpRes ARB on day 3. Day 3 and 5 indicated higher ARB survival in the MP biofilm in TOC 200 mg/L (Fig. B - Supplementary materials).

In the synthetic wastewater of TOC 100 mg/L, simulating a settled influent, all three ARB indicated around 1 log increase per mL filtrate and 7 log increase per mg MP biofilm in the initial period of 3 days (Fig. C - Supplementary materials). ARB distribution between total MP biofilm and the surrounding water indicated higher ARB survival in the MP biofilm on day 3 (Fig. D - Supplementary materials). The highest value of 500 mg/L was included in the present study to evaluate the survival of ARB at high TOC levels. The ARB indicated an initial increasing growth trend; the trend indicated variability afterward (Fig. E - Supplementary materials). Previous studies indicated that for a few microbial species, a nutrient-rich medium is suitable (Govaert et al., 2018) while other studies indicated the suitability of a nutrient-poor medium in biofilm development (Kadam et al., 2013). The distribution of total ARB in the flasks between the surrounding water and MP biofilm indicated higher survival of ARB in MP biofilm on day 3 and day 5 (Fig. F - Supplementary materials).

All over, by day 3, an average increase in ARB was between 0.1 and 2 log value per mL of water and 7 to 9 log per mg of MP in various strengths of synthetic wastewater. In literature, *Pseudomonas* sp. was reported to form biofilm along with *Bacillus* sp. (Tribedi et al., 2015; Ricci et al., 2019). There was a significant difference in viable ARB by day 3 in the MP biofilm in the synthetic wastewater of 15, 100, 200, and 500 mg/L TOC, indicating the bacterial growth preference for MP surfaces. ARB were able to form biofilm on the available MP surface and persisted in the surrounding water. The initial increase and further delayed growth in the surrounding water corresponded with that in the MP biofilm.

In previous studies, the exposure time of MP in seawater indicated a significant impact on the MP biofilms microbial diversity. The total MP biofilm amount increased as a function of exposure time in few studies (Xu et al., 2019; Tu et al., 2020). Two contradictory groups of finding exist on the microbial ecology of plastic biofilm vs. the surrounding water. Few studies indicated that plastic has a specific assembly. Hence, the term plastisphere was used for plastic-specific microbes, different from those in the ambient water (Zettler et al., 2013). In contrast, a study indicated that

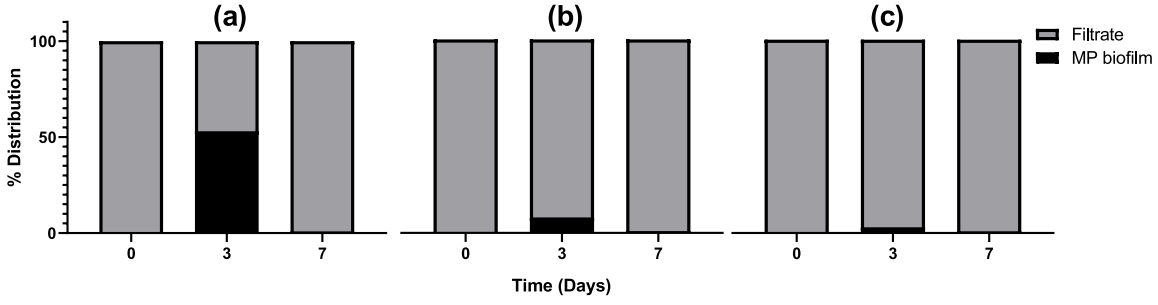


**Fig. 3.** Total viable ARB distribution between the surrounding water (300 mL) and the MP (600 mg) biofilm in the synthetic wastewater of initial TOC of 15 mg/L in: (a) VanRes ARB, (b) SulRes ARB, (c) AmpRes ARB.

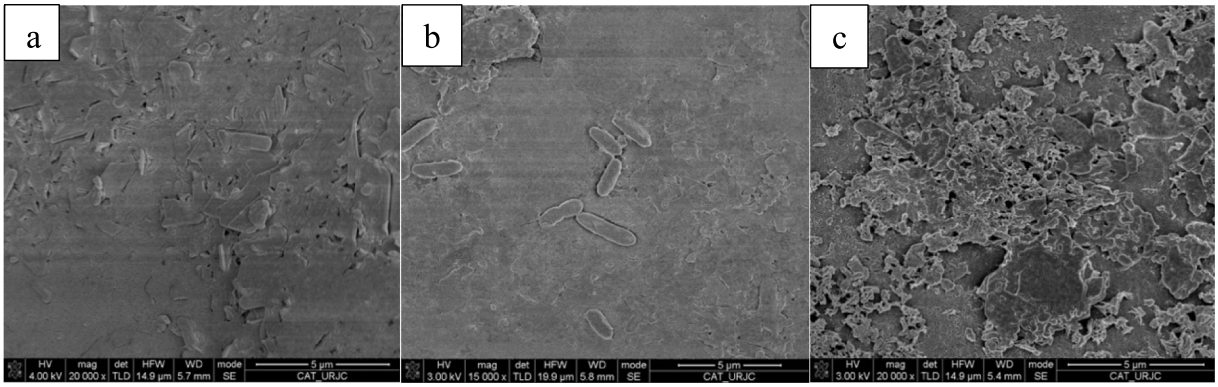


**Fig. 4.** (a) Virgin MP surface. (b) MP after a period of 3 days, incubated with selected ARB. (c) MP after an incubation period of 7 days (15 mg/L TOC).

no plastic-specific bacterial community exists (Pinto et al., 2019). Other studies found consistency in the dominant bacterial classes in the MP biofilm and surrounding water (Xu et al., 2019; Eckert et al., 2018). In the case of specific assembly, the MP surface growth preference for ARB species will pose a risk of ARB transfer to drinking water sources.

*3.2.2. Effluent temperature, MP mass, and antibiotics concentration*

Microbial communities in MP biofilm are context-dependent, and the environmental factors shape the heterotrophic activities of MP biofilm (Arias-Andres et al., 2018a,b; Pinto et al., 2019). At a temperature difference of 4 °C, most ARB at both temperature groups of 26 °C and 30 °C in synthetic wastewater of 200 mg/L TOC, was not found to be significantly different, while only AmpRes ARB indicated significant difference both in the surrounding water and biofilm on day 3 (Fig. G - Supplementary materials).

Minor temperature differences may not impact the ARB number in MP biofilm during shorter storage periods. Similar observations have been made in previous studies. At a temperature of 25 °C and 30 °C, no significant difference was observed between the average optical density (OD) value of *Listeria monocytogenes* species in biofilm (Govaert et al., 2018), while the study was conducted only for a single day on a single bacterial species.

In a batch with double the MP mass as the control flask, the mean variance remained insignificant based on viable cell counts. The total ARB in 1200 mg MP biofilm was not significantly higher than the total ARB in 600 mg (Fig. 5a, b). In another study, bacterial cell numbers and morphologies did not change significantly when increasing MP concentration either (Donlan, 2002).

Antibiotic mixture (20 μg/L of vancomycin, ciprofloxacin, sulfamethoxazole, and ampicillin each) did not induce a significant difference in ARB numbers of planktonic cells in the surrounding water (Fig. 6a). While antibiotics supplemented flasks with 80 μg/L antibiotics showed enhanced growth of VanRes and SulRes ARB on day 3. Contradictory to the observations for ARB in water, in the MP biofilm, lower ARB numbers were acquired, ARB being significantly lower on day 7 (Fig. 6b). Antibiotics adsorption on MP biofilm might impact the ARB numbers. At this moment, the present study is not sufficient to predict the mechanism behind the lower ARB on day 7 under the present experimental conditions.

In contradiction, previous studies found that bacterial growth on surfaces is slow, while they are less sensitive to the effect of antibiotics and physical conditions. For some antibiotics, treatment of the same bacteria in the attached and free state indicated that a thousand times more antibiotics were required to kill the sessile bacteria (Costerton et al., 1995). It is speculated that antibiotics are resisted by microbes via gene mutation or EPS secretion in excess under antibiotic stress.

Other studies found that erythromycin in low concentration ( ≤ 1 mg/L) was resisted by more EPS formation in the biofilm (M. Zhang et al., 2019; X. Zhang et al., 2019; Guo et al., 2015). These studies indicated biofilm as an antibiotic barrier, but the conclusions may not apply to the present study. In this study, the MP biofilm was formed in the presence of very low antibiotic concentration, exposed for a longer period of 7 days. Lower antibiotic concentration and longer exposure time might contribute to the death of ARB in the MP biofilm, resulting in significantly lower ARB on day 7 in the present study.

*3.3. Factors affecting the ARGs accumulation on MP surface*

The incubation period, TOC level, antibiotic pressure, and MP mass were hypothesized to impact the relative abundance of ARGs in water. In the present study, majority ARGs (*sul1*, *van*A) and MGE (*intl1*) were detected in the MP biofilm (Figs. 7, 8), whereas the relative quantities of ARGs remained below detection level in the synthetic wastewater of 100 mg/L and 200 mg/L TOC. ARGs in the surrounding water are only reported for day 3 in synthetic wastewater of 15 mg/L TOC (Fig. H, Table D - Supplementary materials). The distribution of viable ARB numbers was

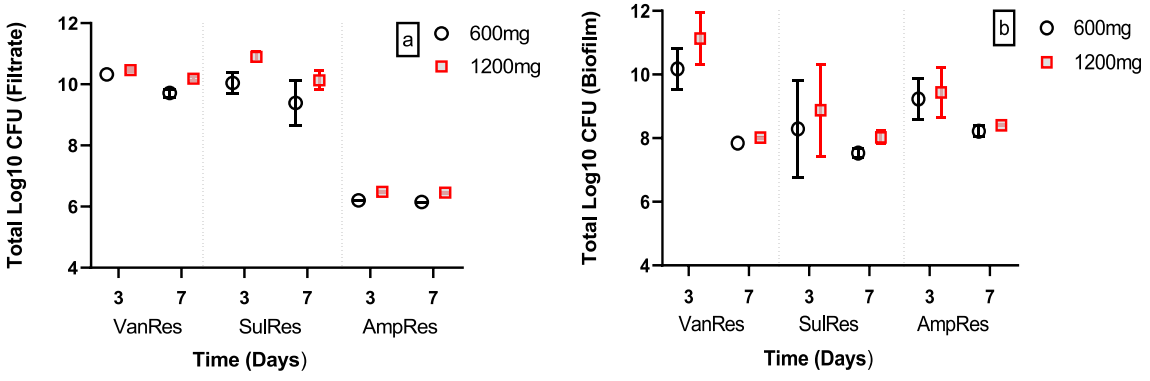


**Fig. 5.** Viable ARB in synthetic wastewater of 15 mg/L TOC at different MP levels; a) in the surrounding water and, b) in the MP biofilm.

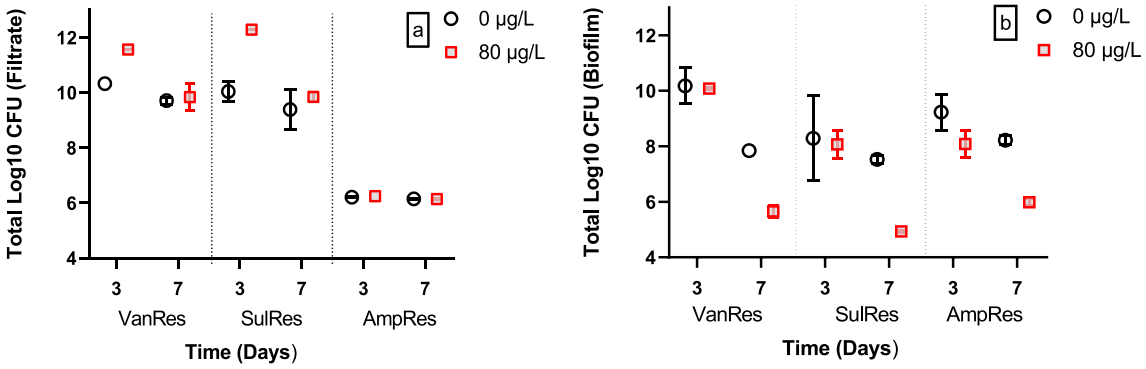


**Fig. 6.** Viable ARB in synthetic wastewater of 15 mg/L TOC with and without antibiotics; a) in the surrounding water and, b) in the MP biofilm.

higher in the surrounding water (Figs. 3, B, D, F - Supplementary materials) while the relative abundances of recovered ARGs and *intl1* are higher in the MP biofilm. The closely packed EPS embedded with a high density of dead cell deposits might contribute to higher relative gene copies in the MP biofilm. Moreover, no direct comparison between surrounding waters and MP biofilm can be established due to different quantification units. Higher DNA recovery is expected from MP biofilm due to dense volume. The surrounding water volume might not have been enough to recover higher DNA concentrations resulting in below detection level ARG copies.

### 3.3.1. Time and TOC level

ARGs and *intl1* were analyzed in the 3 synthetic wastewater matrices (15, 100, 200 mg/L TOC) on day 3 and day 7 or 9 (Fig. 7). In the MP biofilm in 15 mg/L TOC synthetic wastewater, the relative abundance of *sul1* and *intl1* decreased significantly between days 3 and 7. Likewise, in the 100 and 200 mg/L TOC synthetic wastewater, the relative abundances of *sul1* and *intl1* in MP biofilm increased significantly between days 3 and 9 (Fig. 7). There was an exception in the case of *van*A, it was detected only in the MP biofilm in 100 mg/L TOC water, and it decreased from $1.3 \times 10^{-4}$ on day 3 to $8.9 \times 10^{-6}$ on day 9. These results indicated that time had a negative correlation with the relative abundance of *sul1* and *intl1* in MP biofilm of low TOC water matrix (15 mg/L) while positive correlation in high TOC water matrix (100 and 200 mg/L). The *van*A ARG was below detection level in MP biofilm of 15 and 200 mg/L TOC and had the lowest relative abundance in 100 mg/L TOC synthetic wastewater.

A comparison among the 3 TOC levels showed a highest relative abundance of *sul1* and *intl1* in the MP biofilm of 15 mg/L TOC synthetic wastewater. The total biological mass and absolute ARG numbers might remain higher in the high TOC water matrix but the relative abundance of *intl1*, *sul1*, and *van*A were found to be low.

### 3.3.2. Antibiotics and MP mass concentration

The batch experiment spiked with an antibiotic mixture (80 µg/L) in 15 mg/L TOC synthetic wastewater was compared with a control without antibiotics. Both flasks contained 600 mg/L MPs (Fig. 8a, b). In another batch of 15 mg/L TOC synthetic wastewater, 600 mg/L MPs containing the control batch were compared with 1200 mg/L MP containing flask (Fig. 8a, c). The impact of antibiotic pressure and MP mass on the relative abundance of ARGs and *intl1* was evaluated in the study. Under antibiotics pressure, *intl1* was not detected on days 3 and 7 (Fig. 8b) in comparison to the control (Fig. 8a).

The relative concentration of *sul1* significantly decreased under antibiotic pressure to $5.4 \times 10^{-3}$ and $9.2 \times 10^{-3}$ on days 3 and 7, respectively. While *van*A abundance increased on days 3 and 7 (Fig. 8b). Antibiotic pressure might impact different ARGs differently. S. Wang et al. (2020) and Z. Wang et al. (2020) showed that the adsorbed antibiotics on MPs have a significant impact on ARGs' distribution on MP surface, leading to an increased ARGs number under 5 µg/L antibiotic pressure during 30 days. While Cheng et al. (2022) reported that the background antibiotics in municipal wastewater significantly inhibited intracellular ARGs and not the extracellular ARGs in the MP biofilm.

An increase in MP mass indicated an increase in the relative abundance of *van*A on days 3 and 7 (Fig. 8c). While *van*A gene was below the detection level in the control sample (Fig. 8a). MP mass might significantly impact the ARGs abundance in MP biofilm by providing more surface for ARGs accumulation. But in the present study, although *intl1* was inhibited, other competing genes might have concentrated on the MPs with additional MP mass. The low *intl1* abundance in MP biofilm is in contradiction with a study conducted by Eckert et al. (2018). They found that *intl1* increased in

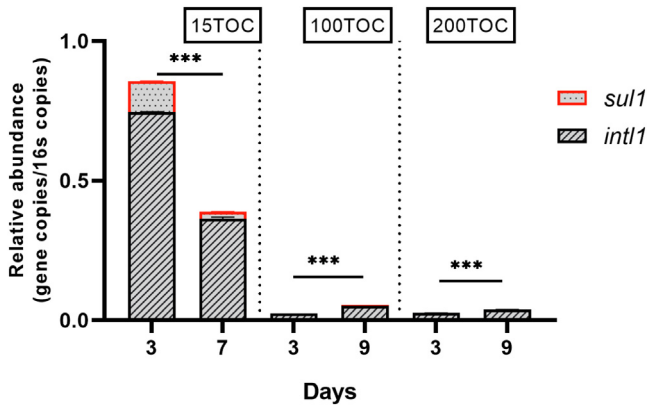


**Fig. 7.** Relative abundance of ARGs and *intl1* in MP biofilm in synthetic wastewater of 15, 100, and 200 mg/L TOC across time.

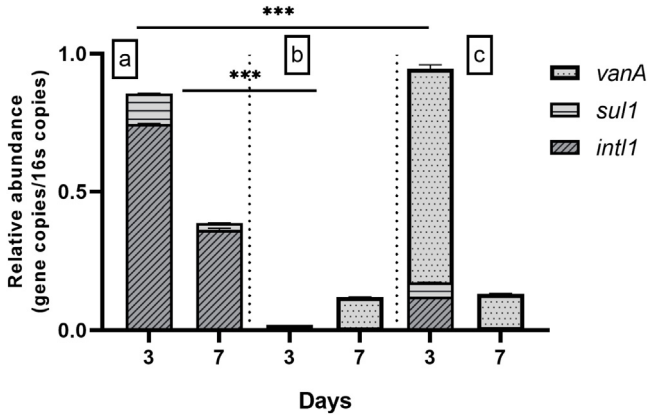


**Fig. 8.** Relative abundance of ARGs in MP biofilm in 15 mg/L TOC synthetic wastewater. a) Control, 0 µg/L antibiotics and 600 mg/L MPs, b) 80 µg/L antibiotic mixture supplemented with 600 mg/L MPs and, c) 0 µg/L antibiotics and 1200 mg/L MPs.

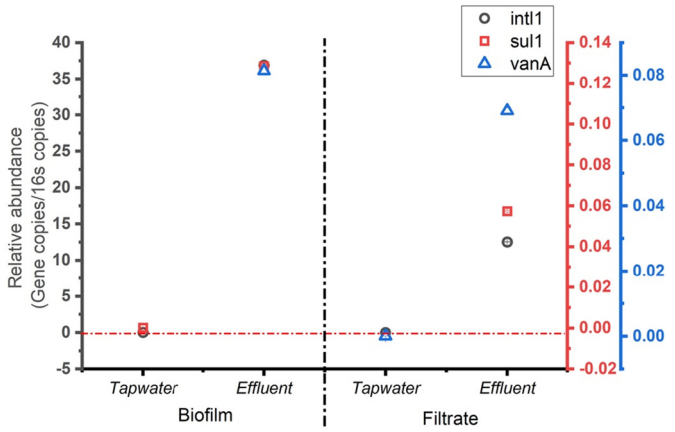


**Fig. 9.** Relative abundance of ARGs and *intl1* in the tap water (TOC 4.3 mg/L) and WWTP effluent (TOC 7.7 mg/L) on day 30.

abundance in MP biofilm with the increasing MP particle number, but not in the surrounding water.

In the synthetic wastewater of 15 mg/L TOC, the ARGs and *intl1* were detected only on day 3 in the control (600 mg MP and without antibiotic mixture), batch containing antibiotic mixture, and without antibiotics and double MPs mass containing batches (Fig. H - Supplementary materials). The ARGs and *intl1* were below detection level in the surrounding synthetic wastewater on day 7. A comparison of the relative abundances indicated low relative abundance for all 3 genes in the control and under antibiotic pressure. While higher relative abundances of *intl1* and *sul1* were found in synthetic wastewater containing higher MP mass (Fig. H - Supplementary materials). Interestingly, despite keeping the filtrate volume the same in all batches, the higher relative abundance of ARGs with higher MP mass indicated MP contribution to increasing ARGs in the surrounding water. Zhao et al. (2021) reported that MPs and the antibiotics in water enriched ARGs in the MP biofilm. The results acquired are summarized in Table D - Supplementary materials.

### 3.4. ARB, ARGs, and intl1 in MP biofilm in WWTP effluent and tap water

Lower bacterial cell numbers were expected and found in the WWTP effluent and tap water on the day of sample collection. The CFUs were reported per liter of filtrate and per mg of MP. The viable bacterial numbers decreased in the WWTP effluent and tap water filtrate by day 30 (Fig. I - Supplementary materials). This might be attributed to an assumed excessive nutrient depletion due to a longer storage period. In contrast, bacterial cells survived in the MP biofilm incubated in tap water and WWTP effluent. The few bacterial cells in poor nutrient conditions might prefer and survive on attached surfaces, for instance, on the MP surface.

The relative abundance of two ARGs, namely *sul1* and *van*A, and one MGE, namely *intl1* are shown in Fig. 9a and b, respectively. A higher accumulation of the ARGs and MGE in the MP biofilm and the surrounding effluent filtrate is observed. Among the three genes, the relative abundance of *intl1* in the effluent was the highest as compared to *sul1* and *van*A. The high relative abundance of all three genes in the MP biofilm of WWTP effluent indicated the ARGs accumulation potential on MP surfaces in treated water. While the relative abundance of ARGs and *intl1* was significantly lower than that of WWTP effluent in the MP biofilm and surrounding tap water (Fig. 9a, b). Prolong storage period of 30 days might not provide favorable conditions for the propagation of biological elements in tap water.

The MP biofilm FEG-SEM images indicated a layer of biofilm growth with visible bacterial cells embedded in the biofilm matrix of WWTP effluent by day 30. Likewise, the bacterial cells were also visible on the MP surface incubated in tap water (Fig. 10). The biofilm developed, irrespective of the initial number of colonizing bacterial cells and organic contents in the treated water.

In a study, MP incubated in river water and wastewater indicated that MP biofilm microbial community was shaped by the source water, rather than by the MP morphology. Location and the season of the year impacted the MP biofilm community (Oberbeckmann et al., 2016; Parrish and Fahrenfeld, 2019).

The present study showed that MPs in treated effluent and tap water become a potential reservoir of ARGs and *intl1* during a prolonged storage period. In literature, plastic materials in the marine environment were considered a reservoir for ARGs, organic pollutants, and metal-resistant genes (Rochman et al., 2013; Yang et al., 2019). Furthermore, Di Cesare et al. (2016) reported that WWTPs are a source of *intl1* in the effluent receiving water bodies.

The plastic degraded to MP and nano-plastics might continue to accumulate ARB and ARGs. As shown in a study conducted by Parrish and Fahrenfeld (2019), the size of the microplastic for a given type in the same source water did not indicate a significant impact on the microbial structure of the MP biofilm. Moreover, ARB colonization is not hindered by the initial number of live bacterial cells, even a small number of bacteria can initiate biofilm formation (Manaia, 2017).

ARB and ARGs have previously been found at the consumers' end and in the water distribution systems. Khan et al. (2016) reported a diverse ARB community in the tap water of Glasgow, Scotland. In their study, *intl1* was detected in eight bacteria (9.2 %), and the coexistence of *intl1* and ARGs in two ARB species. In drinking water resources, multidrug-resistant *Escherichia*, *Shigella*, *Enterobacter*, *Salmonella*, *Klebsiella*, and integrons were also reported by Kumar et al. (2013).

Furthermore, Shi et al., 2013 reported 1.4 % integrons among 148 isolates in drinking water and indicated the enrichment of ARGs by

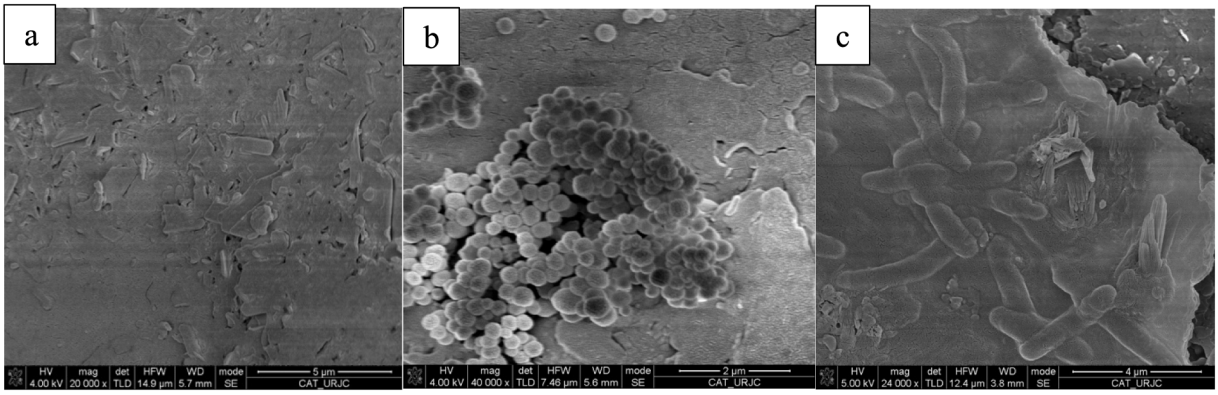


**Fig. 10.** (a) Virgin MP surface. (b) MP biofilm on day 30 in wastewater treatment plant effluent. (c) MP biofilm on day 30 in tap water.

chlorination. Therefore, the biofilm formed in the water distribution systems is expected to contain ARB, ARGs, and transferable markers. Even disinfectant residual of concentration up to 0.5 mg/L (both $Cl_2$ and $NH_2Cl$) in drinking water was found unable to disinfect biofilm (Wang et al., 2020). The biofilm could enhance the survival and spread of antibiotic resistance due to the proximity of bacterial cells, the presence of *intl1*, and the barrier from external stressors.

## 4. Conclusion

ARB resistant to common antibiotics were phylogenetically related and dominated by closely related genus in WWTP effluent. The ARB in WWTP effluent were predominately biofilm-forming bacterial species.

Decreasing TOC level and exposure period impacted the number of viable ARB in MP biofilm. Days 3 and 5 contained a higher percentage of live ARB in the flasks, in comparison to days 7 and 9. But minor temperature difference, MP mass difference, and antibiotic pressure had varying impacts on the ARB community and abundance.

MP was able to accumulate ARB, ARGs, and *intl1* in WWTP effluent and tap water in a nutrient-deprived environment. WWTP effluent disinfection methods should take into consideration the risk of antibiotic resistance spread through low concentration ARB, MPs, and ARGs that survive disinfection. Together the contaminants might persist in treated water.

To better understand the role of MP biofilm as an antibiotic resistance carrier in potable water, it is further recommended to quantify and characterize the biofilm of many different types of common MP in their natural concentration ranges. Furthermore, it is also further recommended to consider the contribution of MP biofilm in disinfection studies to avoid the overestimation of disinfection efficiency usually determined based on free microbial cells removal.

## CRediT authorship contribution statement

**Shabila Perveen:** Investigation, Methodology, Data curation, Writing – original draft. **Cristina Pablos:** Conceptualization, Supervision, Resources, Writing – review & editing. **Ken Reynolds:** Validation, Supervision, Writing – review & editing. **Simon Stanley:** Validation, Supervision, Writing – review & editing. **Javier Marugán:** Supervision, Validation, Resources, Writing – review & editing.

## Data availability

Data will be made available on request.

## Declaration of competing interest

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

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2022.159024.

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