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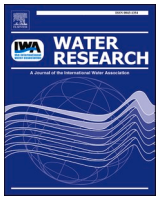

To what extent do water reuse treatments reduce antibiotic resistance indicators? A comparison of two full-scale systems

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ABSTRACT

Water reuse is an essential strategy for reducing water demand from conventional sources, alleviating water stress, and promoting sustainability, but understanding the effectiveness of associated treatment processes as barriers to the spread of antibiotic resistance is an important consideration to protecting human health. We comprehensively evaluated the reduction of antibiotic resistance genes (ARGs) and antibiotic-resistant bacteria (ARB) in two field-operational water reuse systems with distinct treatment trains, one producing water for indirect potable reuse (ozone/biologically-active carbon/granular activated carbon) and the other for non-potable reuse (denitrification-filtration/chlorination) using metagenomic sequencing and culture. Relative abundances of total ARGs/clinically-relevant ARGs and cultured ARB were reduced by several logs during primary and secondary stages of wastewater treatment, but to a lesser extent during the tertiary water reuse treatments. In particular, ozonation tended to enrich multi-drug ARGs. The effect of chlorination was facility-dependent, increasing the relative abundance of ARGs when following biologically-active carbon filters, but generally providing a benefit in reduced bacterial numbers and ecological and human health resistome risk scores. Relative abundances of total ARGs and resistome risk scores were lowest in aquifer samples, although resistant *Escherichia coli* and *Klebsiella pneumoniae* were occasionally detected in the monitoring well 3-days downgradient from injection, but not 6-months downgradient. Resistant *E. coli* and *Pseudomonas aeruginosa* were occasionally detected in the nonpotable reuse distribution system, along with increased levels of multidrug, sulfonamide, phenicol, and aminoglycoside ARGs. This study illuminates specific vulnerabilities of water reuse systems to persistence, selection, and growth of ARGs and ARB and emphasizes the role of multiple treatment barriers, including aquifers and distribution systems.

1. Introduction

It is conservatively estimated that 2.8 million people in the US are sickened each year by infections caused by antibiotic resistant pathogens and 35,000 die as a result (CDC 2019), presenting a growing global health threat (WHO 2015). Antibiotics present a two-edged sword: on one hand they are critical life-saving drugs that must be preserved and protected, while on the other hand, their overuse and misuse contribute to elevated and sustained levels of sublethal concentrations of antibiotics in affected human, agricultural, and natural environments that can contribute to the evolution and spread of resistant bacteria (Bracing for Superbugs). Antibiotic resistance can be intrinsic (e.g., lacking the cell structure targeted by a particular antibiotic), or may be acquired through mutation or horizontal gene transfer. When antibiotic resistance genes (ARGs) occur in pathogens, the expected therapeutic effect of the antibiotic is attenuated or lost, resulting in treatment failure and poor health outcomes. Because antibiotics, antibiotic resistant bacteria (ARBs), and ARGs are excreted in abundance in sewage, there is a need to ensure that wastewater treatment in general (Ashbolt et al., 2018; Manaia et al., 2018), and water reuse treatment in particular (Xi et al.,

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2009), attenuate these constituents and corresponding potential for human exposure, i.e., via ingestion, skin contact, or inhalation (Garner et al., 2016).

Water reuse is an essential element of sustainability (National Research Council 2012), e.g., alleviating water shortages, impacts of climate change, seawater intrusion to groundwater wells, and surface water contamination (U.S. EPA 2012). No federal water reuse regulations exist in the U.S. and regulations for agricultural reuse were recently implemented in the E.U. in 2023 (USEPA 2018; European Commission). Although such regulations do consider some microbial constituents, such as *Legionella* spp. and *Escherichia coli*, pharmaceuticals, ARB, and ARGs are also worthy of consideration due to their ability to persist through treatment and potential exposure routes for downstream users (Kinney et al., 2006; Gerrity and Snyder, 2011; González et al., 2015; Roccaro, 2018). Shoushtarian and Negahban-Azar (2020) noted that most agricultural water reuse regulations across the globe follow conventional wastewater monitoring approaches and do not account for additional contaminants of concern. Wastewater treatment plants (WWTPs), the source water for water reuse, receive inputs from a wide variety of sources containing elevated ARB and ARGs (e.g., hospitals, slaughterhouses, industry) (Shannon et al., 2007; Watkinson et al., 2007; Okoh et al., 2007) and are widely recognized to be ecosystems of concern for ARG mobilization and proliferation (Baquero et al., 2008; Rizzo et al., 2013; Karkman et al., 2018). The activated sludge stage of wastewater treatment has been shown to effectively alter the microbiome of wastewater and broadly reducing co-occurrences of ARGs and mobile genetic elements (MGEs) (Dai et al., 2022; Majeed et al., 2021). However, the effects on specific ARGs of interest are mixed, with some studies reporting removal and other reporting enrichment (Gao et al., 2012; Rafraf et al., 2016; Wen et al., 2016; Di Cesare et al., 2016; Zhang et al., 2009; Börjesson et al., 2009). Additionally, ARB and ARGs of clinical concern can sometimes still be detected in WWTP effluent (Majeed et al., 2021; Korzeniewska et al., 2013; Schages et al., 2020).

Thus, there is a need to establish a knowledge base of the efficacy of water reuse treatments for further attenuating antibiotic resistance, but few studies have examined the fate of ARB and ARG in full-scale facilities (Luprano et al., 2016; Hong et al., 2018; Garner et al., 2018). Goldstein et al. found that sensitive and resistant *Enterococcus* spp. and *Staphylococcus* spp. did not survive treatment when the water reuse facility (consisting of sand filtration, chlorination, dichlorination and discharge of post-secondary clarified WWTP effluent) was operating as designed (Goldstein et al., 2012, 2014). However, *Enterococcus* spp. were found in nasal swabs of exposed spray irrigation workers, suggesting that distribution system management is also important to consider in mitigating antibiotic resistance risk posed by water reuse (Goldstein et al., 2014).

More comprehensive study is needed to better assess the potential for water reuse systems as a post-WWTP barrier to the evolution and dissemination of antibiotic resistance. Culture-based methods can provide essential information regarding survival and growth of clinically-relevant bacteria and are directly informative of human health risk assessment (Zhiteneva et al., 2020; Chaudhry et al., 2017). However, conventional applications of culture-based methods typically capture a small, targeted subset of ARBs existing in complex wastewater treatment system ecosystems. Shotgun metagenomic sequencing can complement information provided by culture by comprehensively profiling corresponding resistomes (i.e., full complement of ARGs carried across a microbial community). Metagenomic sequencing directly profiles DNA, which can persist in extracellular and intracellular forms after disinfection, making ARGs of specific concern given their ability to mobilize via horizontal gene transfer (Berendonk et al., 2015; Martinez, 2009; Mao et al., 2013). Ideally, water reuse treatments could be optimized to minimize ARB and ARGs, in addition to meeting performance expectations for more general water quality measures.

The purpose of this study was to comprehensively evaluate removal of ARB and ARGs through two distinct full-scale water reuse facilities implementing differing treatment technologies, relative to removal achieved by their corresponding activated sludge WWTPs. This research provides valuable insight into how full-scale potable and non-potable water reuse systems can be optimally designed and managed in a way that minimizes concerns about propagation of ARBs and ARGs, and corresponding risks to human health.

2. Materials and methods

2.1. Sample collection

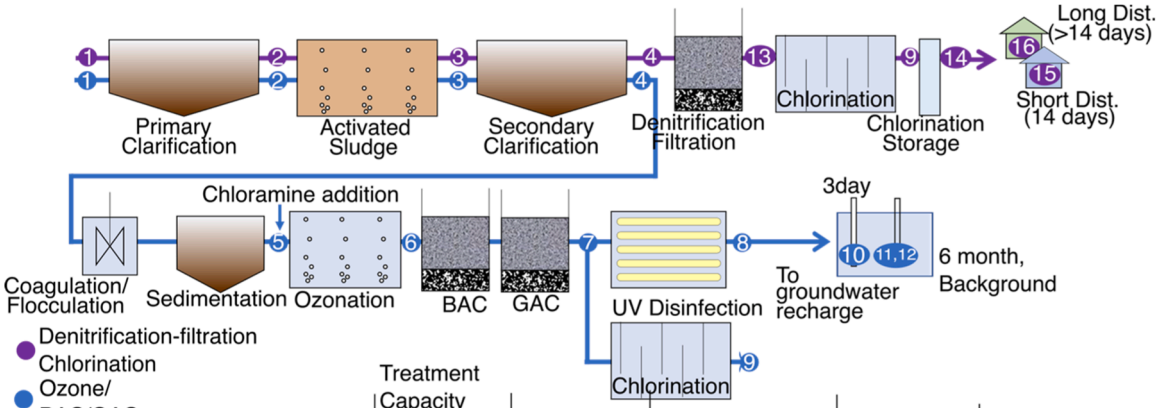
Two full-scale water reuse facilities were the subject of this study, a non-potable treatment plant in Florida and an indirect potable reuse plant in Virginia (Fig. 1). Samples were collected four times at each treatment facility between November 2018 to August 2019 (Table S1). Samples were collected before and after each treatment stage, as well as from two groundwater injection monitoring wells at the "ozone/BAC/GAC" potable reuse plant [one 3-day and one 6-month groundwater travel time from the injection well ("background" for sampling event 1 and "influenced" for events 2–4)](11 sampling locations per event, $n = 4$ per location) and from two distribution system points at the "denitrification-filtration/chlorination" non-potable reuse plant representing short (14 days) and long (> 14 days) residence times (10 sampling locations per event, $n = 4$ per location). Samples were named according to the immediately preceding treatment process/location and processed according to protocol for each analyte (SI Methods 1).

2.2. Culturing and confirmation

Culture methods were employed to quantify six clinically-relevant ARB: cefotaxime-resistant *E. coli,* methicillin-resistant *Staphylococcus aureus,* cefotaxime-resistant *Klebsiella pneumoniae,* imipenem-resistant *Acinetobacter baumannii,* ceftazidime-resistant *Pseudomonas aeruginosa,* and vancomycin-resistant *Enterococcus* spp., which have been identified as ESKAPEE pathogens by the US Centers for Disease Control to be of critical concern for the dissemination of antibiotic resistance (Rice, 2008). Samples for culture analysis were concentrated by membrane filtration by filtering three dilutions onto 0.45-μm mixed cellulose esters membrane filters (Fisher Scientific, Waltham, MA). Duplicate filters were processed for each dilution and for plating on selective-differential media with and without the screening antibiotic (Weinstein, 2019). Media and incubation conditions are provided in Table S2. Because culture conditions were optimized as the study progressed, EPA Methods 1600 and 1603 (US Environmental Protection Agency 2006; US Environmental Protection Agency 2002) with or without the screening antibiotic were applied to isolate *Enterococcus* spp. and *E. coli* in sample events 3 and 4 at the denitrification-filtration/chlorination plant and in sample events 2, 3 and 4 at the ozone/BAC/GAC plant . At least five antibiotic-resistant colonies were streaked for isolation on antibiotic-containing plates, where possible, at each treatment stage for each target ARB. Isolates were confirmed to target species or genus via polymerase chain reaction (Table S3) and were further confirmed for full resistance to antibiotics by the Kirby-Bauer disk diffusion assay. Culture data are reported for sampling trips 3 and 4 for the denitrification-filtration/chlorination plant and for sampling trips 2, 3, 4 for the ozonation BAC/GAC plant, since initial culture conditions yielded insufficient confirmation rates of target bacteria (Table S4).

2.3. Water chemistry

On-site water quality testing included free chlorine, total chlorine, temperature, dissolved oxygen, pH, and turbidity. Additionally, samples were preserved accordingly and transported to the laboratory for analysis of biodegradable dissolved organic carbon and heavy metals. Details on these analyses are provided in SI Methods Section 2. Non-targeted



		Treatment Capacity (Million Gallons/Day; m3/Day)	Hydraulic Residence Time (Hours)	Solids Residence Time (Days)	Empty Bed Contact Time (min)	Free Chlorine Residual (mg/L)
Denitrification-filtration Chlorination		33;125,000	27	16	NA	>1
Ozone/ BAC/GAC	Influent->Secondary	30;114,000	14-16	12	BAC 9.4	0.5-2.5
	Floc Sed->Well	1.0;3,790	3.0		GAC 30	

Fig. 1. Schematic of the denitrification-filtration/chlorination non-potable reuse plant (top/ purple) and the ozone/BAC/GAC indirect potable reuse plant (bottom/ blue), with sampling locations labeled sequentially by number. BAC- Biologically active carbon filtration; GAC- Granular active carbon filtration; Short Dist.- Sampling point in the distribution system with a residence time estimated to be 14 days; Long Dist.- Sampling point in the distribution system with a residence time estimated to be >14 days. Physiochemical process data can be found in Table S7.

pharmaceutical and personal care product screening was carried out using ultra-performance liquid chromatography tandem mass spectroscopy and is the subject of a companion manuscript.

2.4. Shotgun metagenomics and qPCR

Filter membranes were either transported same-day or shipped overnight on ice between labs and stored at −80 °C prior to DNA extraction using the FastDNA SPIN Kit (MP Biomedicals, Solon, OH). 16S rRNA (Suzuki et al., 2000) gene copy numbers were measured via qPCR using the 1369F/1492R primers (F 5′-CGGTGAATACGTTCYCGG-3′, R 5′- GGWTACCTTGTTACGACTT-3′), which have been previously validated for use in wastewater samples (Garner et al., 2019) (SI Methods 3). QA/QC was implemented according to EMMI guidelines (Borchardt et al., 2021), including incorporating blanks and a standard curve in every run and ensuring that samples were measured within the quantifiable range. Samples were checked for inhibition by conducting serial dilutions on all sample types. Dilutions were chosen for qPCR that returned the highest gene copies while remaining above detection. Shotgun metagenomic sequencing was conducted on all DNA extracts (n = 84). Sequencing was conducted across four metagenomic Illumina NextSeq flow cells (2×150-cycle paired end reads) at the Duke Center for Genomics Sequencing Center (Durham, NC). Library preparation was performed using the Kapa DNA Hyper Preparation Kit (Roche; Basel, Switzerland). Field blanks were included in each sequencing lane but contained insufficient DNA and failed at the library preparation stage. All metagenomic data has been uploaded under NCBI BioProject PRJNA669820. Sample accession numbers can be found in SI Table S5. All sequencing results (total reads, reads after trimming, generated scaffolds and resistome composition) can be found in SI II Table S1.

Metagenomic reads were trimmed with Trimmomatic (Bolger et al., 2014) and merged using vsearch (Rognes et al., 2016). ARGs were annotated using DIAMOND (Buchfink et al., 2015) (amino acid identity ≥80 %; e-value cutoff = 1e-10; minimum alignment length = 37 amino acids) against the Comprehensive Antibiotic Resistance Database (Jia et al., 2016) (CARD, version 3.0.8). All gene annotations and normalizations can be found in SI II Table S2. Clinically-relevant ARGs were defined as Rank 1 'current threats' and Rank 2 'future threats' as categorized by Zhang et al. in the SARG database (SI Table S6) (Zhang et al., 2021). Corresponding SARG database genes were manually mapped to the CARD database for further analysis. Reads were assembled using MEGAHIT (Li et al., 2015) and the ecological resistome risk score was calculated using MetaCompare (v2.0) (Rumi et al., 2024). Ecological resistome risk is determined by annotating assembled contigs to DeepARG-DB (Arango-Argoty et al., 2018) for ARG identification, mobileOG-DB (Brown Connor et al., 2022) for MGE identification, and GTDB-TK (Chaumeil et al., 2019) for pathogen marker identification. Human health resistome risk was also calculated and focuses on only Rank 1 ARGs as categorized by Zhang et al (Zhang et al., 2021) and ESKAPEE pathogens. MetaCompare 2.0 Resistome Risk score outputs are reported in SI II Table S3.

2.5. Data analysis and statistics

All tests were performed in R version 3.4.1 (R Core Team 2013) with sampling trips treated as biological replicates. Kruskal-Wallis and Wilcoxon rank sum tests were performed on metagenomic data normalized to 16S rRNA (measured via metagenomics) to identify differences in the detection of total and specific ARG class relative abundance. Non-metric multidimensional scaling (NMDS) ordinations were generated using the "Vegan" package (version 2.5–5) with Bray-Curtis dissimilarity matrices (Beals, 1984). Analysis of similarity (ANOSIM) was used to determine differences in bacterial community and resistome beta diversity. Spearman correlations were used to compare ARGs, physiochemical, antibiotic data and culture data with Bonferroni corrections. Strong correlations were defined for Spearman tests as r^2 >0.7. Statistical grouping was determined for comparing total ARGs assessed with metagenomics using a linear model and the least-square means (R package: emmeans). Statistical significance was set at α=0.05, with each sampling event treated as a biological replicate. All figures can be regenerated with code provided at github.com/ikeenum/Paper_Figure_regeneration/tree/main/Water_reuse_CDC.

3. Results

3.1. Total bacteria trends

Reductions of total bacteria measured via the 16S rRNA gene were apparent in both WWTPs from influent to secondary effluent (Fig. 1c). An additional 4 log gene copies per milliliter reduction was observed from secondary effluent to chlorination in the ozone/BAC/GAC plant (Table S8). Only an additional 0.6 log removal was observed in the denitrification/ filtration/ chlorination plant after secondary clarification.

3.2. Comparison of resistome profiles between and within the WWTPs and water reuse treatment trains

Treatment stage strongly shaped the resistomes ($R = 0.65$), with some separation based on treatment plant as well ($R = 0.14$) (Fig. 2). Similarities in resistome composition across the two WWTPs were notable. There were two predominant clusters apparent in the ARG profiles, one containing the influent and primary-treated effluent of both plants and one comprised of intermediate treatment stages (activated sludge, secondary effluents, flocculation/sedimentation, BAC/GAC filters and the denitrification filter). ARG profiles did not differ between treatment plants within the clusters (influent-primary $p = 0.99$, activated sludge/BAC/GAC cluster: $p = 0.14$, respectively). The chlorination, 3-day, and background groundwater well samples from the ozone/BAC/GAC treatment train were variable in composition and did not cluster with other samples, whereas 6-month groundwater samples clustered with intermediate treatment stages. In the denitrification-filtration/chlorination plant, the resistome profiles did not vary from activated sludge to denitrification ($p = 0.06$); however, a shift in resistome composition after denitrification (i.e., chlorination, chlorination storage, long and short residence times in the distribution system) was apparent.

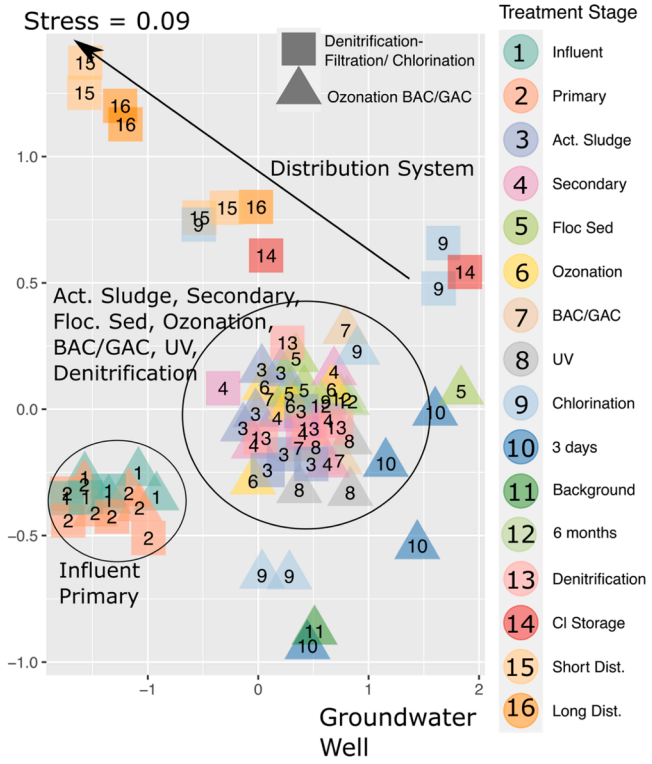


Fig. 2. Non-metric multi-dimensional scaling (NMDS) analysis comparing ARG profiles (i.e., number and types of ARGs pertaining to various classes of antibiotic resistance) through both water reuse treatment trains: Denitrification-filtration/chlorination and ozone/BAC/GAC. Shapes indicate the plant of origin and treatment stages are labelled by number sequentially through the treatment trains (Fig. 1).

3.3. Removal of total ARGs through the WWTPs

Relative abundances of total ARGs were similar in the influent, primary effluent, and secondary clarifier effluent across the two plants and significantly decreased from primary clarification to activated sludge (Fig. 3). These resistomes consisted primarily of MLS, multidrug, aminoglycoside and beta-lactam ARGs. The activated sludge process at the denitrification-filtration/chlorination plant attenuated total ARG relative abundance to a greater extent than at the WWTP serving the ozone/BAC/GAC facility. Relative abundance of ARGs corresponding to most resistance classes decreased from influent to secondary effluent at both WWTPs (Fig. 4a,b). Rifamycin was the only ARG category that exhibited an increase in relative abundance from influent to secondary effluent, a phenomenon observed at both WWTPs.

3.4. Removal of total ARGs through the water reuse treatment stages

In the ozone/BAC/GAC water reuse treatment train, the relative abundance of total ARGs, averaged across the 4 sampling events, increased from secondary effluent to chlorinated effluent. Especially notable was the increase in relative abundance of total ARGs following ozonation, relative to flocculation and sedimentation. ARGs conferring resistance to individual drug classes were differentially affected by various treatment processes (Fig. 4c,d). Decreases were observed in beta lactam ARGs, a drug class containing many clinically-relevant ARGs, in all treatment stages after secondary effluent. ARGs conveying resistance to phenicol; however, were elevated in all stages as compared to the secondary effluent. Ozonation specifically acted to elevate the multidrug resistance class of ARGs, although it reduced peptide and glycopeptide resistance. BAC/GAC filters elevated the "other" resistance class (many of these genes convey resistances to antimicrobials such as triclosan and antifungals) relative to the secondary effluent, but served to reduce sulfonamide ARGs.

The resistome composition also shifted across treatment stages in the denitrification-filtration/chlorination plant, but in a distinct manner relative to the ozone/BAC/GAC plant (Fig. 2). No statistically significant changes were observed in the relative abundance of total ARGs in the post-secondary effluent treatment stages (Fig. 3b). In terms of individual drug classes, aminoglycoside, sulfonamide, and "other," the relative abundances of ARGs were elevated in every treatment stage after secondary effluent.

3.5. Total ARGs in the aquifer

The relative abundance of total ARGs in the ozone/BAC/GAC effluent following chlorination were higher than in the groundwater well that represented 3-days travel post injection. It was also notable that the types and abundances of ARGs in the chlorinated water were distinct from the profile of ARGs observed in the background groundwater monitoring well (Figs. 3a, 4c). Further, there were few observable differences in resistome composition or magnitude between the 3-day well, 6-month well, and the background well. The "other" resistance class (in this case consisting only of *taeA* (fungal resistance) and *triC* (triclosan) was the only one that was higher in relative abundance in the 3-day, 6-month, and background well water when compared to secondary effluent (Fig. 4c).

3.6. Total ARGs in the distribution system

There were no significant differences in resistome composition or magnitude in the denitrification-filtration/chlorination plant after chlorination storage. ARGs pertaining to phenicol, aminoglycoside,

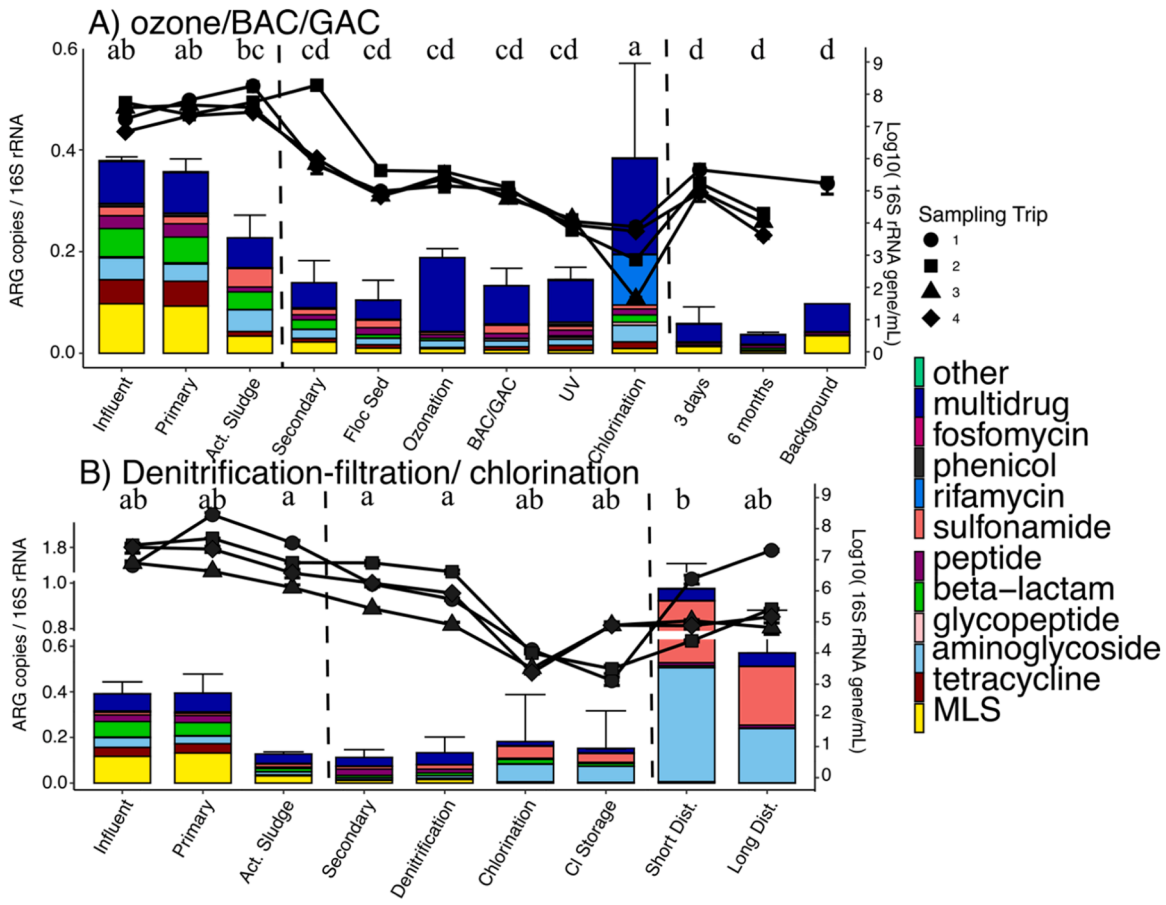


Fig. 3. Average relative abundance of total ARGs detected in the A) ozone/BAC/GAC indirect potable and B) denitrification-filtration/chlorination treatment plants. Gene annotations were grouped together by class of antibiotics to which they encode resistance "3 days" represents the groundwater well with a 3-day travel time, "6 months" represents the groundwater well with a 6-month travel time, and "Background" is a groundwater well where the injected water had not yet reached at the time of Sampling Trip 1. Short and Long Dist. refers to travel times in the distribution network of 14 days and > 14 days, respectively. Stacked bars represent averages across the 4 sampling events and error bars represent the standard deviation in total ARGs. The multidrug category represents ARGs which convey resistance to more than one antibiotic drug class (excluding macrolide-lincosamide-streptogramin (MLS) ARGs). The other category refers to ARGs which convey resistance to antiseptics, biocides, and antitumor medication. Statistical groupings were determined for comparing total ARGs assessed with metagenomics using a linear model and the least-square means (R package: emmeans). Overlaid with the relative abundance of gene annotations is the enumerated 16S rRNA gene data measured via qPCR (right axis). To show variation across sampling events, each trip is plotted separately.

sulfonamide, and "other" resistance notably increased in magnitude in both distribution system sampling points, collectively by ~5× the abundance of the chlorinated storage tank that feeds the distribution system.

3.7. Clinically-Relevant ARGs

The number of ARGs classified as clinically-relevant reduced substantially (30–40 ARGs, ~0.95–0.5 log copies/16S rRNA gene) through both WWTPs from influent to secondary clarification (Figure S2). There was a reduction in the number of clinically-relevant ARGs in the ozone/BAC/GAC plant from secondary effluent to chlorinated effluent, though this was not the case in the denitrification-filtration/chlorination treatment plant. No differences were observed in number of clinically-relevant ARGs as a result of chlorination following the BAC/GAC stage. More clinically-relevant ARGs (n = 40) were identified in the chlorinated effluent of the denitrification-filtration/chlorination plant across all sampling trips and in both distribution system sampling points than in the effluent of the ozone/BAC/GAC plant or groundwater wells (n = 24). Of these, 17 genes (*AAC(6′)-IB, AAC(6′)-IB7, ANT(2″)-IA, APH(3″)-IB, APH(3″)-IC, APH(3′)-IA, APH(6)-ID, bacA, catB2, catB3, cmlA5, floR, mdtG, mphA, tetO, tetW*) were found in the effluents and receiving environments. Most clinically-relevant ARGs did not increase in the distribution system, relative to the chlorinated effluent.

3.8. Attenuation of resistome risk through water reuse treatment

In both WWTPs, activated sludge was responsible for the majority of the reduction in the MetaCompare ecological resistome risk (ERR) and human health resistome risk (HHRR) score, relative to that of the primary clarifier (Figure S3). At the ozone/BAC/GAC treatment plant, a significant increase in the MetaCompare ERR score was noted following ozonation, relative to the flocculation and sedimentation stage, though this was not seen in the HHRR score. No further change in was observed in either score after ozonation and both scores were the lowest in groundwater. No difference in ERR or HRR was observed in the 3-day or 6-month travel time groundwater wells as compared to the background monitoring well, and all injection well resistome risk scores were lower than those of the UV injection water.

At the denitrification-filtration/chlorination plant, no differences were observed in the ERR score from secondary effluent to denitrification, but a decrease was observed after chlorination. However, the ERR increased in the distribution system relative to the plant effluent. The HHRR score did not change significantly after the activated sludge stage.

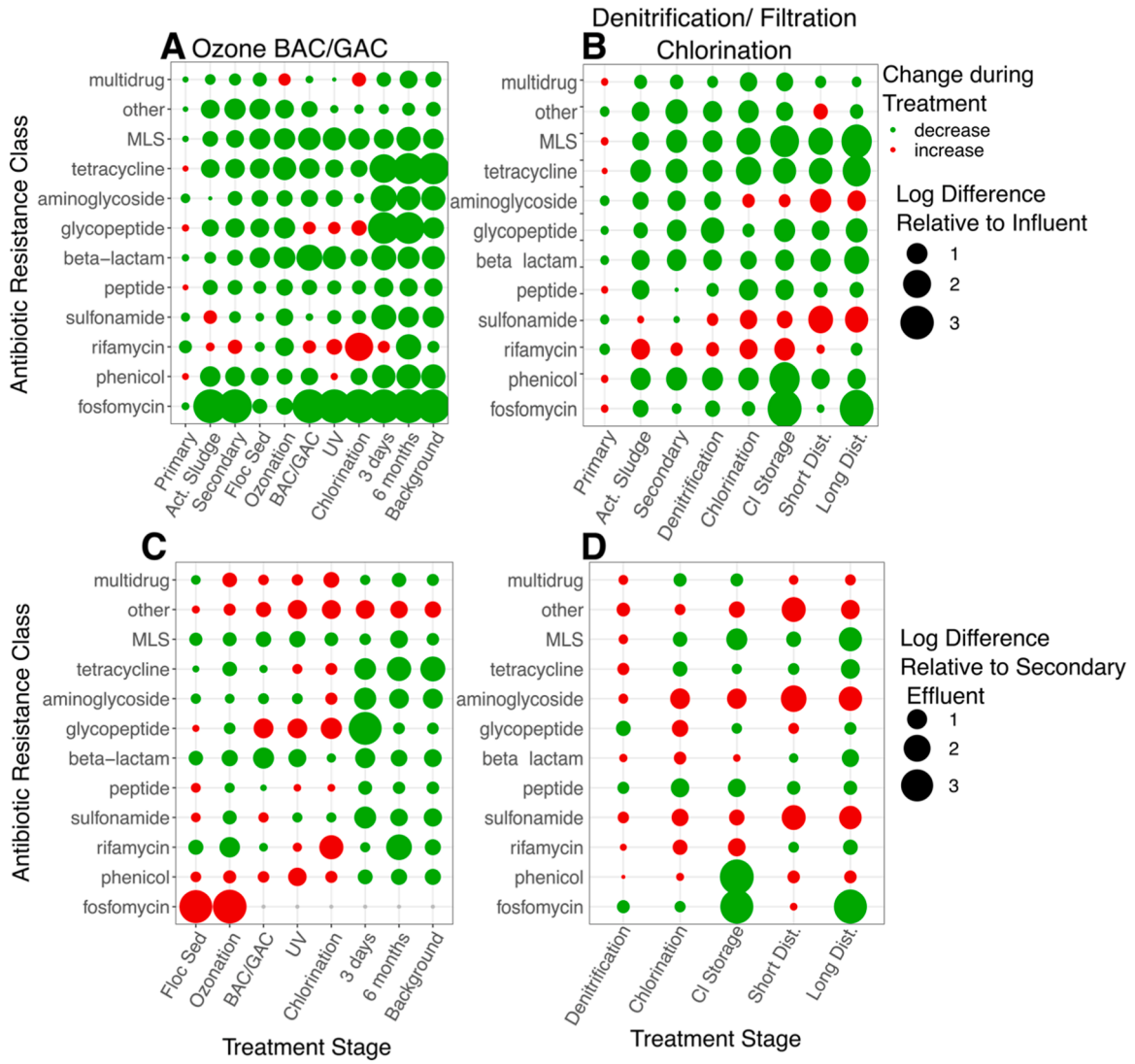


Fig. 4. Log change in average relative abundance of ARGs (ARG copies/ 16S rRNA gene) for each treatment plant sampling location, categorized according to class of antibiotics to which the ARG encodes resistance (MLS = macrolide-lincosamide-streptogramin). A) Ozone/BAC/GAC indirect potable reuse treatment stages relative to the influent raw sewage, B) Denitrification-filtration/chlorination non-potable reuse treatment stages relative to the influent raw sewage, C) Ozone/BAC/GAC indirect potable reuse treatment stages relative to the secondary effluent, and D) Denitrification-filtration/chlorination indirect potable reuse treatment stages relative to the secondary effluent. ARGs were annotated from metagenomic data and categorized by resistance class, as described in Fig. 3.

3.9. Culture-based enumeration of clinically-relevant ARBs

Specificity of the commercially-availaable media used to isolate *A. baumannii, K. pneumoniae, P. aeruginosa* and *S. aureus* (Table S2) were inadequate to isolate target ARB from wastewater and water reuse samples. Only 2.7 % (6 of 219) of imipenem-resistant *A. baumannii,* 11 % (37 of 340) cefotaxime-resistant *K. pneumoniae*, 12 % (26 of 222) ceftazidime-resistant *P. aeruginosa*, and 0 % (out of 200) methicillin-resistant *S. aureus* were confirmed to species. Among these, confirmed, antibiotic-resistant *K. pneumoniae* and *P. aeruginosa* were occasionally isolated post-disinfection (Table S4). Resistant *P. aeruginosa* were confirmed in the distribution system in three of the four sampling events in the denitrification-filtration/chlorination plant while resistant *K. pneumoniae* was detected in the UV and 3-day groundwater well at two sampling events. Further analysis focused primarily on *Enterococcus* spp. and *E. coli*, which yielded much higher confirmation rates, particularly after the isolation medium for *E. coli* was changed from MacConkey agar to mTEC agar, and vancomycin concentration was changed from 4 μg/ml to 32 μg/ml.

The data shown in Fig. 5 are derived from sample events where the specificity of the culture methods was optimized to isolate *Enterococcus* spp. and *E. coli* (sample events 3 and 4 at the denitrification-filtration/chlorination plant and sample events 2, 3 and 4 at the ozone/BAC/GAC plant). The confirmation frequency of vancomycin resistant *Enterococcus* (VRE) and cefotaxime-resistant *E. coli* isolates to genus/species in these sampling events ranged from 89 to 96 % for VRE and 89 to 100 % for cefotaxime-resistant *E. coli* (Table S4). These confirmation rates were then applied to estimate the numbers of target organisms across the data set. Influent concentrations of VRE and cefotaxime-resistant *E. coli* were two to four orders of magnitude lower than total *Enterococcus* and *E. coli*, e.g., total *E. coli* concentrations in the influent of the ozone/BAC/GAC WWTP were $10^{6.7}$ CFU/100 mL, while cefotaxime-resistant *E. coli* concentrations were 10^{5} CFU/100 ml (Fig. 5). In other words, 1 % or less of cultured *Enterococcus* and *E. coli* were phenotypically resistant to the antibiotic included in the media.

VRE were reduced 3.5 and 1 logs, while cefotaxime-resistant *E. coli* were reduced 2.5 and 2 logs, from influent to secondary effluent in the WWTPs serving the ozone/BAC/GAC and denitrification-filtration/chlorination plants, respectively (Fig. 5). In the subsequent reuse treatment stages, the levels of VRE and cefotaxime-resistant *E. coli* were

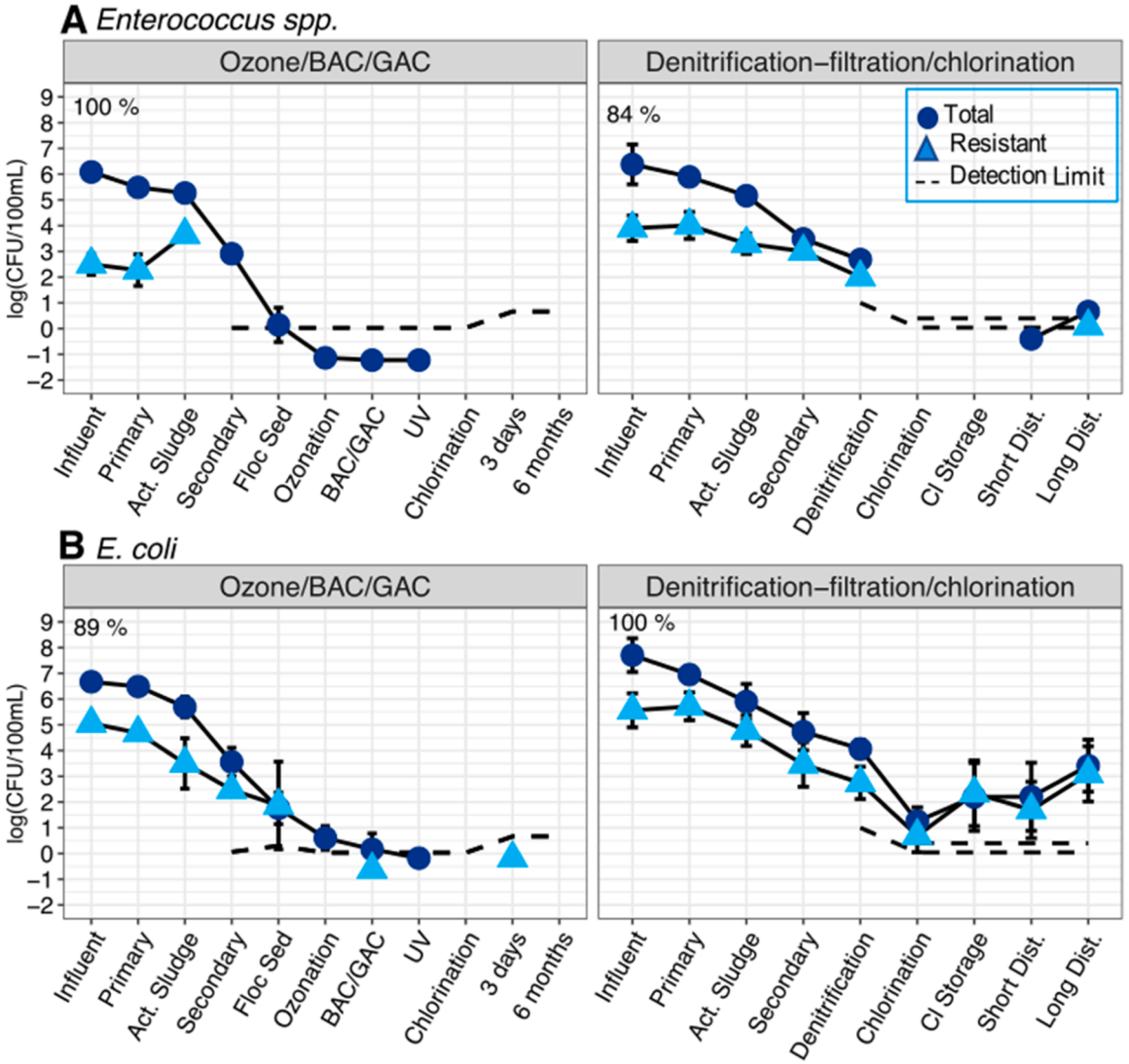


Fig. 5. Concentration of total and vancomycin-resistant *Enterococcus* spp. (A) and cefotaxime-resistant *E. coli* (B) assessed by culture (log_{10} CFU/100 ml) in the ozone/BAC/GAC and denitrification-filtration/chlorination reuse plants. Data as shown are not corrected for genus (*Enterococcus* spp.) or species (*E. coli*) confirmation frequency. Genus/species confirmation rates among resistant isolates that were subject to genus/species confirmation testing by PCR (n = 28, 145, respectively) are reported in the upper left corner of each panel. Treatment stages with no data point plotted represent no target confirmed at that treatment stage. Detection limits are indicated with the dashed line based on the minimum statistically-valid plate count for the lowest dilution applied for that sampling location/event.

reduced below the detection limit (an additional 3 logs from influent) in the ozone/BAC/GAC system after the UV treatment process. In the denitrification-filtration/chlorination treatment plant, the levels of VRE were also reduced below detection after denitrification (an additional 3 logs) and the levels of cefotaxime-resistant *E. coli* and were reduced by an additional 2 logs. No isolates recovered on VRE media post-disinfection were confirmed to genus, while three *E. coli* isolates recovered post-disinfection on media containing cefotaxime were confirmed to species (Table S4). Resistant *E. coli* were detected in the 3-day groundwater well as well as in the distribution system of the denitrification-filtration/chlorination system.

Differential removal of cefotaxime-resistant *E. coli* and VRE was noted in secondary clarification across both plants (Wilcoxon) (Fig. 5). Cefotaxime-resistant *E. coli* were reduced less than total *E. coli* during secondary clarification and flocculation/sedimentation stages of the ozone/BAC/GAC treatment plant. VRE were also removed to a lesser extent by the activated sludge process serving the Ozone/BAC/GAC plant and by the secondary clarifier and denitrification treatments at the denitrification-filtration/chlorination.

3.10. Correlations among the resistome, cultured bacteria and water quality measurements

Positive correlations (Spearman, $p < 0.0001$) were observed between total bacterial 16S rRNA genes and cefotaxime-resistant *E. coli* and VRE (rho = 0.75, 0.59, respectively). The relative abundance of total ARGs also positively correlated with total bacterial 16S rRNA genes (rho = 0.48). Cefotaxime-resistant *E. coli* correlated with total ARGs measured via metagenomics (rho = 0.69), but VRE did not. Examining significant and strong correlations ($r^2 > |0.7|$) between physiochemical data (Table S7, Figure S4 and S5) and metagenomics, free and total chlorine negatively correlated with the relative abundances of beta-lactam, MLS, multidrug, tetracycline and rifamycin (total chlorine only) ARGs. DO was also found to negatively correlate with sensitive *Enterococcus* spp. while phosphate correlated with both *Enterococcus* spp. and *E. coli*.

4. Discussion

4.1. Majority of arb/arg removal achieved by activated sludge treatment

This study provided a comprehensive comparison of the behavior and fate of ARB and ARGs through two distinct field-scale water reuse treatment systems. The raw influent wastewater resistomes were similar in composition and magnitude, which supported the comparability between the two systems. We found that most removal of ARB/ARGs was achieved by activated sludge treatment. The reduction in total ARG relative abundance achieved by activated sludge and secondary clarification is highly comparable to rates reported in the literature (Bengtsson-Palme et al., 2016; Lou et al., 2023; Dias et al., 2022). Additionally, it was found that activated sludge treatment was highly effective in reducing resistome risk scores, which is consistent with prior surveys of activated sludge WWTPs that indicated reduction of both clinical and mobile ARGs (Dai et al., 2022; Ju et al., 2019). Cefotaxime-resistant *E. coli* were also removed by 2–2.5 log and VRE by 1–3.5 log. However, VRE was not removed as well as antibiotic sensitive *Enterococcus* spp. at one of the WWTPS. Cefotaxime-resistant *E. coli* also were not removed as well as sensitive *E. coli* by sedimentation processes. This suggests that in some cases, ARB can be more difficult to remove than their sensitive counterparts, as has been found in other studies (Dias et al., 2022; Machado et al., 2023).

4.2. Additional removal achieved by tertiary water reuse treatments?

One key question is whether post-secondary treatment provides added benefit in terms of antibiotic resistance concerns and what degree of additional removal might be expected from more aggressive treatments employed for the purpose of potable water reuse (Garner et al., 2018; Fahrenfeld et al., 2013). Total ARG relative abundance tended to further reduce during tertiary water reuse treatment, but not to the extent observed via activated sludge, probably in large part because the latter already started with very high levels. This trend was also previously noted by Dias et al. when investigating the use of UV for tertiary treatment (Dias et al., 2022). Some reuse treatments were associated with increases in certain ARGs. ARGs categorized as “clinically-relevant, ” current and future threats as designated by Zhang et al. (2021) generally decreased in both reuse systems. However, ozonation followed by BAC tended to enrich total ARG relative abundance, which was mainly driven by enrichment of multidrug ARGs. Application of MetaCompare 2.0 resistome risk analysis revealed that this increase was reflected in an increase in the ERR score, but not the HHRR score, indicating that human pathogens and clinically-relevant ARGs are not contributing to the increases in total ARGs observed following ozonation.

Interestingly, chlorination had differing effects when comparing the two treatment plants. In the ozone/BAC/GAC plant, where chlorination followed BAC/GAC filtration, relative abundance of total ARGs significantly increased, but ERR and HRR scores were unaltered. At the denitrification-filtration/chlorination treatment train, chlorination immediately followed the denitrification filter and was not associated with any change in total ARGs and ERR and HRR scores markedly reduced. It is important to also take into account that chlorination also acts to reduce the total microbial numbers, as observed in this study from the 16S rRNA gene measurements. Thus while chlorination can sometimes increase relative abundance of ARGs across the microbial community, as observed in this study and other studies of wastewater and drinking water systems (Jia et al., 2015), ARG concentrations (i.e., ARGs/mL) generally reduce. Zheng et al. noted how chlorine doses can contribute to whether ARG relative abundances increase (Zheng et al., 2017).

The behavior of the resistome in biological treatment via BAC/GAC filters is also of interest. Here we found that the BAC/GAC filters in the ozone/BAC/GAC plant reduced the relative abundance of ARGs measured via metagenomics. While some have suggested that biological treatment will increase the mobility of ARGs (Petrovich et al., 2018), this was not found in this study. Ozonation increased the ERR score, but subsequent biological treatment reduced both the ERR and HHRR scores.

Ultimately, the more aggressive treatments employed at the ozone/BAC/GAC plant intended to produce water of potable reuse quality did present benefits relative to the non-potable denitrification-chlorination plant. Given that the ARB and ARGs measured here correlated well with total bacteria (SI Results 1), this suggests that the aggressive removal of bacteria by the ozone/BAC/GAC plant may be the main driver of removal of ARB and ARGs. For example, the flocculation/sedimentation process achieved measurable reduction ahead of the ozone/BAC process.

4.3. Distribution system and groundwater as receiving environments

One striking finding of this study was that non-potable reuse distribution systems are vulnerable to re-growth of ARB. This highlights that there may still be concerns at the point of use for ARB exposure in nonpotable systems. Even though the water is not intended for drinking, aerosols and skin contact remain a concern (Garner et al., 2016; Hong et al., 2018). Sulfonamide, aminoglycosides, glycopeptide, beta-lactam, rifamycin and phenicols classes of ARGs were enriched in the distribution system, to a level such that average relative abundances of total ARGs rivaled levels in the raw influent sewage. The ERR and HHRR scores in the distribution system were also elevated, to levels higher than the activated sludge. These observations are consistent with a prior study of this system’s distribution system, which was included in a field survey of ARGs in non-potable reuse distribution systems (Garner et al., 2018). The present study further corroborated these findings, with antibiotic resistant *E. coli* and *P. aeruginosa* recovered from the non potable reuse distribution system on more than one occasion. These findings are consistent with understanding that chlorine-based disinfectants can favor re-growth of ARBs relative to sensitive counterparts (Fahrenfeld et al., 2013; Khan et al., 2016). Non-potable water reuse distribution systems may be even more vulnerable to re-growth of ARB than potable systems given that they may be challenged by higher levels of ARB/ARGs to begin with, while also requiring higher doses of chlorine to maintain residual due to higher chlorine demand (Garner et al., 2018; Fahrenfeld et al., 2013; Al-Jassim et al., 2017; Brienza et al., 2022).

Groundwater, on the other hand, was the most depleted of ARGs and in ERR/HHRR scores than any other sample type included in this study. The affected groundwater well did not have significantly differ from the background well, in total ARG abundances or when evaluating individual drug classes, although the composition of the resistome was shaped by the injected potable reuse water. Nonetheless, some ARGs were detected, even in the background well sampling, including occasional detection of ARGs classified as clinically-relevant as has been seen previously when advanced potable reuse was used to recharge an aquifer in Orange County, CA (Harb et al., 2019). Further resistant *E. coli* and *K. pneumoniae* were cultured from the 3-day groundwater well sample, though none were seen in the 6-month or Background groundwater samples. Overall, the findings suggest the importance of leveraging aquifer storage as an additional barrier in attenuating ARB and ARGs.

4.4. Comparison of culture and metagenomics approaches

Culturable ARBs were reduced below quantification in the final treated reuse water of each facility, but resistant *P. aeruginosa, E. coli* and *K. pneumoniae* were still recovered in the receiving waters. Metagenomic profiling of total ARGs mirrored the overall trends of the culture-based targets, including reduction during treatment. This study further highlighted the need for improved methods for culture of antibiotic-resistant *A. baumannii, K. pneumoniae, P. aeruginosa,* and *S. aureus* from

wastewater. Selectivity of the isolation methods used for these ARB was very low, even though relatively expensive, chromogenic media were used for *S. aureus* and *A baumannii*. New methods for *K. pneumoniae* isolation in water have been proposed by the MedVetKlebs consortium (Consortium, 2020), and the specificity of methods for *A. baumannii, Aeromonas* spp. and *P. aeruginosa* in wastewater and environmental water has been recently reviewed (Milligan et al., 2023), therefore, using the more selective media is recommended. Low genus/species confirmation rates via PCR were seen for all target organisms where an EPA standard method did not exist. While culture methods are ideal to inform existing risk assessment models, issues in specificity proved an insurmountable barrier for detecting certain antibiotic-resistant pathogens in the complex matrix of wastewater. On the other hand, metagenomic sequencing circumvents culture bias, but with trade-offs being lack of ability to confirm viability and elevated detection limit (Davis et al., 2023).

5. Conclusions

Wastewater is known to contain pathogenic ARB and ARGs encoding resistance to clinically-important antibiotics. This especially presents a concern when the treated wastewater is intended for reuse (Garner et al., 2018; Fahrenfeld et al., 2013), but currently it is unknown which treatments most reliably attenuate antibiotic resistance (Rizzo et al., 2013; Hong et al., 2018). This study provides a comprehensive assessment of the fate and behavior of key ARGs via metagenomics and ARB via culture methods and identifies key vulnerabilities, such as regrowth in non-potable reuse distribution systems and the benefits of aquifer storage as an additional reuse barrier. Effective treatments, and combinations of treatments, for reducing clinically-relevant ARGs and overall resistome risk scores are identified. The following is a summary of key conclusions:

- The majority of ARB and ARG removal occurs during activated sludge treatment, relative to subsequent tertiary water reuse treatments. In some cases, resistant forms of *Enterococcus* spp. and *E. coli* were not removed to the extent as corresponding sensitive strains.
- ARGs, especially multi-drug ARGs, can become enriched across the microbial community following ozonation or chlorination. ERR score increased following ozonation as well, but HHRR was not affected and both ERR and HHRR, as well as ARGs of clinical concern, reduced after chlorination.
- The groundwater resistome composition was influenced by the injected potable reuse water, as evidenced by shifts observed in the background well as the injected water reached it with time, but levels of ARGs and resistome risk scores remained the lowest of any water type examined in this study. Occasional detections of resistant *K. pneumoniae,* and *E. coli* were noted after disinfection processes and in the 3-day groundwater monitoring well, emphasizing the importance of aquifer storage as a reuse barrier.
- The non-potable reuse distribution system was found to be vulnerable to regrowth of organisms that tended to carry ARGs, especially sulfonamide and aminoglycoside ARGs. Occasional positive detections of resistant *P. aeruginosa* and *E. coli* were noted in the distribution system, emphasizing the need for guidance on how to better manage distribution systems as an antibiotic resistance barrier

CRediT authorship contribution statement

Ishi Keenum: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Jeanette Calarco:** Investigation, Visualization, Writing – original draft, Writing – review & editing. **Haniyyah Majeed:** Investigation, Methodology. **E. Eldridge Hager-Soto:** Investigation, Methodology. **Charles Bott:** Investigation, Resources, Writing – review & editing. **Emily Garner:** Conceptualization, Formal analysis, Methodology, Project administration, Writing – original draft. **Valerie J. Harwood:** Conceptualization, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Amy Pruden:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Validation, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Amy Pruden reports financial support was provided by National Science Foundation. Amy Pruden reports financial support was provided by Centers for Disease Control and Prevention. Amy Pruden reports financial support was provided by US Bureau of Reclamation. Amy Pruden reports financial support was provided by Hampton Roads Sanitation District. Charles Bott is an an employee of Hampton Roads Sanitation District. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All figure regeneration and metagenomic annotations can be found at https://github.com/ikeenum/Paper_Figure_regeneration/tree/main/Water_reuse_CDC. All metagenomic files can be found in NCBI BioProject PRJNA669820.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2024.121425.

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