



Microplastic biofilm, associated pathogen and antimicrobial resistance dynamics through a wastewater treatment process incorporating a constructed wetland

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ARTICLE INFO

Keywords:

Microplastics

Biofilm

Colonization

Antimicrobial resistance

Constructed wetland

ABSTRACT

Microplastics in wastewater are colonized by biofilms containing pathogens and antimicrobial resistance (AMR) genes that can be exported into receiving water bodies. This study investigated establishment and changes in microplastic-associated biofilm and AMR during a conventional full-scale 2100 population equivalent wastewater treatment process combined with a free water surface polishing constructed wetland. Sequential microplastic colonization experiments were conducted at different stages of the wastewater treatment process, including in raw sewage, treated effluent and the constructed wetland. Two scenarios were tested in which the constructed wetland served as either (i) a polishing step or (ii) as primary recipient of sewage inoculated microplastics. Bacterial 16S rRNA gene sequencing was carried out for qualitative bacterial community analysis. qPCR was applied for quantitative analysis of AMR genes (*sul1*, *ermB*, *tetW*, *intI1*), bacterial biomass (16S rRNA) and a human fecal marker (HF183). Microbial diversity on microplastics increased with incubation time. The initial sewage-derived biofilm composition changed more significantly in the wastewater effluent compared to the constructed wetland. Pathogen and AMR load decreased by up to two orders of magnitude after coupled conventional and constructed wetland treatment, while less impact was observed when sewage-inoculated microplastic material was directly transferred into the constructed wetland. *Aeromonas*, *Klebsiella*, and *Streptococcus* were key pathogenic genera correlated with AMR in microplastic-associated biofilms. Despite decreasing trends on human pathogens and AMR load along the treatment process, microplastic-associated biofilms were a considerable potential hotspot for AMR (*intI1* gene) and accommodated Cyanobacteria and fish pathogens.

1. Introduction

Tackling plastics pollution and uncontrolled spread of human-induced antimicrobial resistance (AMR) are critical global challenges. Plastic pollution severely damages aquatic life (MacLeod et al., 2021) and transforms ecosystems (Oberbeckmann et al., 2018). As a subcategory of plastic pollution, microplastics (MPs), plastic particles in the micrometer to lower millimeter size range (Frias and Nash, 2019) negatively affect aquatic and terrestrial wildlife due to disrupting animal

digestive and immune systems, and reproductive success (Li et al., 2018; Sharifinia et al., 2020). MPs and smaller sized nanoplastics also pose potential hazards to human health (Revel et al., 2018). AMR is among the top ten global public health problems. There are more fatalities caused by antibiotic resistant bacteria than by HIV or malaria (Murray et al., 2022).

Despite different origins and characteristics, MP pollution and AMR spread share similar environmental dispersion routes. Both, MPs and AMR bacteria occur simultaneously in wastewater, while wastewater

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<https://doi.org/10.1016/j.watres.2023.119936>

Received 12 December 2022; Received in revised form 5 March 2023; Accepted 1 April 2023

Available online 3 April 2023

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treatment plants (WWTPs) are hubs for both contaminants (Pazda et al., 2019; Ziajahromi et al., 2017). Along sewage and wastewater treatment, MPs are colonized by microbial biofilms (McCormick et al., 2014), including bacteria populations with high relative AMR gene abundance (Lai et al., 2022), especially in antibiotic-rich wastewater (Berglund, 2015).

Wastewater treatment plants (WWTPs) are not specifically designed for complete MPs or AMR removal. Attenuation of MPs by WWTPs depends on treatment stage and technology; ranging from 50% for basic secondary treatment towards >99% for advanced tertiary treatment (Blair et al., 2019; Raju et al., 2020; Talvitie et al., 2017). Despite high MPs removal (>95%) achieved in large municipal WWTPs (Conley et al., 2019; Kim et al., 2022) the estimated release of MP via WWTPs, for example in Germany, is $0.6 \cdot 10^6$ to $1.2 \cdot 10^6$ MP particles per person annually (Schmidt et al., 2020).

A typical conventional WWTP would be expected to deliver 1-3 log (90-99.9%) removal of AMR genes; advanced treatment such as ozonation or membrane filtration may lead to 4 log (99.99%) removal (Hiller et al., 2019; Wang and Chen, 2020). However, WWTP effluent can still reach >104 AMR gene copies (GC) mL⁻¹ for the most prevalent AMR genes such as tetracycline or sulfonamide resistance (Wang et al., 2020), 1-3 orders of magnitude higher than background concentrations of AMR genes upstream WWTPs (Berglund et al., 2015; Marti et al., 2013).

MPs and AMR risks need to be also addressed in non-conventional treatment such as constructed wetlands (CWs). CWs are a nature-based low-energy water treatment technology that mimic the purification processes of natural wetlands. Despite apparent limitations due to the low-tech design and operation, CWs can treat various types of industrial, agricultural and municipal wastewater (Chowdhury et al., 2022; Parde et al., 2021). MPs removal in CW from wastewater streams varies widely between 20% and 95% (Wang et al., 2020; Wei et al., 2020; Zhou et al., 2021). AMR removal in CWs is comparable to conventional WWTPs (Ma et al., 2022).

Few studies have investigated biofilm growth on MPs and related pathogen and AMR spread in sewage and WWTPs. MPs collected from WWTP effluent have a distinctively different bacterial community than the planktonic community (Kelly et al., 2021; Martínez-Campos et al., 2021), including pathogenic bacteria (Kelly et al., 2021) and AMR genes. Presence of MPs in wastewater effluent increased survival and spread of pathogens (Oberbeckmann et al., 2018; Proia et al., 2016). Moreover, disinfection had little effect on microplastic-associated biofilm composition and their ability to accommodate intact AMR (Galafassi et al., 2021; Shen et al., 2021; Yang et al., 2022). In summary, there is strong evidence on the possible risk of increased AMR spread through MPs released by WWTPs. Studies investigating MPs biofilm and resistance in WWTPs are limited to single step incubation times (i.e. 24h, 48h, 21d) (Miao et al., 2019; Parrish and Fahrenfeld, 2019; Pham et al., 2021), single step treatment (e.g. raw sewage, WWTP effluent) (Lai et al., 2022; Martínez-Campos et al., 2021; Yang et al., 2022) or laboratory studies (Eckert et al., 2018; Wang et al., 2021; Wu et al., 2019). Few field studies were carried out, and none investigated time-dependent colonization dynamics (Galafassi et al., 2021; Kelly et al., 2021), or the role of CW systems in mitigating MP associated pathogen and AMR hazard.

The aim of this study was to address this knowledge gap by looking at the combined effect of colonization time and treatment step-dependent dynamics of MPs associated AMR and pathogens in wastewater. Colonization and AMR evolution patterns on sterile MPs were studied including how these patterns change along different stages of a conventional wastewater treatment process combined with a polishing CW. Downstream treatment of wastewater effluent via a polishing CW may alter MPs biofilm composition and AMR levels and minimize the synergy of MPs pollution and AMR spread, beyond simple physical MPs retention. To test this hypothesis, batches of sterile microplastics were successively incubated and transferred between different stages of a full-scale treatment process equipped with a CW at varying incubation

times and genetically analyzed. This is the first MPs sequential colonization experiment that provides both temporal and spatial analysis of MP biofilm alteration during a full-scale wastewater treatment process, additionally incorporating a CW.

2. Methods

2.1. Sampling site

The study site was Cromhall Water Recycling Centre (WRC) located at Cromhall, South-West Gloucestershire, UK. Cromhall WRC receives an average daily discharge of 1.4 ML/d of a combined sewerage from a rural catchment with a pollution load of 2100 population equivalent (PE). Cromhall WRC consists of Sewage Treatment Works (STW) providing secondary treatment (oxidation ditch) and surface flow constructed wetlands acting as a polishing step (Fig. 1A). The STW was operated since 1980s. The CW operation was added in 2020 for enhanced phosphorus removal. STW effluent and CW water quality data are available in supplementary data (Table 1S). Due to Covid pandemic related restrictions in 2021 water quality monitoring of STW inflow raw wastewater was not possible. Cromhall CW has a surface of 0.8 ha and consists of 12 treatment cells of variable size, depth, and vegetation cover (Table S2). Plant composition is dominated by the emergent macrophytes, *Schoenoplectus lacustris* and *Typha angustifolia*. Open water zones are covered primarily with duck weed and low-growing *Apium nodiflorum*.

2.2. Experimental design

The colonization experiment used four types of plastics including high density polyethylene (HDPE), polyvinyl chloride (PVC), polyethylene terephthalate (PET) and polystyrene (PS) (Fig. S1A) which represent major polymer types present in municipal wastewater, including diversity of plastics physicochemical properties affecting biofilm formation (Azizi et al., 2022; Xu et al., 2021). Microplastics were produced via freeze-grinding of commercial plasticware made from the selected polymers (as indicated by the composition label). Produced microplastics were sieved to obtain a uniform specimen range of 0.50-0.75 mm. Approximately 400 mg of each polymer type was prepared in triplicates and each MP type loaded into separate stainless steel mesh tea infusers (Fig. S1B) and sterilized in an autoclave. Loaded infusers were attached to a steel chain and additionally wrapped into a coarse mesh bag to prevent accidental loss of samples. Infusers containing MPs were transferred alongside the wastewater treatment process at three locations (Fig. 1A) to simulate successive transport and sequential colonization of MPs through the wastewater treatment system (Fig. S2 A-D). Throughout the manuscript sampling locations are denoted as follows: WWTP inflow point containing raw sewage, RWW; effluent from secondary clarifier comprising of a final effluent from the WWTP, SCE; treatment cell 6 at the CW, CW. Three separate colonization experiments, indicated with indexes 1, 2 and 3, respectively, were carried out simulating different travel routes of MPs (Fig. 1B).

Colonization experiment 1 was based on successive transition of MPs from sewage (RWW MPs incubation point), through the conventional wastewater treatment process (SCE) followed by the polishing CW. Experiment 2 simulated a case when CW acts as primary recipient of wastewater by transferring MPs directly from raw sewage (RWW) into the CW, while bypassing the secondary treatment step. In experiment 3, sterile MPs were deployed into CW as a quasi-control to assess independently the effect of CW microbial community on MPs colonization without sewage pre-inoculation.

The study was carried out in a period of stable summer weather conditions (July-September 2021) without prolonged dry periods or heavy rain events reflected by water flow readings (Fig. S3). Each experiment was carried out at two colonization/incubation times of 4 days and 20 days. The selected incubation times were chosen to simulate

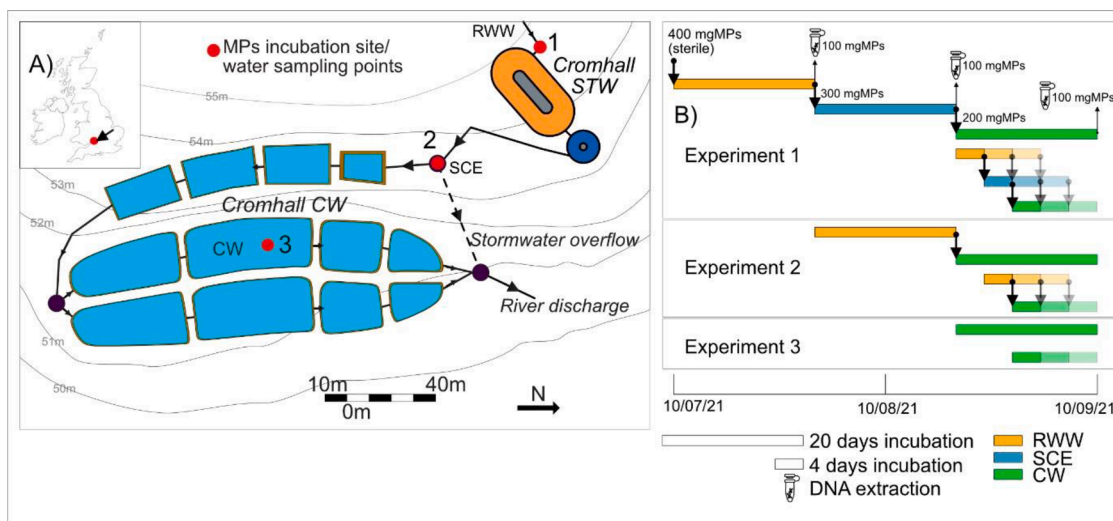


Fig. 1. (A) Schematic view of Cromhall Water Recycling Centre with marked water sampling and MPs deployment points which include (1) WWTP inflow point (RWW), (2) WWTP final effluent following secondary clarifier (SCE) and (3) treatment cell 6 at the CW (CW). (B) Incubation and sampling timeline for experiments 1, 2 and 3. The 20-day replicates were deployed simultaneously, while the 4-day replicate deployment were staggered to minimize possible effects of variable wastewater quality on biofilm growth and composition. Exemplary transitions and subsampling for DNA extraction of MPs are shown for experiment 1 with 20-day incubation.

short (4d) and long (20d) retention times applicable to MPs in both conventional WWTP and CWs while providing enough time span to ensure visible temporal shift in colonization patterns. In conventional WWTPs MPs retention often correlates with suspended (Frehland et al., 2020) solids retention time (SRT) which varies between few days up to 20 d and beyond depending on the treatment process (Vieno and Sillanpää, 2014). For CWs use of HRT instead of SRT to determine MPs retention might be more adequate (Bydalek et al., 2023). For the CW system studied here HRT ranges between 2d and 15d depending on the weather conditions and was typically 5-10 days during the study period.

The experimental timeline is shown in Fig. 1B. The applied timeline synchronized MPs deployment in the CW rather than in sewage (RWW) to accommodate for more pronounced temperature fluctuation in the CW while also considering the relatively stable composition of municipal wastewater. MPs were first deployed into raw wastewater at the inflow of the WWTP (RWW). Following selected incubation times at each location, MPs were removed from infusers and washed with sterilized deionized water. Subsequently, 100 mg MPs from each infuser were transferred into separate 2 mL sterile centrifuge tubes and stored at -20°C until the further processing. The remaining MPs were transferred into new sterile infusers and then deployed into the next location. MPs of the four polymer types were incubated in separate infusers during the *in-situ* colonization phases but were pooled together for the DNA extraction to create an equal-weight composite sample consisting of all 4 polymer types. Note, that polymer type may affect colonization patterns on MPs (Ramsperger et al., 2020; Tu et al., 2021). We decided on pooled analysis of a representative plastics mixture in WWTPs due to analytical cost considerations. Safety regulations did not allow deployment of infusers into the oxidation ditch and secondary clarifier. Instead infusers were placed in the WWTP final effluent chamber receiving wastewater from secondary clarifier (SCE). CW treatment cell 6 served as a deployment site for MPs due to its well-established vegetation cover and position in the middle of the CW. In the CW, infusers were attached to a foam floater and submerged approximately 5-10 cm below water surface (Fig. S2 D). Direct dispersion of MPs onto the water surface was also considered but turned out unfeasible due to a large wildfowl population dwelling in the CW over the study period. Additional water samples were collected to provide information on planktonic fraction of the microbial community background. Each sampling site was subsampled 3 times a day at 1000 mL sample size. Subsamples were later combined into a quasi-daily

composite sample. A total of 3 quasi-daily composites were collected for the SCE (secondary clarifier effluent) and CW (treatment cell TC6) each while the raw sewage (RWW) was collected only once due to sampling restriction imposed by the Covid pandemic. Water samples were immediately filtered on $0.45\ \mu\text{m}$ nitrate cellulose (NC) membranes. Membranes containing DNA material were stored at -20°C until downstream processing but not longer than 2 weeks.

2.3. Microbial community and AMR analysis

2.3.1. DNA extraction

Under aseptic conditions microplastics were transferred into the DNA extraction tubes, 25 mg of each polymer type were mixed to reach a total of 100 mg of MPs in Lysis Matrix E extraction tubes (MP Biomedicals, USA, 2022). For water sample analysis, frozen $0.2\ \mu\text{m}$ nitrocellulose membranes were cut into small pieces and loaded into the extraction tubes. DNA was extracted using the FastDNA SPIN Kit for Soil (MP Biomedicals, USA, 2022) following manufacturer's protocol, applying $2 \times 30\ \text{s}$ at speed 5.5 m/s bead beating with a FastPrep 24 system (Webster et al., 2003). Following extraction, DNA stock solution was stored at -80°C . Control PCR and gel electrophoresis was run to assess quality and optimum dilution for DNA stocks to minimize PCR inhibitory effect in downstream processing.

2.3.2. 16S rRNA gene profiling

The genomic library construction protocol was based on single-step amplification of the V4 region of the 16S rRNA using tagged primers described by Kozich et al. (2013). Two 96-well PCR plates were prepared with A5/B7 and B5/B7 indexed primers, respectively. Each PCR reaction contained 17 μL of Accuprime Pfx Supermix, 1 μL of template DNA, and 2 μL of each paired set of index primers. Each plate contained negative and positive controls with PCR grade H₂O and 1:3 dilution of Mock Community (BEI Resources, VA, USA). Additionally, a blank DNA extraction served as a DNA extraction process quality control. The following PCR conditions were applied: initial 2 min. at 95°C ; 30 cycles of 20 s at 95°C , 15 s at 55°C and 5 min. at 72°C ; and a final step of 72°C for 10 min. All samples were run in triplicates. All PCR products were analyzed on 1.2% (w/v) agarose gel electrophoresis for an initial quality check and if successful were further purified using SPRI magnetic beads (Beckman Coulter, IN, USA). Poor quality replicates were reamplified by

PCR until satisfactory quality was obtained. Once purified, selected replicates underwent final quality-check via automated electrophoresis tool (4200 TapeStation, Agilent, CA, USA) and subsequently triplicates were pooled together. DNA concentration of the 16S rRNA library pools were quantified using Qubit dsDNA high-sensitivity assay (Qubit 4, Invitrogen, UK) and later equalized to create a normalized pool using the lowest concentration. Sequencing was performed on Illumina MiSeq platform at the Cardiff University Genomic Research Hub, using 2 × 250 bp paired-end flow cells and reagent cartridges.

2.3.3. AMR qPCR

Quantitative analysis of gene abundance using qPCR targeted genes associated with antimicrobial resistance (*tetW*, *ermB*, *sul1* and *int11*) as well as the bacterial 16S rRNA gene, used as a proxy for bacterial biomass and HF183 marker gene for human fecal contamination. Selected *tetW*, *ermB* and *sul1* AMR genes code resistance mechanisms against high-usage broad-spectrum antibiotics belonging to tetracycline, erythromycin and sulfonamide respectively which are widely present in municipal wastewater and represent genetic diversity of the AMR mechanisms (Hendriksen et al., 2019; Reygaert, 2018). Primers and qPCR conditions used in the study are presented in Table S3. AMR gene standards were made of plasmids extracted from respective AMR *E. coli* clones (Xu et al., 2019). 16S rRNA gene standard was prepared as 27F-1492R PCR amplicon amplified from pure culture of *E. coli*. HF183 gene standard was prepared via cloning of HF183 amplicons obtained from raw wastewater PCR into JM109 competent cells using pGEM-T Easy Vector System (Promega, France). Standard DNA was quantified (Qubit 4, Invitrogen, UK) and serially diluted to generate qPCR standard curves. QPCR reactions were made up in 10 µL and consisted of 6 µL of Luna Universal Master Mix (NEB, UK), 0.25 µL of each primer, 0.5 µL of PCR grade H₂O and 4 µL of DNA template. QPCR condition are presented in Table 1S in supplementary materials. All samples were run in triplicates. Quantifications were accepted if reaching between 90 and 110% efficiency and R² >98% for a 6-point calibration curve. Each qPCR run included negative control with PCR grade H₂O as a DNA template. QPCR was carried out on (Stratagen Mx3000P, Agilent, CA, USA) and the amplification results were analyzed via (MxPro qPCR, Agilent, CA, USA) and converted into gene copy number (GC) per either 1 mL (water sample) or 1g of MP (GC / 1 mL and GC / 1g MP respectively) (Webster et al., 2015).

2.4. Data processing

2.4.1. Sequencing data pre-processing

QIIME 2 (Quantitative Insights Into Microbial Ecology 2) pipeline was used for demultiplexing and quality filtering (DADA2 truncating was carried out with forward sequences trimmed at 180 position and reverse sequences at 140 position), taxonomy assignment (Greengenes 16S rDNA sequences database (DeSantis et al., 2006)) and phylogenetic tree creation (q2-phylogeny plugin with MAFFT and FastTree2 programs). Obtained QIIME artifacts were imported into and further processed using phyloseq package (v 1.38.0) in R software (v 4.1.3) (McMurdie and Holmes, 2013). Amplicon sequence variants (ASV) with total abundance of less than 10 were removed. Taxonomic filtering excluded non-bacterial and unknown sequences such as chloroplast, Archaea or mitochondria. Samples were rarefied to a 35000 reads per sample to normalize all samples to a same sequencing depth. The applied rarefaction depth led to exclude one sample due to low number of reads (<10000). Post-processing taxonomic coverage reached a minimum of 0.9893 of original taxonomic coverage, thus data pre-processing led to a loss of a maximum ca 1% of taxonomic information. To assess pathogen dynamics taxonomic composition was screened for presence of pathogenic genera and species representing priority pathogens established by the WHO (Asokan et al., 2019).

2.4.2. Statistical analysis

Microbial diversity indices including Chao1 richness and Shannon and Simpson community diversity were calculated using phyloseq package (v 1.38.0) in R software (v 4.1.3) and q2-diversity plugin in QIIME2 pipeline. Chao1 index quantifies total number of species observed in a sample, while Shannon and Simpson indexes qualitatively assess heterogeneity of the community based on richness and evenness of the community respectively. Taxonomic composition was visualized using ggplot R package (v 3.3.6). Constrained analysis of principal components (CAP) inbuilt in phyloseq package (v 1.38.0) was used to find association between experimental design parameters and community composition. Bacterial assemblages developed on respective MPs were compared based on unweighted UniFrac distances (phyloseq v1.38.0) conducted on ASV abundance. Similarities and difference between respective microbial assemblages were visualize via principal coordinates analysis (PCoA) plot (phyloseq v1.38.0). Correlation analysis was performed to screen for relationship between AMR genes and selected functional and pathogenic taxa on genus level using corplot R package (v0.92). Regression random forest machine learning method based on randomForest R package (v4.7-1.) was further applied to assess the impact of microbial community onto AMR genes variability observed in the system (Liaw and Wiener, 2002). Quantification of changes in AMR genes abundance was calculated (Eq. (1)) as log absolute abundance difference between two respective inflow and outflow incubation points.

$$\log \text{ ARG removal} = \log(\text{ARG}_n) - \log(\text{ARG}_{n+1}) \quad (1)$$

Where ARG_n is the absolute abundance of AMR genes at the upstream incubation point and ARG_(n+1) is abundance at the next downstream incubation point.

3. Results and discussion

3.1. Microplastic-associated biofilm diversity and spatio-temporal dynamics

DNA was extracted from a total of 34 MP samples and seven quasi daily composite water samples collected at three different steps for the wastewater treatment process and at two different incubation times (MPs only). Sequencing revealed the presence of a total of 4690 taxa across all samples, showing spatial and temporal gradients of taxonomic diversity after successive inoculation at different wastewater treatment stages (Table S4). In experiments 1 and 2 MPs inoculated in sewage developed the least diverse microbial communities (Shannon, Chao1) indicating biofilm maturation with increasing diversity at longer incubation time. The highest community diversity was observed for MPs inoculated for 20 days in secondary clarifier effluent (SCE) and the CW, respectively, which carried over already established biofilms from previous treatment stages. CW water samples showed a much lower diversity (Shannon = 3.35) than the biofilm developed on MPs in the CW (Shannon = 4.44 ÷ 5.78) during experiment 3 (Table S4) possibly due to the low suspended solids concentration and turbidity in the water column (Table S1). In the same environment, the free-floating bacterial community is typically less diverse than particle-associated assemblages forming the biofilm (Liu et al., 2019; Savio et al., 2015).

Biofilm communities on MPs in experiments 1 and 2 formed distinctive groups reflecting spatial and temporal diversity as visualized by an unweighted UniFrac PCoA plot (Fig. 2). In experiment 1 (Fig. 2a), the 20-day grown biofilm showed close similarities with planktonic communities present at RWW and SCE while CW MPs showed closer similarities to SCE biofilm than CW planktonic composition. For 4 d incubation qualitative similarities between raw sewage, SCE and CW were apparent. After a combined retention of 8 d in SCE and CW, MPs carried raw wastewater derived microbial community, with little alteration caused by the respective treatment process. In experiment 2 (Fig. 2b),

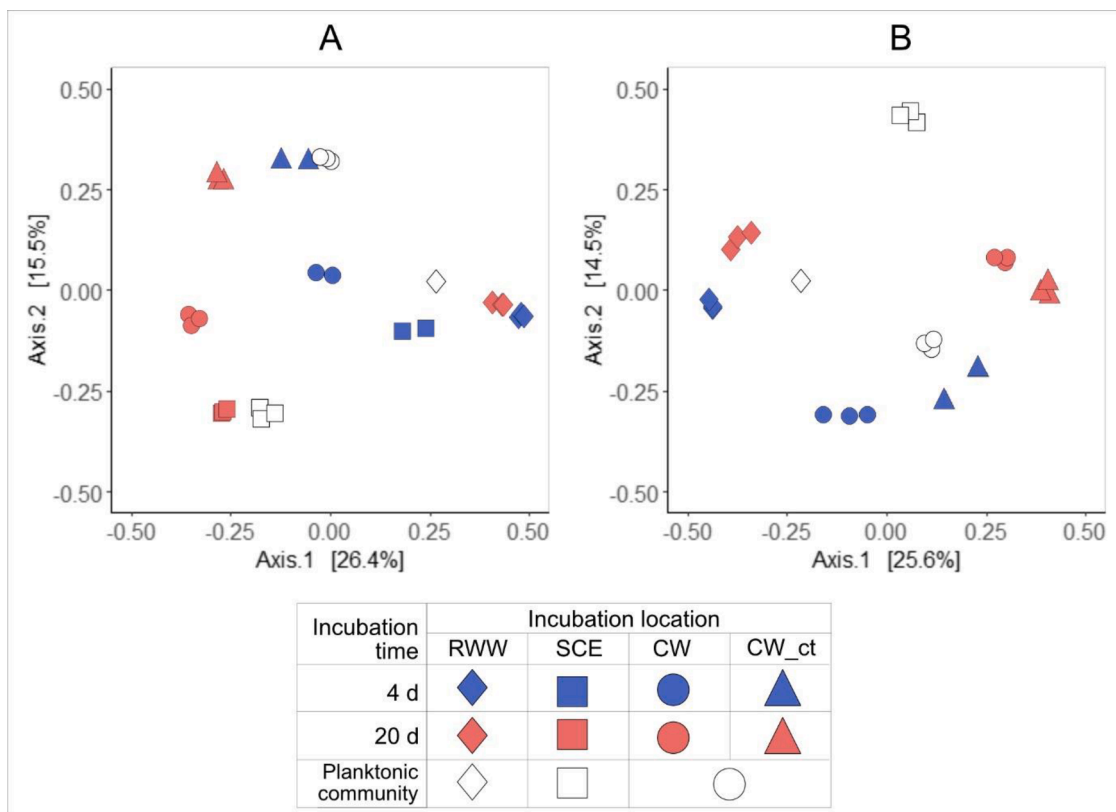


Fig. 2. PCoA analysis (based on UniFrac distances) of spatial (treatment step) and temporal (incubation time) characterization of MP-associated biofilm, and planktonic communities observed during experiment 1 (A) and experiment 2 (B). Data from experiment 3 included as CW_ct for reference. The eigenvalues of axis 1 and axis 2 presented in square parenthesis indicate the % variation of the dataset captured by the given axis. The distance between the data points on the ordination plain indicate the similarity between the points: the smaller the distance the greater the similarity of bacterial community composition between a given pair of samples.

biofilm composition comprised 2 groups separating CW and RWW results.

biofilm evolution in the SCE. Forced aeration in wastewater treatment provides different aerobic-anaerobic metabolic niches in a high nutrient environment, supporting diverse consortia development (Cao et al.,

The overall distribution of data points on the PCoA plot may indicate

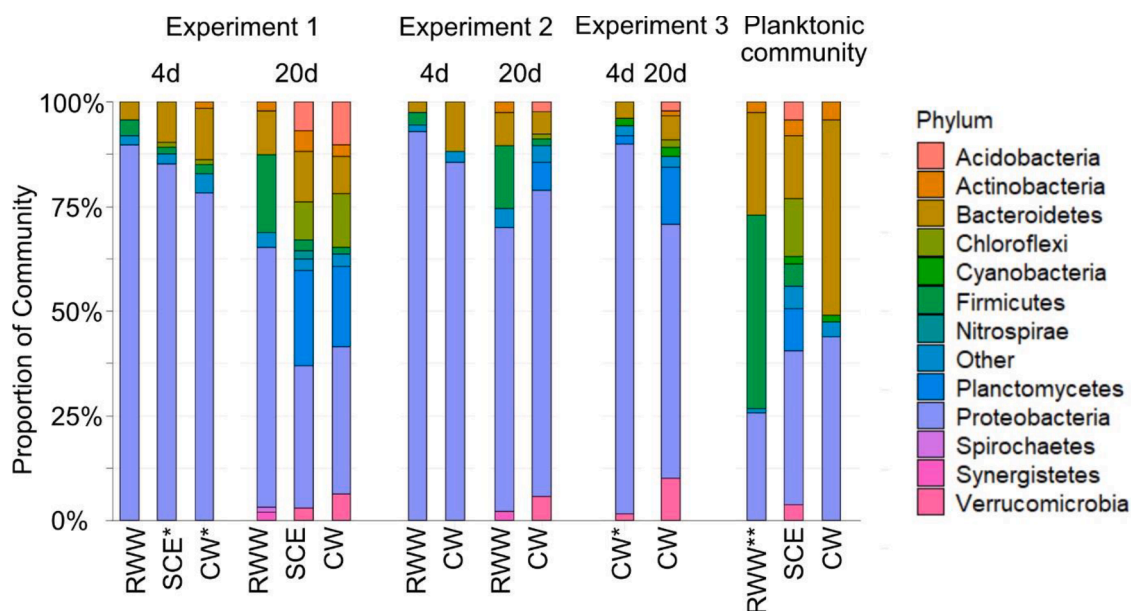


Fig. 3. Phylum level community composition of MPs associated biofilm and water samples collected at different stages of wastewater treatment (RWW- raw wastewater, SCE- secondary clarifier effluent, CW- constructed wetland) and incubation times for MPs (20 d and 4 d). Presented Phyla groups constitute >1% of community composition, otherwise grouped as “Other”. All samples were analyzed in triplicates (n = 3), unless marked otherwise: * =2 replicates; ** = single replicate. Analytical error bars omitted for clarity, mean standard deviation for replicates was 1.53 ± 0.20% for the normalized data for each Phylum shown.

2017; de Kreuk et al., 2005; Wilén and Balmér, 1999). Our data show that some of the original sewage microbial community is preserved on the 4-day incubated MPs. However, biofilm communities that developed initially on MPs immersed in sewage are less adaptable when transferred directly into the CW as indicated by the dissimilarities shown on the PCoA plain (Fig. 2b). This is probably due to fast transition from a nutrient rich low oxygen environment (RWW) into an environment with higher oxygen concentrations and fewer readily available nutrients (CW) (Ghattas et al., 2017; Santos Soares et al., 2022).

Despite indications of potential biofilm preservation, each treatment step left a unique footprint on a microbial community on the MPs, as shown by constrained analysis of principal component (CAP; text S2.2) which was used to assess the impact of experimental design variables on the microbial community composition. CAP showed the combined effect of incubation time and location (treatment step) which explained 83.5% and 79.8% of community composition variability for experiment 1 and experiment 2, respectively.

3.2. Microplastic-associated biofilm community composition

Proteobacteria emerged as primary colonizers of MPs making up ca. 90% of the biofilm community composition after 4 d contact time with raw sewage (RWW) (Fig. 3). With increasing incubation time (20 d), relative abundance of Proteobacteria decreased to 60%, with distinct changes in major proteobacterial families. Moraxellaceae and Campylobacteraceae were dominant (>65%) after 4 d incubation while 20 d incubation shifted proteobacterial representation towards Comamonadaceae and Rhodocyclaceae (60%) (Figs. S4 and 5). Proteobacteria represent a wide range of different types of metabolism and have been observed as key biofilm forming microbial taxa on MPs in various environments (Guo et al., 2022; Lee et al., 2008; Miao et al., 2019). That includes also known plastic degrading species as discussed in more detail in supplementary material (text S1, Fig. S8). The raw wastewater inoculated biofilm showed a significant increase in gram-positive bacteria after 20 d representing Firmicutes (16.7%) and Actinobacteria (2.3%). Subsequent incubation of MPs at SCE for 4 d did not affect the phylum level diversity but showed a significant decrease of Campylobacteraceae (<5%) and emergence of Pseudomonadaceae (15.8%). The taxonomic shift observed after 20 d in SCE was more pronounced and resulted in acquisition of Planctomycetes (22.7%), Chloroflexi (9.0%) and Acidobacteria (6.9%). Planctomycetes, Chloroflexi and Acidobacteria are key phyla in biological wastewater treatment, including within oxidation ditches, as studied here (Zhang et al., 2019). Planctomycetes comprised mainly of Pirellulaceae (37%) and Planctomycetaceae (36%), which include known ammonia oxidizers critical also in the Anammox process (dos Santos et al., 2021). Filamentous Chloroflexi were represented by the A4b family (36%). Chloroflexi form and stabilize microbial flocks in activated sludge, while Acidobacteria species largely contribute to phosphorus and nitrogen removal processes (Kristensen et al., 2021; Nierychlo et al., 2019). MPs transferred from WWTP into the CW (experiment 1) showed minor community shifts with acquisition of Verrucomicrobiaceae and photoautotrophic Rhodocyclaceae. Both families are widely found in sediments of wetland ecosystems (Ansola et al., 2014) indicating population exchange and migration between wetland sediment and planktonic communities.

Direct transfer of MPs from RWW into the CW (experiment 2) manifested in shifts in the anaerobic population. This included obligate anaerobic Firmicutes (Veillonellaceae, Fusibacter, Anaerovorax, Trichococcus) replaced by Planctomycetes and Bacteroidetes (Fig. 3). Proteobacteria (75-90% of population) were maintained irrespective of the incubation time and unlike to experiment 1, where the Proteobacteria decreased sharply (85% to 34%). Family Comamonadaceae showed to be most adapted to CW environment almost doubling its relative abundance (ca 30%) irrespective the incubation time. At direct deployment of MPs into the CW (experiment 3) biofilms after 4 d were initially dominated by Proteobacteria and Bacteroidetes (92.2% total).

After 20 d, the community also comprised of Planctomycetes (13.7%), Verrucomicrobia (10.1%), Chloroflexi (1.8%) and Acidobacteria (2.1%). MPs also carried Cyanobacteria (1.8-2.1%) presumably due to algal blooms observed in the CW (Fig. S7). Algal blooms are a common operational problem of CWs often indicating hydraulic mismanagement (i.e. stagnant water zones) or lack of adequate plant shading (Healy and Cawley, 2002). MPs harboring Cyanobacteria may facilitate spread of undesired algal blooms in downstream freshwater ecosystems (Curren and Leong, 2020).

The planktonic community was dominated by Bacteroidetes with significantly ($p < 0.05$) lower abundance of Proteobacteria (Fig. 3). The raw wastewater planktonic community was dominated by four bacterial families (Lachnospiraceae (23.8%), Ruminococcaceae (13.9%), Comamonadaceae (13.7%) and Prevotellaceae (11.9%)) (Fig. S6). Water samples collected from WWTP effluent were diverse with appreciable population of Comamonadaceae (7.8%), Flavobacteriaceae (4.8%), Procabacteriaceae (4.8%), Pirellulaceae (4%) and Moraxellaceae (3.4%) and 40 different families contributing 1-2% to community composition. The diversity of clarifier effluent (SCE) was in sharp contrast to the CW community composition where Flavobacteriaceae and Comamonadaceae comprised 75% of community composition and ten bacterial families >1% relative abundance (Fig. S6).

3.3. Fate of pathogens in microplastic-associated biofilms

MPs in raw sewage were colonized by up to 25% in relative abundance by genera comprising antimicrobial resistant ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (de Oliveira et al., 2020; Lai et al., 2022). Some WHO list priority pathogens. (*Neisseria*, *Campylobacter* and *Aeromonas*) were also found (Fig. 4). The overall relative abundance of pathogenic genera was highest for MPs incubated in raw wastewater. Transition of MPs through wastewater treatment stages led to a steady decrease of this potentially pathogenic population. At 20 d incubation, relative abundance of *Acinetobacter* species decreased over 2 orders of magnitude at SCE and CW passage (Fig. 4) and a 5-10-fold decrease of relative abundance of *Klebsiella* species. The observed loss of *Klebsiella*, contrasts a study reporting high survival on MPs in WWTPs (Kelly et al., 2021). Compared to planktonic bacterial communities, MPs biofilm had a 1-2 orders of magnitude higher relative abundance of pathogens (Fig. 4), supporting observations that MPs may aid the survival of pathogens (Shen et al., 2021). Nevertheless, the studied WWTP-CW system lowers pathogen export via MPs. This observation in line with typical trends for microbial pathogens reduction of conventional wastewater treatment processes and in constructed wetlands (Alexander et al., 2015; Wu et al., 2016).

Predatory activity might also play role in shaping pathogenic population on MPs biofilms. MPs biofilms at SCE and CW showed high populations of Bdellovibrionaceae. This family contains several predatory/parasitic genera such as *Vampirococcus* or *Bdellovibrio* (Guerrero et al., 1986) which prey on gram-negative bacterial hosts including human pathogens such as *E. coli*, *Salmonella*, *Legionella* and *Pseudomonas* species (Sokkett and Lambert, 2004). MPs incubated in treated wastewater at SCE and CW sampling points had an observable relative abundance (0.1-0.6%) of Bdellovibrionaceae dominated by aerobic genus of *Bdellovibrio* with *B. bacteriovorus* species. The relative abundance of Bdellovibrionaceae increased with incubation time (experiment 1 and 2) and along treatments stages (experiment 1). Bdellovibrionaceae were also observed in the planktonic community of SCE and CW (0.14% and 0.02%, respectively) and control MPs (0.08-0.21%) in experiment 3.

A screening for known fish pathogens found a minor population (0.1-1%) of *Aeromonas* species identified as *Aeromonas hydrophila* or *Aeromonas salmonicida*, both known to cause ulcer disease in infected fish (Viršek et al., 2017). *Aeromonas* species were present after 4 d incubation at WWTP (SCE) and CW but were not observed after 20 d of

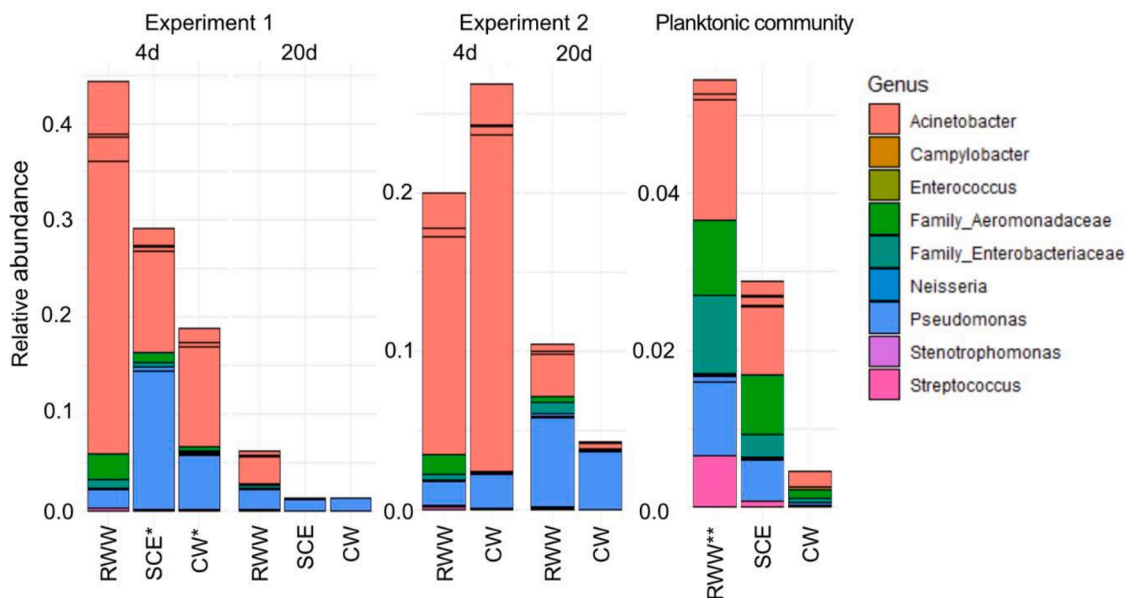


Fig. 4. Relative abundance of genera comprising key pathogenic species (i.e. ESKAPE group) observed in MPs associated biofilm and water samples at different stages of wastewater treatment (RWW- raw wastewater, SCE- secondary clarifier effluent, CW- constructed wetland) and incubation times (20 d and 4 d). All samples were analyzed in triplicates ($n = 3$), unless marked otherwise: (*) – 2 replicates; (**) – single replicate. Analytical error bars omitted for clarity, standard deviation for replicates was $0.48 \pm 0.13\%$ for the non-normalized data.

incubation. Sequencing data also revealed presence of *Stenotrophomonas* and *Lactococcus* genera that include known fish pathogens (Pękala-Safińska, 2018). Latter were found in very small numbers (<0.01%). Previous studies found fish pathogens (i.e. *Aeromonas salmonicida*) on MPs incubated in raw municipal wastewater (Lai et al., 2022). These findings confirm that MPs carrying pathogens are not only

posing threat for human health but also for other aquatic organisms (Li et al., 2022).

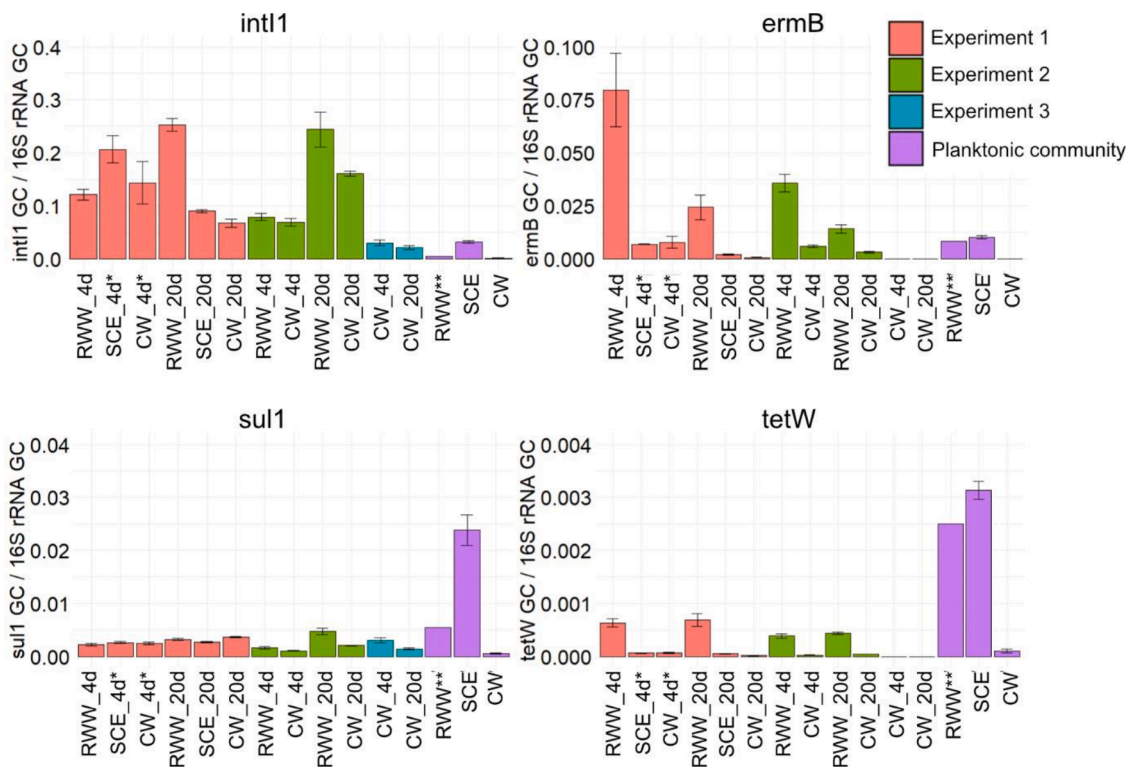


Fig. 5. Relative abundance of selected AMR genes (*ermB*, *tetW*, *sul1*) and AMR associated *int11* gene observed in MPs associated biofilm and water samples at different stages of wastewater treatment (RWW- raw wastewater, SCE- secondary clarifier effluent, CW- constructed wetland), incubation times (20 d and 4 d) and experiments (1-3; water samples). All samples were analyzed in triplicates ($n = 3$), unless marked otherwise: (*) – 2 replicates; (**) – single replicate. Absolute abundance data with standard errors are presented in SM Table S5.

3.4. AMR dynamics in microplastic-associated biofilms

3.4.1. Fate of AMR on MPs associated biofilm

MPs transferred through all three stages of wastewater treatment developed a stable biofilm biomass ranging between 107 and 109 16S rRNA gene copies (GC)/1g MP (Table S5). At equal incubation time the number of 16S rRNA gene copies was almost 2 orders of magnitude higher on MPs inoculated in raw sewage compared to MPs incubated in the CW only (experiment 3). Fig. 5 shows the relative AMR gene abundance of each investigated AMR gene in relation to the respective 16S rRNA gene copies. Throughout the treatment process (experiment 1 and 2), *intI1* relative abundance varied by the factor of 4, ranging between 6.68×10^{-2} and 2.55×10^{-1} . Similar dynamics were observed for the *sul1* gene, in which *sul1* gene levels remained within the range of 1.09×10^{-3} to 4.58×10^{-3} and relative abundance never exceeded a factor of three between treatment steps. Neither *intI1* nor *sul1* showed a stable decrease of relative abundance following treatment processes indicating lack of treatment-step specific selective pressure. Between treatment steps *ermB* and *tetW* dynamics exhibited more than one order of magnitude variability. The loss of *ermB* and *tetW* genes was most pronounced between RWW and SCW in both experiment 1 and 2. In experiment 1, SCE showed a relative abundance of *tetW* and *ermB* in downstream CW without significant changes ($p > 0.05$) between treatment steps. In experiment 3, MPs incubated in the CW had negligible presence of *ermB* and *tetW* (relative abundance $< 10^{-6}$ ARG GC/16S rRNA gene), 2 orders lower than respective CW samples (experiment 1 and 2). The *sul1* gene relative abundance was similar across all three experiments. AMR gene dynamics of the planktonic community were dominated by the WWTP which enriched the final effluent AMR (SCE). However, the CW planktonic community exhibited a 1-3 orders lower relative abundance of AMR genes than the SCE.

Changes in relative AMR abundance may serve as indicator for MPs biofilm resistome alteration during treatment. To assess the role of MPs

as a transport route for AMR bacteria, the ratio of AMR relative abundance in biofilms and the respective planktonic community was calculated (Fig. 6). The calculated ratio indicates whether there is a selective growth and enrichment of AMR bacteria on either MPs or in the planktonic community. Data show no clear enrichment pattern across all investigated AMR genes but rather gene-specific behavior. Depending on treatment stage and incubation time, *ermB* and *intI1* genes were between 2 and 100 times (< 2 log ratio) enriched on MPs, while *tetW* relative abundance was always lower on MPs (2-50-fold lower) compared to the planktonic community. No clear pattern was found for *sul1*.

Ambiguous fate of AMR on MPs was reported in disinfected wastewater effluent with higher abundance of *qnrS* and *ermB* genes in the planktonic community compared to the MPs biofilm, while *sul2* showed the opposite trend (Galafassi et al., 2021). Similarly, Martínez-Campos et al. (2021) found that depending on polymer type and applied treatment process, presence of *sul1* and *tetM* AMR genes varies between MPs biofilm and planktonic community without clear pattern. The dynamics of AMR acquisition by biofilm can be determined by MPs sorption of antibiotics or slow-release toxic additives and intermediates with often antimicrobial properties, thus creating a selective growth environment promoting specific AMR bacteria and triggering different resistance mechanisms (Atugoda et al., 2021; Syranidou and Kalogerakis, 2022; Su et al., 2021). Based on findings of this study it cannot be excluded that MPs biofilm may exert a positive effect on AMR spread at gen-level, considering that the observed relative abundance of *intI1* was consistently higher (2-100-fold difference; Fig. 6, Table S8) on MPs. Future studies should incorporate high-throughput sequencing to obtain full insight into a biofilm resistome responses to the temporal and treatment-specific variability in wastewater treatment process

3.4.2. AMR removal and community composition correlation

Regression random forest analysis shows AMR gene-specific

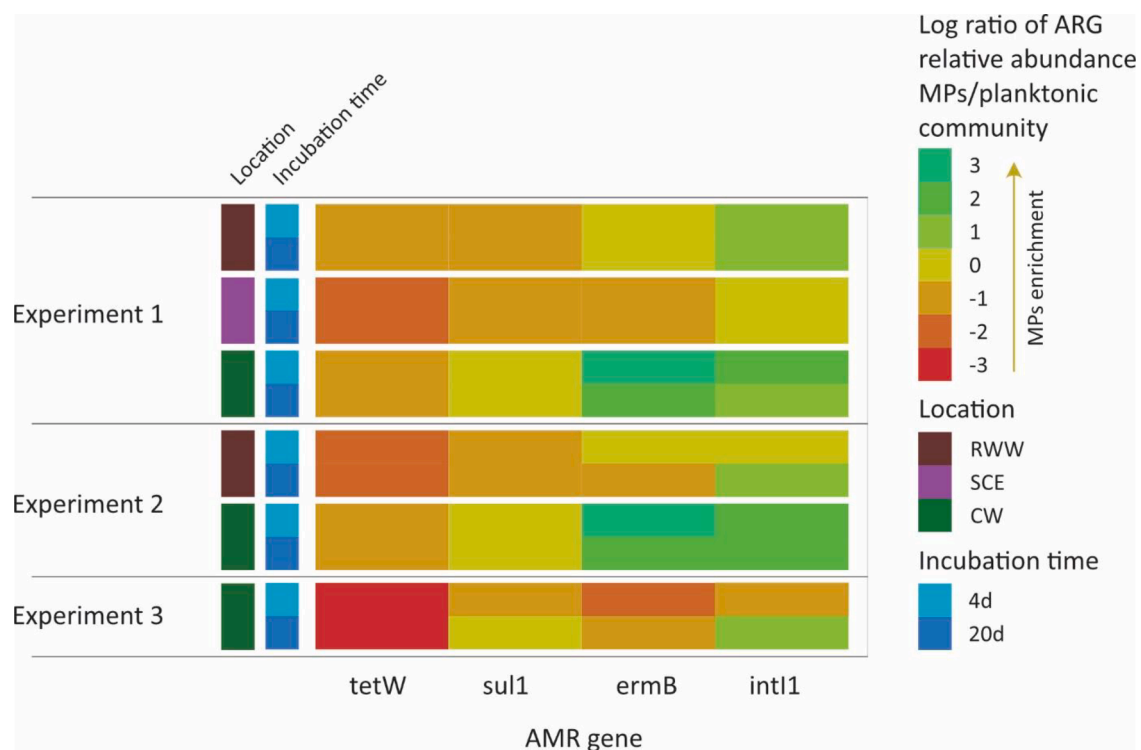


Fig. 6. Heatmap of AMR (*tetW*, *ermB*, *sul1*) and AMR associated (*intI1*) relative gene abundance ratio (relative abundance within biofilm community divided by relative abundance within planktonic community) at the different treatment stages (RWW- raw wastewater, SCE- secondary clarifier effluent, CW- constructed wetland) during colonization experiments. Log ratio values > 0 and < 0 correspond to higher relative abundance of the respective gene on MPs biofilm and in the planktonic community, respectively. See Table S8 in supplementary material for numeric values.

dynamics (Table S6). Changes of the microbial community during the treatment process explained almost 80% (79.55%) of tetW observed variability, compared to 54% of sul1 gene dynamics. ErmB and intI1 dynamics were both equally affected (69-70%) by differences in community composition between treatment stages. Only six unique genera were strongly correlated ($r > 70\%$, $p < 0.05$) with sul1; none of them shared correlation with other AMR genes (Fig. 7). Sul1 was the least affected by the shifting environmental conditions between treatment steps with the absolute abundance never decreasing more than 1 log unit (<90%) (Table 1). The observed relative persistence of sul1 can be attributed to persistence of sulfonamides (resistance determinants) which are poorly removed in WWTPs thus triggering and maintaining selective pressure on the microbial community throughout the treatment process (Zhou et al., 2022). Sulfonamides were present in the studied system (unpublished data). Additionally, sul1 has high dissemination potential due to its mobility associated with broad range of plasmid types and relatively low metabolic burden for the host ensures preservation even in the absence of sulfonamides (Jiang et al., 2019; Wu et al., 2010). TetW and ermB strongly correlated ($r > 70\%$, $p < 0.05$) with 47 genera, while sharing 23 genera including Bacteriodes, Faecalibacterium or Lactococcus. Both tetW and ermB exhibited similar dynamics decreasing by 1-2 log units (90-99%) along treatment stages and with incubation time. Higher tetW and ermB decrease might be due to the physicochemical properties of tetracycline and erythromycin respectively which are typically effectively immobilized in WWTPs due to its high adsorption affinity and biodegradation potential (Liu et al., 2019; Shao and Wu, 2020). The fact that ermB and tetW dynamics were explained in high degree by the community composition shifts indicates on host-specific relationships. These are determined by environmental/treatment conditions that also affects concentrations of antibiotics (Oberoi et al., 2019). IntI1 had a total of 28 correlated genera, primarily anaerobic species belonging to Fusibacter, Desulfobacter or Anaerovorax. Dissemination of IntI1 was limited (>1 log reduction) by the WWTP environment but only at prolonged incubation (20 d), otherwise the intI1 gene abundance was relatively unaffected (>0.5 log decrease) or occasionally increasing (Table 1). AMR genes were also correlated with pathogenic bacteria (Table S7). tetW and ermB correlated with Aeromonas, Klebsiella and Streptococcus ($r > 50\%$, $p < 0.05$). ermB correlated with Acinetobacter ($r = 74\%$, $p < 0.05$). IntI1 correlated only with Pseudomonas ($r = 46\%$, $p < 0.05$). sul1 did not show significant correlation with any investigated pathogenic genera (Table S7), except negative correlation with Acinetobacter ($r = (-51\%)$, $p < 0.05$). Human fecal marker (HF183) correlated with tetW but not

with ermB. In our study presence of pathogenic genera did not provide clear predictive measure for presence of AMR. Increased sequencing depth could potentially uncover more detailed dynamics in future studies. Nevertheless, the observed level of correlation emphasizes the extend of AMR acquisition and transfer beyond the obvious pathogenic hosts (Dionisio et al., 2023). That coincides with several reports showing complexity of AMR surveillance programs which need to consider new dissemination factors for predictive analysis and control measures (Oniciuc et al., 2018; Pehrsson et al., 2016). This is particularly important since AMR host identification may play a critical role helping to target key AMR reservoirs via pathogen-specific phage therapy or engineered lysins (Rice et al., 2020).

3.5. The effect of CW on mitigation of microplastic-associated pathogen and AMR spread

Unlike some modern conventional WWTPs, CWs are not equipped with dedicated disinfection steps to control pathogen and AMR export. Nevertheless, there are several processes that can effectively limit survivability and dissemination of pathogens and AMR in CWs, including photoinactivation, sedimentation and predation (Wenk et al., 2019; Wu et al., 2016). In this study, the role of CW as a primary recipient of sewage contaminated MPs was simulated in experiment 2. The results show that observed decreases of MP-associated AMR in CW were less affected by the incubation time (4 d vs 20 d) compared to the WWTP (SCE samples in experiment 1). The average difference between 20 d and 4 d in the CW was (-0.1) log decrease in comparison to 0.91 log decrease observed at WWTP in experiment 1 (Table 1). Although, the CW exhibited a higher decrease in MP-associated AMR in the first 4 d of contact time, the WWTP outperformed the CW when MPs were incubated for a longer period. The WWTP exerted increasing pressure on the MP associated AMR community with increasing incubation time, while the CW showed gene- and time-dependent variability. The best performance was achieved when combining the WWTP with the CW polishing treatment that led to over 99% decrease of AMR carried by MPs (experiment 1). This strengthens the case for using CWs as polishing units coupled with conventional WWTPs (Liu et al., 2015). Complete retention of MPs in CWs is not feasible due to the design limitations of the system (Bydalek et al., 2023). However, the observed pathogen and AMR neutralizing capacity on MP biofilms during CW passage may reduce the immediate microbiological threats on receiving water bodies' ecosystems. The observed effectiveness of CW could be compromised by less favorable weather conditions in the wintertime due to lower

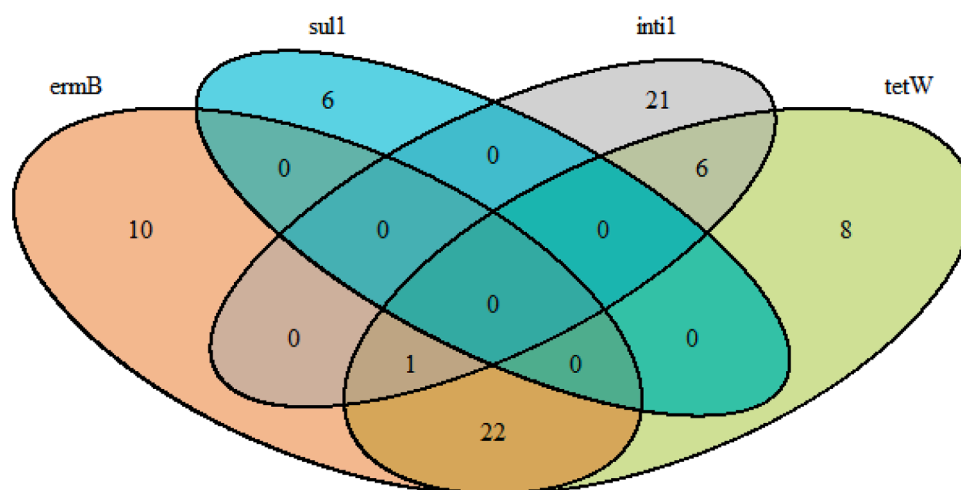


Fig. 7. Graphical representation (Venn diagram) of number of shared and unique bacteria genera showing strong correlation ($r > 70\%$, $p < 0.05$) with dynamics of AMR genes (tetW, ermB, sul1) and AMR associated intI1 gene in MPs associated biofilm during wastewater treatment in the investigated treatment. Correlations were made based on the relative abundances of selected genus and AMR gene.

Table 1

Log unit downstream decrease of AMR genes (tetW, ermB, sul1) and AMR associated intI1 gene along treatment stages based on absolute abundance of MPs associated biofilm and planktonic AMR genes at respective stages (SCE- secondary clarifier effluent, CW- constructed wetland).

Experiment	Incubation time	Treatment stage			AMR gene decrease (log unit decrease)			
					tetW	sul1	intI1	ermB
1	20 d	RWW	→	SCE	1.91 ± 0.04	0.88 ± 0.05	1.26 ± 0.08	1.88 ± 0.08
		SCE	→	CW	0.57 ± 0.16	0.04 ± 0.13	0.21 ± 0.23	0.58 ± 0.20
	4 d	RWW	→	SCE	1.12 ± 0.08	0.05 ± 0.07	-0.1 ± 0.1	1.17 ± 0.05
		SCE	→	CW	0.20 ± 0.17	0.24 ± 0.13	0.35 ± 0.24	0.15 ± 0.20
2	20 d	RWW	→	CW	1.28 ± 0.10	0.61 ± 0.05	0.43 ± 0.09	0.91 ± 0.04
	4 d	RWW	→	CW	1.58 ± 0.06	0.57 ± 0.05	0.46 ± 0.07	1.18 ± 0.04
Planktonic sample	N/A	RWW	→	SCE	2.65 ± 0.03	2.12 ± 0.05	1.91 ± 0.02	2.67 ± 0.05
	5.3 ± 0.2 d*	SCE	→	CW	1.32 ± 0.16	1.48 ± 0.08	1.23 ± 0.10	3.25 ± 0.08

*Hydraulic retention time estimated based on the CW design dimensions and average flow measurement during July-September 2021.

temperatures, limited solar irradiation and possible hydraulic overload due to rain events which would lower retention time. Therefore, future studies should address seasonal variability of the fate of MP-associated biofilm in wastewater treatment systems including specifically CWs which are known to show seasonal performance variability (Myszograj et al., 2018; Varma et al., 2021).

4. Conclusions

- The position of CW as a polishing step was beneficial to further neutralize the MP-associated AMR load exported from the WWTP.
- Increased MP incubation time within the WWTP and CW help eliminate both AMR contamination and potentially pathogenic bacteria belonging to the genera *Pseudomonas*, *Arcobacter*, *Acinetobacter* and *Streptococcus* and *Klebsiella*.
- MPs associated with AMR showed gene-specific dynamics. *Aeromonas*, *Klebsiella*, and *Streptococcus* were key pathogenic genera correlated with presence of AMR in MP biofilms.
- Despite observed reduction in pathogens and AMR load, MPs exported from WWTPs and CWs can be a source of fish pathogens (*Aeromonas*, *Stenotrophomonas* and *Lactococcus*) and Cyanobacteria potentially affecting downstream ecosystems.
- As a primary treatment unit, the CW delivered a consistent AMR decrease independent from MPs retention time (4 d or 20 d), however the CW showed a variable effect on removal of pathogenic genera.
- As a primary treatment step the CW was less efficient in neutralizing AMR and pathogens on MPs than the WWTP equipped with an oxidation ditch.
- In combination the WWTP and the CW can provide over 99% decrease of AMR and pathogenic load carried with MPs from sewage.

Funding and acknowledgments

Franciszek Bydalek was supported by a Natural Environment Research Council (NERC) studentship at the GW4 Centre for Doctoral Training in Freshwater Biosciences and Sustainability (GW4 FRESH CDT, Grant number NE/RO11524/1).

CRediT authorship contribution statement

Franciszek Bydalek: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Gordon Webster:** Methodology, Visualization, Writing – original draft, Writing – review & editing. **Ruth Barden:** Writing – review & editing. **Andrew J. Weightman:** Conceptualization, Resources, Supervision, Writing – review & editing. **Barbara Kasprzyk-Hordern:** Conceptualization, Supervision, Writing – review & editing. **Jannis Wenk:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing –

original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request. Raw genetic sequencing data are available at NCBI repository <https://www.ncbi.nlm.nih.gov/bio-project/940639> Accession: PRJNA940639

Acknowledgments

The authors would like to express their gratitude for Dr Like Xu from the University of Bath for providing AMR *E. coli* clones containing plasmids with AMR genes (intI1, sul1, tetW and ermB) to prepare qPCR standards.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.watres.2023.119936](https://doi.org/10.1016/j.watres.2023.119936).

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