Changes in prevalence but not hosts of antibiotic resistance genes during the COVID-19 pandemic versus pre-pandemic in wastewater influent

Deshpande, Aishwarya S.[1], *Ehasz, Genevieve*[2], *Eramo, Alessia,*[2] *Fahrenfeld, Nicole.*[2*]

[1]*Department of Biochemistry and Microbiology*, *Rutgers University, New Brunswick, NJ 08901*

[2]*Department of Civil and Environmental Engineering*, *Rutgers University, Piscataway, NJ 08854*

[*]*Corresponding author, nfahrenf@rutgers.edu, 848-445-8416*

Abstract

The COVID-19 pandemic offered a unique opportunity to study shifts in environmental antibiotic resistance that could be associated with the changes in disinfectant and/or antibiotic usage patterns, co-infections, or other behaviors. The aim of this study was to document temporal changes (pre-, early-, versus later-pandemic) in antibiotic resistance genes (ARGs), ARG hosts, biomarkers of potential co-infections, and the total microbiome in municipal wastewater influent from one separate sanitary and one combined sewer system. The 16S rRNA gene copy normalized concentration of *qac*E was higher in early- than pre-pandemic samples, and *sul*1 and *tet*(G) were higher in early- than later-pandemic samples. Metagenomics revealed significant changes in the abundance of the macrolide and sulfonamide ARG classes. COVID-19 cases positively correlated with the disinfectants/antiseptics group of ARGs and negatively correlated with the sulfonamide and aminoglycoside resistance classes. Discussion is provided regarding the correspondence of these observations with antibiotic prescription pattern changes during the study period. Putative waterborne pathogens were identified, which is of potential

interest for understanding the prevalence of community co-infections. No changes in host-ARG associations were observed. Overall, the results of this study may help in understanding the impact of the pandemic/lack thereof on another public health crisis: antibiotic resistance.

Keywords

Nanopore sequencing, ARGs, microbiome, co-infection, waterborne pathogens, wastewater-based epidemiology

Synopsis: Using wastewater-based epidemiology to measure changes in antimicrobial resistance during the COVID-19 pandemic can guide how to protect public health.

1 Introduction

The recent COVID-19 pandemic may have direct implications for antimicrobial resistance. Many of the EPA-recommended disinfectants for SARS-CoV-2 contain quaternary ammonium compounds (QACs) as their active ingredient, and the use of these disinfectants increased significantly during the pandemic.[1] Apart from QACs, disinfectants can also contain other biocides that are antimicrobials.[2] During the pandemic, a wastewater treatment plant (WWTP) in Greece was reported to have received 152% more biocides as compared to pre-pandemic time in 2019 [3]! Likewise, a high concentration of disinfectants and their positive correlation with COVID-19 cases was observed in residential dormitory wastewater in Singapore.[4] Studies measuring the concentration QAC residues in wastewaters in USA during the pandemic are presently not available. Given the concern with increased used of disinfectants during the pandemic, several researchers have reviewed the potential implications on wastewater treatment and receiving waters.[5, 6] Of particular concern is whether the increased use of disinfectants

resulted in increases in antimicrobial resistance (AMR). Notably, if the biocide concentrations reach the sub-minimum inhibitory concentration for most of the bacteria found in the system, this increase in selection pressure can promote AMR evolution [as postulated by several researchers reviewed by McBain et al.[7]].

The consumption of antibiotics surged in hospitalized patients during the initial months of COVID-19 pandemic to treat or prevent other secondary infections. During the early stages of the pandemic, 72% of COVID-19 patients were prescribed and using antimicrobials even though the incidence of bacterial or fungal co-infection in COVID-19 infected patients was around 8%.[8] The lack of decision support and rapid diagnostic tools early in the pandemic also lead to increased and unnecessary use of antimicrobials.[9] Some antibiotics that were prescribed and used commonly during this time were penicillin, meropenem, moxifloxacin, cephalosporins, macrolides, and quinolones.[10, 11] However, for outpatient-health care, the usage of antibiotics dropped in 2020 compared to 2019, which could be attributed to lower incidences of other respiratory diseases.[12] Given that a range of antibiotic concentrations can select for antibiotic resistance,[13] the pandemic offers an opportunity to study the potential effects of changes in antibiotic consumption on antibiotic resistance in the wastewater environment.

Wastewater-based epidemiology (WBE) has extensively been used to study the spread of SARS-CoV-2 during the COVID-19 pandemic.[14-22] WBE is also a useful tool for tracking the spread of antibiotic resistance in communities with sewage collection systems and the environment.[23-27] Pre-pandemic studies from our team showed that the variance in wastewater influent abundance of ARGs such as *sul*1, *tet*(G), *tet*(W), *tet*(O), *van*A and *erm*F was explained by season as well as water quality factors such as pH and heavy metals.[28] In addition to ARG monitoring, WBE also

allows for studying the total microbial community[29] and can aid in our understanding the co-infecting pathogens and other changes in the sewage microbiome.[30]

WBE studies monitoring ARG during the pandemic are emerging in the literature with varying durations, ARG targets, and methods. A wastewater surveillance study from Las Vegas, Nevada demonstrated that the abundance of ARGs belonging to fluoroquinolone and beta-lactam classes increased during a COVID-19 surge in December 2020 compared to November 2020. The study used a combination of qPCR and metagenomic sequencing for analyzing the samples.[31] Using metagenomics, a positive correlation was also observed between the macrolide, tetracycline, sulfonamide and some beta-lactamase ARG classes and time over three months in hospital wastewater from Saudi Arabia.[32] Likewise, a study from India noted significant increases in antidrug resistance of *E.coli* in 2020 compared to 2018 using the culture based Kirby-Bauer disk diffusion method.[33] To our knowledge, there are no reports to-date of the relative abundance of ARGs and their hosts in wastewater influent nor comparisons of pre- and during-pandemic concentrations of ARGs and their hosts in wastewater influent. Understanding not only the abundance and diversity of antibiotic resistance genes, but also their hosts and genetic context is of interest for risk assessment.[34, 35]

The objectives of this study were to (O1) study the abundance of ARGs in wastewater across time at two WWTPs during the COVID-19 pandemic, (O2) determine if there were microbial community changes during the pandemic and (O3) identify the hosts of these ARGs and determine whether a shift in ARG-hosts is observable before/during the pandemic. Wastewater influent samples were analyzed using qPCR for selected ARGs and long read nanopore sequencing to study the wastewater resistome and microbiome, the latter with a focus on

identifying putative pathogens. Overall, the results presented seek to help in understanding the potential impact of the pandemic on another public health crisis: antibiotic resistance.

2 Materials and Methods

2.1 Sampling, DNA extraction, and qPCR

Composite Wastewater influent samples were collected from two WWTPs every week from June 2020 to March/May 2021.[36] WWTP-A has a design flow <10 MGD and is connected to a separate sanitary sewer system. WWTP-D has a design flow of 330 MGD and is connected to a combined sewer system (these WWTP names are selected to match those used in Fahrenfeld et al.[36]). Samples were collected every week on Tuesdays/Wednesdays. Automatic samplers were used for collecting composite 24-hour samples (1 Liter) from the WWTPs. All samples were transported on ice to the lab. Before filtering the samples, they were pasteurized to inactivate viruses [37]. Around 200-300 mL of each composite sample was filter concentrated using 0.22 μm mixed cellulose ester membrane filters (Millipore Sigma, St. Louis, MO, USA), and the filters were stored at -80°C until DNA extraction. One sample per month was chosen for DNA extraction from each WWTP, avoiding the days on which rainfall was recorded. DNA extraction from the selected filters was performed using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH, USA).

Archived wastewater influent sample DNA extracts stored at -20°C collected in 2015- 2016 from WWTP-D were retrieved for comparison. Briefly, composite samples were composed of WWTP grab samples (500-mL each) collected at 8 am, 10 am and 12 pm. All samples were collected during baseflow conditions in sterile 500-mL Nalgene bottles, transported to the lab on ice, and

stored at 4°C prior to filter concentration (90-230 mL) using 0.22 µm nitrocellulose filters (Millipore Corporation, Billerica, MA). These DNA extracts were categorized as pre-pandemic samples.

Water quality parameters monitored in the pandemic wastewater samples included pH, total suspended solids (TSS), and chemical oxygen demand (COD), as previously described.[36] qPCR was performed for 16S rRNA gene[38] and for select resistance genes. ARGs analyzed included *sul*1,[39] *tet*(G),[40] *bla*TEM,[41] and *qac*E.[42]

Details on COVID-19 cases estimation for each sampling event at each WWTP are provided in a previous study by the lab.[36] In brief, publicly available county data (NJ COVID-19 Dashboard) was used for estimating the COVID-19 cases. The population of towns served by the WWTP were estimated using US census data. The number of COVID cases was obtained for each sampling event by multiplying the percentage of the county population residing in the towns served by the WWTP (Table S2).

qPCR reactions were prepared with 0.4 µM forward and reverse primers, 5 µl SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA), and 1 µl sample to make a total of 10 µl reaction volume per well. All DNA extracts were diluted by a factor of 1:100 to reduce the inhibitors present in the samples. No-template controls (NTC) consisting of sterile molecular biology grade water were included in every run. Pre-quantified standards were diluted 10-fold to produce a seven-point calibration curve from 10^8 to 10^2 gene copies for ARGs or to 10^3 gene copies for 16S rRNA. The standards, samples, field blanks and the NTC were run in triplicate (i.e., technical replicates). Melt curve analysis was performed for each gene to check the

specificity of the reaction. The primers, annealing temperatures, efficiencies, and R^2 values are listed in Table S3.

Samples were assigned to seasons based on the date of sampling: Spring season being from March 1 to May 31, Summer from June 1 to August 31, Fall from September 1 to November 30 and Winter from December 1 to February 28 (Table S2).

2.2 Nanopore Sequencing & Bioinformatics

Eight samples from WWTP-D along with four archived samples from the same WWTP were selected for long-read shotgun sequencing using the Minion MK1C platform. Samples were chosen from the same months as that of pre-pandemic samples when possible or randomly. All samples were purified using the Genomic DNA Clean & Concentrator Kit (gDCC, Zymo Research, Tustin, CA) and quantified using Qubit (Invitrogen) before library prep. The native barcoding kit 24 (SQK-NBD112.24) was used for library prep and three samples were barcoded per run. At least 1000 ng DNA was used per sample, with the exception of two samples with low DNA concentrations as measured via Qubit (Table S4). Library prep was performed following the manufacturer's protocol with the following minor modifications to reduce DNA losses and avoid size selection. In the DNA repair and end prep stage, AMPure beads (1.8X concentration) were added and after resuspending the pellet in nuclease free water, the incubation time was increased to 10 minutes at 37°C. AMPure beads (0.8X concentration) were used in the Native barcode ligation and for the final Adapter ligation and clean up stage. During all stages of the AMPure bead clean-up, when the pellet was resuspended in nuclease-free water to elute the DNA, gentle pipette mixing was done before incubation to encourage the elution of DNA. At the end of each step, Qubit analysis was performed to quantify the DNA remaining at each stage.

Loading beads were used for loading samples on the flow cell. Barcoded samples were loaded onto a flow cell (FLO-MIN106-D) following the manufacturer's instructions and run for 72 hours, until the flow cells were used up. A total of four runs were performed with three barcodes per run, resulting in a total of 12 samples. The flow cell pore count was checked before starting each run.

DNA extracts were prepared, cleaned, barcoded, and sequenced (details in Supplemental Information). Sequences were deposited in the National Centre for Biotechnology Information Sequence Read Archive (NCBI-SRA-PRJNA977080).

Raw reads were generated in fast5 format and all details of quality and depth are provided in Table S5. The average sequencing data generated was 1.46 Gb per sample. Recent literature reporting the resistome and microbiome of wastewater influent samples through nanopore sequencing reported 1.25-6.07 Gb per sample.[43-45] Notably, Dai et al. (2022)'s results suggested that rate of ARG detection did not change with the sequencing depth.[43]

Reads were basecalled using Guppy basecalling software (v6.4.6, GPU version) to return fastQ reads. Barcoding, demultiplexing, and barcode trimming were done using the Guppy barcoding software (v6.4.6). N50 and quality of the fastQ reads was checked using stats.sh command from BBMap.[46] Samples were then analyzed using the FastQ Antimicrobial Resistance pipeline (v2022.08.16-15679) in EPI2ME software. The FastQ Antimicrobial resistance pipeline has been designed for reads generated through the Oxford Nanopore platform. The pipeline uses the Centrifuge database for taxonomic classification and the comprehensive antimicrobial resistance database (CARD) for antimicrobial resistance genes identification [47] and has been used in previous studies to identify ARGs and their hosts.[43, 45]. However, the classification of reads

provides the likely host-ARG association and there is an uncertainty in these analyses,[48] which is greater for ARGs associated with mobile genetic elements [49]. Hence, the term "putative host-ARG association" has been used in this study.

Lineage was derived from the taxids provided in the output file using the NCBITax2Lin code (https://github.com/zyxue/ncbitax2lin). Custom python scripts were used for data filtering and sorting for ARGs and hosts. Only the hits obtained from the Kingdom Bacteria were retained for further analysis. The percent abundance of different genera was found by dividing the number of reads classified by the total number of reads for the sample after QC filtering.

Only the ARG hits identified through the protein homolog model were considered for further analysis.[43] ARGs were then grouped into categories based on The CARD database (https://card.mcmaster.ca/, downloaded on 3/25/23[50]). The abundance of ARGs was found using the following formula.[43]

$$ARG\ Abundance\ (gene\ copies/Gb) = \frac{\frac{Alignment_{end} - Alignment_{start}}{Length_{reference}}}{\sum_1^n length_{read}(Gb)}$$

Here, $Alignment_{end}$ and $Alignment_{start}$ are the positions in the read where alignment started and ended, $Length_{reference}$ (bp) is the length of the respective ARG and n represents the total number of reads present in the sample after QC. All the parameters for calculating ARG abundance were obtained from the output file from the EPI2ME pipeline.

The abundance of each ARG type associated with a particular host was calculated by summing up the abundances of each ARG (of that ARG type) that was assigned to the particular host. For example, for sulfonamide ARG type linked to the putative host *Vibrio,* the abundances of all

ARGs belonging to the sulfonamide category (such as *sul*1, *sul*2 etc.) and classified as *Vibrio* were summed.

2.3 Data Analysis

All statistical tests were performed in RStudio version 4.1.3 (www.r-project.org). Normality of data was checked using a Shapiro-Wilk test. Homogeneity of variances was confirmed using a Bartlett test. Two-way ANOVA with a posthoc TukeyHSD was performed on parametric qPCR data (i.e., *qac*E, *tet*(G), *bla*(TEM)). For non-parametric qPCR data (i.e.,16S rRNA, *sul*1*)*, the Kruskal-Wallis test followed by the post-hoc Dunn test was with Bonferroni correction performed. Oneway.test was used with a posthoc pairwise t-test (pairwise.t.tet) with Bonferroni correction for qPCR data that were normal but did not have equal variance (i.e., *tet*(G)). Spearman's correlations were tested between the qPCR measured concentration of ARGs and the number of COVID-19 cases reported in the sewer catchment one week prior to the date of sampling (previously shown to have moderate correlation with the SARS-CoV-2 N1 gene copies per capita per day[36]). PERMANOVA (adonis) analysis was performed to understand the relative importance of factors (pH, conductivity, COD, TSS, COVID cases, season, time phase) impacting qPCR measured ARG concentrations.[51]

For the NGS data, a one-way ANOVA was performed on all ARG types (i.e., ARG classes such as macrolide, sulfonamide, etc.) against different pandemic phases (i.e., pre-, early-, and later-pandemic), followed by TukeyHSD posthoc test if the p-value was significant (<0.05). Samples collected during the pandemic from June 2020 to March/May 2021 were divided into two time phases: early second wave designated as early-pandemic (June 2020-December 2020) and later

second-wave designated as later-pandemic (January 2021- March/May 2021). A one-way ANOVA was also performed on the total abundance of all ARGs to test for differences in the sampling periods. Shannon, Simpson and InverseSimpson diversity indices were calculated for ARGs and the total microbial community. A Kruskal-Wallis rank sum test was performed on ARG diversity indices. Linear discriminant analysis effect size (LEfSe) test was performed on ARG hosts and total microbial community to identify the biomarkers[52] in the pre-, early-, and later-pandemic samples. Spearman's correlations were performed between ARG abundances and the number of COVID-19 cases reported in the sewer catchment one week prior to the date of sampling (non-parametric correlation test). Bray-Curtis dissimilarity matrices were calculated for (1) the total microbial community at genus level and (2) ARG abundances. Then, non-metric multidimensional scaling (nMDS) and otherer plots were made using ggplot2 package.[53]

3 Results and discussion

3.1 Differences in normalized ARG concentration observed in qPCR analysis

Differences were seen for selected ARGs normalized to 16S rRNA gene copies when comparing pre- early-, and later-pandemic samples (Fig. 1, Fig S1). For WWTP-D, *qac*E was significantly higher in early-pandemic samples than pre- and later-pandemic samples from WWTP-D (TukeyHSD, both $p<0.04$) and *tet*(G) was significantly higher in early pandemic samples compared to pre-pandemic samples (post hoc pairwise t-test, $p=0.002$). The 16S rRNA gene copy normalized concentration of *sul*1 gene was higher in early- than later-pandemic samples (Dunn test, $p= 0.003$). No differences were observed between *bla*TEM gene normalized concentrations comparing pre- and early- and later- pandemic samples (2-way ANOVA, all $p> 0.7$). For WWTP-A, where only early- and later-pandemic samples were available, no

differences were observed for normalized *qac*E, *tet*(G), *sul*1 and *bla*TEM gene copies between early- and later- pandemic samples (TukeyHSD, all p> 0.059) (Fig. 1).

The absolute concentration of 16S rRNA gene copies/mL was higher in Pre-pandemic than early- and later pandemic samples (Dunn Test, both p< 0.02), while there were no differences in WWTP-A (Kruskal-Wallis test, p= 0.3).

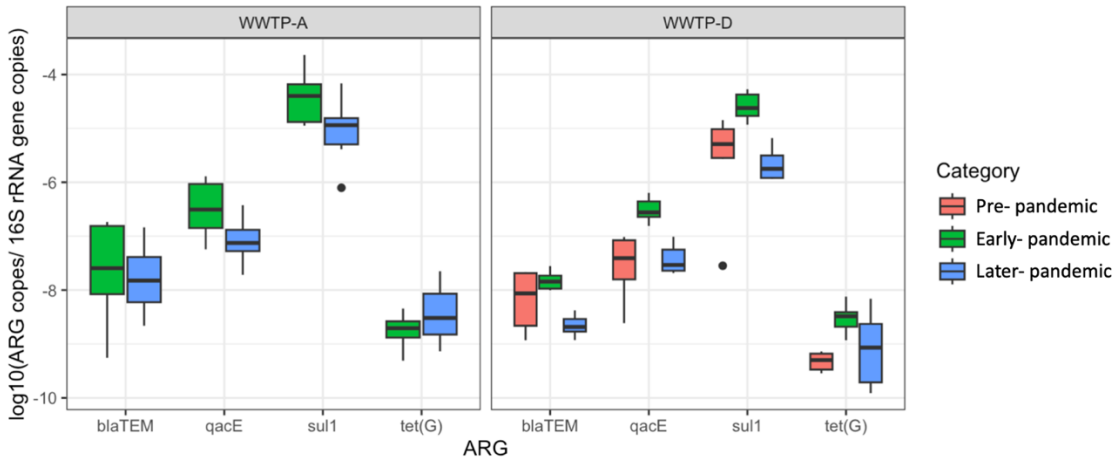


Fig. 1 *Normalized ARG abundances (ARG gene copies/ 16S rRNA gene copies) for blaTEM, qacE, sul1, and tet(G) in wastewater observed in pre-, early-, and later-pandemic samples. Pre-pandemic samples were collected in 2015-16 while early- and later-pandemic samples were collected during the early second wave and the later second wave during the pandemic in 2020-21. Boxplots represent N=5 samples for each time phase and gene combination.*

A moderate negative correlation was observed between the 16S rRNA gene copy normalized concentrations for both *sul*1 and *bla*TEM and the total number of COVID-19 cases for WWTP-D samples (Fig. S2, Spearman's rho=-0.59 and -0.74, respectively and both p < 0.043). Similarly, a

moderate negative correlation was observed between the 16S rRNA gene normalized concentrations of *sul*1, *qac*E and *bla*TEM and the total number of COVID-19 cases one week before the sample collection date for WWTP-A samples (Fig. S2, Spearman's rho=-0.66, -0.78, -0.72, respectively, all $p< 0.01$). [No correlations were observed for *tet*(G) with either WWTP nor *qac*E and WWTP-D and total COVID-19 cases, Fig. S2.] Including pre-pandemic samples in addition to early- and later-pandemic samples for WWTP-D resulted in no significant Spearman's correlations between normalized ARG concentrations and COVID-19 cases one week prior to sampling (all $p>0.1$, Fig. S3).

PERMANOVA analysis performed on all samples (pre-, early-, and later pandemic) indicated that pandemic phase 16S rRNA gene copy normalized ARG concentrations were associated with pandemic phase (i.e., pre-, early, or later; $p=0.001$), but not with COVID-19 cases, WWTP, and season (all $p>0.06$). PERMANOVA analysis of samples for which water quality data were available (i.e., early- and later-pandemic) indicated that pH had a significant effect on ARG concentrations (PERMANOVA, $p=0.039$) while other factors (conductivity, COD, TSS) did not (PERMANOVA, $p>0.054$).

Given the availability of pre-pandemic and pandemic samples, the results from WWTP-D could support the predictions that the increased use of cleaning agents[54] and increases in antibiotic prescriptions and residues in wastewater[55] may select for increases in antibiotic resistance during the pandemic.[5, 55] The subsequent decrease in the concentration of *qac*E could possibly be due to changes in behavior as the pandemic progressed due to lesser usage of disinfectants, as was predicted by [56]. Measuring the selecting agents (which was beyond the scope of the present study) in parallel with ARGs would be needed to demonstrate a direct relationship.

The variation in the ARG abundances observed in this study could also be potentially attributed to other factors such as seasonal variations[57] and/or changes in the movement of people in/out of the sewer catchment. We previously observed season was an important factor for explaining the variation in ARGs measured via qPCR in wastewater influent sampled in 2016-2017 from WWTPs with >100 MGD design flows.[28] Such seasonal variations were speculated to follow changes in antibiotic prescription rates[58] and therefore selective pressure in the gut microbiome[59] and antibiotic fluxes in wastewater.[60]

The demographics of the sewage catchment were likely different for the pre- versus the early- and later-pandemic samples, which may also have impacted our observations given that the while the sewage microbiome is markedly similar around the globe[61] it does reflect population gut microbiome.[62] There was a consistent drop in U.S migration from 2019 to 2021 and the local mobility (movement within the country) also dropped.[63] Drastic changes in movement compared to baseline were reported in the sewer catchments studied here for residential, workplace, and transit (Fig. S11[36]) during the pandemic. Notably, both WWTP studied here have regional rail lines, airports, and travelers/commuters in the densely populated study region. Human mobility has been linked to increase in antimicrobial resistant organisms and the antimicrobial resistant infections.[64] In-system sampling to differentiate transient and/or transportation associated populations (for e.g. [65]) and larger/longer data sets could aid in disentangling any potential human movement impacts.

System-to-system differences were demonstrable in the present study through comparison of the early- and later-pandemic qPCR results between the two WWTPs that showed inconsistent results for *qac*E and *sul*1. The differences could be due to several system-specific factors

295 including the differences in design flow, hydraulic residence time, sewer type, and water
296 quality.[28, 66, 67] Since samples from wet weather days were not included in this study, rainfall was
297 assumed to not have any impact on the observations. WWTP-D has a design flow over one order
298 of magnitude greater with a travel time travel 1.5-2hrs longer than WWTP-A. There is potential
299 for but limited evidence to support ARG selection in sewers (as reviewed by[27]) but ARG
300 transcription in sewers has been demonstrated,[68] which may imply that travel time could impact
301 observations of ARGs in wastewater influent. Further research would be useful in confirming the
302 impact of travel time on ARG concentrations. The sewer type could also impact our observations
303 given that WWTP-D is a combined sewer system while WWTP-A is separate sanitary system
304 and we previously reported significant differences in the abundances of some ARGs via qPCR as
305 a function of sewer type (all with >100 MGD design flow) sampled in 2016-2017.[28] Not
306 including samples from wet weather days may have reduced the impact of sewer type. Again, a
307 larger number of samples from more treatment plants would be useful for disentangling these
308 potential impacts.

309 Differences in water quality as noted above through random forest analysis may also affect the
310 concentrations of ARGs in WWTPs. We had previously found that variation in ARGs could be
311 explained by water quality parameters such as conductivity, pH, COD, TSS, and heavy metals [28].
312 Likewise, significant correlations were observed between some ARGs and ammonium,
313 phosphate, and COD in wastewater influent from eight WWTPs in China.[67] Some of these
314 observations could be due to co- and cross-selection (e.g., heavy metals) whereas others may be
315 more related to shifts they cause in microbial community composition.

316 3.2 Metagenomic insights into ARG abundance pre- and during the pandemic

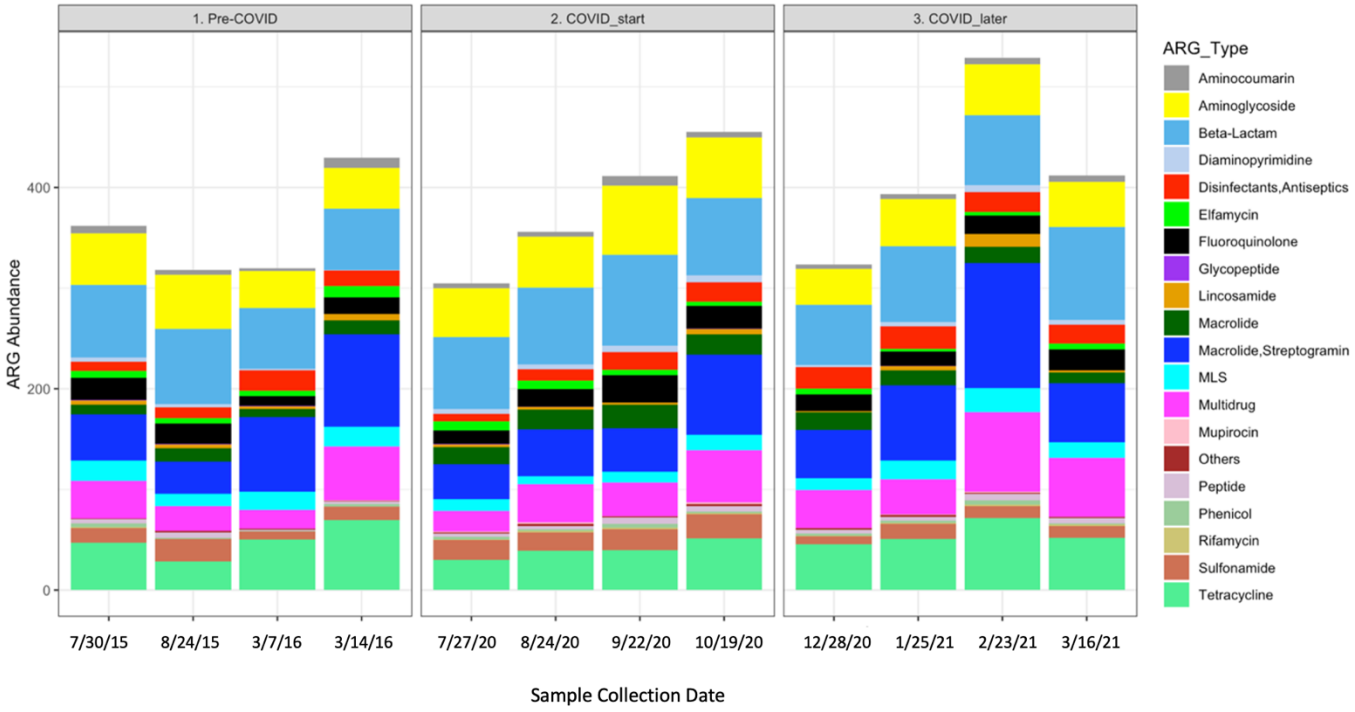
317 In total, 625 different ARGs were identified in the samples analyzed from pre-, early-, and later-
318 pandemic samples from WWTP-D. ARGs were grouped into 20 categories based on drug class
319 (Fig. 2A). On an average, the ARG types that were most abundant in all samples (abundance
320 greater than 10% of total average abundance) were beta-lactam, macrolide-streptogramin,
321 multidrug, aminoglycoside and tetracycline. Details of raw reads obtained after sequencing are
322 provided in Table S5.

323 The abundance of macrolide ARGs was significantly greater in early- than pre-pandemic
324 samples (TukeyHSD, p= 0.004, Fig. 2B). Interestingly, there were no significant differences in
325 the concentration of macrolide ARGs between the pre- and later-pandemic samples. Further, the
326 abundance of sulfonamide ARGs was significantly greater in the early- than the later-pandemic
327 samples (TukeyHSD, p= 0.03, Fig. 2B). No differences were observed in other ARG types
328 across the pre-, early-, and later-pandemic samples.

329 While antibiotics were not measured in the wastewater in the present study, information on
330 antibiotic prescriptions in the US is available. The macrolide azithromycin was the most
331 commonly prescribed antibiotic in the initial months of the COVID-19 pandemic in USA[69, 70]
332 and could be related to the higher abundances of macrolide ARGs observed via metagenomics.
333 The prescription rate decreased in May 2020 but remained higher than the pre-pandemic time in
334 2019.[12, 70] In contrast, the changes in tetracycline and sulfonamide genes do not correspond with
335 prescription rates for sulfonamide and tetracyclines, which decreased during the study period.[71]
336 This was mainly due to a decrease in outpatient health care for other respiratory illnesses during
337 the pandemic. CDC report indicates that although the outpatient antibiotic usage resumed to

normal rate in 2021, it was still lower overall in 2021 compared to 2019.[12] Thus, other potential factors should be explored (See Section 3.1).

A)



B)

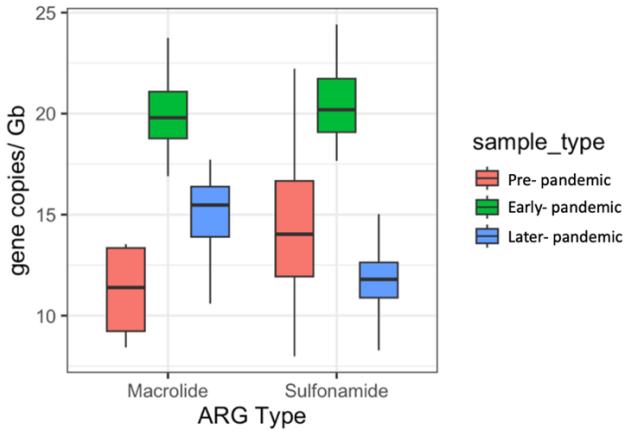
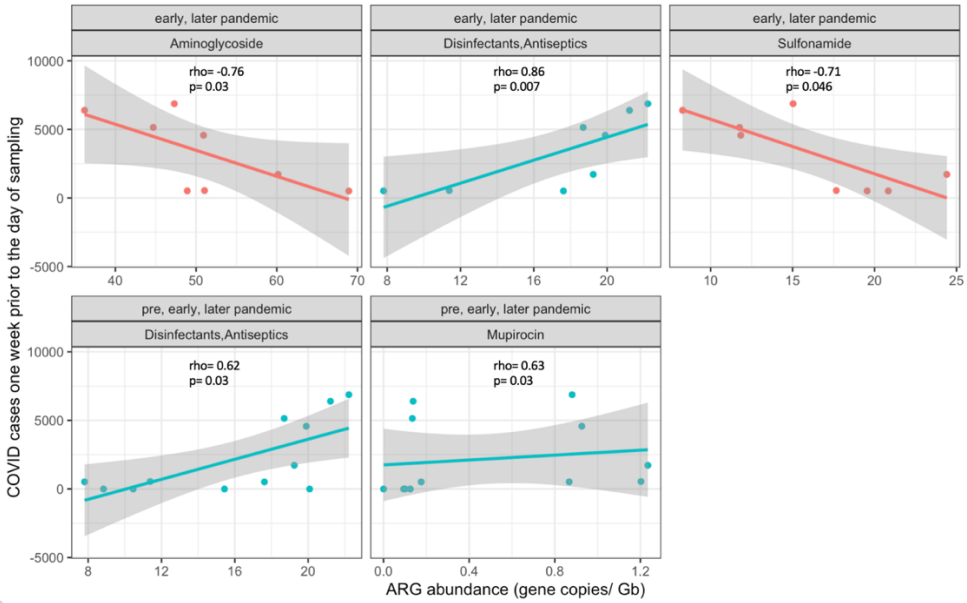


Fig. 2 *A) Stacked bar graph representing the cumulative ARG abundance in pre-, early-, and later-pandemic samples. Colors represent the respective ARG Types as per the legend. B) ARG class abundance from metagenomes from pre-, early-, and later-pandemic samples.*

No differences were observed in the alpha diversity of ARGs between time phases (pre-, early-, and later-pandemic; Kruskal Wallis test, $p>0.2$, Fig. S5A). Similarly, no differences were observed in the profile of ARGs between time phases and seasons (PERMANOVA, $p=0.26$, Fig.S5B). A strong positive correlation was observed between the disinfectants-antiseptics class and COVID-19 cases one week prior to sampling (Spearman's rho=0.86, $p=0.006$). The sulfonamide and aminoglycoside resistance classes showed a moderate negative correlation with COVID-19 cases (Spearman's rho= -0.71 and -0.76, respectively, $p <0.028$; Fig.3). Note that a significant negative correlation was also observed between the *sul*1 gene, conferring resistance to sulfonamides, quantified through qPCR and COVID-19 cases (See Section 3.1). Similar results were observed in a study of hospital wastewater, where a negative correlation was reported between ARGs belonging to drug classes aminoglycoside, peptides, beta-lactam, fluoroquinolone, and lincosamide and the number of COVID-19 patients.[32] This negative correlation could be attributed to a variety of reasons, including high rates of antibiotic prescription in the initial months of the pandemic followed by the decrease in overall antibiotic usage as the pandemic progressed.[12] Interestingly, the correlation was not significant for either aminoglycoside and sulfonamide when pre-pandemic samples were included (COVID-19 cases for pre-pandemic samples were considered zero), underscoring the need for inclusion of samples from a longer time period to understand any pandemic-related changes.

Other ARG classes that did not have significant correlations include macrolide and MLS, that may be worth exploring with larger pandemic data sets (Spearman, both $p>0.057$, Fig. S6). Correlations were also tested including pre-, early-, and later-pandemic data for ARG abundances and COVID-19 cases. (Note, COVID-19 cases for pre-pandemic samples were considered to be zero). Including pre-pandemic samples, disinfectants-antiseptics and mupirocin categories of ARGs showed a moderate positive correlation with COVID-19 cases (Spearman rho= 0.62, 0.63, respectively, both $p=0.03$ which are inexact due to the data having ties; Fig. 3). Note, however, that for the mupirocin correlations, that the ARG abundance range was quite small. Mupirocin is used to treat hospital acquired infections such as MRSA. Strikingly, the incidence of MRSA infections increased by 13% from 2019 to 2020 in USA.[12] Similarly, significant increases in MRSA infections were reported in Turkey during 2020 as compared to 2019 from nasal swabs of around 2700 participating patients.[72] Compiled data from various reports around the world suggests that MRSA was one of the most frequent pulmonary infection causative agent in patients with COVID-19.[73] More localized prescription rates and measurement of the antibiotic residues in the wastewater could help substantiate any relationships and other drivers for these observations such as ARG-host selection and/or co- and cross-selection. Other ARG classes did not show significant correlation with the COVID-19 cases (Fig. S7).



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384 **Fig. 3** *ARG abundance in metagenomes versus COVID-19 cases one week prior to the day of*
385 *sampling. Each plot header lists data from the time periods included in the plot (i.e., pre-, early-,*
386 *and later-pandemic or early- and later-pandemic only). Note that X-axis range is different for*
387 *each ARG Type. Significant positive and negative correlations are shown in teal and coral color*
388 *respectively, and grey shading represents the 95% confidence interval. (Results for insignificant*
389 *correlations are shown in Fig. S7.)*

390 3.3 Microbiome observations pre-, early-, and later pandemic

391 The most abundant genera present in the total microbial community across all samples were
392 *Aliarcobacter, Aeromonas, Acinetobacter, Arcobacter, Cloacibacterium* and *Pseudomonas* (Fig.
393 4). The profile of total community is shown in nMDS plot in Fig. S8B. No differences were
394 observed in the microbial community structure between time phases and seasons
395 (PERMANOVA, all $p>0.08$). There were no differences in the alpha diversity of the total
396 microbial community at the genus level between the different pandemic phases (PERMANOVA,

p=1, Fig. S8A). Studying the wastewater influent microbiome can (1) help in determining the rate of co-infections in the community and (2) be used to evaluate the relation between microbiome shifts and COVID-19 prevalence in the community.[30]

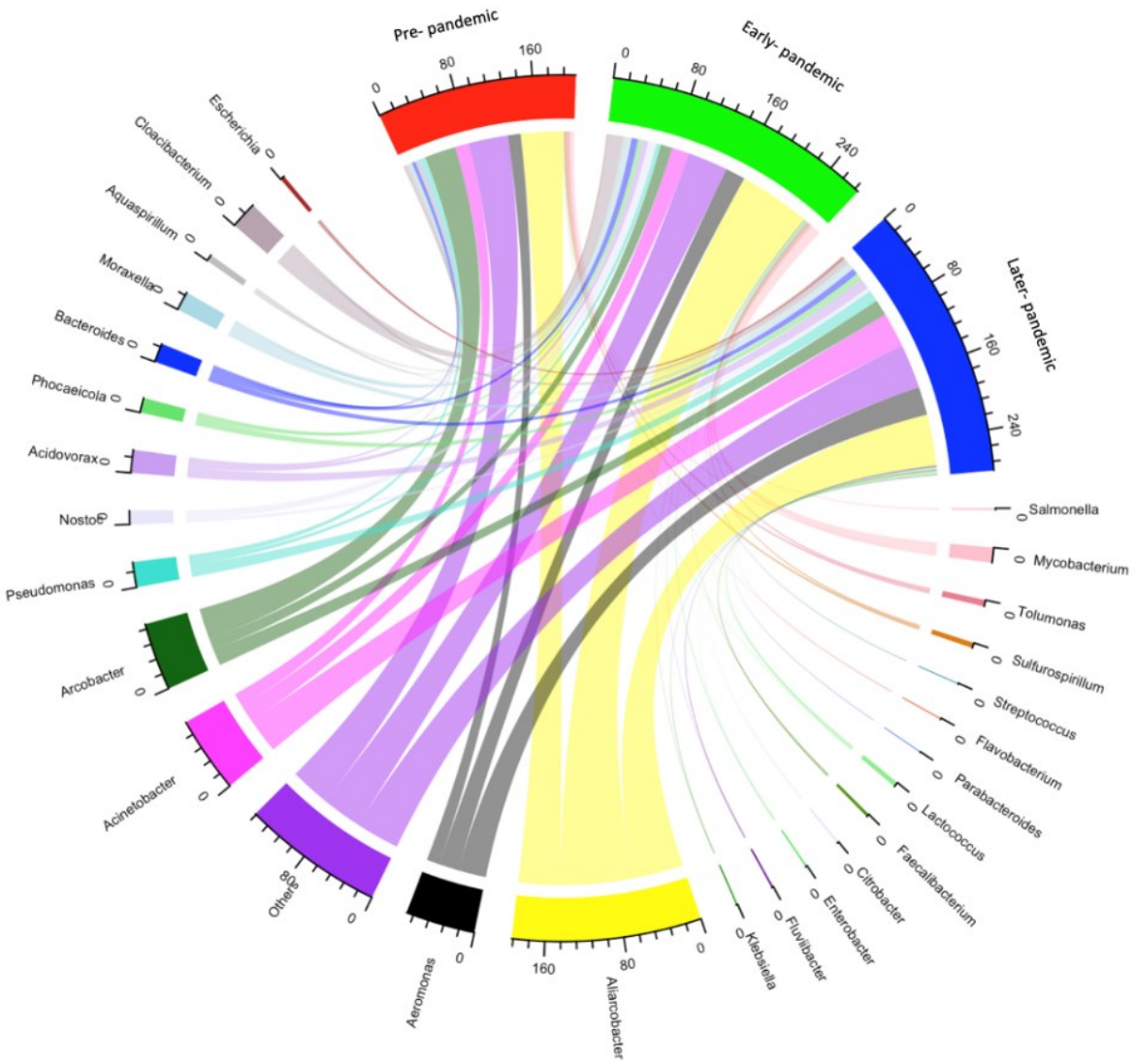
The LEfSe analysis indicated specific biomarkers (LDA score >3) in all the pandemic phases (Fig. S9). Specifically, eight genera were differentially abundant in pre-pandemic samples. Three genera were biomarkers for the early- and seven genera were biomarkers for later-pandemic samples. Among the genera that contain waterborne and opportunistic pathogens, *Salmonella* was biomarker in pre-pandemic samples. Other reports of *Salmonella* associated with co-infection were not identified in the literature. Interestingly, there were 22% less *Salmonella* infections in 2020 compared to pre-pandemic in the US, probably due to pandemic-related behavioral changes such as fewer restaurant meals.[12]The genus Enterobacter was a biomarker in early-pandemic samples. Rapid diagnostic tests from a study from Italy identified *Enterobacter cloacae* as a co-infecting organism during the COVID-19 outbreak.[74] Another study from Maryland, USA identified a significant positive correlation between *Enterobacter cloacae* and SARS-CoV-2 in wastewater during the pandemic in 2021 through next generation sequencing.[30]

The genus *Escherichia*, which contains waterborne pathogens, was a biomarker in our later-pandemic samples. There is the possibility that these are markers of the co-infections or even hospital-acquired superinfections during COVID-19 caused by *Escherichia coli*[75-77] as there were health care facilities in the sewer catchments for the present study. Whether healthcare wastewater impacts the municipal WWTP microbiome depends on several factors including the dilution[78] and both WWTPs in the present study had healthcare facilities in the catchments. Specifically, *E.coli* was a common pathogen causing secondary bloodstream infections in

COVID-19 patients at three medical centers in the study region from March- May 2020.[79] The most abundant genus *Aliarcobacter*, includes the emerging foodborne and zoonotic pathogen *Aliarcobacter butzleri.*[80]

It is also possible that the biomarker observations could indicate other pandemic-related health changes: a study conducted from May to July 2020 in wastewater influent from Chile observed through metagenomics that the wastewater microbiome associated with gastrointestinal disorders preceded the SARS-CoV-2 detection in untreated wastewater, suggesting that wastewater microbiome could be an indicator for COVID-19 surveillance.[81]

Apart from the biomarkers discussed above, there were no differences in the alpha diversity nor the structure of the microbiome, indicating that the pandemic phases studied here were not associated with a shift in the microbial community. This was contrasting to the results observed in a recent study where temporal shifts in wastewater microbiome were observed through metagenomics during an increase in COVID-19 cases in Maryland.[30] Studies comparing the wastewater influent microbiome between the pandemic and pre-pandemic time are not available in literature.



435

436

437 **Fig. 4** *Major genera (>0.5% abundance in all samples) observed in the total microbial*

438 *community from the pre-, early-, and later- pandemic samples. Genera with abundances <0.5%*

439 *have been grouped into "other" category.*

3.4 ARG-host association in pre-, early-, and later- pandemic samples

In total, 58 different genera were identified as the hosts of ARGs. Ubiquitous waterborne putative pathogenic hosts [as defined by[82]] identified in the samples included the genera *Campylobacter, Mycobacterium, Burkholderia, Aeromonas, Vibrio, Escherichia* and *Salmonella*. The most frequent hosts of ARGs belonging to the category of disinfectants/antiseptics were *Pseudomonas* and *Vibro* (Fig. 5). Specifically, the hosts were *Pseudomonas aeruginosa* and *Vibrio cholerae*. *Vibro* was also observed to be the host of most of the sulfonamide resistance genes (~99%).

Previous studies from around the world including clinical reports and data from broiler farms have shown that generally the common hosts of *qac*E / *qac*Edelta1 gene were *Klebsiella, Pseudomonas* and *E.coli*[83, 84] while *qac*A/B genes were associated with *S. aureus* and *E. faecalis* (reviewed by [84]). All *qac* genes belong to the disinfectants-antiseptics class and confer resistance to quaternary ammonium compounds.[85] The genus *Vibro* has been found to contain multidrug and toxic compound extrusion (MATE) pumps, conferring resistance to biocides such as chlorhexidine, benzalkonium chloride, and triclosan.[86] A review on environmental *Vibrio* species spanning two decades (2000- 2019) found that the most of the ARGs carried by this genus belonged to tetracycline (21%) and beta-lactam and sulfonamide (20%) drug classes.[87] This observation partly aligns with the result of this study that *Vibrio* genus was the host of sulfonamide resistance genes.

ARGs belonging to aminocoumarin drug class were exclusively linked to *Escherichia* and *Streptomyces* genera. In contrast, the ARG classes tetracycline, beta-lactam, multidrug, and aminoglycoside each had more than ten hosts. Among the waterborne putative pathogenic hosts,

462 *Mycobacterium* was exclusively linked to multidrug resistant genes, *Campylobacter* was linked
463 to aminoglycoside, diaminopyrimidine and tetracycline ARG classes, and *Burkholderia* was
464 linked to multidrug and beta-lactam ARG classes. The rest of the putative pathogenic genera
465 hosts were linked to more than three ARG classes. LEfSe analysis on ARG-hosts at the genus
466 level as a function of ARG abundances did not show any differentially abundant features
467 between pre-, early-, and later-pandemic samples (Fig. 5). This indicates that no changes in the
468 putative host-ARG linkages were identified during the different time phases. Thus, while there
469 were changes in the antibiotic resistance concentrations for select ARG classes, there is no
470 evidence for significant ARG transfer to new hosts despite the potential for selective pressure
471 (Section 1). The lack of differences in the ARG-hosts also indicates that the host diversity did
472 not change when comparing samples from 2015-26 and 2020-21, despite the changes in the
473 movement of people noted above. It would be interesting to see how conserved these results are
474 across sewer sheds and geographies, for healthcare associated wastewater (rather than the
475 municipal WW sampled here), and for longer or different phases of the pandemic (notably this
476 study did not capture the first wave). Studies using high throughput methods in wastewater to
477 identify ARG hosts during the pandemic are not available in literature for comparison. Several
478 studies have reported an increase in antimicrobial resistance and multidrug resistance organisms
479 (MRDOs) during the pandemic (reviewed in [88] based on clinical data).While several multidrug
480 resistant organisms were identified in this study, evidence for significant increases during the
481 pandemic was not observed.

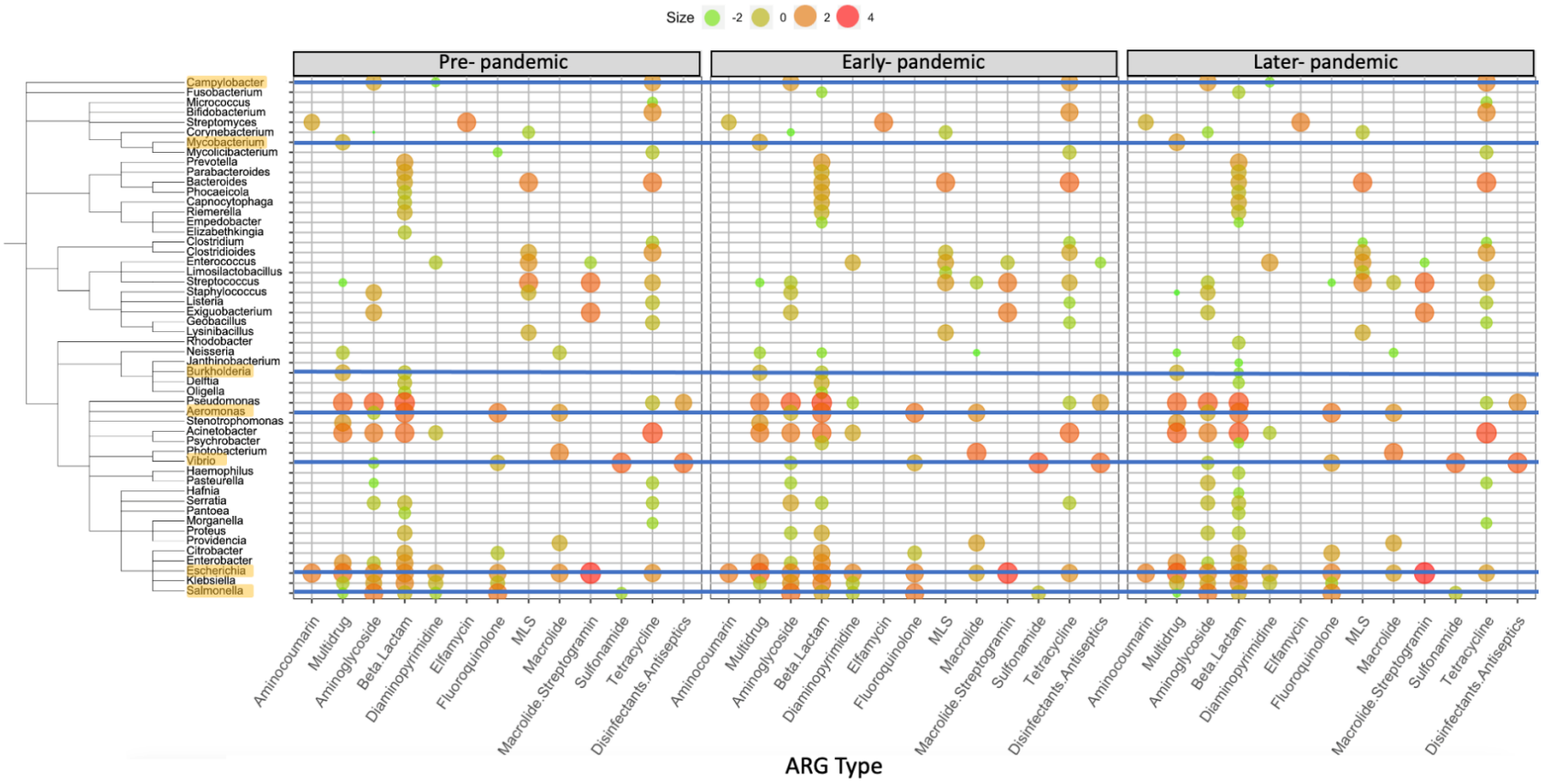


Fig. 5 *Bubble plot showing hosts linked to ARG classes as a function of ARG abundances. Size and color of bubble indicate the log abundance of ARGs (gene copies/ Gb) associated with the particular host. Genera highlighted in yellow represent the ubiquitous waterborne pathogens [as defined by [82]]. Note that ARG types having average abundance lesser than <5 gene copies/Gb have been omitted from the figure. (Fig. S10 includes all ARG types.)*

5 Conclusion

Overall, the results of this study indicate that the pandemic was associated with some changes in antibiotic resistance and microbial community in wastewater influent samples. The normalized concentration of *qac*E gene, which confers resistance to quaternary ammonium compounds was higher in WWTP-D in early pandemic samples, compared to pre- and later-pandemic samples. Further, a positive correlation was observed between disinfectants/antiseptics class of ARGs and COVID-19 cases, providing evidence to support the hypothesis that increased use of disinfectants resulted in higher residues in the WWTPs. Studies measuring the concentration of antibiotics and biocides will be useful in future for finding the direct relationships of biocide concentration with abundance of other ARGs. The abundance of macrolide class of ARGs was higher in early-pandemic than pre- and later-pandemic samples, potentially due to the increased prescription of azithromycin antibiotic in the initial months of the pandemic. Similar observations were noted for the *tet*(G) and *sul*1 genes through qPCR, potentially attributed to other factors such as seasonal variation and changes in human movement during the pandemic given that prescriptions of the associated antibiotic decreased during the pandemic. A negative correlation was observed between sulfonamide and aminoglycoside ARG types and the number of COVID-19 cases. Several correlations between ARGs and COVID-19 cases in the present study had p-values between 0.05- 0.06 and would be worth exploring in the future with a larger sample size. Analysis of the total microbial community revealed that the pathogenic genera *Enterobacter* and *Escherichia* were the biomarkers in early and later pandemic samples respectively, shedding some light on the co-infecting organisms during the pandemic. Among the hosts of ARGs, *Pseudomonas* and *Vibrio* were the most common hosts of the disinfectants/antiseptics class of ARGs, of particular interest given the increased use of biocides

during the pandemic and the potential resulting selective pressure [7, 89, 90]. Overall, while differences were observed in the resistome, the pandemic was not associated with shifts in the microbiome nor hosts of ARGs. The results of this study help in understanding the impact of the pandemic on antibiotic resistance, ARG-hosts, and co-occurring putative pathogens in wastewater.

Supporting Information: Figures with additional qPCR and metagenomic ARG data, correlations between ARGs and COVID-19 cases, and biomarker analysis results. Tables with additional details/data for sampling, water quality analyses, qPCR primers and QA/QC, and metagenomic sequencing.

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