Disentangling abiotic and biotic effects of treated wastewater on stream biofilm resistomes enables the discovery of a new planctomycete beta-lactamase

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Abstract

Antibiotic resistance, mediated by environmental reservoirs, poses a threat to human and animal health. Aquatic biofilms impacted by treated wastewater (WW) are known environmental reservoirs for antibiotic resistance, but it remains unclear if primarily biotic or abiotic factors from WW influence the abundance and dissemination of antibiotic resistance genes within biofilm communities. To disentangle these factors, natural biofilms were previously grown in flume systems with different proportions of stream water and either ultrafiltered or non-ultrafiltered treated WW. In this study, we conducted deep shotgun metagenomic sequencing of 75 biofilm, stream, and WW samples from these flume systems and compared microbiome and resistome composition. Our analysis revealed a strong relationship between the resistome and prokaryotic microbiome composition which was also associated with experimental treatment. Within biofilm communities exposed to increasing percentages of WW, we observed a decrease in the relative abundances of Cyanobacteriota and Verrucomicrobiota, while Bacteroidota increased. Notably, several antibiotic resistance genes (ARGs) exhibited a significant increase in abundance in biofilms grown with increasing WW%, while a small number of sulfonamide and beta-lactamase ARGs showed higher abundance when biofilms were instead grown in ultrafiltered WW. Overall, our results pointed toward the dominance of biotic over abiotic factors in determining ARG abundances in WW-impacted stream biofilms. However, some ARGs exhibited divergent abundance patterns through proposed gene family-specific mechanisms. To investigate these gene families experimentally, we biochemically characterized a new, sequence-divergent beta-lactamase from planctomycetes abundant in stream biofilms and in WW. This ARG discovery is noteworthy, not only due to its unusual source organism belonging to the phylum Planctomycetota, but also because it was not detected by standard computational ARG tools.

This underpins the need for continued development of ARG detection methods using workflows with *in silico* sequence and structure analysis coupled to *in vitro* functional characterization. In summary, this study assessed the relative importance of biotic and abiotic factors in the context of WW discharge and its impact on antibiotic resistance genes in aquatic ecosystems.

Keywords

Antibiotic resistance genes, Stream resistomes, Biofilms, Flume systems, Metagenomics, Beta-lactamases

Introduction

Antibiotic resistance is a major threat to public health. Pollution resulting from anthropogenic activities alters the abundance and proliferation of antibiotic resistance genes (ARGs) in environmental reservoirs (Singer et al., 2016). The rapidly increasing consumption of antibiotics and release of micropollutants (MPs) into the environment (Rizzo et al., 2013) stimulates the spread of antibiotic-resistance genes (ARGs), posing a threat to modern medical procedures and treatments that rely on effective antibiotics (Shallcross and Davies, 2014).

Aquatic ecosystems that receive effluent from wastewater treatment plants (WWTPs) are influenced by biotic factors, such as antibiotic-resistant bacteria released from WWTPs (Alexander et al., 2020) and abiotic factors (Rodriguez-Mozaz et al., 2015; Rogowska et al., 2020), such as nutrients and MPs that are not completely eliminated by conventional WWTPs (Mukherjee et al., 2021). Several recent studies demonstrated that exposure to WWTP effluent alters the taxonomic and ARG composition of microbial communities in downstream rivers (Carles et al., 2021; Desiante et al., 2022; Lee et al., 2023; Matviichuk et al., 2022; Perveen et al., 2023). However, disentangling the effects of abiotic and biotic factors on ARG abundance is challenging in the field. Here, to systematically distinguish abiotic and biotic factors, we built on a previous research study which used controlled flume systems to grow biofilms in ultrafiltered and non-ultrafiltered WWTP effluent diluted with different proportions of stream water (Carles et al., 2021; Desiante et al., 2022). Ultrafiltration (UF) treatment (pore size 0.4 μm) removed most microorganisms and particles (biotic factors) while maintaining consistent nutrient and MP levels, including antibiotics. Therefore, the UF treatment separating biotic from abiotic factors enabled investigation of the factors shaping the ‘resistome’, i.e., the total composition of antibiotic resistance genes (ARGs), of stream biofilms influenced by WWTP discharge.

Many studies on stream biofilms have relied on amplicon sequencing methods for taxonomic and/or ARG profiling (Carles et al., 2021; Depetris et al., 2022; Haenelt et al., 2023; Romero et al., 2019). As opposed to amplicon sequencing, short-read and long-read metagenomic sequencing studies of streams and stream biofilms (Ju et al., 2022; Lee et al., 2023; Reddington et al., 2020) do not introduce a polymerase chain reaction (PCR) amplification bias. As we demonstrate in this study, total metagenomic sequencing also enables the identification of sequence-divergent ARGs not detected by conventional primer pairs. Amplicon sequencing is

more sensitive in that it can aid in the detection of low abundance species, but it is also biased towards specific taxa during the PCR process (Brumfield et al., 2020; Tremblay et al., 2015). The PCR bias also limits the detection of some bacterial phyla such as the Planctomycetota for which the commonly-used 16S rRNA PCR primers do not properly bind (Klindworth et al., 2013; Oyarzúa et al., 2021). The Planctomycetota are a specific focus in this paper as an abundant phylum in WWTPs (Y. Zhang et al., 2023), lacustrine (Andrei et al., 2019), and riverine biofilm (periphyton) communities (Cai et al., 2021), which are undersampled by classical amplicon-based methods (Oyarzúa et al., 2021). We therefore conducted deep shotgun metagenomic sequencing of stream biofilms and water samples in this study, enabling us to sample less well-studied microorganisms and their functional genes including ARGs.

Here we demonstrated a combined computational and biochemical workflow to investigate the effects of treated wastewater (WW) on microbiome taxonomic composition and ARG composition in flume biofilms grown in natural stream water mixed with different proportions of ultrafiltered and non-ultrafiltered WW (Carles et al., 2021; Desiante et al., 2022). Specifically, we leveraged the functional genes identified through shotgun metagenomic sequencing to heterologously express an uncharacterized ARG from the phylum Planctomycetota. The enzyme displayed beta-lactamase activity despite sharing low sequence identity with previously identified ARGs. Overall, this study enabled dissection of the influences of biotic and abiotic factors on stream biofilm resistomes in order to characterize both known and new ARGs.

Methods

Biofilm cultivation and sampling

The flume systems and biofilm cultivation procedures used in two independent experiments to generate the samples sequenced in this study were described previously (Carles et al., 2021; Desiante et al., 2022). Briefly, the system consisted of flow-through channels fed with different mixtures of two different water sources: (1) a peri-urban stream (Chriesbach; Dübendorf, Switzerland) and (2) treated wastewater from a pilot-size wastewater treatment plant (100-person equivalents). In the first experiment (Exp. 1), the stream water was mixed with treated wastewater (WW) at the following percentages: 0% (control), 10%, 30%, and 80% WW. In the second experiment (Exp. 2), the 10% treatment was omitted and replaced by additional treatments with 30% and 80% ultrafiltered WW (UF), using a filter with a pore size of 0.4 µm to remove particles, e.g., suspended solids and microorganisms. Glass slides were added to the flow-through channels and biofilms naturally forming on the slides were allowed to develop for a period of four weeks. Water parameters (pH, temperature, conductivity, dissolved oxygen) were monitored weekly (Carles et al., 2021; Desiante et al., 2022).

DNA extraction and metagenomics assessment

Stream water, WW, UF WW samples and biofilms from glass slides were sampled as described previously (Desiante et al., 2022; Carles et al., 2022). Total genomic DNA was extracted with the DNeasy PowerBiofilm kit (QIAGEN) for a total of 75 samples to be submitted for metagenomic sequencing (**Table S1**). The quality and quantity of the DNA extracts were measured using a

NanoDrop One spectrophotometer. Extracted DNA samples were stored at -20 °C and shotgun metagenomic sequencing was performed using Illumina platform NovaSeq with a paired-end (2×150 bp; average of 20 Gbp raw data per sample) strategy. Library construction and sequencing was performed by Novogene (Cambridge, UK). The reads obtained from shotgun metagenomics and containing adapters and low-quality reads (N > 10% and quality score ≤ 5) were removed by Novogene, and the read qualities were double-checked by the authors using Fastp v.0.20.0 (https://github.com/OpenGene/fastp). After quality controls of metagenome reads, de-novo assembly was performed using MEGAHIT v1.2.9 (Li et al., 2015). Open reading frames (ORFs) were predicted from the assembled contigs using Prodigal v2.6.3 (Hyatt et al., 2010). Although metagenomes from both Exp. 1 and Exp. 2 were sequenced and the data for both are re-reported here, we focused on Exp. 2 for analysis since it included UF treatment, unlike Exp. 1.

Taxonomic profiling

The taxonomic composition of the metagenomes was profiled using a degenerate consensus marker-gene profiling tool, mTAGs v1.0.4, with 1000 maximum accepts and 1000 maximum rejects (Rognes et al., 2016; Salazar et al., 2021). mTAGs profiles for all samples were merged using mTAGs' merge function. Genus- and phylum-level per-sample count tables were used for subsequent analysis.

Reads-based ARG detection and ordination analyses

The ARG resistome was profiled using the DeepARG v1.0.2 short reads pipeline with default settings (Arango-Argoty et al., 2018). ARG counts normalized by the 16S-rRNA counts within the output files for each sample were merged into an abundance table. All data analysis and visualization were done in R v4.3.0. Data manipulation and visualization were done using the tidyverse package (Wickham et al., 2019). Principal coordinate analysis (PCoA) of microbiome composition was done using the genus abundance table. Unaligned and unassigned reads were removed. Genus counts were normalized using the trimmed mean of M-values (TMM) method within the EdgeR package (Pereira et al., 2018; Robinson et al., 2010; Robinson and Oshlack, 2010). Normalized genus counts were square-root-transformed. PCoA analysis of ARG resistome composition was done using the 16S-normalized abundance data. For both data sets, pairwise Bray-Curtis distances between samples were computed using the 'vegdist' function from the vegan package (Oksanen et al., 2007). PCoA was performed using the 'pcoa' function from the ape package (Paradis et al., 2004). Procrustes analysis comparing the PCoA vectors of the two data sets was done using the 'procrust' and 'protest' functions from the vegan package (Oksanen et al., 2007). The Kruskal-Wallis test (McKnight and Najab, 2010) was applied to test for significant correlations between the normalized average coverage of different ARGs and wastewater effluent proportions (WW%), and p-values were corrected for multiple hypothesis testing using the Benjamini-Hochberg method (Benjamini and Hochberg, 1995).

163 **ARG identification and coverage analysis from metagenome assemblies**

164 For a complementary and separate approach to the metagenomic short reads pipeline for ARG
165 analysis, predicted open reading frames (ORFs) from assembled metagenomes were identified
166 by protein BLAST v2.9.0 (McGinnis and Madden, 2004) search against the Structured Antibiotic
167 Resistance Genes (SARG) protein database v2.2, with e-value and query coverage cut-offs of
168 0.1 and 20, respectively (Yin et al., 2018). Using methods established by (Lee et al., 2021) for
169 detecting ARGs in riverine metagenomes, BLAST hits with an amino acid percent identity of over
170 85% and an alignment length of 100 amino acids were retained. BLAST hits were then clustered
171 using CD-HIT v4.6.8 (Fu et al., 2012; Li and Godzik, 2006), with parameters –n 5 and –c 0.7. The
172 102 non-singleton cluster representatives of SARG hits were selected for further analysis.
173 Metagenomic coverage of selected (clustered) BLAST hits within all metagenomes were
174 calculated based on methods previously described (Lee et al., 2021). Briefly, raw reads were
175 mapped back to BLAST hits using bowtie2 v2.4.4 (Langmead and Salzberg, 2012) using the
176 parameters shown in **Table S2**. Coverage depth was calculated using samtools v1.12 and
177 average coverage was calculated as described by (Albertsen et al., 2013). The average
178 coverages of the 102 ORFs were normalized to reads per million using the method described by
179 (Lee et al., 2021).

180

181 **Bioinformatic analyses of beta-lactamases**

182 Homologs of Planctomycetota ARGs were identified by BLAST v.2.9.0 (Camacho et al., 2009)
183 querying assembled proteins from the 75 metagenomes. Trends across different WW and UF
184 WW conditions were examined leading to prioritization of two metallo beta-lactamase homologs
185 as described in the results (NCBI accession numbers MBX3358097.1 and MBX3359331.1)
186 assigned to the *Phycisphaeraceae* (Planctomycetota). Structural modeling of MBX3358097.1 was
187 conducted using ColabFold AlphaFold 2.0 (Mirdita et al., 2022) with default parameters. Models
188 were assessed for quality based on predicted local distance difference test (pLDDT) scores and
189 aligned pairwise with the reference structure in the Protein Data Bank (PDB ID: 3NKS) using the
190 PyMOL 'super' command with default parameters.

191

192 **Cloning and protein expression**

193 For functional characterization, two full-length proteins (NCBI accession numbers
194 MBX3358097.1 and MBX3359331.1) were codon-optimized for *E. coli* codon usage using the
195 Integrated DNA Technologies Codon Optimization Tool (https://eu.idtdna.com/pages/tools/).
196 Signal peptides were detected using SignalP v6.0 (Teufel et al., 2022) and removed where
197 present. Genes (**Table S3**) were synthesized as gBlocks by Integrated DNA Technologies. The
198 genes were cloned individually by Gibson Assembly into the multiple cloning site 1 of the
199 pETDuet-1 vector (*ampR* resistance marker) with N-terminal hexahistidine tags (**Table S3**).
200 Constructs were transformed into *E. coli* DH5α cells and verified by Sanger sequencing
201 (Microsynth, Balgach, Switzerland). Sequence-verified constructs were transformed into *E. coli*
202 BL21(DE3) cells (New England Biolabs Frankfurt, Germany). Starter cultures (5 mL) were
203 grown in LB with 100 µg/ml ampicillin overnight at 37°C. From the starter cultures, 2.5 mL was
204 added to 400 mL LB with 100 µg/mL ampicillin in a 1 L baffled Erlenmeyer flask and grown to an

205 optical density of 0.5-0.8 at 37°C. Protein expression was induced by adding 0.1 mM isopropyl
206 β-D-1-thiogalactopyranoside (IPTG) and cultures were incubated for 18h at 16°C.
207
208 **Protein purification**
209 Expression cultures were poured into 50 mL Falcon tubes and centrifuged at 4000 rcf for 15 min.
210 Supernatants were decanted, and cell pellets were resuspended in 4 mL buffer A (20 mM Tris,
211 500 mM NaCl, 20 mM imidazole, 10% glycerol, pH = 8) per gram of pellet weight. Cells were lysed
212 by 2 cycles of sonication using a 6 mm needle, 20% amplitude and 10 x 10 second pulses with a
213 rest time of 10 seconds in between (total sonication time per cycle = 3 minutes). Lysates were
214 centrifuged at 20,000 rcf at 4°C for 1h. Cleared lysate was filtered through a 0.2 µm syringe filter,
215 loaded into a ÄKTA Pure system (Cytiva Europe GmbH, Glattbrugg, Switzerland) and injected
216 onto a HisTrap FF 5 mL column (Cytiva, Europe GmbH, Glattbrugg, Switzerland). The column
217 was washed with 20 column volumes of buffer A. His-tagged proteins were eluted in 3 column
218 volumes of buffer B (same as buffer A but with an imidazole concentration of 500mM), and 8 x 2
219 mL fractions were collected. The fractions with the highest protein concentrations were
220 determined qualitatively by Bradford assay (Bradford, 1976). Imidazole was removed by running
221 the sample over a PD-10 desalting column (Cytiva Europe GmbH, Glattbrugg, Switzerland) and
222 eluting in buffer (5 mM Tris, 30 mM NaCl, 10% glycerol, 0.1 mM $ZnCl_2$, pH = 8). $ZnCl_2$ was added
223 because the enzyme of interest is suspected to be Zn^{2+}-dependent. The purified protein was flash
224 frozen using liquid nitrogen in 200µl aliquots and subsequently stored at -80°C.
225
226 **β-Lactamase activity assay**
227 Purified MBX3358097.1 was tested with standard β-lactamase activity detection assays using the
228 chromogenic cephalosporin analog, nitrocefin (O'Callaghan et al., 1972). Nitrocefin was prepared
229 in a 40 mM stock solution in DMSO and then diluted in Tris buffer to prepare a 1 mM working
230 solution. Briefly, standard nitrocefin assays were run in a 96-well plate by combining 180µl 20 mM
231 Tris buffer with 0.1 mM $ZnCl_2$ (pH = 7.0) with 10 µL purified enzyme and 10µL nitrocefin working
232 solution. The plate was incubated in a plate reader (BioTek Synergy H1, Agilent) at 25°C, and
233 absorbance at 482 nm was measured every minute for 2h. As negative controls, boiled enzyme
234 and buffer-only conditions were included in every assay. To obtain the boiled enzyme control,
235 aliquots of purified protein were incubated at 100°C for 15 minutes. Reaction rates were quantified
236 from continuous measurements as described previously by Ryu et al. using the molar extinction
237 coefficient of nitrocefin (12,124 M^{-1} cm^{-1}) in 20 mM Tris (Ryu et al., 2016).

238 Results and discussion

239 Biotic factors appear to be predominantly responsible for altered community 240 composition in biofilms grown in WWTP effluent

241 We deeply sequenced metagenomes of 75 total biofilm and water samples to an average
242 sequencing depth of 138 million reads per sample (max. 204 million reads). After assembly, we
243 first used the metagenomic data to analyze the taxonomic composition of stream biofilm
244 communities to assess whether community composition was correlated with experimental

treatments: 30% WW, 80% WW, 30% UF WW, 80% UF WW, and stream water (0% WW). Experimental treatment was correlated with taxonomic composition for both prokaryotes (PERMANOVA, $R^2 = 0.65$, $p < 0.001$) (**Figure 1**) and eukaryotes (PERMANOVA, $R^2 = 0.70$, $p < 0.001$) (**Figures S1-S3**) at the genus level (Salazar et al., 2021). Growth in 80% WW relative to stream water (0% WW) altered prokaryotic biofilm community composition, namely we observed a decrease in the relative abundance of *Cyanobacteria* and *Verrucomicrobiota* and an increase in the relative abundance of *Bacteroidota* (**Figure 1**), in agreement with Carles et al. (2022). These shifts also mirror changes in microbial communities previously described downstream of WWTPs relative to those upstream (Matviichuk et al., 2022). Fold changes between UF and non-UF WW biofilm samples, and in other lower abundance taxa, e.g., the Planctomycetota, are further discussed by Carles et al. (2022).

The taxonomic composition of biofilms grown in UF wastewater is more similar to biofilms grown in 0% WW than biofilms grown in the same fraction of non-UF wastewater, suggesting that biotic factors from WWTPs are responsible for community changes as opposed to abiotic factors (**Figure 2, Figure S3**). This metagenomic analysis highlights that WW alters stream biofilm composition and supports the findings of Carles et al. (2022) that some microorganisms from WW were able to colonize and modify biofilm community composition.

Resistome composition correlates with microbiome composition

We next examined how the total set of ARGs detected in each sample - the ‘resistome’ - varied with experimental treatment (e.g., WW% and non-UF vs. UF). Resistome composition was significantly affected by experimental treatment (PERMANOVA, $R^2 = 0.60$, $p < 0.001$), much like microbiome composition (**Figure S4**). Biofilms exposed to 30% or 80% UF WW clustered with 0% WW samples as opposed to samples exposed to equivalent amounts of non-UF WW (**Figure 2**).

To test for a relationship between microbiome and resistome composition, we used Procrustes superimposition of resistome PCoA vectors onto prokaryotic microbiome PCoA vectors (**Figure 2**). Procrustes analysis revealed that the composition of the resistome was structurally related to the composition of the prokaryotic microbiome. The low Procrustes sum of squares was 0.1889 which indicated a strong alignment together with the calculated correlation value of 0.9 in a symmetric Procrustes rotation (p-value < 0.001). The alignment between microbiome and resistome has been previously observed in microbial communities downstream of WWTPs (Lee et al., 2023; Matviichuk et al., 2022), but local environmental features of different sampling sites are potential confounding factors that may have determined both microbiome and resistome composition in the field. Here we demonstrated a microbiome-resistome relationship in a controlled experiment with biofilms grown in a defined gradient of stream water to WW and ultrafiltered WW matrices. Generally, the rotated PCoA vectors for the resistome are closer to the prokaryotic microbiome PCoA vectors for biofilm samples than they are for water samples (**Figure 2**). These results further suggest that changes in resistome composition in WW-impacted stream biofilms are likely due to changes in colonization of the biofilm communities by WWTP bacteria.

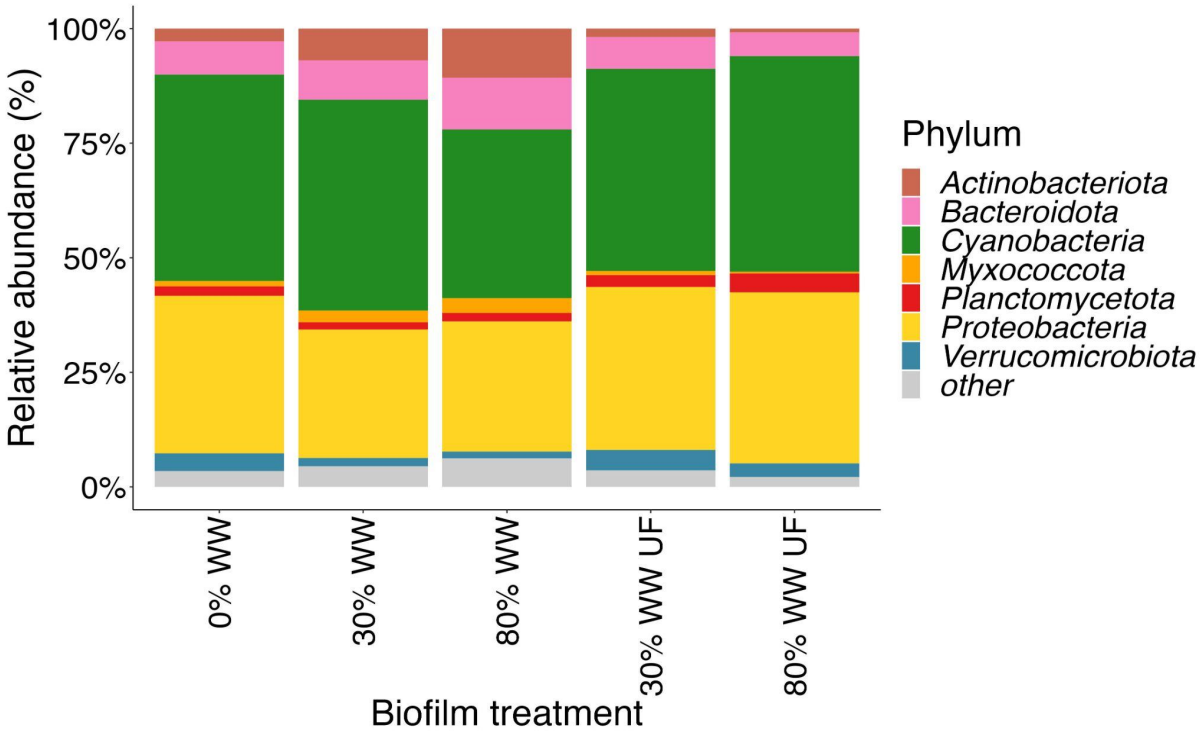

Figure 1. Mean relative abundance of prokaryotic phyla in metagenomes of stream biofilms grown with different percentages of treated wastewater (WW) with or without ultrafiltration (UF) treatment. Taxa which were in the upper decile of abundance in at least one experimental treatment were retained while the remaining phyla were binned as 'other'.

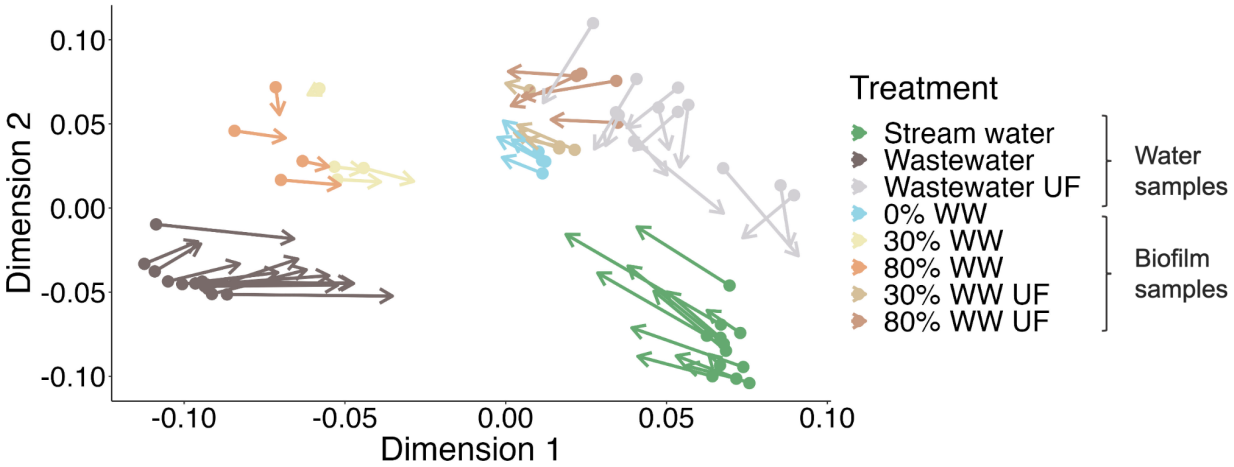

Figure 2. PCoA-based Procrustes analysis showing a structural alignment between microbiome and resistome composition. Points indicate position on PCoA axes 1 and 2 of prokaryotic composition (microbiome), while arrow tips indicate position on PCoA axes 1 and 2 of resistome composition following Procrustes rotation. The correlation coefficient in a symmetric Procrustes rotation was 0.901, the sum of squares (m12 squared) was 0.1889), and p-value was less than 0.001.

298 **WW ultrafiltration treatment has ARG class-specific effects on biofilm resistomes**

299 A total of 570 ARGs across 41 different higher-level ARG classes were identified in the biofilm
300 metagenomes using a short reads pipeline for ARG detection (Arango-Argoty et al., 2018). In
301 biofilms, the abundances of ARGs conferring multidrug resistance, in addition to ARGs conferring
302 resistance to beta-lactams, fluoroquinolones, peptides, rifamycins, and tetracyclines, differed
303 significantly between conditions **(Table S5)**. The normalized read counts of several, but not all,
304 ARG classes increased with increasing WW%, as seen in **Figure 3**. In biofilms grown in
305 comparable percentages of UF WW, the abundance of ARGs from the abovementioned classes
306 did not increase (**Figure 3**). The UF treatment results thus further support that biotic factors in
307 WW increased the abundance of many ARG classes in biofilms downstream.

308

309 As a notable exception, sulfonamide ARGs showed increasing abundance with higher WW% yet
310 displayed the highest abundance in 80% WW UF conditions. The unusual abundance in UF-
311 grown biofilms suggests that in addition to being impacted by biotic factors, abiotic factors such
312 as micropollutants (MPs) and nutrients may play a role in sulfonamide ARG abundance.
313 Additionally, ARG abundance in UF-grown biofilms may relate to gene-family specific
314 mechanisms such as horizontal transfer which may occur through transient contact or free DNA
315 passing through the ultrafiltration membrane. The sulfonamide resistance, ARGs *sul1-4,* are
316 known to be "highly mobilized" in WWTP effluent microbiomes (Ju et al., 2019). Although many
317 ARGs are mobile, *sul1* ARGs, in particular, are notable for their tight association with the class 1
318 integrons (Lee et al., 2021) which are among the most common multiresistance cassette systems
319 found in biological systems (Gillings et al., 2008, 2015). In support of the mobility of the
320 sulfonamide resistance genes, *sul1* genes detected in our metagenomes were co-localized on
321 the same metagenomic contigs as mobile genetic elements including *int1* integron-integrases as
322 well as recombinases and transposases (**Figure S5**). Notably, the presence of *intI1* genes in
323 environmental samples has been suggested as a gene proxy to estimate levels of anthropogenic
324 pollution including MPs (as proposed by (Gillings et al., 2015). Our results further support this
325 relationship between *int1* genes, sulfonamide ARGs typically associated with *int1,* and abiotic
326 factors including pollutants (Gillings et al., 2008).

327

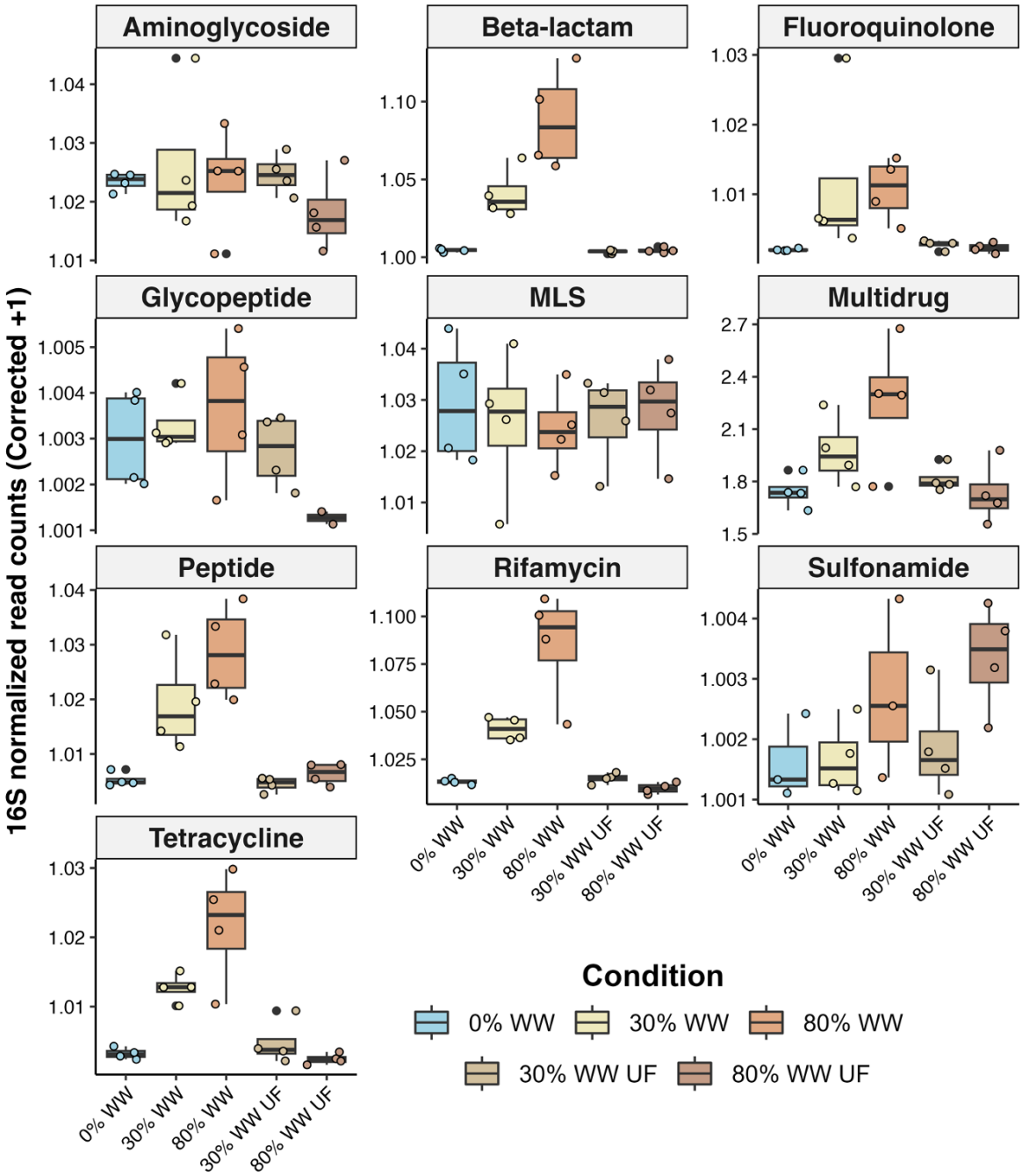

Figure 3. Abundance (16S-normalized read counts) of the DeepARG hits assigned to ARG classes which varied significantly with adjusted p-values ≤ 0.001 (Kruskal-Wallis test + Benjamini-Hochberg multiple hypothesis correction) between different treatment conditions. MLS = macrolide-lincosamide-streptogramin.

Association between assembled ARG abundances and WW percentage

To obtain more detailed information on the sequence identity of abundant ARGs in the biofilm communities, we assembled the metagenomes into contigs and used stringent criteria (see

Methods) to query called ORFs against the Structural Antibiotic Resistance Genes (SARG) database. Assembly-based approaches are likely to have lower sensitivity but higher specificity than short read-based approaches (Tamames et al., 2019, Peng et al., 2021). The results of querying 75 assembled metagenomes against the SARG database yielded 2,240 ARG hits. We clustered ARGs at the 70% amino acid sequence similarity level to obtain 102 ARG 'cluster representatives.' Annotating the cluster representatives by ARG class resulted in 15 beta-lactam, 14 multidrug, 9 aminoglycoside, 5 tetracycline, 3 macrolide-lincosamide-streptogramin (MLS), 2 sulfonamide, 1 spectinomycin, and 1 vancomycin ARG clusters, in addition to 51 other cluster representatives that were classified by SARG into the 'other' ARG category.

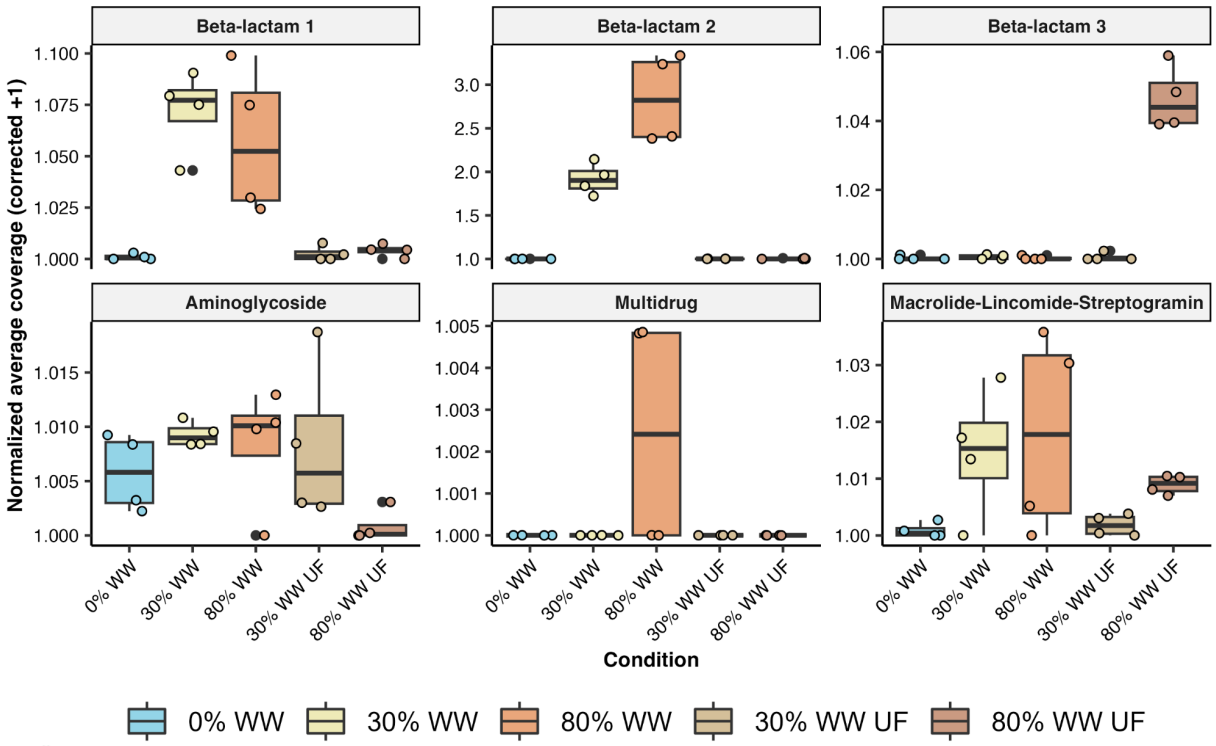

Figure 4. Corrected normalized average coverage of ARG cluster representatives identified using an assembly-based ARG detection method. ARG clusters with significant differences in abundances between treatment conditions are displayed (adjusted p-values ≤ 0.05 using Kruskal-Wallis test + Benjamini-Hochberg multiple hypothesis correction).

To quantify relative ARG abundances across treatment conditions, we mapped raw reads back onto the 102 ARG cluster representatives. Ten ARG cluster representatives differed significantly (Kruskal-Wallis test, $p < 0.05$) in abundance between at least one experimental treatment condition (**Table S6**). One each of the multidrug, MLS, and aminoglycoside class ARG representatives, in addition to two beta-lactam resistance ARGs, displayed the trend of increasing abundance with increasing WW% previously found for most of the ARGs identified with the short-reads pipeline. Two ARG classes encoding for beta-lactam resistance displayed the greatest increase in abundance with %WW (Class-A beta-lactamase gene "beta-lactam 1", $p = 0.0054$ and

359 a Class-D beta-lactamase bla OXA-4 gene “beta-lactam 2”, p = 0.0054, **Figure 4**). These ARGs
360 have significant increases in average coverage in biofilms with increasing WWTP effluent. In
361 contrast, a third beta-lactam ARG (extended-spectrum beta-lactamase BEL-2 “beta-lactam 3” p-
362 value = 0.0488) displayed the highest average coverage exclusively in the 80% UF WW condition.
363 These results suggest the abundance of this cluster of beta-lactamase ARGs may have been
364 affected mainly by abiotic factors such as MPs in the UF WW-grown biofilms, the effects of which
365 may have been outcompeted by biotic effects in the non-UF WW grown-biofilms.
366
367 Our combined results from short read-based and assembly-based ARG analysis generally aligned
368 with the exception of the sulfonamide ARGs which did not show a significant difference in the
369 assembly-based approach. This may be due to limitations or artifacts of the metagenomic
370 assembly. The significance of beta-lactamase ARGs in the short read-based approach was also
371 captured in the assembly-based approach. Overall, our results also align well with previous
372 studies indicating that exposure to WWTP effluent alters resistome composition and increases
373 ARG abundance in surface waters in Switzerland (Lee et al., 2023) and globally (Czekalski et al.,
374 2015; Matviichuk et al., 2022; Mukherjee et al., 2021; Thornton et al., 2020). Beta-lactamase
375 ARGs were also previously shown to increase in abundance in microbial communities exposed
376 to WWTP effluent (Fadare and Okoh, 2021; Mughini-Gras et al., 2019) and are consistently the
377 most abundant class of ARGs detected in surface waters (Laht et al., 2014; Osińska et al., 2020;
378 Rodriguez-Mozaz et al., 2015; Stoll et al., 2012). The prevalence is attributed to a variety of
379 factors, including the early discovery and continued widespread overuse of beta-lactam antibiotics
380 for humans and animals (Agarwal et al., 2023; Sta Ana et al., 2021). Moreover, one of the beta-
381 lactam clusters (“beta-lactam 3”) in our dataset showed an unexpectedly high abundance only in
382 80% WW UF (**Figure 4)**, making beta-lactamases a target in this study for further investigation.

383 **Micropollutants as an abiotic factor which may influence ARG transfer**

384 We next examined MP concentrations as abiotic factors which may influence gene-family specific
385 ARG abundances, such as the *sul* and “beta-lactam 3” ARG clusters which showed unexpectedly
386 higher abundances in the UF WW over non-UF conditions (**Figure 3, Figure 4)**. Based on
387 previous chemical analysis by liquid chromatography-tandem mass spectrometry (Desiante et al.,
388 2022), MP concentrations measured in the flume biofilm samples increased with increasing WW%
389 (**Table S4**), but MP concentrations did not significantly differ between the UF and non-UF WW-
390 grown biofilms. Other antibiotics targeted, including sulfamethoxazole and sulfapyridine (**Table**
391 **S4**), were below or near the limit of quantification in WW-grown biofilms, preventing further
392 analysis. The macrolide antibiotic, clarithromycin, had the highest concentrations in 80% WW and
393 80% UF WW grown biofilms (**Table S4**). Clarithromycin concentrations increased with WW% and
394 did not vary significantly between UF and non-UF treatments. In contrast, the metagenomic
395 abundances of macrolide-lincosamide-streptogramin (MLS) genes - the ARGs typically conferring
396 resistance to clarithromycin - were significantly lower in UF compared to non-UF conditions
397 (**Figure 4**). This example represents a case of an antibiotic-ARG pair where ARG abundances
398 did not correlate with the expected abiotic factor (antibiotic concentration). However, this does not
399 preclude that other MPs which are not classified as antibiotics may have influenced MLS ARG
400 abundance.

A growing body of evidence supports that many anthropogenic pollutants which do not have known antimicrobial bioactivities nonetheless influence ARG transfer and proliferation (Alderton et al., 2021; Kimbell et al., 2023; Qiu et al., 2022; Wang et al., 2021; Yu and Guo, 2022; H. Zhang et al., 2023). However, there is generally a lack of knowledge about how such 'non-antibiotic' MPs may still be selective agents for ARG abundance. Previously, 75 different MPs were measured in the flume biofilms, stream water, and WW (Desiante et al., 2022) including artificial sweeteners (sugar replacements, e.g., acesulfame-K, saccharin and cyclamate), non-antibiotic pharmaceuticals (e.g., antihypertensives), corrosion inhibitors, and pesticides (**Table S4)**. Artificial sweeteners were noteworthy as the most abundant MPs detected in µg/L concentrations in WW samples (Desiante et al., 2022) and exhibiting a pattern of increasing concentrations with increasing WW% (**Table S4**). Notably, the artificial sweeteners measured here (acesulfame K, cyclamate, and saccharin) all contain sulfonamide moieties, which have a chemical resemblance to the moieties found in sulfonamide antibiotics (**Figure S6**). Sulfonamides were also one of the few ARG classes with significantly higher abundances in UF vs. non-UF WW-grown biofilms (**Figure 3**). These observations led us to speculate that abiotic factors, such as sulfonamide-containing MPs, may play a role in promoting gene-family specific ARG abundances. In support of this, several experimental studies have shown that artificial sweeteners promote high rates of horizontal gene transfer and natural uptake of ARGs in bacteria (Yu et al., 2022; Yu and Guo, 2022). Further work has demonstrated that acesulfame-K and other artificial sweeteners directly enhance the rate and quantity of horizontal plasmid-mediated transfers of *sul* genes (Li et al., 2022). Interestingly, a plasmid-encoded microbial hydrolase capable of cleaving the sulfonamide moiety of acesulfame-K was recently identified and shown to have a beta-lactamase-like structural fold (Bonatelli et al., 2023). Taken together, these observations prompted us to conduct functional investigation of beta-lactamase-fold genes that increased in abundance with WW% and sulfonamide-containing MPs.

Planctomycetota beta-lactamases display differential abundances in response to sulfonamide-containing MPs and WWTP effluent

To investigate the relationship between ARG abundances and sulfonamide-containing MPs, we consulted previous reports of enrichment studies of activated sludge communities grown on sulfonamide-containing MPs including artificial sweeteners (Deng et al., 2019; Huang et al., 2021). Multiple independent enrichment studies on either acesulfame-K (Huang et al., 2021), saccharin, or cyclamate (Deng et al., 2019) all reported a significant increase in the relative abundance of bacteria belonging to the phylum Planctomycetota and particularly the planctomycete family *Phycisphaeraceae*. The *Phycisphaeraceae* are known to colonize the surfaces of aquatic macroalgae (Rambo et al., 2020) and share a common evolutionary ancestor with the anaerobic ammonia-oxidizing (anammox) planctomycetes promising in wastewater treatment processes (Andrei et al., 2019; Wu et al., 2022). Previously, Desiante et al. (2022) also identified significant differences in relative abundances of *Phycisphaeraceae* in biofilms grown in 80% WW and 80% WW UF in two independent flume biofilm experiments ($\log_2$ fold-change > 4; $p < 0.05$). Through differential abundance analysis conducted here, the Planctomycetota also emerged as a phylum with statistically-signiTficant differences in relative abundances between

WW and UF WW treatments (**Figure 5A**). In terms of their ecological roles, taxa belonging to the phylum Planctomycetota compose up to 2.1% relative abundance of the biofilm, WW, and stream water metagenomes, confirming previous reports of the Planctomycetota as a numerically-relevant member of both freshwater and wastewater microbial communities (Andrei et al., 2019; Cai et al., 2021). Based on metagenomic analysis, algae-associated *Phycisphaeraceae* are likely to impact the fitness of their algal hosts (Rambo et al., 2020) e.g., in stream biofilms, but the current unculturability of most symbiotic microorganisms in the *Phycisphaeraceae* limits our knowledge of their ARG phenotypes. We therefore targeted *Phycisphaeraceae* ARGs for functional characterization.

Due to challenges in the laboratory cultivation of the *Phycisphaeraceae* (Rambo et al., 2020), we opted for a heterologous expression approach to functionally validate *Phycisphaeraceae* ARGs. We focused on beta-lactamases for four reasons: (1) the beta-lactamases were identified in our ARG analysis as the most abundant and significantly-different class of ARGs affected by the percentage of treated wastewater, (2) beta-lactamase fold enzymes were also previously identified as plasmid-encoded enzymes active in the biotransformation the sulfonamide MP acesulfame-K (Bonatelli et al., 2023), revealing the 'structural overlap' of a versatile beta-lactamase fold capable of cleaving both sulfonamide- and beta-lactam moieties, and (3) previous evidence of sulfonamide cleavage in the sweetener cyclamate was shown by enzymes which were metal-dependent hydrolases but their sequences were not determined (Nimura et al., 1974). Finally, (4), full-length homologs with >50% amino acid identity to the sulfonamide resistance genes *sul1-4* encoding drug resistant variants of the sulfonamide target dihydropteroate synthase were not detected in the *Phycisphaeraceae*. For these reasons, we hypothesized that metallo-beta-lactamase-fold enzymes may play an overlooked role in sulfonamide resistance in these planctomycetes.

Functional characterization of a new beta-lactamase ARG from Planctomycetota

To identify metallo-beta-lactamase fold enzymes assigned to the *Phycisphaeraceae*, we queried the 75 metagenomes sequenced in this study as well as metagenome assembled genomes (MAGs) from previous sulfonamide-containing MP enrichment studies (Deng et al., 2019; Huang et al., 2021). We identified two full-length metallo beta-lactamase fold protein hits from the *Phycisphaeraceae* which showed a trend of increasing abundance patterns with percentage of wastewater relative to UF controls (**Figure 5B**). Both shared low similarity (less than 30% amino acid identity and between 20-50% query cover) with known beta-lactamases. We heterologously expressed each of the *Phycisphaeraceae* genes in *E. coli.* One protein did not express in *E. coli* and despite optimization could not be produced or purified to homogeneity with nickel-affinity chromatography. The second gene (MBX3358097.1) expressed sufficiently for purification with an average yield of 500 µg/mL pure protein (**Figure S7**), therefore we proceeded with the functional analysis of this protein.

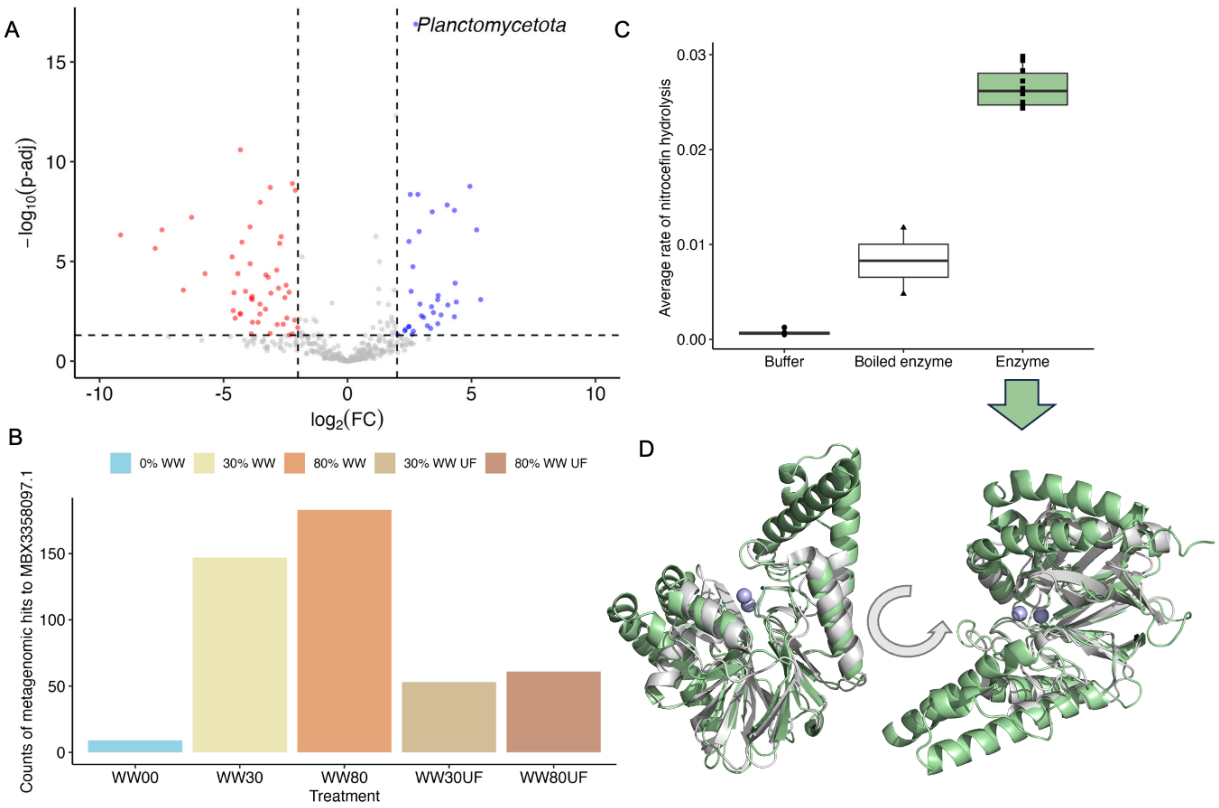

Figure 5. A) Volcano plot displaying $\log_2$-fold change of differentially abundant taxa between WW30UF and WW30. Similar significant trends, although with a lower fold-change difference, were observed in the Planctomycetota in WW80UF vs. WW80 samples. B) Counts of hits showing significant homology to the putative beta-lactamase MBX3358097.1 in biofilm metagenomes. C) Purified enzyme assays demonstrated beta-lactamase activity of MBX3358097.1 with the colorimetric cephalosporin analog, nitrocefin, compared to boiled enzyme and enzyme buffer only controls. D) Two different views of a high-confidence AlphaFold2 model (mean pLDDT = 96.5) of MBX3358097.1 structurally-aligned with its closest structural homolog in the Protein Data Bank, the *Bacillus cereus* beta-lactamase (PDB ID: 3KNS), identified by FoldSeek (van Kempen et al., 2023) as of 20.11.2023.

Next, we tested whether the purified *Phycisphaeraceae* protein encoded a functional beta-lactamase despite sharing low sequence similarity to known beta-lactamases. The protein cleaved the cephalosporin analog, nitrocefin, at significant levels above the boiled enzyme and buffer control (**Figure 5C**), indicating beta-lactamase activity. We next tested its substrate specificity for sulfonamide containing compounds. Using HPLC analysis we determined that the *Phycisphaeraceae* enzyme did not degrade the sulfonamide-containing MPs acesulfame-K, saccharin, or cyclamate, nor did it degrade sulfamethoxazole (data not shown). Although this planctomycete-derived beta-lactamase was sequence divergent from known beta-lactamases (less than 22% amino acid identity) with known beta-lactamases in the PDB, AlphaFold2 models indicated the protein shared structural similarity (RMSD of 1.53 Å) with class B2 beta-lactamases (**Figure 5D**). This analysis therefore also exemplified the potential of AlphaFold2 to identify proteins with low sequence similarity yet higher structural similarity performing conserved

functions such as beta-lactam cleavage. More broadly, this result highlights how sequence-structure-function analysis can be used to mine microbial functions of interest from metagenomes (Robinson, 2023; Ruppé et al., 2019).

There are few previous reports of ARGs from the phylum Planctomycetota (Ivanova et al., 2021). Planctomycetes were previously reported to have an unusual proteinaceous cell wall composition devoid of peptidoglycan (König et al., 1984), however later findings demonstrated planctomycetes do indeed produce peptidoglycan and are thus susceptible to cell-wall targeting antibiotics including beta-lactams (Jeske et al., 2015). To our knowledge, this is the first biochemical characterization of an active metallo-beta-lactamase from the *Phycisphaeraceae*. This ARG was also not found by current computational ARG detection tools, including the SARG database used in this study (Yin et al., 2018) due to its low sequence identity with known ARGs. This suggests that functional antibiotic resistance determinants, particularly from understudied microbial taxa, may be overlooked by state-of-the-art ARG detection methods. A knowledge gap remains whether such sequence-diverse ARGs from the Planctomycetota and other difficult-to-culture bacteria make significant contributions to environmental ARG reservoirs.

Conclusions

Our results from a controlled flume system showed that WWTP effluent altered both the microbiome and resistome composition of stream biofilms. Through ultrafiltration treatment of WWTP effluent, we could attribute changes in taxonomic and resistome composition primarily to biotic rather than abiotic factors. Comparing the abundances of ARG classes in UF vs non-UF conditions revealed that many ARGs had significantly higher normalized metagenomic abundances in non-UF WW as opposed to UF WW conditions which can be attributed to the introduction new microbial taxa from the WWTP effluents (biotic factors). Conversely, some sulfonamide and beta-lactamase ARGs were more abundant in biofilms grown in UF WW. We speculated this may be linked to the presence of sulfonamide-containing MPs that were not removed by ultrafiltration, such as artificial sweeteners present at high concentrations in WW. We therefore aimed to exemplify a metagenome mining approach to test hypotheses emerging from relative abundance observations since previous work had shown that the planctomycete *Phycisphaeraceae* were enriched with sulfonamide-containing sweeteners (Deng et al., 2019; Desiante et al., 2022; Huang et al., 2021). Here, we demonstrated that a sequence-divergent beta-lactam resistance gene, reconstructed from our metagenomes and originating from *Phycisphaeraceae*, was not involved in sulfonamide artificial sweetener degradation but shows beta-lactamase activity. This highlights the need to characterize new ARGs from understudied taxa to create a more comprehensive ARG catalog for querying complex microbial communities such as stream biofilms. Overall, this study investigated the interplay between MPs, ARGs, and their microbial hosts, and exemplified new functional workflows to shed light on antibiotic resistance determinants in aquatic environments.

Data availability

The metagenomes have been deposited in the NCBI Sequence Read Archive under BioProject: PRJNA733009. Scripts used for analysis and figures are available at: https://github.com/MSM-group/AMR-ecoimpact-paper. Data and supplemental figures are also available in the Supplemental Information associated with this manuscript.

CRediT authorship contribution statement

Conceptualization: Mustafa Attrah, Milo Schärer, Serina L. Robinson, Kathrin Fenner, Helmut Bürgmann. Methodology: Mustafa Attrah, Mauro Esposito, Milo Schärer, Giulia Gionchetta, Jack van de Vossenberg, Serina L. Robinson. Investigation: Mustafa Attrah, Milo Schärer, Mauro Esposito, Serina L. Robinson. Writing - Original draft: Mustafa Attrah, Milo Schärer, Serina L. Robinson, Jack van de Vossenberg. Writing - Review & editing: All authors. Visualization: Milo Schärer, Mustafa Attrah, Serina L. Robinson. Project administration: Serina L. Robinson, Piet Lens, Jack van de Vossenberg. Funding acquisition: Mustafa Attrah, Kathrin Fenner, Serina L. Robinson

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Declaration of competing interests

None

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