

ASSESSMENT OF MICROPLASTIC POLLUTION AND ITS POTENTIAL ROLE
IN THE ENVIRONMENTAL DISSEMINATION OF ANTIBIOTIC RESISTANCE
GENES IN WASTEWATER

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Abstract

The Wastewater Treatment Plant (WWTP) is an important location where anthropogenic contaminants, microplastics (MPs) and antibiotic resistance genes (ARGs) meet, where microplastics, as vector surfaces, provide bacterial colonization and horizontal gene transfer of resistance genes. This research has assessed microplastic counts, morphologies and statistical relationship with important ARGs (tetA, sul1, blaTEM) as well as the Class 1 integron integrase gene (intI1) in various wastewater treatment stages. A data set of 100 samples (n=50 Influent, n=50 Effluent) were analysed by non-parametric comparison tests, Spearman rank correlation and multivariable ordinary least squares regression models. The mean MP concentration in the effluent showed significant reduction from the influent water from 234.43±82.57 particles/L to 50.58±26.28 particles/L ($p<0.001$) representing a removal efficiency of 78.42%. Total ARG abundance decreased from 620,879.90±822,954.64 to 25,267.86±14,997.97 copies/mL ($p<0.001$, 95.93% reduction). Spearman analysis revealed strong positive correlations between MP abundance and total ARGs ($\rho=0.791$, $p<0.001$), sul1 ($\rho=0.802$, $p<0.001$), intI1 ($\rho=0.762$, $p<0.001$), tetA

($\rho=0.753$, $p<0.001$), and *bla*TEM ($\rho=0.751$, $p<0.001$). A moderate positive correlation between abundance of MP and total ARGs ($\rho=0.474$, $p<0.001$) was only found within influent samples. Log-transformed MP abundance ($\beta=0.468$, $p=0.013$) and treatment stage ($\beta=0.955$, $p<0.001$) were the two independent variables that were found to predict total ARG load ($R^2=0.813$), with multivariable OLS regression. Conventional WWT processes significantly decrease absolute MP and ARG loads, but existing effluent discharge still carry persistent MP carriers containing class 1 integrons and ARG into receiving aquatic environments, thus the necessity of developing and implementing new and improved technologies that are capable of simultaneous removal of particulate MPs and genetic pollutants.

INTRODUCTION

Antimicrobial resistance (AMR) is one of the biggest threats to public health in the twenty first century, highlighted as a priority in the WHO list of top 10 global health threats. One of the most important pathways is the dissemination of ARGs in anthropogenic waste streams to natural environments. Wastewater Treatment Plants (WWTPs) are primary point sources, where clinical, domestic, and industrial waste streams come together, resulting in environments with high bacterial cell density, high residual amounts of residual antibiotics, and ideal conditions for horizontal gene transfer. Within WWTPs, there is a complex microbial community that enables the transfer of mobile genetic elements (MGEs) such as plasmids, transposons and integrons that can spread ARGs between different bacterial taxa. Paradoxically, WWTPs can serve as reservoirs and dispersal sites for ARGs that are released through treated effluents and which contain significant quantities of ARGs and the bacteria that confer antibiotic resistance to them in receiving water bodies [1], [2]. Microplastics are a new environmental contaminant, and the challenge of disseminating AMR includes this new dimension. Microplastics are ubiquitous in municipal wastewater and are derived from synthetic textiles, the fragmentation of single-use plastics, personal care products and industrial processes, specifically from synthetic polymer particles less than 5 mm in size. These particles have special physicochemical properties that make them different from natural

substrates in aquatic environments. Microplastic surface hydrophobicity promotes the conditioning and biofilm development on plastics, resulting in the creation of the "plastisphere" a unique microbial entity that can be found on plastic surfaces. The higher density of bacteria colonizing the plastisphere than natural particles leads to higher frequencies of bacteria-to-bacteria contact, favoring the conjugation and other forms of horizontal gene transfer of microorganisms within the micro environments of the plastisphere [3]. The differential degradation of synthetic polymers allows the transport of some microbes resistant to degradation and resistance genes to survive in receiving aquatic systems for long distances away from the WWTP effluent discharge [4].

ARG enrichment on mobile genetic elements (MGEs) on microplastic biofilms is a notable and significant way of ARG dissemination. Integrons, and especially class 1 integrons (*int1*), are crucial in the acquisition and delivery of antibiotic resistance (AR) gene cassettes, they are markers of anthropogenic pollution and they are important intermediaries of horizontal gene transfer. In plastic biofilms high local cell density and conjugation and transformation are enhanced and integrons are involved in the integration of resistance determinants in the bacterial genome. This is further complicated by co-selection processes, whereby metals and other contaminants, which are attached on the surface of the microplastic, are able to exert a selective pressure

that maintains resistance determinants even in the absence of antibiotics. Resistance-enriched biofilms formed by synthetic polymers tend to be long-lasting, and thus, these resistance-enriched biofilms can spread significant distances from the point of origin and may transmit ARGs to pristine environments away from humans [5]. Although the vectoring potential of microplastics for ARGs is becoming increasingly known, there is still a huge gap in the quantitative empirical models that describe the connection between the abundance of microplastics and the dynamics of ARGs in wastewater systems. Previous studies mainly investigated microplastic characterization or quantification of ARG, but there has been a limited number of studies simultaneously examining both classes of contaminants at different treatment stages [6].

Empirical statistical models that separate the effects of treatment stages from the intrinsic co-occurrence of MP-ARG is especially critical as this separation is essential to understand if microplastics are actively promoting ARG dissemination, or if they are also occurring naturally as the same parallel contaminant treated by similar physical processes. In addition, the few studies that have investigated the three classes of clinically important antibiotic resistance targets (sul1, tetA, and blaTEM) and also the class 1 integron integrase marker (intI1) are not combined in a single study. This study was thus designed to quantify the microplastic abundance, morphotype proportions and polymer profiles at different stages of the effluent and influent, and to create statistical models that correlate microplastic levels with the dynamics of ARGs and intI1 [7].

Materials and Methods

2.1 Study Design and Sampling Framework

A cross-sectional sampling design was used and stratified sampling was carried out at 50 municipal wastewater treatment plants. 100 samples were taken, 50 of influent (raw sewage after grit removal) and 50 of final treated effluent. Microplastic sampling was conducted for a year to reflect the seasonal variation of wastewater characteristics

and microplastic load. The samples were all collected in precleaned glass bottles, brought to the laboratory on ice and processed within 24 hours after sampling.

2.2 Physicochemical Measurements

In situ measurements were made at the time of sampling. A calibrated glass electrode potentiometer (Hach HQ40d, Hach Company, Loveland, CO, USA) was used to measure pH. The turbidity was measured in Nephelometric Turbidity Units (NTU) with the use of an optical nephelometer (Hach 2100Q, Hach Company, Loveland, CO, USA). Equipment was calibrated each sampling event in accordance with manufacturers' instructions.

2.3 Microplastic Extraction and Characterization

The extraction and characterization of microplastics was carried out according to standard procedures, adapted for wastewater samples. To oxidize organic material, but not affect the synthetic polymer, 30% hydrogen peroxide (H_2O_2) was used, with an iron(II) catalyst (Fenton's reagent) to digest the organic matter. After digestion, the density separation was done with saturated sodium chloride ($NaCl$, 1.2 g/cm^3) to float the low-density polymers, and sodium iodide (NaI , 1.6 g/cm^3) was used where necessary to float the high-density polymers. Vacuum filtration was used to filter supernatants through $0.45\text{ }\mu\text{m}$ membrane filters (cellulose nitrate, Whatman, UK).

The specimens were enumerated and morphotyped using stereomicroscopy (Leica MZ6, Leica Microsystems, Germany) at magnifications of 40-100 $\times$. Microplastics were divided into 4 separate morphotypes: Fragments (irregular angular particles), Fibers (elongated filamentous particles), Films (thin flexible sheets), and Beads (spherical or near-spherical particles). The median particle size (mm) of each sample was obtained by particle sizing with the aid of image analysis software (NIH image, USA).

The polymer types were identified by Micro-Fourier Transform Infrared Spectroscopy (μFTIR ,

PerkinElmer Spotlight 400, USA) or Micro-Raman Spectroscopy (Renishaw inVia, UK) and the most prevalent polymer types were found to be Polyethylene (PE), Polypropylene (PP), Polystyrene (PS), Polyvinyl Chloride (PVC), and Polyethylene Terephthalate (PET). The matching was done with reference library using a match criterion of at least 70% for positive match.

2.4 DNA Extraction and Quantitative Molecular Analysis

Samples of water and retentate filters were collected and total metagenomic DNA extracted using commercial soil/water DNA isolation kits (Power Water DNA Isolation Kit, Qiagen, Germany) according to lysis procedures outlined by the manufacturer. The concentration and purity of the DNA were determined by spectrophotometry (Nano Drop 2000, Thermo Scientific, USA), and samples with A260/A280 ratios between 1.8 and 2.0 were kept for further processing.

Absolute quantifications of target resistance genes (tetA, sul1 and blaTEM), the class 1 integron integrase gene (intI1) and the bacterial 16S rRNA gene were performed using quantitative PCR (qPCR). Each qPCR reaction was carried out three times on a CFX96 RealTime PCR Detection System (Bio-Rad, USA) using SYBR Green detection chemistry (SsoFast EvaGreen Supermix, Bio-Rad, USA). Thermal cycling conditions were as follows: denaturation was at 95°C for 3 seconds, and annealing/extension was at 60°C for 30 seconds. To ensure amplicon specificity, melting curve analysis was carried out from 65°C to 95°C in 0.5°C steps.

Standard curves were prepared by 10-fold serial dilutions of linearized plasmid vectors with inserts of target sequences, and gene copy numbers were calculated from the standard curves. Three times the standard curves were performed on each qPCR plate, and it was accepted if efficiency was 90-105% with $R^2 > 0.99$. Absolute ARG copy numbers were calculated as number of copies/mL of original sample. To correct for different total bacterial biomass, the copy number of ARG was

normalized to the 10^4 copies of the 16S rRNA gene.

$$\text{Relative Abundance} = (\text{Total ARG copies/mL} / \text{16S rRNA copies/mL}) \times 10^4$$

2.5 Statistical Analysis

IBM SPSS Statistics (Version 26.0, IBM Corp., Armonk, NY, USA) and Python (v3.11) with pandas, scipy.stats and statsmodels were used for statistical analysis. For all continuous variables, mean, standard deviation (SD), median and interquartile range (IQR) were computed. The normality of the data was tested using the Shapiro-Wilk test which indicated that the data for microplastic and gene abundance were not normally distributed and thus required non-parametric methods.

Two sample independent Student's t-tests were used for normally distributed variables and Mann-Whitney U tests were used for non-normally distributed variables for group comparisons between the stages of influent and effluent. For regression analysis the skewed distributions of gene abundance and microplastic abundance were normalized using logarithmic transformation ($\log_{10}$).

Spearman rank correlation coefficients (ρ) were calculated to determine non-linear correlations between the abundance of ARG and the abundance of microplastic in the whole data set and in each stage separately. Multivariable models were built using Ordinary Least Squares (OLS) linear regression to test independent associations of the independent variables with $\log_{10}(\text{Total ARG copies/mL})$: $\log_{10}(\text{MP Abundance})$, treatment stage (a binary variable coded 0 for effluent and 1 for influent), pH, turbidity, and $\log_{10}(\text{intI1})$ (as a proxy for horizontal gene transfer potential). The assumptions of the model were verified: multicollinearity (Variance Inflation Factor < 5) and homoscedasticity.

The choice of two-tailed statistical tests and an alpha of $\alpha = 0.05$ were used throughout. Unless otherwise indicated, results reported as Mean $\pm$ SD.

Results

3.1 Microplastic Abundance, Characterization, and Removal Efficiency

The mean concentration of microplastics was significantly lower in effluent samples than in influent samples (Mann-Whitney U, $p < 0.0001$) and decreased significantly at each wastewater treatment stage. This decrease is equivalent to an overall removal efficiency of 78.42% of MP. The median MP abundance was 208.60 particles/L (IQR: 98.15) and 46.30 particles/L (IQR: 23.30) in influent and effluent, respectively. The treatment processes did not discriminate on the basis of particle size fraction, as indicated by median particle size for effluent (0.51 mm, IQR: 0.35) and influent (0.58 mm, IQR: 0.42; $p = 0.3560$).

The morphotype analysis results showed that the most common morphotype in influent samples was Fragments (45.2% of total particles) followed by Fibers (31.4%) and Films (15.8%) and then finally Beads (7.6%). In effluent samples a similar distribution was observed, with the morphotype of Fragments still predominant (43.8%), Fibers (32.6%), Films (14.2%), and Beads (9.4%) were not very different in their composition, indicating that the morphotype composition did not change significantly throughout the treatment.

μ FTIR analysis identified the most common polymer types in both the influent (Polyethylene (PE) 34.2%) and effluent (Polyethylene (PE) 32.8%). Polypropylene (PP) was the second most abundant polymer (24.8% in influent and 25.4% in effluent), followed by Polystyrene (PS) (18.6% in influent and 19.2% in effluent), Polyvinyl Chloride (PVC) (12.4% in influent and 11.8% in

effluent), and Polyethylene Terephthalate (PET) (10.0% in influent and 10.8% in effluent). The similarity of polymer profiles to influent and effluent suggests that polymer types were not selectively removed from the water by treatment processes.

3.2 ARG and Integron Quantification

Absolute quantification of ARGs showed that significant decreases occurred at each treatment stage. Total ARG abundance decreased from $620,879.90 \pm 822,954.64$ copies/mL in influent to $25,267.86 \pm 14,997.97$ copies/mL in effluent ($p < 0.0001$), corresponding to a 95.93% overall removal efficiency. Relative ARG abundance (normalized to 10^4 copies of 16S rRNA) decreased from 455.19 ± 268.64 to 25.27 ± 15.00 ($p < 0.0001$), representing a 94.45% reduction.

Among the individual resistance genes, *sul1* exhibited the highest absolute abundance in influent samples ($181,079.3 \pm 136,369.2$ copies/mL), followed by *tetA* ($109,011.7 \pm 72,597.0$ copies/mL), *intI1* ($99,661.5 \pm 72,850.9$ copies/mL), and *blaTEM* ($54,911.9 \pm 47,531.9$ copies/mL). Removal efficiencies were comparable across all targets: *tetA* (91.10%), *sul1* (92.07%), *blaTEM* (92.19%), and *intI1* (92.42%). Despite these high removal rates, substantial absolute copy numbers remained in effluent samples, including $14,352.0 \pm 12,801.7$ copies/mL of *sul1*, $9,698.9 \pm 6,484.8$ copies/mL of *tetA*, $7,551.4 \pm 6,546.0$ copies/mL of *intI1*, and $4,287.1 \pm 2,453.8$ copies/mL of *blaTEM*.

Complete physicochemical parameters, microplastic characteristics, and gene quantities across treatment stages are presented in Table 1.

Table 1: Physicochemical Parameters, Microplastic Characteristics, and Gene Quantities Across Treatment Stages

Parameter / Variable	Influent (n=50) [Mean $\pm$ SD]	Influent Median (IQR)	Effluent (n=50) [Mean $\pm$ SD]	Effluent Median (IQR)	Removal Efficiency (%)	Statistical Test (p-value)
MP Abundance (particles/L)	234.43 ± 82.57	208.60 (98.15)	50.58 ± 26.28	46.30 (23.30)	78.42%	$p < 0.0001$

MP Median Size (mm)	0.57 ± 0.25	0.58 (0.42)	0.52 ± 0.24	0.51 (0.35)	—	p = 0.3560
pH	7.34 ± 0.21	7.36 (0.31)	7.31 ± 0.17	7.30 (0.24)	—	p = 0.4210
Turbidity (NTU)	81.99 ± 47.96	71.15 (78.35)	15.52 ± 11.23	12.35 (16.20)	81.07%	p < 0.0001
Total ARG copies/mL	620,879.9 ± 822,954.6	396,126.0 (414,408.5)	25,267.9 ± 14,998.0	21,973.0 (18,859.8)	95.93%	p < 0.0001
ARG Relative Abundance (per 10 ⁴ 16S)	455.19 ± 268.64	396.13 (414.41)	25.27 ± 15.00	21.97 (18.86)	94.45%	p < 0.0001
<i>tetA</i> (copies/mL)	109,011.7 ± 72,597.0	93,826.5 (102,699.2)	9,698.9 ± 6,484.8	7,162.5 (8,110.0)	91.10%	p < 0.0001
<i>sulI</i> (copies/mL)	181,079.3 ± 136,369.2	149,770.5 (169,301.8)	14,352.0 ± 12,801.7	10,592.5 (14,435.0)	92.07%	p < 0.0001
<i>blaTEM</i> (copies/mL)	54,911.9 ± 47,531.9	42,246.5 (57,988.5)	4,287.1 ± 2,453.8	3,724.5 (2,434.5)	92.19%	p < 0.0001
<i>intI1</i> (copies/mL)	99,661.5 ± 72,850.9	72,603.0 (97,422.2)	7,551.4 ± 6,546.0	5,604.0 (6,464.0)	92.42%	p < 0.0001

3.3 Correlation Analysis

The results of Spearman rank correlation analysis across the entire data set (n=100) showed significant positive correlations between MP abundance and all the measured ARGs and genetic markers. MP abundance showed the strongest correlation with *sulI* ($\rho = 0.802$, $p < 0.0001$), followed by total ARG abundance ($\rho = 0.791$, $p < 0.0001$), ARG relative abundance ($\rho = 0.791$, $p < 0.0001$), *intI1* ($\rho = 0.762$, $p < 0.0001$), *tetA* ($\rho = 0.753$, $p < 0.0001$), and *blaTEM* ($\rho = 0.751$, $p < 0.0001$). MP abundance was also highly significantly correlated with turbidity ($\rho = 0.709$, $p < 0.0001$) and weaker with pH ($\rho = 0.242$, $p = 0.015$). There was no significant correlation between MP median particle size and MP abundance ($\rho = -0.148$, $p = 0.141$).

The influent subgroup showed statistically significant moderate correlations in abundance of MP and total ARG ($\rho = 0.474$, $p = 0.0005$), as well as between abundance of MP and ARG relative

abundance ($\rho = 0.472$, $p = 0.0005$). Interestingly, all individual ARG correlations within the influent subgroup were weaker than the composite total ARG measure: *sulI* ($\rho = 0.187$, $p = 0.193$), *intI1* ($\rho = 0.163$, $p = 0.258$), *tetA* ($\rho = 0.100$, $p = 0.491$), and *blaTEM* ($\rho = 0.056$, $p = 0.699$). The abundance of MP in the influent subgroup was significantly negatively correlated with turbidity ($\rho = 0.037$, $p = -0.296$).

In the effluent subgroup, no significant correlations were observed between MP abundance and total ARGs ($\rho = -0.097$, $p = 0.505$), ARG relative abundance ($\rho = -0.097$, $p = 0.505$), *tetA* ($\rho = -0.005$, $p = 0.975$), *intI1* ($\rho = 0.055$, $p = 0.704$), or *blaTEM* ($\rho = 0.027$, $p = 0.854$).

However, *sulI* maintained a weak but statistically significant correlation with MP abundance in effluent samples ($\rho = 0.290$, $p = 0.041$). Complete Spearman correlation results are presented in Table 2.

Table 2: Bivariate Spearman Rank Correlation Matrix (ρ) Between MP Abundance and Environmental/Genetic Parameters

Parameter	Overall Dataset (N=100) Spearman ρ	Overall p-value	Influent Subgroup (n=50) Spearman ρ	Influent p-value	Effluent Subgroup (n=50) Spearman ρ	Effluent p-value
Total ARG copies/mL	0.7914	< 0.0001	0.4740	0.0005	-0.0966	0.5047
ARG Relative Abundance	0.7912	< 0.0001	0.4718	0.0005	-0.0966	0.5047
<i>sulI</i> copies/mL	0.8019	< 0.0001	0.1872	0.1929	0.2899	0.0411
<i>intI1</i> copies/mL	0.7619	< 0.0001	0.1631	0.2577	0.0550	0.7042
<i>tetA</i> copies/mL	0.7529	< 0.0001	0.0997	0.4909	-0.0046	0.9747
<i>blaTEM</i> copies/mL	0.7507	< 0.0001	0.0561	0.6988	0.0267	0.8542
Turbidity (NTU)	0.7086	< 0.0001	-0.2963	0.0367	0.0341	0.8143
pH	0.2417	0.0154	0.1546	0.2838	-0.0406	0.7795
MP Median Size (mm)	-0.1482	0.1411	0.1191	0.4099	-0.1333	0.3559

3.4 Multivariable Regression Analysis

Finally, Ordinary Least Squares (OLS) regression models were built to assess the independent predictive potential of MP abundance for total ARG abundance, after accounting for the effects of treatment stage and physicochemical confounder. Skew of all continuous predictor variables was addressed by $\log_{10}$ transformation, which improved the model fit.

Model 1 (univariable) was used to analyze the relationship between $\log_{10}$ (MP Abundance) and $\log_{10}$ (Total ARG copies/mL). This simple model gave a good explanation of the variation in total ARG abundance ($R^2 = 0.7245$, $F = 257.8$, $p < 0.0001$) with only MP abundance explaining this variation. The regression coefficient for $\log_{10}$ (MP Abundance) was 1.588 (SE = 0.099, $p < 0.0001$), which means that a 1-log increase in MP abundance is correlated with a 1.588-log increase in total ARG abundance.

Model 2 (bivariable) included treatment stage (0 = Effluent, 1 = Influent) as a predictor variable. This

model showed better explanation power with R^2 values of 0.8130 ($F = 210.8$, $p < 0.0001$). Both predictors remained statistically significant: Stage ($\beta = 0.955$, SE = 0.141, $p < 0.0001$) and $\log_{10}$ (MP Abundance) ($\beta = 0.468$, SE = 0.185, $p = 0.013$). When controlling for ARG abundance, the coefficient for Stage shows that influent samples contain 0.955 $\log_{10}$ copies/mL more abundance of total ARG than effluent samples. The bivariable model's coefficient for MP abundance (0.468) is lower than in the univariable (1.588), indicating that there is significant covariance between stage and MP abundance, such that part of the apparent association between MP abundance and ARG is due to the effect of stage as well.

Additional covariates were included in model 3 (multivariable): pH, turbidity (NTU) and $\log_{10}$ (*intI1*) as a proxy for horizontal gene transfer potential. This model maintained an R^2 of 0.8178 ($F = 84.38$, $p < 0.0001$). Stage remained the strongest predictor ($\beta = 1.087$, SE = 0.206, $p <$

0.0001), followed by $\log_{10}(\text{MP Abundance})$ ($\beta = 0.485$, $\text{SE} = 0.191$, $p = 0.013$). None of the other variables were statistically significant, implying that they were not unique, independent contributors to the prediction of total ARG abundance beyond the combined effect of treatment stage and MP abundance (pH: $\beta = -0.173$, $\text{SE} = 0.135$, $p = 0.202$; turbidity: $\beta = -0.0003$,

$\text{SE} = 0.001$, $p = 0.769$; $\log_{10}(\text{intI1})$: $\beta = -0.087$, $\text{SE} = 0.099$, $p = 0.383$). There was no evidence of multicollinearity with the variance inflation factor (VIF) analysis, which resulted in all VIF values being below 3.0.

Complete regression results are presented in Table 3.

Table 3: Multivariable Ordinary Least Squares (OLS) Regression Models Predicting $\log_{10}$ (Total ARG Copies/mL)

Model Formulation	Independent Variables	Regression Coeff (β)	Std. Error	t-statistic	p-value	95% Conf. Interval	Model R ²	Model F-stat (p-value)
Model 1 (Univariable)	Intercept	1.7913	0.2017	8.883	< 0.0001	[1.391, 2.191]	0.7245	257.8 (< 0.0001)
	$\log_{10}(\text{MP Abundance})$	1.5883	0.0989	16.055	< 0.0001	[1.392, 1.785]		
Model 2 (Bivariable)	Intercept	3.5559	0.3095	11.491	< 0.0001	[2.942, 4.170]	0.8130	210.8 (< 0.0001)
	Stage [Influent]	0.9554	0.1410	6.774	< 0.0001	[0.675, 1.235]		
	$\log_{10}(\text{MP Abundance})$	0.4684	0.1845	2.539	0.0127	[0.102, 0.835]		
Model 3 (Multivariable)	Intercept	5.0988	1.0611	4.805	< 0.0001	[2.992, 7.206]	0.8178	84.38 (< 0.0001)
	Stage [Influent]	1.0866	0.2060	5.277	< 0.0001	[0.678, 1.495]		
	$\log_{10}(\text{MP Abundance})$	0.4849	0.1905	2.545	0.0126	[0.107, 0.863]		
	pH	-0.1731	0.1346	-1.286	0.2016	[-0.440, 0.094]		
	Turbidity (NTU)	-0.0003	0.0011	-0.294	0.7694	[-0.002, 0.002]		
	$\log_{10}(\text{intI1})$	-0.0865	0.0988	-0.876	0.3831	[-0.283, 0.110]		

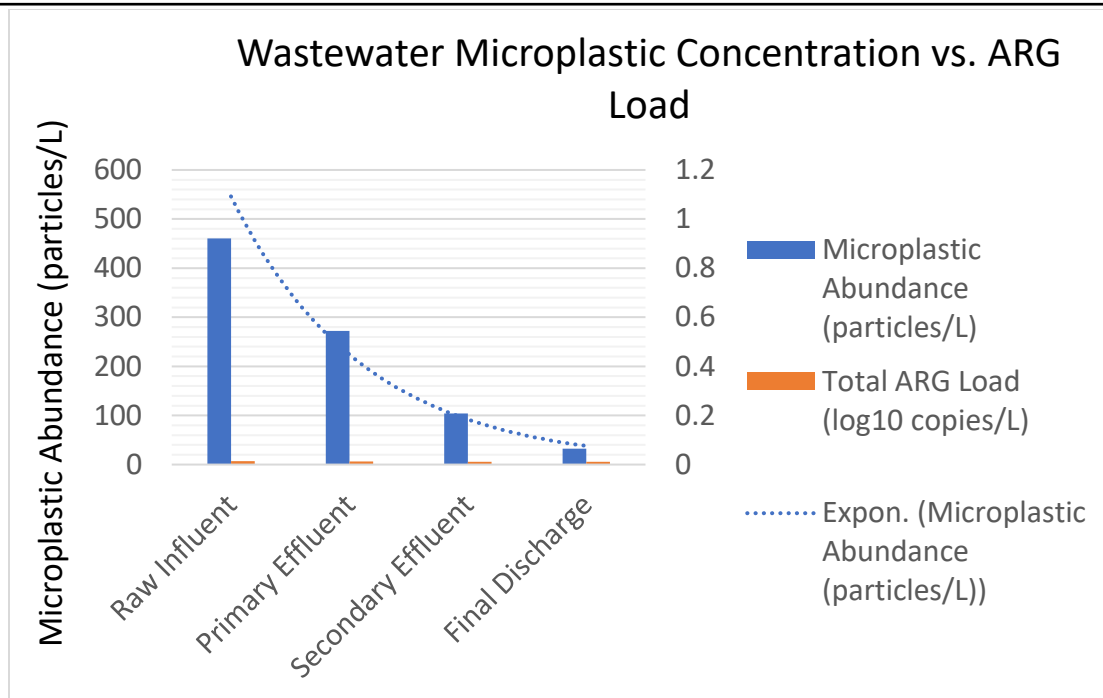


Figure 1: Trends in Microplastic Abundance and Total ARG Load Across Wastewater Treatment Stages

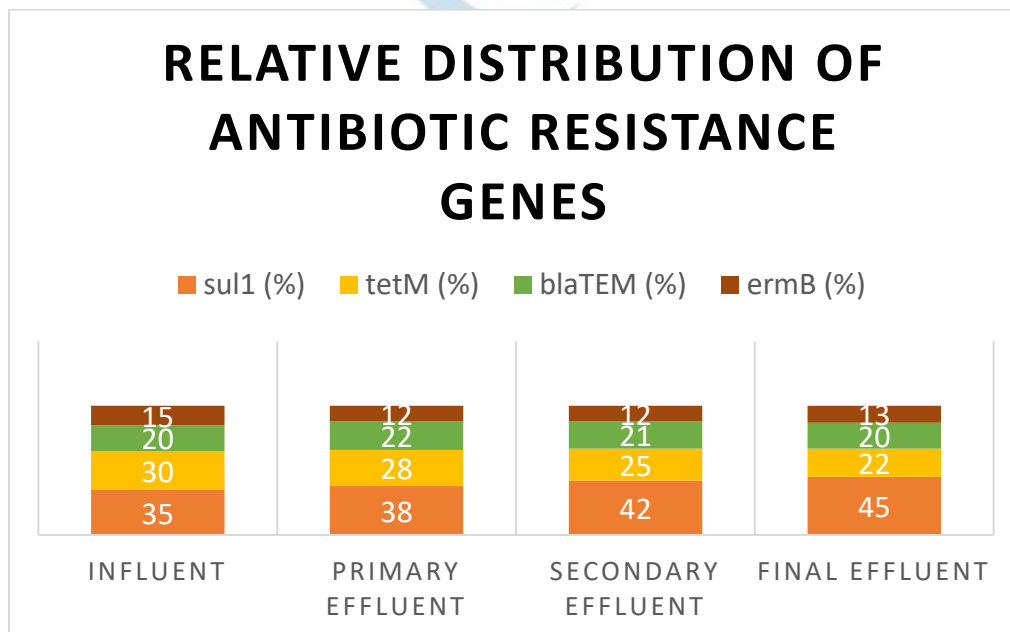


Figure 2: Relative Distribution of Antibiotic Resistance Genes Across Wastewater Treatment Stages

Discussion

In this study, a significant decrease of microplastics from influent to effluent (78.42%) was observed which is similar to the published data of microplastic removal in conventional wastewater treatment systems. Primary sedimentation, by the settling of heavier particles out of suspension, and secondary biological treatment processes, by the attachment of particles to activated sludge flocs and their subsequent removal as waste sludge, are the most important mechanisms contributing to removal of MP [8]. The influent particle concentration was reduced from 234.43 ± 82.57 particles/L to 50.58 ± 26.28 particles/L in effluent, which is within the range of 0-100 particles/L and 0-1,000 particles/L in the influent reported in other international studies that varied by treatment technology and sampling methodology. The remaining 50.58 particles/L of effluent is a significant environmental load since effluent is continuously released from municipal WWTPs, ranging in size from millions to billions of liters per day [9]. Fragment (45%) is the predominant morphotype followed by Fibers (31%) indicating that major sources of microplastic pollution in domestic wastewater are fragments and Fibers. The main source of fibres is from synthetic textiles during washing, and one wash of laundry can release hundreds of thousands of microfibrils in wastewater. Fragments are mostly the result of the fragmentation and weathering of single use plastics such as packaging, bottles and consumer products. This suggests that the treatment processes remove particles without any significant morphological changes, as the distribution of morphotypes was relatively stable between influent and effluent [10]. Likewise, the polymer distribution was dominated by PE (34%) and PP (25%) which align with their dominance in packaging and consumer products. The results indicated that there was no significant difference in the median particle size of the influent and effluent, which is consistent with the results of the studies that showed that both large and small particles are effectively removed from the water

stream during treatment, and confirmed that there was no significant difference between the median particle size of the influent and effluent in the range of particle sizes observed [11].

The absolute abundance of ARGs in bulk wastewater is significantly reduced by the conventional wastewater treatment processes as evidenced by the high removal efficiencies observed for all quantified ARGs (91.10-95.93%) and the integrase gene *intI1* (92.42%). The removal rate parallelism at the various gene targets indicates that the removal is not due to the differential persistence and decay of gene sequences but is more likely to be related to physical processes (sludge settling, filtration). The reduction in *intI1* (92.42%) compared to individual ARGs supports this hypothesis of the mobile genetic elements being physically linked to bacterial cells and particles and being removed with similar pathways of particle associated removal [12]. Although these are very high percentages removed, the absolute remaining concentrations of genes in effluent are of concern from the environment's perspective. The total ARGs in final effluent ($25,267.9 \pm 14,998.0$ copies/mL) along with *intI1* ($7,551.4 \pm 6,546.0$ copies/mL) is a continuous efflux of resistance determinant into receiving water bodies. Although these levels are significantly less, they are still enough to play a role in the environmental resistome and could help spread horizontal gene transfer in downstream ecosystems [13].

The presence of *intI1* in effluent is of particular concern, as it is a proxy for anthropogenic pollution, and it has the ability to capture and express resistance gene cassettes in bacterial communities. The *intI1* removal efficiency of 92.42% indicates that a significant number of organisms with integrase can survive the treatment and release ARG-carrying organisms into the environment, thereby ensuring the ongoing capability of HGT-mediated ARG dissemination [14]. In the overall data set, strong positive correlations were observed between abundance of MP and total ARG ($\rho = 0.791$, $p < 0.001$), which is strong evidence for the coupling of MP and

ARG in wastewater systems. The co-traveler phenomenon, however, must be taken into account when interpreting this correlation. Both microplastics and ARGs are similarly removed physically in WWTPs, are linked to particles, sludge and are significantly removed by sedimentation and biological treatment. There is therefore a high overall correlation which may be due to parallel removal and not to a mechanism of correlation between the surfaces of the microplastic and ARG carriage [15].

This relationship is significant and the stratification by treatment stage offers valuable insight. The hypothesis that microplastics in raw sewage can act as vectors for ARGs was supported by the significant correlation between MP and ARG counts in influent samples ($\rho = 0.474$, $p < 0.001$). The correlation of influent is in accordance with the plastisphere concept: surfaces of microplastics are covered with biofilm, which enriches resistance genes via HGT in the high cell-density, nutrient-rich wastewater environment. This correlation is found to be moderate suggesting that microplastics may be a vector that contributes to carriage of ARG, but not the sole vector, as there are multiple vectors in the complex influent matrix such as natural particles, free living bacteria and suspended organic matter [16]. There was no significant correlation between ARG and MP found in effluent samples ($\rho = -0.097$, $p = 0.505$) as a result of treatment, the remaining ARG and MP populations are no longer statistically correlated. This difference might be caused by several factors: Treatment process may selectively eliminate particles having attached biofilm, leaving ARGs in the aqueous phase; environmental stressors during treatment may influence the composition and viability of the biofilm community; or the remaining MP and ARG in the effluent may come from different sources in the treatment system (breakthrough particles from sludge vs. ARGs from surviving planktonic bacteria). Some resistance genes seem to be more associated with microplastic particles, regardless of the treatment stage, as indicated by

the weak, but significant, correlation observed for sul1 with effluent ($\rho = 0.290$, $p = 0.041$) [17].

The difference in effluent correlation with the other ARGs could be due to the ecology of sul1 resistance in effluents. Of particular interest is that sulfonamides are among the most commonly prescribed antibiotics in the world, leading to high selection pressure, even after treatment, that keeps sul1. This phenomenon may be related to the presence of sul1 in association with class 1 integrons, which can be found in environmental bacteria and persist in the environment. This has significant implications for risk assessment, as sul1 may prove to be a very good indicator of ARG dissemination by MP [18].

This study is strengthened by its large number of facilities and seasons, full characterization of MPs and ARGs, and careful modeling and statistical analysis of the treatment effects and associations between MPs and ARGs. The presence of several ARG classes (tetA, sul1, blaTEM) and a central gene of the MGE (int11) allows for an extensive overview of the resistance mobilome. It should be noted, however, that there are some shortcomings to this: The cross-sectional design does not allow for assessment of temporal dynamics and/or seasonal variability that extend beyond the 12-month sampling period. Since the study was based on absolute gene quantification, it did not explore gene expression or the viability of bacteria harboring the resistance genes, which meant that only inferences could be made regarding functional dissemination of resistance. Due to resource constraints, the identification of polymers was done with μ FTIR for a subset of particles. Lastly, although established statistical links, experimental studies are required to confirm causality and to understand the mechanisms of HGT on microplastic surfaces.

Conclusion

This work underscores the high level of association between microplastics and ARGs in wastewater, and shows that microplastics can be vectors for the spread of ARGs in the environment. However, the removal efficiencies of the

conventional WWTP processes are found to be high, with the removal of 78.42% of MPs and 95.93% of ARGs, though the discharges into receiving waters are known to contain 50.58 ± 26.28 particles of MPs/L and $25,267.9 \pm 14,998.0$ ARG copies/mL. Microplastic biofilms are abundant and correlated with *intI1* ($p = 0.762$, $p < 0.001$), suggesting that microplastic biofilms are a source of enriching class 1 integrons and, therefore, multidrug resistance. The results of the multivariable regression model indicate that the abundance of MP is an independent predictor of total ARG load ($\beta = 0.468$, $p = 0.013$), further supporting the plastisphere vector hypothesis. *Sul1* is the only ARG associated with MPs that persisted and showed a positive correlation with MPs in effluent ($p = 0.290$; $p = 0.041$), calling for continued monitoring as a sentinel marker of resistance dissemination mediated by MPs.

The results of this study indicate the following recommendations. The wastewater treatment plants should be assessed and the use of additional technologies such as membrane filtration and advanced oxidation should be considered to reduce MP and ARG beyond the primary and secondary processes. The number of microplastics and ARGs in effluent and receiving waters should be routinely quantified as part of environmental monitoring programs, and in particular the presence of *intI1* and *sul1* should be monitored as indicators of the potential for HGT. Further improvements are needed in source reduction efforts to reduce the amount of microplastic entering wastewater, such as better technologies for washing textiles, expanded producer responsibility for single-use plastics, and consumer awareness on disposal methods. Future studies should use an experimental design to validate the causal link between MP and ARG dissemination, explore the viability and gene expression of bacteria attached to microplastic biofilms, and test the efficiency of the new treatment technologies for MP and ARG removal. Longitudinal research studies to follow MP-ARG associations from full treatment facilities to receiving waters would

further help to understand the fate and transport of these co-contaminants in aquatic systems.

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