Influence of DNA from non-viable sources on the riverine water and biofilm microbiome, resistome, mobilome, and resistance gene host assignments

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Abstract

Shotgun metagenomic studies have revealed the diversity, relative abundance, and hosts of antibiotic resistance genes (ARGs) across environmental matrices. There is motivation to combine this method with viability-based techniques to better define ARG hazard. The objectives of this study were to evaluate the performance of different methods for extracellular DNA (eDNA) and putative "non-viable" cell DNA separation and understand the influence on ARG-host assignments. Paired water and biofilm samples were collected along a land use gradient. To study putative "viable-cell" DNA, samples were treated with propidium monoazide (i.e., PMA-DNA). To study eDNA, intracellular and extracellular DNA were separated. qPCR revealed differences in total 16S rRNA gene copies in water for filter vs. centrifuge-concentrated samples, but otherwise there were no differences in gene copy concentrations between DNA fractions. Next, metagenomic sequencing was performed on PMA-DNA and total DNA extracts revealing significant differences between the two for bacterial community structure and ARG profiles. Putative viable taxa containing pathogenic ARG hosts were identified in biofilm and

water. Removing PMA-bound DNA improved N50 and assembly mapping compared to total DNA extracts. This study demonstrates the impact of different sample preparation methods on informing the hazard potential associated with riverine ARGs in water and biofilm.

Keywords: eDNA, propidium monoazide, ARG, metagenomics

Environmental Implication

Antibiotic resistance is global health challenge with environmental dimensions given that antibiotic resistant infections can be community acquired. In this study methods for separating extracellular DNA and putative "viable-cell" (i.e., PMA treated) DNA were compared to understand their impact on quantification of the *sul*1 antibiotic resistance gene (ARG), which is detected frequently in the environment. Next, the PMA-DNA technique was combined with shotgun metagenomics to better define ARG hazard in riverine waters and biofilms. The microbiome, resistome, mobilome, and ARG-host assignments were compared for total DNA and PMA- DNA to understand the impact of sample preparation to inform environmental hazard identification for ARGs.

1. Introduction

Microbial DNA in environmental matrices consists of both extracellular DNA (eDNA) and intracellular DNA (iDNA) (Nagler et al., 2018; Nielsen et al., 2007), the latter of which may be present in non-viable cells including those with compromised membranes. eDNA is ubiquitous in environmental samples and is a mixture of DNA derived from various sources (such as autolysis and disinfection) (Bairoliya et al., 2021). Both the e- and iDNA fractions may be collected using standard total DNA extraction methods, potentially providing a biased snapshot of microbial communities. The effect of eDNA on bioinformatics analyses may differ for

44 different types of environmental samples with respect to microbial communities, as recently
45 reviewed by Bairoliya et. al. (Bairoliya et al., 2021).

46 Multiple methods exist for differentiating eDNA and putative "non-viable" cell DNA from total
47 DNA. Separation and extraction of eDNA and iDNA from environmental samples can be done
48 via filtration, centrifugation, and/or column-based protocols (Alawi et al., 2014; Corinaldesi et
49 al., 2005; Mao et al., 2014; Wang et al., 2016). Alternatively, extracellular DNA can be removed
50 using nucleases prior to cell lysis or be bound by dyes such as propidium monoazide (PMA) in
51 the presence of light which then interferes with amplification by polymerases and therefore
52 reduces the signal in many biomolecular assays (Lennon et al., 2018; Nocker et al., 2007b;
53 Sakcham et al., 2019; Villarreal et al., 2013). PMA dye also can penetrate cells with sufficiently
54 compromised membranes (Nocker et al., 2007a). Notably, PMA treatment will not differentiate
55 DNA from cells with intact membranes that are not viable (Joux and Lebaron, 2000). eDNA and
56 iDNA extraction methods depend on the recovery of the targeted DNA fraction, which in turn
57 could be dependent on different sample properties such as sediment clay content (Mao et al.,
58 2014), and water pH, COD, turbidity, etc. (Wang et al., 2016). Estimating the adsorbed eDNA
59 and DNA present within cells with compromised membranes has only been attempted in a few
60 studies (Liu et al., 2020). While DNase treatment is effective in eliminating eDNA, the presence
61 of DNase inhibitors in environmental samples can interfere with the effectiveness of eDNA
62 removal (Bairoliya et al., 2021). PMA treatment is relatively easy and fast to perform, has been
63 extensively optimized (Nocker et al., 2006; Nocker et al., 2007b), and has been used in the past
64 in conjunction with shotgun metagenomics sequencing (Be et al., 2017; Weinmaier et al., 2015).
65 However, the utility of the method could be limited due to variations in PMA penetrability based
66 on cell membrane or spore formation and the matrix properties (pH, turbidity, or for matrices

that adsorb PMA such as soils) (Shen et al., 2021). Of particular interest is understanding how removal of eDNA or application of PMA impacts our understanding of environmental microbial hazard, specifically here applied for understanding environmental antibiotic resistance in river water and biofilm.

Antibiotic resistance is a major 21st century global health challenge with environmental dimensions (Programme, 2017). Environmental risk assessment is needed and would benefit from understanding the genetic context of the wide range of ARGs in complex environmental samples (Zeng et al., 2019). Identifying the hosts of ARGs and host-viability is important because these factors indicate the hazard posed by ARGs (He et al., 2019; Zeng et al., 2019). Given that culture-based methods cannot capture the true microbial diversity in a sample and result in an underestimation of the total-viable bacteria in environmental samples (Kumar and Ghosh, 2019), there is motivation to apply biomolecular methods. Describing the host diversity of ARGs can aid in the identification of hazards that ARGs pose to humans. (Zeng et al., 2019). Viable ARG hosts, which when capable of phenotypic expression of antibiotic resistance are referred to as antibiotic-resistant bacteria (ARB) and pose varying risks (Vaz-Moreira et al., 2014).

Separation of eDNA and iDNA (Alawi et al., 2014; Deshpande and Fahrenfeld, 2022; Dong et al., 2019; Liu et al., 2020; Mao et al., 2014; Zhang et al., 2013; Zou et al., 2022) or application of PMA (Eramo et al., 2019; Garcia-Armisen et al., 2013) to understand environmental antibiotic resistance has been performed for a limited number of freshwater aquatic systems. Comparing riverine matrices, biofilm had a higher abundance of ARGs than sediment and water according to qPCR quantification from Yangtze estuary, China (Guo et al., 2018) and sediment samples had

higher concentrations of ARGs in eDNA than iDNA as quantified by qPCR in the Haihe river basin in China (Mao et al., 2014). A significant influence of wastewater discharge was observed on ARGs in eDNA and iDNA fractions in Tama river water, Japan (Liu et al., 2020). With respect to ARG host assignments, the eDNA microbial community in Raritan River sediments was significantly different from iDNA and total DNA, and potential pathogenic hosts were identified in all the DNA fractions (Deshpande and Fahrenfeld, 2022). Application of PMA-qPCR demonstrated significant amounts of non-viable cell ARGs in chlorinated wastewater effluent but no differences >1.8 km downstream in the receiving rivers through qPCR (Eramo et al., 2019). Comparison of the PMA method to eDNA/iDNA extraction and the impact of extracellular DNA and DNA from cells with compromised membranes on the resulting understanding of ARG hazard in environmental matrices has yet to be studied.

The objectives of this study were to (O1) evaluate the performance of different methods for eDNA and PMA-DNA DNA separation, and (O2) understand the influence of PMA bound DNA on bacterial community, ARG profiles, mobilome, and ARG and host assignments in river water and biofilm. Water and biofilm samples were collected along the Raritan River, NJ, USA and samples processed in parallel using PMA and eDNA/iDNA for qPCR analysis for *sul*1 and 16S rRNA gene copies. The PMA samples were selected for further study via metagenomic sequencing with assembly to characterize antibiotic resistance genes and perform host assignment. The ultimate aim was to improve our understanding of the viable riverine microbiome and its resistomes and mobilome towards improving environmental microbial hazard assessment.

2. Material and Methods

2.1 Sampling

Paired water and biofilm samples were collected from five sites with varying influences by wastewater effluent, urban activities, agricultural activities, and tides along the Raritan River in New Jersey, USA (Table S1) (Deshpande and Fahrenfeld, 2022). Composite water samples were collected every hour for three hours in sterile 1-liter Nalgene bottles. Composite biofilm samples were collected from the surfaces of rocks and leaves (8×16.5 cm^2 per swab using a sterilized stencil (Fig.S1)) at the site using sterile cotton swabs and stored in sterile 15 mL Falcon tubes post collection. Two field blanks were collected per matrix. Field blanks for water consisted of autoclaved deionized water and for biofilm consisted of sterile cotton swabs. The field blanks were left open in the field for the duration of sampling. Samples were stored and transported to the lab in coolers on ice and then stored at 4°C until processing. All samples were processed within one week of collection with all eDNA extractions and PMA treatments completed in the first three days of collection (Day 1- water eDNA extraction (8-10 hours), day 2- biofilm sample preprocessing and PMA treatment (6-7 hours), day 3- biofilm eDNA extraction (7-8 hours)). Weather conditions were measured at each site using a portable weather station (WM-4, ambient weather®, AZ) and the water temperature was measured using a thermometer. pH and conductivity were measured using a multimeter (Orion Star A329, Thermo Scientific, Waltham, MA) for water (Allison and Richards, 1954; EPA, 2004). Chemical oxygen demand (COD) for water samples was measured using the Hach Method 8000 and DR2700 spectrophotometer (Hach, Loveland, CO). Environmental Sciences Section (ESS) Method 340.2 was performed to analyze the total suspended solids (TSS) for water samples (Table S2).

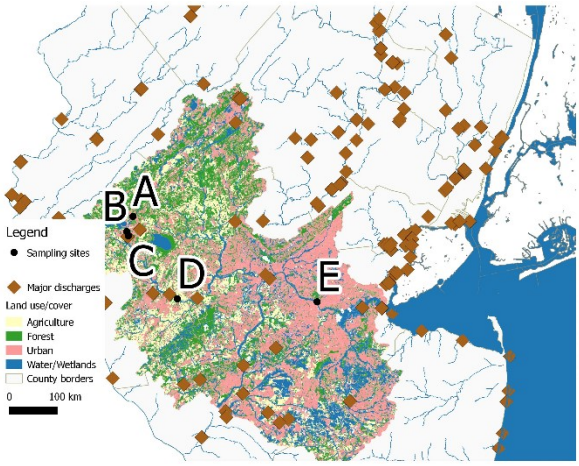


Fig. 1 *Map showing locations of sampling sites (black circles) and major (brown) surface water discharges along a land-use gradient. Land use and land cover is displayed only for the Raritan River catchment, located in central New Jersey, USA.*

2.2 Sample pre-processing

Composite water samples were mixed in equal volumes to create one combined sample for each site. Swabs with biofilm samples were suspended in 0.1M phosphate buffer (0.093 M Na_2HPO_4, 0.007 M NaH_2PO_4, pH 8.0) (Zhang et al., 2013) and sonicated on ice for 5 minutes at 500 watts, 20kHz and 20% amplitude (Qsonica sonicators, Model Q500, 20 kHz converter, Model CL-334 and a 12.7 mm probe, CT) (Branck et al., 2017; Foladori et al., 2010; Medina et al., 2020). After sonication, the solutions were combined to create one sample for each site. The composite samples from both matrices were used for subsequent analyses.

2.3 Separation and extraction of eDNA and iDNA

eDNA was extracted from sample splits of composite pre-processed water and biofilm samples according to an established protocol (Corinaldesi et al., 2005; Mao et al., 2014) with minor modifications. Water samples (200 mL) were divided into four 50 mL aliquots and centrifuged at

7100×g for 25 minutes to concentrate the iDNA and adsorbed eDNA (Fig.2). The pellets were washed with phosphate buffer and polyvinyl polypyrrolidone (PVPP) and centrifuged to separate the adsorbed eDNA from iDNA. The supernatants containing eDNA were pooled in each aliquot and filtered through polycarbonate filter (0.2 µm, Whatman). 1% CTAB buffer (in 50 mM Tris-10 mM EDTA, pH 8) was added to the supernatant of each aliquot.

PVPP was added to sonicated biofilm samples in phosphate buffer (8 mL), which were then centrifuged at 7100×g for 25 minutes (Fig.2). Supernatants were collected and pellets were again washed with phosphate buffer and centrifuged. Supernatants were combined, filtered (0.22µm Polyethersulfone, VWR) and 1% CTAB buffer was added to the filtrate. Following CTAB buffer addition, the protocol was similar for water and biofilm samples, which included incubation, centrifugation, and alcohol precipitation steps (Deshpande and Fahrenfeld, 2022). Before alcohol precipitation steps, GlycoBlue™ coprecipitant (Invitrogen™, Thermo Fischer Scientific) was added aid in precipitation. Pellets obtained after centrifugation of water and biofilm samples contained iDNA and were stored at -20°C until iDNA extraction. The FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH) was used for iDNA extraction from stored pellets.

To obtain "total DNA," water samples were filtered through 0.22 µm filter (Nitrocellulose, Millipore Corporation, Billerica, MA) (filter-concentrated DNA or t_{filt} DNA). Sonicated biofilm samples were centrifuged at 6000×g for 10 minutes (centrifuge-concentrated DNA or t_{cent} DNA) (Fig.2). "Total" DNA was extracted from these filter and centrifuge concentrated samples using the FastDNA® SPIN Kit for Soil (MP Biomedicals, Solon, OH).

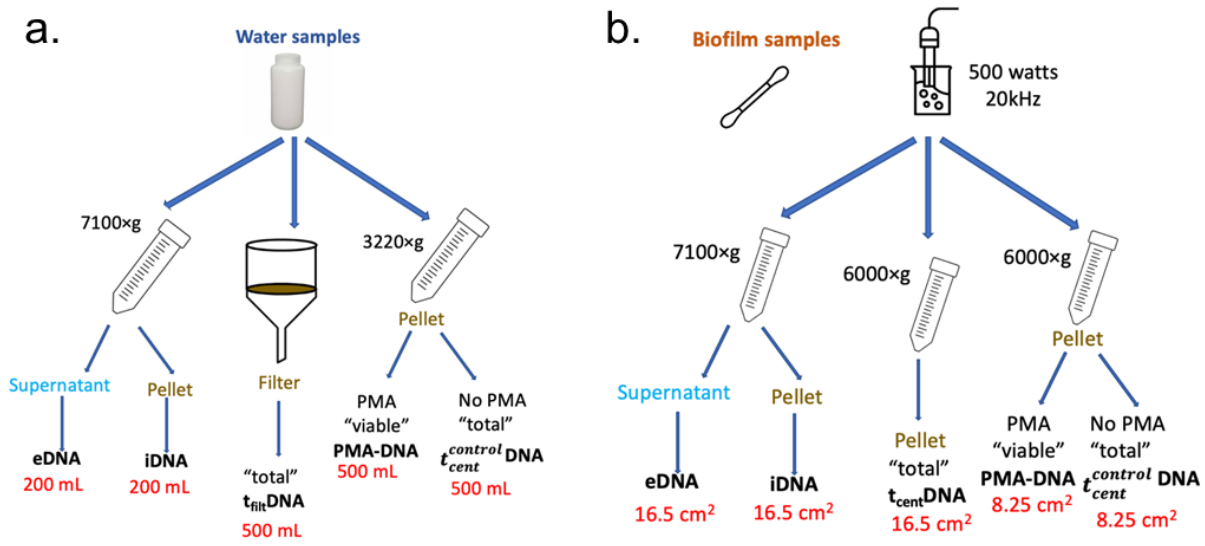


Fig. 2 *Flowchart of methodology for different sample processing approaches used in the study for a) water and b) biofilm samples. Values in red represent the sample volume (mL) or area (cm^2) used for processing of water and biofilm samples respectively. Words in bold represent the terminology used for each fraction of DNA.*

2.4 Reduction of signal from PMA-bound DNA treatment

Water samples (1000 mL) were centrifuged at 3220×g for 25 minutes and the supernatant was removed except for the last 10-15 mL (Fig.2). Samples were vortexed briefly (10-15 s) in pulses at maximum speed (Vortex-Genie 2) to resuspend pellet (Truchado et al., 2020), transferred to 50 mL tubes, and centrifuged again at 6000×g for 10 minutes. One-eighth of the volume (equivalent to one swab) of biofilm sample was centrifuged at 6000×g for 10 minutes to obtain a pellet (Fig. 2).

Cell pellets of water and biofilm samples were resuspended in 1 mL 1X phosphate-buffered saline (PBS), mixed, and split equally into two tubes (500 µL in each tube). One tube was treated with 50 µM PMA (PMA-DNA) while the other was left untreated, representing the total

183 community ($t_{cent}^{control}$DNA). All tubes were incubated in the dark for 5 minutes at room
184 temperature and then photoactivated using the PMA-Lite™ LED Photolysis Device (Biotium,
185 Fremont, CA) (Nocker et al., 2010; Nocker et al., 2007b). DNA was extracted from PMA treated
186 and untreated cells in separate lysing matrix tubes using FastDNA® SPIN Kit for Soil (MP
187 Biomedicals, Solon, OH).

188 **2.5 QA/QC and matrix Spikes**

189 Positive controls for eDNA extraction included pUC19 plasmids containing the *van*Z gene
190 (Deshpande and Fahrenfeld, 2022; Gallego et al., 2020), while *Escherichia coli* (*E. coli*) DH5a
191 cells were used as positive controls for iDNA. Controls were prepared and quantified based on a
192 recent study (Deshpande and Fahrenfeld, 2022; Gallego et al., 2020).These pre-quantified
193 controls were added to sample splits from all sites after sample pre-processing and prior to
194 eDNA and iDNA separation. The pUC19 plasmids containing *van*Z gene also served as positive
195 controls for PMA treatment and were spiked into water and biofilm sample splits from one site
196 prior to PMA treatment. *van*Z gene is rarely detected in environmental settings, and it was not
197 detected in our water and biofilm samples (Deshpande and Fahrenfeld, 2022). Negative controls
198 consisted of field blanks, which were extracted as described above for eDNA, iDNA, “total”
199 DNA, and PMA treated and non-treated samples.

2.6 Quantitative PCR (qPCR) and shotgun metagenomic sequencing

qPCR was performed for *sul*1 (Pei et al., 2006), *van*Z (Jensen et al., 1998) and 16S rRNA genes (Muyzer et al., 1993). *sul*1 is very commonly observed in the environment (Deng et al., 2022; Jiang et al., 2021; Miao et al., 2021; Munir et al., 2011) and is considered to be an indicator of antibiotic resistance in the environment (Berendonk et al., 2015). DNA extracts were diluted (1:20 to 1:100) to decrease the concentration of inhibitors. The qPCR total reaction volume was 10 µl with 5µl SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA), 0.4 µM forward and reverse primers, and 1 µl diluted sample. All reactions included three technical replicates (triplicate) for each sample and a negative template control (NTC) consisting of molecular biology grade water. Each qPCR plate also included a seven-point calibration curve of pre-quantified standards of the target gene ranging from 10^8 to 10^2 gene copies. QA/QC for the qPCR reactions included melt curve analysis and confirmation of the PCR product length via gel electrophoresis for 20% of samples run. Details of qPCR runs including primers, annealing temperature, R^2, and efficiencies are provided in Table S3.

For O2, samples from PMA treatment study (PMA-DNA and $t_{cent}^{control}$DNA) were chosen for shotgun metagenomic sequencing. DNA extracts from the PMA treated and untreated samples underwent multiple displacement amplification (MDA) using the REPLI-g® Midi kit (Qiagen, Germantown, MD) to amplify the PMA-DNA treated and total DNA in the untreated samples. The Repli-G kit has been demonstrated to amplify DNA samples with minimal bias (Hosono et al., 2003). A study conducted on assessment of Repli-G kit for metagenomics sequencing found that the genomic composition of samples was consistent after MDA. However, the study also noted some losses in species diversity after MDA (Ahsanuddin et al., 2017). The rationale for amplifying all samples via MDA apart from enriching the PMA-DNA concentration was that the

biases would be uniform across all samples (matrices and treatments), facilitating cross-matrix comparisons.

MDA was performed on all biofilm and water samples along with one MDA replicate each from PMA treated and untreated samples of water and biofilm ((5 sites × 2 matrices × 2 treatments) + 4 replicates = 24 samples). Each MDA reaction included the REPLI-g Human Control (positive control, Qiagen, Germantown, MD) and negative control which consisted of sterile nuclease-free water. Additionally, the matrix spikes of samples (which had been spiked with plasmids containing *van*Z prior to PMA treatment) in PMA treated and untreated samples were also subjected to MDA by REPLI-g to determine whether the PMA bound DNA was amplified through MDA.

MDA samples were submitted for shotgun metagenomic sequencing (DNA link USA, Inc., Los Angeles, CA). The samples were purified by AMPure beads (Beckman Coulter, Indianapolis, IN, USA), DNA quantity was measured by Qubit and quality was confirmed by Nanodrop and gel electrophoresis before sequencing (Table S4). The DNA library was constructed using a TruSeq DNA library preparation kit (Illumina, San Diego, CA). Paired-end sequencing (150-bp) was performed using the NovaSeq6000 platform. Sequences have been deposited in the National Centre for Biotechnology Information Sequence Read Archive (accession number: PRJNA872572).

2.7 Processing of raw reads

Reads obtained after sequencing were trimmed using the default parameters of Trimgalore (Krueger, 2015). Trimmed reads were classified using Kaiju 1.7.4 software (Progenomes database) to study the total microbial community (Menzel et al., 2016). The abundance of

245 microbial taxa was determined by the percentage of reads assigned to the taxon. Reads were
246 assembled using the default parameters of Megahit assembler v1.2.9 (Li et al., 2016). BBmap
247 was used to map contigs to the raw reads to determine assembly quality (Bushnell, 2014)
248 Prodigal v2.6. 3 (Hyatt et al., 2010) was used for predicting open reading frames (ORF) from the
249 resulting contigs and BLASTP was performed on the identified ORFs against the Comprehensive
250 Antibiotic Resistance Database (CARD) database to classify ARGs (Alcock et al., 2020) and
251 identify ARG carrying contigs (ACCs). The abundance of ARGs was calculated using the
252 following formula:

253 $$\sum_{1}^{n} \frac{N\ mapped\ reads\ X\ 150/L\ ARGlike\ ORF}{S} \qquad \text{(Eq.1)}$$

254 where, $N_{\text{mapped reads}}$ is the number of reads mapped to ARG-like ORFs, 150 is the length of
255 paired-end reads (bp), $L_{\text{ARG-like ORFs}}$ is the length of the ARG-like ORF (bp) and S is the
256 sequencing depth (in GB) (Ma et al., 2016).

257 ACCs were filtered from the contigs using a customized python script. ARGs associated with
258 mobile genetic elements (MGEs) were identified in ACCs through BLASTP against the
259 ACLAME database (http://aclame.ulb.ac.be). The cutoff criteria for ARGs as well as MGEs was
260 80% identity and 70% query coverage with an e-value 1e-10 (Ma et al., 2016). Finally, the
261 taxonomic classification of ACCs was performed using the contig annotation tool (CAT) v5.0.5
262 5 (von Meijenfeldt et al., 2019). N50 and percent of reads mapped are reported. The N50 is a
263 statistical parameter that examines assembly quality, while mapping percentage indicates the
264 amount of metagenomics raw data present in the assembly. Assembly performance is often
265 evaluated based on these two parameters indicating that a good assembly should not only have

high average size statistics but also high amount of included information content (Vollmers et al., 2017).

2.8 Data analysis

All statistical tests were performed in RStudio version 3.6.2 (www.r-project.org). Data visualization, sorting, filtering, and grouping were performed in R or Excel. A Shapiro-Wilk test was used to confirm the normality of data and a Bartlett test was performed to check equal variance of residuals. Censored data analysis was performed on qPCR data by imputing non-detect values using the ros command in NADA package (Lee, 2020). One-way ANOVA was performed on parametric 16S rRNA and *sul*1 qPCR data from biofilm samples, with Tukey HSD as posthoc test. A one-way.test was performed for 16S rRNA qPCR data from water samples (which did not have equal variances) followed by pairwise t-test as a posthoc test. A Kruskal-Wallis test followed by Dunn test was used for non-parametric qPCR data (absolute and normalized *sul*1 gene copies). PERMANOVA test from the vegan package was performed for normalized qPCR data, followed the pairwise Adonis test from pairwiseAdonis package as the posthoc test (Arbizu, 2019; Jari Oksanen, 2022). Bray-Curtis dissimilarity matrices were created for the total bacterial community and ARGs. Non-metric multidimensional scaling (nMDS) was performed to visually represent the bacterial community structure and ARG profiles. Shannon, Simpson, and Inverse Simpson diversity indices were calculated for total microbial community and ARGs and their values were compared across matrices and treatments using the PERMANOVA test followed by pairwise Adonis. Random forest analysis was performed to understand the influence of factors including PMA-treatment, sampling location, wastewater influence, and water quality on the variance in alpha diversity of the samples (randomForest package) (Liaw and Wiener, 2002).

Linear discriminant analysis effect size (LEfSe) test (Segata et al., 2011) was performed for identifying biomarkers (i.e., discriminatory features) in the total microbial community and for ARGs as a function of matrix and treatment. Heatmaps were made using the ggplot2 (Wickham, 2016) and reshape (Wickham, 2007) packages to represent the ARG relative abundances across sites. ARG-host assignments in the two matrices were represented using chord diagrams using the circlize package (Gu et al., 2014). Two-way ANOVA was performed for ARGs associated with MGEs to test for changes due to PMA treatment.

3. Results

3.1 Comparison of river water and biofilm DNA fractions by qPCR

3.1.1 Quality assurance and control

Recoveries were determined for each DNA fraction with controls as described in Section 2.5. For the water samples, eDNA recovery was low at 21.2±17.7 % and iDNA recovery was suitable at 75.3 ± 25.3% (Table S6). For the biofilm samples, recovery of both eDNA at 81.6±13.1 % and iDNA at 100.4±6.5% were suitable for downstream analyses (Table S5).

QA/QC for PMA treatment included matrix spikes of plasmid-cloned *van*Z genes to test if PMA treatment inhibited qPCR for extracellular DNA. A matrix spike study for PMA treatment with live and heat-inactivated *E.coli* cells has been reported by our lab previously, where it was observed that PMA dye successfully suppressed 16S rRNA gene signal from heat-inactivated cells, but did not affect the live *E.coli* cells. (Eramo et al., 2019) After PMA treatment, the *van*Z gene copies were either below the detection limit (5.3 log copies per 500 mL water or 8.25 sq. cm biofilm) or not detected in samples with matrix spikes, indicating that PMA treatment inhibited amplification of the gene by qPCR. *van*Z gene copies were well above the detection

limit (6.45±0.4 log copies per 500 mL water or 16.5 sq. cm biofilm) in samples not treated with PMA dye.

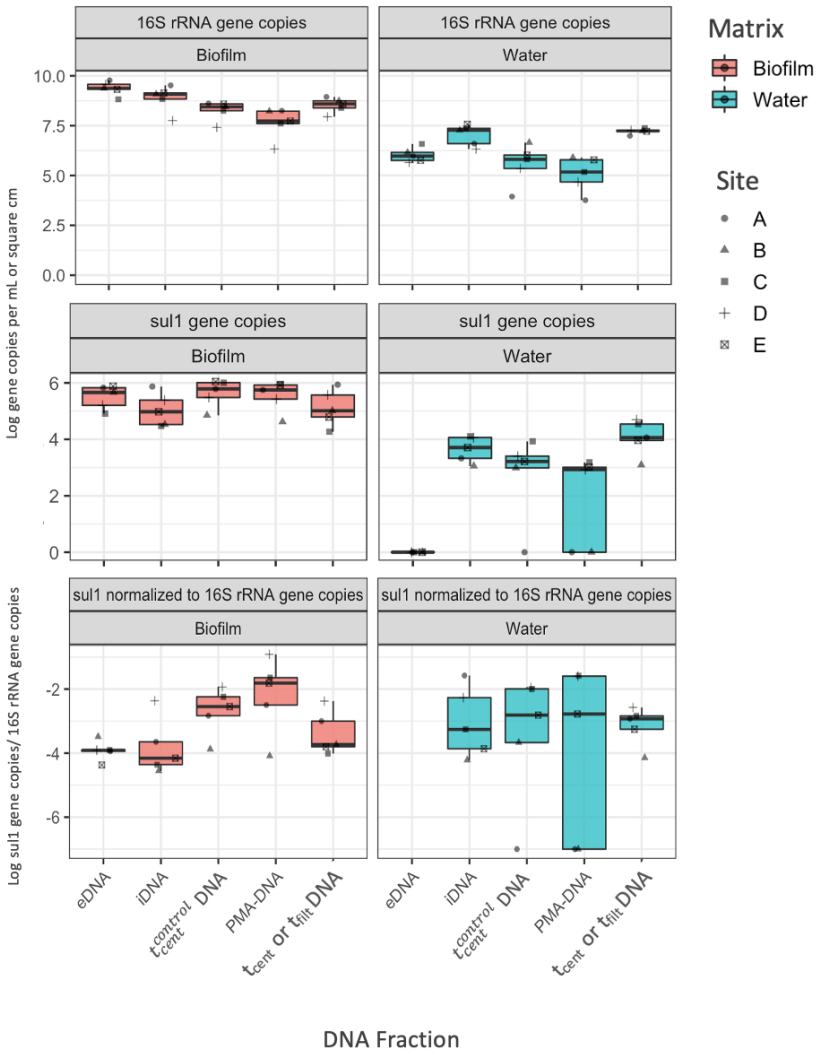
3.1.2 Extracellular, intracellular or PMA-treated versus "total" sul1 in water and biofilm

River water and biofilm samples were extracted using multiple techniques to improve understanding of the loading of *sul*1 and 16S rRNA in different DNA fractions. For water samples, there were 1-3-$\log_{10}$ unit higher concentrations of 16S rRNA gene copies observed in the t_{filt}DNA than the $t_{cent}^{control}$ DNA samples (Fig. 3, post hoc pairwise t-test, p=0.008). Likewise, iDNA concentrations of 16S rRNA gene copies were higher than the $t_{cent}^{control}$ DNA concentrations in water samples (Fig. 3, post hoc pairwise t-test, p=0.02). There were no differences observed in the absolute 16S rRNA gene concentration between iDNA and the t_{filt}DNA nor between PMA-DNA and the $t_{cent}^{control}$ DNA, the latter of which served as the control for the PMA treatment (Fig. 3, post hoc pairwise t-test, p=1).

For the biofilm samples, eDNA concentrations of 16S rRNA gene copies were significantly greater than the PMA-DNA and the $t_{cent}^{control}$ DNA (Fig. 3, Tukey HSD, p=0.00072 and 0.038, respectively). Likewise, iDNA concentrations of 16S rRNA gene copies was greater than PMA-DNA (Tukey HSD, p=0.017). There were no differences between the 16S rRNA gene copy concentrations for the t_{cent}DNA, iDNA, nor the $t_{cent}^{control}$ DNA used in PMA treatment (Fig. 3, Tukey HSD, all p>0.1).

The *sul*1 gene was quantifiable in most of the water sample fractions and all biofilm samples. For water samples, *sul*1 was below detection in all the water eDNA extracts (low recovery of eDNA introduced in Section 3.1.1) and 2/5 of the water PMA-DNA extracts. Significantly higher *sul*1 gene concentration was observed in t_{filt}DNA than the PMA-DNA fraction (Fig.3, Tukey

HSD, p=0.008). Otherwise, similar absolute *sul*1 gene copy concentrations were observed the iDNA, $t_{cent}^{control}$DNA, and t_{filt}DNA fractions (Fig. 3, Tukey HSD, all p>0.053). For the biofilm samples, there were no differences in absolute *sul*1 gene copies between eDNA, iDNA, t_{filt}DNA, PMA-DNA, nor t_{cent}DNA (Fig.3, Kruskal-Wallis test, p=0.33). Normalizing *sul*1 gene copies to 16S rRNA gene copies, thereby providing an estimate of relative abundance of *sul*1 in the community, resulted in no differences between iDNA, t_{filt} DNA, t_{cent} DNA nor PMA-DNA samples for water and biofilm (Fig.3, PERMANOVA, adjusted p=1). Also, no differences were seen in the 16S rRNA gene normalized *sul*1 copies between the two matrices (Fig. 3, PERMANOVA, adjusted p=1).



342

343 ***Fig. 3*** *Absolute 16S rRNA gene and sul1 ARG copies, and normalized sul1 gene copies (to 16S*
344 *rRNA gene) in different DNA fractions of biofilm and water obtained via different protocols.*
345 *Absolute gene copies are represented as gene copies per square centimeter and per mL for*
346 *biofilm and water respectively.* t_{cent} *or* t_{filt} *DNA stands for centrifuge (for biofilm samples) or*
347 *filter (for water samples) concentrated "total" DNA. "ND" stands for not detected. Note the*
348 *different scale in each row of the figure.*

349 **3.2. PMA-DNA versus "total" microbial community: Microbiome**

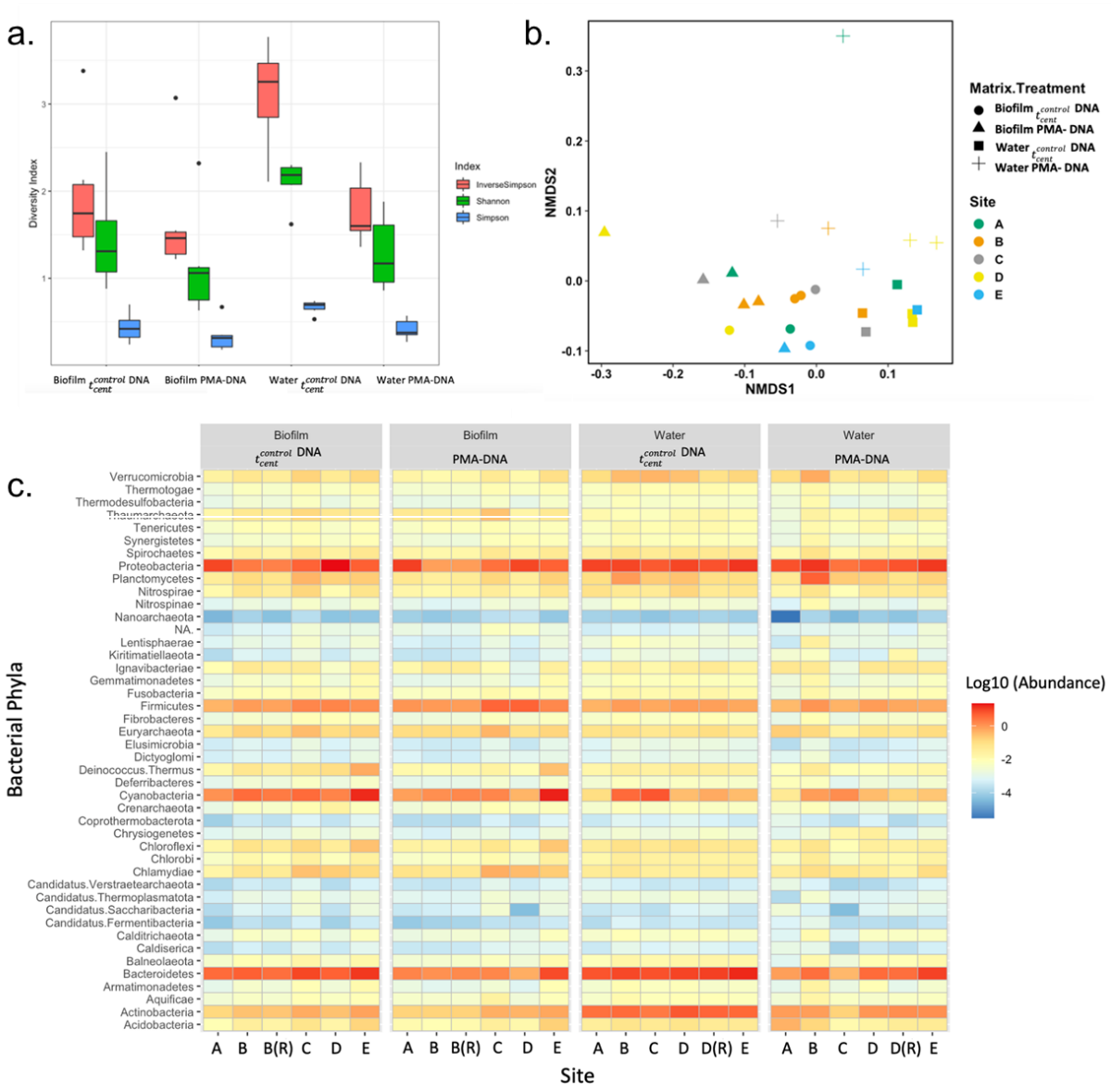
For quality control prior to shotgun sequencing, qPCR was performed on the matrix spike PMA-DNA and $t_{cent}^{control}$ DNA from water and biofilm samples. When the samples with matrix spikes underwent multiple displacement amplification (MDA), the concentration of *van*Z gene in samples not treated with PMA increased over four orders of magnitude (from 6.45 ± 0.4 $\log_{10}$ gene copies to 11.8 ± 0.01 $\log_{10}$ gene copies), but this gene was still below detection or not detected in PMA treated samples. This provided evidence that MDA increased the concentration of the DNA that was not bound with the PMA-dye ("viable-cell" DNA).

Shotgun metagenomic sequencing of the MDA samples generated an average of 12.03±1.2 Gb data per sample (Fig. S2). Bacterial phyla observed in the $t_{cent}^{control}$ DNA community are shown in Fig. 4c. The relative percentage difference (RPD) in the abundance of each phylum in replicates of water and biofilm samples (one replicate of each) ranged from 18 ±15 to 50 ± 48 % (average ± standard deviation) whereas the RPD between PMA-DNA and $t_{cent}^{control}$ DNA samples ranged from 15 ± 13 to 103 ± 41%. The most abundant phyla were similar for both PMA-DNA and $t_{cent}^{control}$ DNA (i.e., Proteobacteria and Bacteroidetes) in water as well as in biofilm (i.e., Proteobacteria, Cyanobacteria and Bacteroidetes).

The total microbial community structure at the family level in biofilm and water samples (Fig 4b) was significantly different for PMA-DNA and $t_{cent}^{control}$ DNA samples and the total DNA samples had greater alpha diversity than "viable-cell" water samples (post hoc pairwise Adonis, R^2=0.33, p= 0.03). No differences were observed in the alpha diversity of PMA treated and total biofilm samples (posthoc pairwise Adonis, p=1). LEfSe test revealed 14 biomarkers of water PMA-DNA and 76 families as biomarkers of water $t_{cent}^{control}$ DNA. In biofilm samples, PMA-DNA had 55 biomarkers while $t_{cent}^{control}$ DNA had 22 biomarkers at the family level. The families *Flavobacteriaceae, Methylophilaceae, Cytophagaceae, Comamonadaceae and*

373 *Microbacteriaceae* in water $t_{cent}^{control}$ DNA and *Burkholderiaceae* in water PMA-DNA had linear
374 discriminant analysis (LDA) score greater than 4. In biofilm samples, the families *Moraxellaceae*
375 and *Hyellaceae* in $t_{cent}^{control}$ DNA and *Bacillaceae* and *Clostridiaceae* in PMA-DNA had LDA
376 score greater than 4.

377 Random forest analysis indicated that PMA treatment (PMA-DNA vs $t_{cent}^{control}$ DNA) (13.97%)
378 had a greater increase in mean square error to explain the alpha diversity at the family level than
379 other factors including matrix (11.52%), site (9.07%), pH (5.86%), TSS (5.5%), and wastewater
380 inputs (5.27%). Overall, these factors explained 51.2% of the variance in alpha diversity at
381 family level.



382

383 ***Fig.4:*** *a. Boxplots of alpha diversity at family level, b. nMDS plot (stress=0.11) of the total*

384 *microbial community in biofilm and water samples at family level and c. heatmap of abundance*

385 *of bacteria phyla present in PMA-DNA and* $t_{cent}^{control}$ *DNA samples. Replicates (R) (N=2) are also*

386 *shown for Site B for biofilm and Site D for water.*

3.3 Total and PMA-treated ARG profiles and diversity: Resistome

Assembly of trimmed reads across samples generated 19,522,851 contigs (Table S7). Treatment with PMA dye reduced the community complexity as measured by alpha diversity and increased mapping percentage compared to the $t_{cent}^{control}$ DNA samples (paired Wilcoxon test, p=0.001). N50 of the total DNA extracts was significantly less than that of PMA-DNA extracts (paired Wilcoxon test, p=0.0002) (Table S7).

A total of 292 and 614 different ARGs were observed across the water and biofilm samples, respectively. ARGs were grouped into 18 drug classes (Fig.5a) and eight resistance mechanisms (Fig.S3). Significant differences were observed in ARG profiles for PMA-DNA and $t_{cent}^{control}$ DNA from water samples and for total DNA between the two matrices (posthoc pairwise Adonis, R^2 = 0.26 and 0.33 respectively, both p<0.024) while no differences were observed in alpha diversity of ARGs (PERMANOVA, p>0.3) (Fig.S4). LEfSe test results indicated that $t_{cent}^{control}$ DNA of water had 3 biomarkers while PMA-DNA had none. For biofilm samples, $t_{cent}^{control}$ DNA and PMA-DNA had 5 and 9 biomarkers respectively (Fig. 5b). ARGs belonging to Rifamycin group, and the ARG *mtr*A were the biomarkers in $t_{cent}^{control}$ DNA of water samples. Biomarkers in in $t_{cent}^{control}$ DNA of water samples were ARGs belonging in aminoglycoside, rifamycin and multidrug resistance groups. Most of the biomarker ARGs from PMA-DNA of biofilm samples belonged to the beta-lactam group (Fig. 5b).

Multidrug resistance was the only ARG type observed in all of the sequenced samples. The aminoglycoside drug class was detected in all total DNA biofilm and water samples and most (83.3 %) of the PMA treated samples from biofilm and water. Genes encoding for resistance to the Rifamycin group of drugs were present in all total water samples and most (~92 %) of the biofilm samples. The *sul*1 gene was detected at 4/5 sites in biofilm samples and 3/5 sites in water

samples, despite being observed at 4 sites via qPCR. Interestingly, MCR ARGs were observed at one of the sites in biofilm. These genes confer resistance to colistin, which is one of the last-resort drugs used for treatment of multidrug resistant infections (Hussein et al., 2021; Poirel et al., 2016).

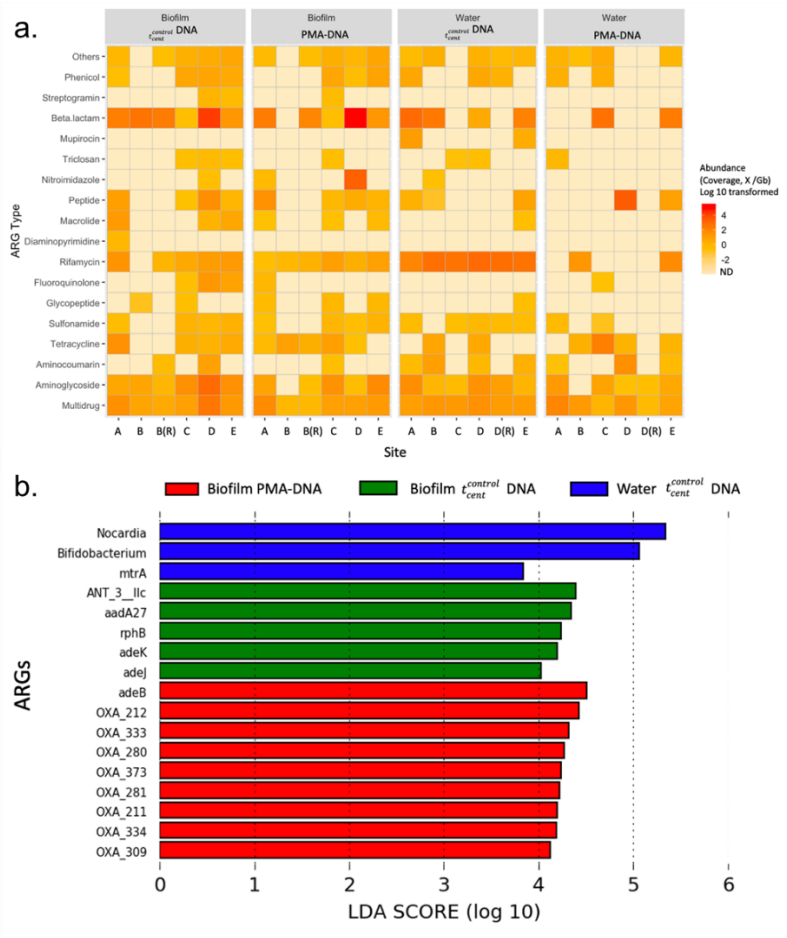


Fig.5. *a. Heatmap showing the relative abundance of ARGs grouped by drug class for biofilm and water PMA-DNA and* $t_{cent}^{control}$ *DNA samples. Replicates (R) of MDA for biofilm (site B) and water (site D) are also shown. b. Plot of LEfSe test results showing biomarkers in samples and their LDA score.*

3.4 Total and PMA-treated mobile genetic elements: Mobilome

To understand the genetic context of the resistance genes, ARGs associated with MGEs were identified using the ACLAME database (Fig. S5). No differences were observed in the percentage of ARGs associated with mobile genetic elements between matrices and PMA treatment (2-way ANOVA, all p>0.05). ARGs were assigned to MGEs for 57.5 ± 31.1% of the resistance genes observed in PMA-DNA and 44.4 ± 22.7% of the ARGs observed for $t_{cent}^{control}$ DNA. The relative percentage difference (RPD) for the replicates ranged from 5.8% to 19.6% for $t_{cent}^{control}$ DNA and from 64.4% to 125% for PMA-DNA samples. No significant correlation was observed between the percentage of ARGs associated with MGEs for the different DNA fractions and the wastewater effluent inputs (Spearman, p>0.12).

3.5 Total and PMA-treated ARG hosts in river water and biofilm

Hosts of ARGs were identified using CAT software and host-ARG linkages are shown in Fig.S6 and Fig.S7. The putative pathogenic ARG-host containing genera identified included *Pseudomonas, Acinetobacter, Bacillus, Clostridium*, and *Coxiella*.

There were no differences in the number of ARG-host linkages between PMA-DNA and $t_{cent}^{control}$ DNA in water and biofilm (Dunn test, adjusted p = 1). Among the putative "viable-cell" hosts of ARGs, pathogenic genera were present in both biofilm and water samples. There were no differences in the total abundance of ARGs assigned to putative pathogenic genera between PMA-DNA and $t_{cent}^{control}$ DNA (Dunn test, adjusted p = 1).

ARG types assigned to hosts, including putative pathogenic hosts, at the genus level included multidrug, aminoglycoside, aminocoumarin, beta-lactam, fluoroquinolone, glycopeptide, macrolide, peptide, tetracycline, rifamycin, and others. Notably, more ARGs were assigned to

hosts at the genus level in biofilm (N=521) than in water (N=29) in total. Biofilm also had more ARG hosts (N=18) than water (N=8) at the genus level.

4. Discussion

4.1 qPCR on riverine DNA fractions

Paired comparison of PMA-qPCR and methods to separate eDNA and iDNA for water and biofilm in the present study demonstrated that the *sul*1 hazard was relatively conserved in PMA-DNA, iDNA, and total DNA for water and for all fractions including eDNA for biofilm. This would appear to be consistent with why *sul*1 is often an ARG of interest for monitoring: it is widespread in the environment. Whether this gene was representative of other ARGs across DNA fractions and matrices was addressed via metagenomic sequencing and is discussed in Section 4.2.

Centrifugation speed and time, sonication, and sample storage have the potential to increase or reduce eDNA and iDNA concentrations. The centrifugation speeds selected were directly from the methods developed for eDNA/iDNA extraction (Mao et al., 2014; Zhang et al., 2013) or PMA-DNA studies (Eramo et al., 2019) and were previously shown to have low impact on cell integrity. When the recommended centrifugation speed could not be met due to instrument limitations, time was increased. Matrix spike recoveries provide a second line of evidence for the times and speeds reported here. Sonication was used for sample pre-processing and the length and energy applied was from studies using sonication with viability-based techniques (i.e., live-dead staining) (Foladori et al., 2010; Foladori et al., 2007) . Further, prolonged sonication for 8 hours at 20 kHz was previously shown to not cause denaturation of DNA (Davis and Phillips, 1978). Hence, 5 minutes of sonication at the selected energy applied was not expected to result in denaturation of eDNA. The potential impact on eDNA and iDNA dynamics during the sample

464 hold time and change from storage at 4°C to room temperature, which was needed to run these
465 analyses in parallel, may be of interest to investigate in future studies. Other researchers
466 performing eDNA extraction on environmental samples stored samples at -20°C (Alawi et al.,
467 2014; Zhang et al., 2013; Zhao et al., 2020) or 4°C (Mao et al., 2014; Zhang et al., 2018) before
468 eDNA extraction. We selected to store out samples at 4°C given that storing at -20°C can reduce
469 viability (Metzger et al., 2015).
470 Direct comparisons of eDNA, iDNA and PMA-DNA methods are not present in the literature to
471 our knowledge, but results obtained here can be put into context of studies that used one of these
472 methods. First, for studies applying eDNA separation methods in surface water, Yangtze estuary
473 biofilms had higher abundances of ARGs generally than water and sediment (Guo et al., 2018).
474 In biofilm and sediment samples, most of the ARGs quantified through qPCR were higher in
475 eDNA than iDNA. In the Raritan River we saw no differences in the abundance of *sul*1 gene
476 between eDNA and iDNA of biofilms. This could possibly be due to differences in location or
477 temporal variation across the study period. The Yangtze study also reported lower abundance of
478 extracellular ARGs than intracellular ARGs including *sul*1 in water samples, which the
479 researchers believed was underestimated due to some limitations of the filtration method
480 (filtration fails to separate adsorbed eDNA which could be bound to colloids and other particles
481 found in the river water and therefore be trapped on the filter. Hence, in the present study, water
482 samples were centrifuged to separate free eDNA and then washed with sodium phosphate buffer
483 to separate the adsorbed eDNA). Second, for the PMA-DNA method, other investigations using
484 qPCR for ARGs were completed by our team in NJ rivers downstream of WWTP outfalls and in
485 WW effluent. Similar to the results for the present study, there were no differences in *sul*1 gene
486 copy concentrations in the surface water PMA-DNA and $t_{cent}^{control}$ DNA collected downstream

from wastewater effluent discharge, despite differences in the chlorinated WW effluent itself (Eramo et al., 2019).

Results of the present study indicated that for 16S rRNA gene copies in river water, the removal of eDNA and/or putative "non- viable" cell DNA did not significantly affect the gene copy concentration in the paired control tDNA samples (t_{filt}DNA for eDNA and t_{cent}DNA for PMA treated samples). Similar results were observed for a study on Arctic and Pacific sediment samples treated with PMA dye, where no significant differences in the 16S rRNA gene abundance were observed between PMA treated and non-treated samples through qPCR in samples collected from greater depths and most of the sediment samples collected from shallow depths (Ramírez et al., 2018). Likewise, only a marginally significant difference was observed in the proportion of eDNA (as measured by the 16S rRNA gene abundance through qPCR) between different ecosystems, including water, sediment, soil, and mammalian gut (Lennon et al., 2018).

Interestingly, a higher 16S rRNA gene copy concentration was observed in iDNA than t_{cent}DNA for water, likely explained by that fact that the centrifuge-concentrated water samples for PMA treatment were centrifuged at a lower speed (3220×g) than iDNA samples (7100×g). The lower speed for the PMA treatment study was selected based upon work showing that it is suitable for preventing cell membrane damage prior to PMA treatment (Eramo et al., 2019). However, it likely indicates that sample concentration method should be considered for water when comparing qPCR results between studies. Likewise, differences were observed in 16S rRNA gene copy concentrations in water for tDNA (t_{filt}DNA, t_{cent}DNA) concentrated via different methods. A recent study from Morocco compared membrane filtration and centrifugation for concentrating surface and wastewater samples and reported that membrane filtration through 0.45 μm pore size filter gave better recovery and concentration of microbes through qPCR as

compared to centrifugation (8000×g). (El Boujnouni et al., 2022). Results from a study conducted by our team indicated that there were no significant differences in 16S rRNA gene and *sul*1 gene copies between centrifuge (4000×g) and filter concentrated wastewater samples nor did it affect the wastewater microbial community structure observed through amplicon sequencing (unpublished data, Eramo and Fahrenfeld). The probable reason for lower centrifuge speed affecting our qPCR results here in surface water could be the difference in matrix, that is, wastewater having a higher suspended solid loading than surface waters. In contrast to the water samples, the biofilm iDNA was similar to the t_{cent}DNA likely because all biofilm samples included the same pre-treatment to resuspend the biofilms from the swabs prior to any further treatments to separate DNA fractions. For riverine biofilm, the absolute concentration of 16S rRNA gene and *sul*1 gene copies, and normalized *sul*1 gene copy concentration of biofilm eDNA were similar to iDNA and total DNA indicating that eDNA is a significant fraction of biofilm DNA samples. Here eDNA was precipitated from the supernatant and therefore may not be captured in studies using centrifuge concentrated DNA as total DNA.

4.2 Sequencing of PMA-DNA and t_{cent}DNA

4.2.1 Microbial community structure

The results of metagenomic sequencing indicated that the microbial diversity of river water could be overestimated by including putative "non-viable" cell DNA. Previous studies have indicated that the effects of eDNA on microbial community vary by environmental matrices. eDNA removal through DNase treatment significantly shifted the monochloramine disinfected drinking water microbiome (Bairoliya et al., 2021). In contrast, eDNA had minimal effect on the microbial community beta diversity in water, sediment, mammalian gut, (Lennon et al., 2018) and soil (Carini et al., 2020; Lennon et al., 2018) samples from the USA.

533 eDNA is known to be an important component of biofilms (Montanaro et al., 2011), especially
534 during the initial formation (Flemming et al., 2016), and is actively released by the organisms
535 producing the biofilm (Barnes et al., 2012; Kilic et al., 2017; Liao et al., 2014; Rose and
536 Bermudez, 2016; Zafra et al., 2012). The lack of differences in alpha diversity of the biofilm
537 PMA-DNA and $t_{cent}^{control}$ DNA would imply that eDNA of the 16S rRNA gene is produced by the
538 organisms in the biofilm and a relatively consistent amount from each, rather than eDNA sorbed
539 or captured from the environment or select biofilm members out-producing others in terms of
540 16S rRNA gene eDNA.

541 There were differences in microbial community structure between PMA-DNA and $t_{cent}^{control}$ DNA
542 in water and biofilm. Of particular interest is the observation of some families that contain
543 pathogenic species were also identified as biomarkers. For example, the family *Clostridiaceae*,
544 which includes the pathogenic species *Clostridioides difficile,* had significantly higher
545 abundance in biofilm PMA-DNA than biofilm $t_{cent}^{control}$ DNA. The significance of *Clostridioides*
546 *difficile* in antibiotic resistance hazards is discussed in section 4.2.2. Further study would be
547 needed to confirm the presence of pathogens with the metagenomic data guiding which
548 pathogens to target.

549 **4.2.2 Resistome, mobilome, and ARG-host assignments**

550 The N50 (assembly quality) and mapping percentage of assembly improved in the PMA-DNA
551 samples as compared to $t_{cent}^{control}$ DNA. This observation is likely due to the treatment with PMA
552 dye reducing the community complexity, as confirmed by the lower diversity indices of water
553 samples. Therefore, removal of putative "non-viable" cell DNA significantly impacted
554 metagenomic assembly and may be desirable for studies where assembly of short read shotgun
555 sequencing should focus on the viable community, such as for microbial risk assessment.
556 Whether these results would hold true for sequencing via platforms generating long reads (e.g.,
557 nanopore) would require future study.

558 Different ARG profiles in PMA-DNA and $t_{cent}^{control}$ DNA from water samples gives a different
559 view of ARG hazards. For example, the beta lactam class was observed in total but not PMA-
560 DNA for Sites A and B. ARGs belonging to Rifamycin group were biomarkers in $t_{cent}^{control}$ DNA
561 of water. The Rifamycin group ARGs were either not detected, or present at lower abundance in
562 PMA-DNA. Rifamycin antibiotic is widely used for treatment of travelers' diarrhea (DuPont et
563 al., 2014; Steffen et al., 2018), and its resistance genes are considered to be universally present in
564 diverse environmental samples (Ma et al., 2014). The multidrug resistant ARG *mtr*A, which was
565 also identified as a biomarker for $t_{cent}^{control}$ DNA, confers resistance to penam and macrolide
566 antibiotics. These results imply that these ARGs were primarily present in the PMA-treated
567 fraction of DNA, and might have been a result of cell lysis, susceptibility of hosts to wastewater
568 disinfection, etc. The ARGs in PMA-treated fraction of DNA pose a potential threat if
569 transformed by pathogenic bacteria or by environmental bacteria that then through HGT pass
570 these genes to pathogens.

The ARGs in PMA-DNA pose greatest hazard when present in pathogens. Specifically, in some PMA-DNA samples, *Acinetobacter* was linked to carbapenem resistance (included in the beta-lactam category) and *Pseudomonas* was linked to multidrug resistance. *Clostridioides difficile* and carbapenem resistant *Acinetobacter* have been included under "urgent threats" category by the US CDC, while multidrug resistant *Pseudomonas aeruginosa* is a "serious threat" (CDC, 2019). Further study using cultivation based and/or pathogen specific biomolecular techniques are suggested to confirm the presence of these potential health threats. Recently, carbapenem resistant *Acinetobacter* were identified in most of the Pilica River, Poland, water samples through culture-based methods (Serwecińska et al., 2021) and in discharged effluent of a WWTP in Portugal (Oliveira et al., 2021). Carbapenem resistant *Acinetobacter* was not detected in our river water samples, but it was identified in some of the PMA-treated biofilm samples. Multidrug resistant *Pseudomonas* was present in some of the PMA-DNA of biofilm and one sample of water. Recently, multidrug resistant *Pseudomonas* spp. has been isolated from combined sewer overflows in Virginia, USA (Balasa et al., 2021) and from dams and rivers in Tunisia (Adhimi et al., 2022).

ARG abundance was not necessarily the highest at the sampling sites most influenced by wastewater effluent. This observation was consistent for both total and PMA-treated samples potentially due to dilution and/or control by other selective forces. Similar results were observed in a study on Mississippi river where high amounts of ARGs were present in wastewater effluent but they had no effect on ARG concentrations in river as measured by qPCR (LaPara et al., 2015). Wastewater disinfection inactivates bacteria but does not destroy the ARGs, thereby releasing extracellular ARGs in the environment through treated effluent (Eramo et al., 2019; Yuan et al., 2019).

Results of mobilome analysis indicated that (1) the PMA-DNA did not consistently have more ARGs associated with MGEs than chromosomal or unclassified across the sampling sites (2) nor did the PMA-DNA have more ARGs associated with MGEs than the $t_{cent}^{control}$ DNA. Identifying ARGs associated with MGEs is especially important for understanding potential hazards given that MGEs facilitate HGT of ARGs and impact their persistence and prevalence (Barnes et al., 2014; Klümper et al., 2019). This study identified no correlation between the percentage of ARGs assigned to MGEs in water or biofilm and wastewater effluent inputs. In a recent study on riverine sediment samples, a positive correlation was observed between MGEs and the wastewater effluent inputs in iDNA of sediment samples that we collected paired with the water and biofilm samples presented here (Deshpande and Fahrenfeld, 2022). This would seem to indicate that river matrix mattered more than land use regarding the association of ARGs with MGEs. This is potentially due to the fact that microbial communities are distinct between the matrices studied or other ARG fate and transport processes are driving their fate (e.g., sorption, settling, etc.). The variation could also be due to biases introduced by the MDA treatment which was not used with the sediment samples, although the kit used for the water and biofilm in this study was selected because it was shown to introduce minimal bias (Hosono et al., 2003).

The greater number of ARGs hosts in biofilms compared to water was expected since biofilms often contain more complex microbial communities (Costerton et al., 1978; Wimpenny et al., 2000). However, the alpha diversity of the total microbial community of water samples was higher than biofilm (Section 3.2). One possible reason for this could be that different software and databases were used for classification of the total microbial community and the hosts (Kaiju *vs* CAT). It could also imply that the less diverse community of biofilm compared to water (see section 3.2) resulted in better coverage for each organism, thus resulting in more hosts.

5.0 Conclusion

Overall, these results indicate a potential advantage of studying PMA-DNA from water and biofilm samples: the improved assembly for short reads, which may also have utility for informing hazard potential and therefore environmental risk assessment for ARGs. Using assembled contigs, it was possible to identify the genera containing potentially viable putative pathogenic hosts in both the matrices that can be further studied using targeted techniques such as qPCR or cultivation-based methods. Application of viability-based metagenomics indicated that the microbial diversity was overestimated for water samples by analyzing "total" DNA compared to PMA-DNA. Likewise, PMA-DNA provided a different measure of the potential for hazards associated with ARGs as indicated by shifts in ARG biomarkers and host assignments. Similar to studies of receiving waters with significant dilution, wastewater effluent inputs did not necessarily impact the microbiome, resistomes, and mobilome in river water and biofilm, even when sampled near an outfall. Further study would be required to determine if these results are reproducible in different sequencing platforms and across different environmental matrices and locations.

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Declaration of competing interest

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

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