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Authors

Malayil, Leena
Ramachandran, Padmini
Chattopadhyay, Suhana
et al.

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Article

Variations in Bacterial Communities and Antibiotic Resistance Genes Across Diverse Recycled and Surface Water Irrigation Sources in the Mid-Atlantic and Southwest United States: A CONSERVE Two-Year Field Study

Leena Malayil, Padmini Ramachandran, Suhana Chattopadhyay, Sarah M. Allard, Anthony Bui, Jicell Butron, Mary Theresa Callahan, Hillary A. Craddock, Rianna Murray, Cheryl East, Manan Sharma, Kalmia Kniel, Shirley Micallef, Fawzy Hashem, Charles P. Gerba, Sadhana Ravishankar, Salina Parveen, Eric May, Eric Handy, Prachi Kulkarni, Brienna Anderson-Coughlin, Shani Craighead, Samantha Gartley, Adam Vanore, Rico Duncan, Derek Foust, Joseph Haymaker, Walter Betancourt, Libin Zhu, Emmanuel F. Mongodin, Amir Sapkota, Mihai Pop, and Amy R. Sapkota*

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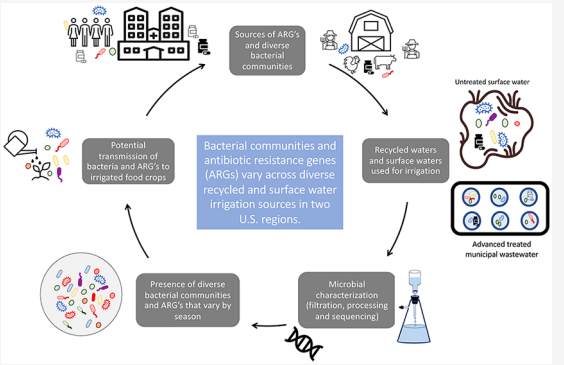
ABSTRACT: Reduced availability of agricultural water has spurred increased interest in using recycled irrigation water for U.S. food crop production. However, there are significant knowledge gaps concerning the microbiological quality of these water sources. To address these gaps, we used 16S rRNA gene and metagenomic sequencing to characterize taxonomic and functional variations (e.g., antimicrobial resistance) in bacterial communities across diverse recycled and surface water irrigation sources. We collected 1 L water samples (n = 410) between 2016 and 2018 from the Mid-Atlantic (12 sites) and Southwest (10 sites) U.S. Samples were filtered, and DNA was extracted. The V3−V4 regions of the 16S rRNA gene were then PCR amplified and sequenced. Metagenomic sequencing was also performed to characterize antibiotic, metal, and biocide resistance genes. Bacterial alpha and beta diversities were significantly different (p < 0.001) across water types and seasons. Pathogenic bacteria, such as *Salmonella enterica*, *Staphylococcus aureus*, and *Aeromonas hydrophilia* were observed across sample types. The most common antibiotic resistance genes identified coded against macrolides/lincosamides/streptogramins, aminoglycosides, rifampin and elfamycins, and their read counts fluctuated across seasons. We also observed multi-metal and multi-biocide resistance across all water types. To our knowledge, this is the most comprehensive longitudinal study to date of U.S. recycled water and surface water used for irrigation. Our findings improve understanding of the potential differences in the risk of exposure to bacterial pathogens and antibiotic resistance genes originating from diverse irrigation water sources across seasons and U.S. regions.



KEYWORDS: *recycled water, reclaimed water, wastewater, irrigation water quality, pathogens, water microbiome, antibiotic resistance genes, antibiotic-resistant bacteria, metagenomics, food safety*

1. INTRODUCTION

Water scarcity and the reduced availability of agricultural water in key food production areas have spurred increased interest in the use of recycled irrigation water (e.g., advanced treated municipal wastewater, return flows, harvested rainwater) in U.S. food crop production. At the same time, the Food Safety Modernization Act, Produce Safety Rule places significant emphasis on ensuring that preharvest agricultural water does not introduce microbiological contaminants onto food crops.[1] Hence, it is critically important that as the use of recycled irrigation water increases any microbiological water quality issues associated with the water are identified and remediated. Issues of potential concern include the possible persistence of bacterial, viral, and protozoan pathogens in recycled irrigation water that has not been adequately treated.[2−7] Moreover, there

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is concern that antibiotic resistance genes (ARGs) persisting in recycled irrigation water could augment environmental reservoirs of resistance and potentially contribute to the growing overall public health challenge of increasing rates of antibiotic-resistant bacterial infections.[8−10]

Pathogenic bacteria[4,11−17] (e.g., *Salmonella enterica*, pathogenic and enterohemorrhagic *Escherichia coli*, *Listeria monocytogenes*, and *Campylobacter jejuni*), viruses[5,18−20] (e.g., norovirus, hepatitis E virus, and hepatitis A virus), and protozoa[5,18,21] (e.g., *Cryptosporidium parvum* and *Giardia intestinalis*) have been detected in diverse irrigation water sources including freshwater (ponds and nontidal rivers), brackish water, advanced treated municipal wastewater, harvested rainwater, and return flows. In some instances, contaminated irrigation waters—such as surface water—have been implicated in foodborne illnesses and multistate outbreaks;[5,7,22,23] however, to our knowledge, there have been no reported foodborne outbreaks associated with the use of recycled irrigation water on food crops in the U.S.

Nevertheless, our group and others have demonstrated that recycled irrigation water and untreated surface water can harbor diverse ARGs. For example, a recycled irrigation water source and a nontidal freshwater body in the Mid-Atlantic region were identified by our group to harbor diverse ARGs including those coding for resistance against tetracyclines, aminoglycosides, and macrolides.[3,6] Similarly, a study of recycled irrigation water utilized to irrigate urban park soils demonstrated that between six and 60 ARGs were statistically significantly enriched among tested recycled irrigation water samples.[24] These studies and others have employed high-throughput quantitative PCR or metagenomic sequencing approaches that enable the simultaneous characterization of a large number of ARGs via a single experiment compared to performing multiple PCR/qPCR assays targeting individual or small groups of ARGs.[25,26]

However, the majority of previous studies that have focused on evaluating ARGs, as well as pathogenic bacteria, viruses, and protozoa, in recycled irrigation water sources have been largely cross sectional in nature, capturing the microbiological quality of sampled water during only one time period at a very limited number of sites. To address this data gap, our team of researchers from the CONSERVE Center of Excellence (conservewaterforfood.org) carried out the most comprehensive longitudinal study to date of U.S. recycled water and surface water used for irrigation, employing multiple laboratories and analytical approaches to understand the overall microbiological, chemical, and physical water quality of sampled sites. Here, we report on the findings from our 16S rRNA gene sequencing and metagenomic sequencing approaches describing the overall taxonomic and functional variations (e.g., levels of ARGs) in bacterial communities across diverse recycled and surface water sources used for irrigation in the Mid-Atlantic and Southwest regions of the U.S.

MATERIALS AND METHODS

Sampling Sites. The Mid-Atlantic and Southwest recycled and surface water sampling sites (n = 22) included in this study are either currently used for irrigation of food crops or have potential as future sources of irrigation water. In the Mid-Atlantic region, there were 12 sites of five different water types: nontidal freshwater river (NF: Sites MA03, MA04, MA05, MA07, and MA09), on-farm pond water (PW: Sites MA10 and MA11), vegetable processing water (VP: Site MA12), advanced treated municipal wastewater from three tertiary wastewater treatment plants (RW: Sites MA01, MA02, and MA06), and tidal brackish water (TB: Site MA08). In the Southwest region, there were 10 sites of three different water types: harvested rainwater (RAW: Sites SW07 and SW08), advanced treated municipal wastewater (RW) including secondary (Site SW04) and tertiary (Site SW01) treated wastewater effluent as well as reverse osmosis concentrate (Sites SW03, SW09, and SW10), and return flows (RF: Sites SW02, SW05, and SW06). Detailed descriptions of these sites and treatment processes (if applicable) appear in Table S1.

Sample Collection and Processing. A total of 410 water samples (n = 346 from the Mid-Atlantic and n = 64 from the Southwest) were collected from these sites over a two-year period (September 2016 to October 2018). Throughout this time period, samples were collected bimonthly from May to October (the peak growing season at the majority of the sites) and monthly from November to April (the off-season at the majority of the sites). At each site, 1 L of water was collected into sterile polypropylene environmental sampling bottles (Thermo Fisher Scientific, Waltham, MA, U.S.). For surface water sites, sterile 1 L bottles were submerged 15−30 cm below the water surface using a sampling stick (Zenport Industries, Portland, OR, U.S.) to collect water samples. For recycled water sites (e.g., advanced treated municipal wastewater facilities), water was collected from spigots located close to field release sites (e.g., sprinklers used for groundwater recharge or irrigation of crops). Before sample collection from spigots, water was allowed to run for 1 min. For all water types, bottles were immediately transferred to coolers containing ice packs for transport to the laboratory.

Within 24 h of sample collection, samples were filtered in the lab. To collect and concentrate bacteria present in each water sample, bottles were inverted three times before a total of 500 mL was split in half and filtered through 0.22 μm, 47 mm filters (Pall Corporation, Port Washington, NY, U.S.) in sterile filter funnels (Thermo Fisher Scientific, Waltham, MA, U.S.). Filters were aseptically transferred to sterile Petri dishes and stored at 4 °C while all filtrations were completed. Within 6 h of filtration, all filters were folded in half, cut into equal-sized strips using sterile scissors, and transferred to two Lysing Matrix B tubes (MP Biomedicals, Solon, OH, U.S.) using sterile forceps. In most cases, processing of each 500 mL water sample resulted in two filters (250 mL/filter). However, for very turbid samples, filtration was halted due to clogging at a lower volume; therefore, a third filter was utilized to achieve the full 500 mL filtration. In these cases, the third filter was cut in half and split between the two lysing matrix tubes, such that each tube still contained material from 250 mL of water. Tubes (two per 500 mL sample) were stored at −80 °C until DNA extractions could be performed.

DNA Extraction. DNA extractions of all water samples (n = 410) were performed using protocols previously published by our group.[27−36] Briefly, 1 mL of PBS was added to lysing matrix B tubes (MP Biomedicals, Solon, OH, U.S.) containing the 0.22 μm filters. The tubes were then incubated with freshly prepared enzyme cocktails (lysozyme, mutanolysin, proteinase K, and lysostaphin) after which the cells were mechanically lysed using an MP Biomedical FastPrep 24 (Santa Ana, CA, U.S.). The DNA was then purified using the Qiagen QIAmp DNA mini-kit (Germantown, MA, U.S.) per the manufacturer's protocol. We also included negative extraction controls

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during water filtration, DNA extraction, and sequencing to check for any potential exogenous contamination. Additionally, to assess the quality of the purified DNA, gel electrophoresis and a NanoDrop (Thermo Scientific, Waltham, MA, U.S.) were used.

DNA Purification and 16S rRNA Gene PCR Amplification and Sequencing. DNA purification and pooling were performed before PCR amplification. From each of the two DNA extractions from each original 500 mL water sample, 50 μL was pooled, and the combined 100 μL was purified using the PowerClean Pro DNA Clean Up Kit (MoBio, Carlsbad, CA, U.S.). For all combined and purified samples, DNA quality control was again performed using gel electrophoresis and a NanoDrop (Thermo Scientific, Waltham, MA, U.S.).

Purified DNA (n = 410) was then PCR amplified targeting the V3−V4 hypervariable region of the 16S rRNA gene using the universal primers 319F (ACTCCTACGGGAGGC-AGCAG) and 806R (GGACTACHVGGGTWTCTAAT) and sequenced on an Illumina HiSeq2500 (Illumina, San Diego, CA) using a method developed at the Institute for Genome Sciences[37] and previously applied by our team to study the microbiomes of environmental samples.[3,6,38]

Metagenomic Library Preparation and Sequencing. A subset of 110 (n = 101, Mid-Atlantic; n = 9, Southwest) samples was selected for metagenomic sequencing and analysis. The Mid-Atlantic samples were selected from sampling dates across all sampling years (2016−2018) that were within the growing season when irrigation water would likely be applied. The sampling dates selected for 2016 are later in the season compared to 2017−2018 because sampling started in October of that year. Regarding the Southwest samples, all samples from SW04 were included due to consistent, repeated sampling at that site, and single samples from three other sites were included for comparison. The Nextera XT kit (Illumina, San Diego, CA, U.S.) was used to prepare DNA libraries for each of the selected samples per the manufacturer's specifications. The samples were then sequenced on the Illumina Hiseq X Ten System targeting 100 bp paired-end reads (Illumina, San Diego, CA, U.S.).

16S rRNA Sequence Quality Filtering and Analysis. The 16S rRNA paired-end reads were assembled using PANDAseq,[39] demultiplexed, trimmed of primers, and assessed for chimeras using UCHIME in Quantitative Insights Into Microbial Ecology (QIIME; release v.1.9.1).[40] Quality reads were then clustered *de novo* into operational taxonomic units (OTUs) at 97% sequence similarity using the pick_otus.py command on QIIME. The taxonomic assignments were then performed using the Greengenes database implemented in QIIME. Data were then imported in RStudio (v.1.1.423) using R packages biomformat[41] and phyloseq.[42]

Prior to normalization, alpha diversity was measured using both the observed richness metric and the Shannon diversity index.[43] Data were normalized using cumulative sum scaling (CSS) in the R package metagenomeseq[44] when necessary, and packages vegan,[45] ggplot2,[46] and metagenome-Seq[44] were used for downstream analysis and plot visualizations. Bray−Curtis dissimilarity was used for calculating beta diversity, and statistical analyses were completed using analysis of similarities (ANOSIM) on normalized data (999 permutations). A linear discriminant analysis (LDA) effect size (LEfSe) analysis was used to compare relative abundance profiles at genus and species levels between the different water types using the MicrobiomeAnalyst tool.[47,48] This analysis performs a non-parametric factorial Kruskal−Wallis (KW) sum-rank test to detect genus or species with significant differential abundance with respect to the different water types, followed by linear discriminant analysis to estimate the effect size of each differentially abundant bacterial genus or species.

Metagenomic Data Analysis. Bacterial taxonomic abundance profiles for the metagenomic data were determined using the Kraken 2 classification tool[49] and Bracken abundance estimator[50] from quality trimmed raw paired-end sequences. Additionally, to identify ARGs present in different water samples, the quality checked raw sequencing reads were processed via the AMR++ bioinformatic pipeline[51] that uses the MEGARes 2.0 database (https://megares.meglab.org)[52] to produce a count matrix of alignments to ARGs. The matrix was then filtered to have counts with minimum coverage and identity of 85% of target genes. The database includes published resistance gene sequences for antimicrobial, metal, and biocide resistance determinants. Downstream data analysis and visualization of the bacterial taxonomic profiles and the various resistance genes were carried out in RStudio (v.1.3.1093) using several R packages (vegan,[45] ggplot2,[46] dplyr,[53] circlize,[54] reshape2,[55] and stringr[56]).

Raw metagenomic reads were also searched against the Comprehensive Antibiotic Resistance Database (CARD) using BLASTn (BLASTn ver. 210 2.7.1+).[57] Queried reads were filtered based on coverage and percent identity above 90%. Using the ARO (Antibiotic Resistance Ontology) index, the ARG identified was assigned to a class. Predicted ARG hosts were then identified by classifying the reads using the taxa information within the CARD database. The results were visualized using a Sankey plot created in Power Bi visuals (ver. 3.0.3.0).

Availability of Data. Data generated in this study were deposited in the NCBI BioProject database under the BioProject accession number PRJNA473136.

RESULTS

16S rRNA Sequencing Data Set Summary. A total of 410 samples were successfully PCR amplified and sequenced, generating a total of 31,010,761 sequences across all samples and 109,676 operational taxonomic units (OTUs). To ensure that all samples in the final data set were sequenced to an appropriate coverage level across samples, the Good's estimate of coverage was calculated according to the formula by Good[58]: Good's Coverage = 1 − (F1/N), where F1 is the number of singletons, and N is the total number of individuals (sum of abundances for all OTUs).[58] Samples with Good's values < 0.85 were removed from the data set for further downstream analysis. These included four nontidal freshwater river samples (MA03, MA05, MA09 (2)), three reverse osmosis concentrate samples (SW03, SW09, SW10), and one tidal brackish water sample (MA08). After pruning of low abundance taxa (OTUs with less than 10 sequences), the final data set analyzed contained 30,741,275 sequences clustered into 54,145 operational taxonomic units from 402 (n = 341 Mid-Atlantic; n = 61 Southwest) samples with a minimum read of 434, maximum read of 200,944, and an average number of sequences per sample of 76,470.83 (± 44,840.38 SD). Our final data set had 341 Mid-Atlantic and 61 Southwest samples which comprised 162 nontidal freshwater samples, 69 pond water samples, 92 advanced treated municipal wastewater samples (secondary and tertiary treated wastewater and reverse osmosis concentrate), 33 tidal brackish water samples, 13

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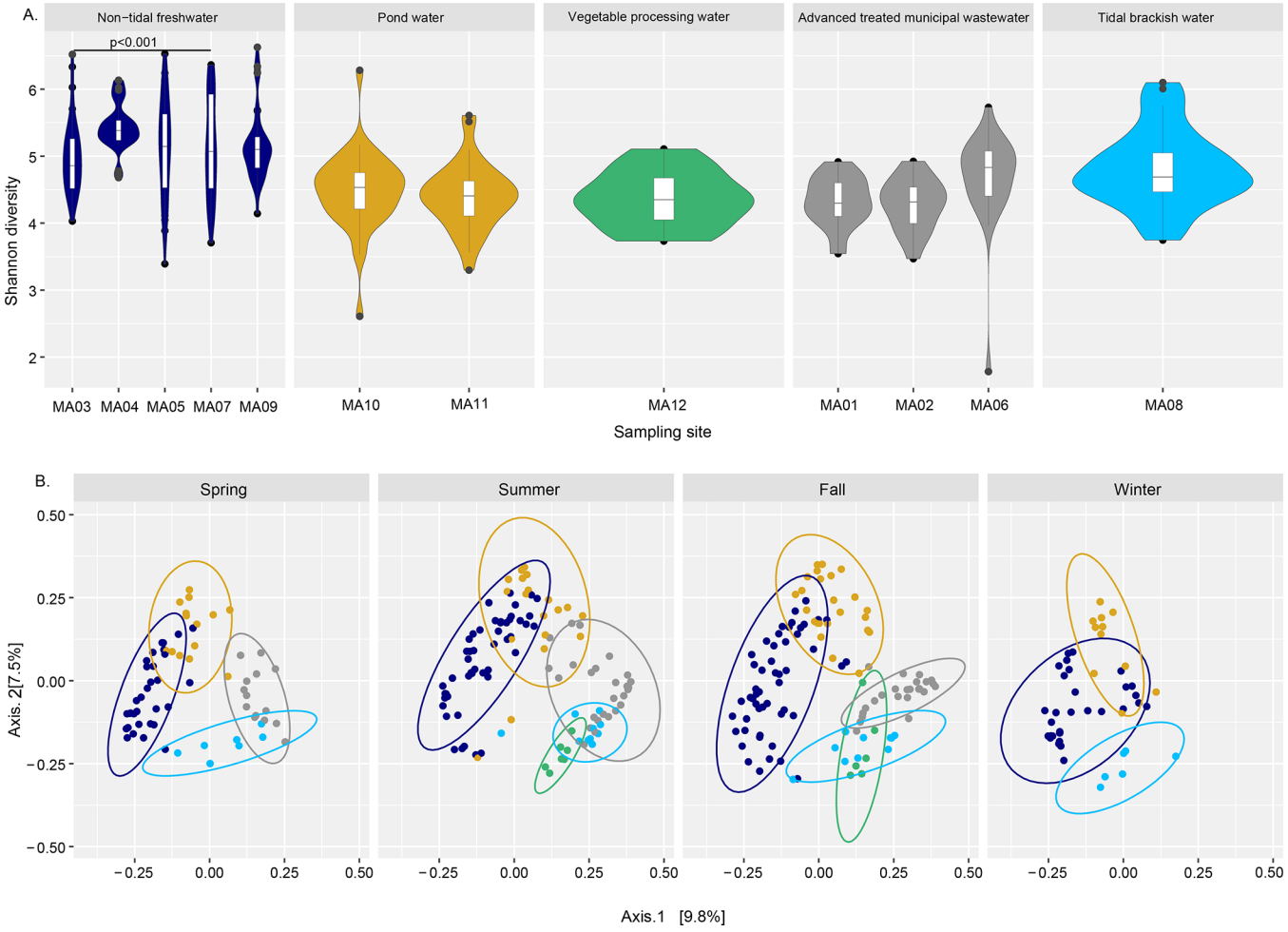

Figure 1. Bacterial diversity within and between water types in the Mid-Atlantic region. (A) Violin box plots representing alpha diversity metrics (Shannon diversity) across rarefied data, colored by water types: nontidal freshwater (dark blue), pond water (brown), vegetable processing water (green), advanced treated municipal wastewater (dark gray), and tidal brackish water (light blue). (B) PCoA plots of Bray−Curtis computed distances between the seasons among the different water types.

vegetable processing water samples, 23 return flow samples, and 10 harvested rainwater samples.

Bacterial Diversity. The Shannon alpha diversity metric was calculated on both rarefied (after down-sampling the Mid-Atlantic samples and Southwest samples to 1816 and 434, respectively) and nonrarefied data. We only present our rarefied data analysis since no differences were observed between the rarefied and nonrarefied analyses. Within the Mid-Atlantic region, alpha diversity of nontidal freshwater samples was significantly higher ($p < 0.0001$) than that of other sample types, and in the Southwest region, return flows had a significantly higher ($p < 0.0001$) alpha diversity when compared to the other water types (Figure S1). Among the nontidal freshwater sites from the Mid-Atlantic region, we observed that sampling site MA03 had a significantly lower ($p < 0.001$) alpha diversity compared to MA07 (Figure 1). Likewise, in the Southwest region, we observed that among the advanced treated municipal wastewater sites, sampling site SW03 had a significantly lower ($p < 0.05$) alpha diversity compared to SW01 and SW10 (Figure 2).

Beta diversity analyses also confirmed that the bacterial microbiota of tested water samples is heavily influenced by water type. In the Mid-Atlantic region, bacterial community structures were significantly different between the water types (ANOSIM R: 0.7027, $p = 0.001$) (Figure 1). Similar to the Mid-Atlantic region, significant differences in beta diversity between the water types within the Southwest region were also observed (ANOSIM R: 0.4756, $p = 0.001$) (Figure 2). In addition, we observed that season had a significant impact on bacterial communities among water types within the Mid-Atlantic region (ANOSIM R: 0.1218, $p = 0.001$) (Figure 1) but not within the Southwest region (ANOSIM R: 0.01237, $p = 0.284$) (Figure 2).

When comparing the advanced municipal wastewater facilities between the two different regions via alpha (Figure S2) and beta diversity measures (Figure S3), we observed that the sampling sites within the Southwest region were more diverse when compared to the sampling sites within the Mid-Atlantic region.

Overall Bacterial Composition via 16S rRNA Sequencing. The predominant bacterial phyla in both regions irrespective of water type and season was *Proteobacteria*, while *Firmicutes* had a lower relative abundance in all water types except the vegetable processing water in the Mid-Atlantic region (data not shown). A deeper look into the top 25 bacterial taxa from the Mid-Atlantic region (Figure S4) revealed that *Limnohabitans* spp. and Uncl. ACK-MI (an unclassified *Actinobacteria*) seemed to be dominant across

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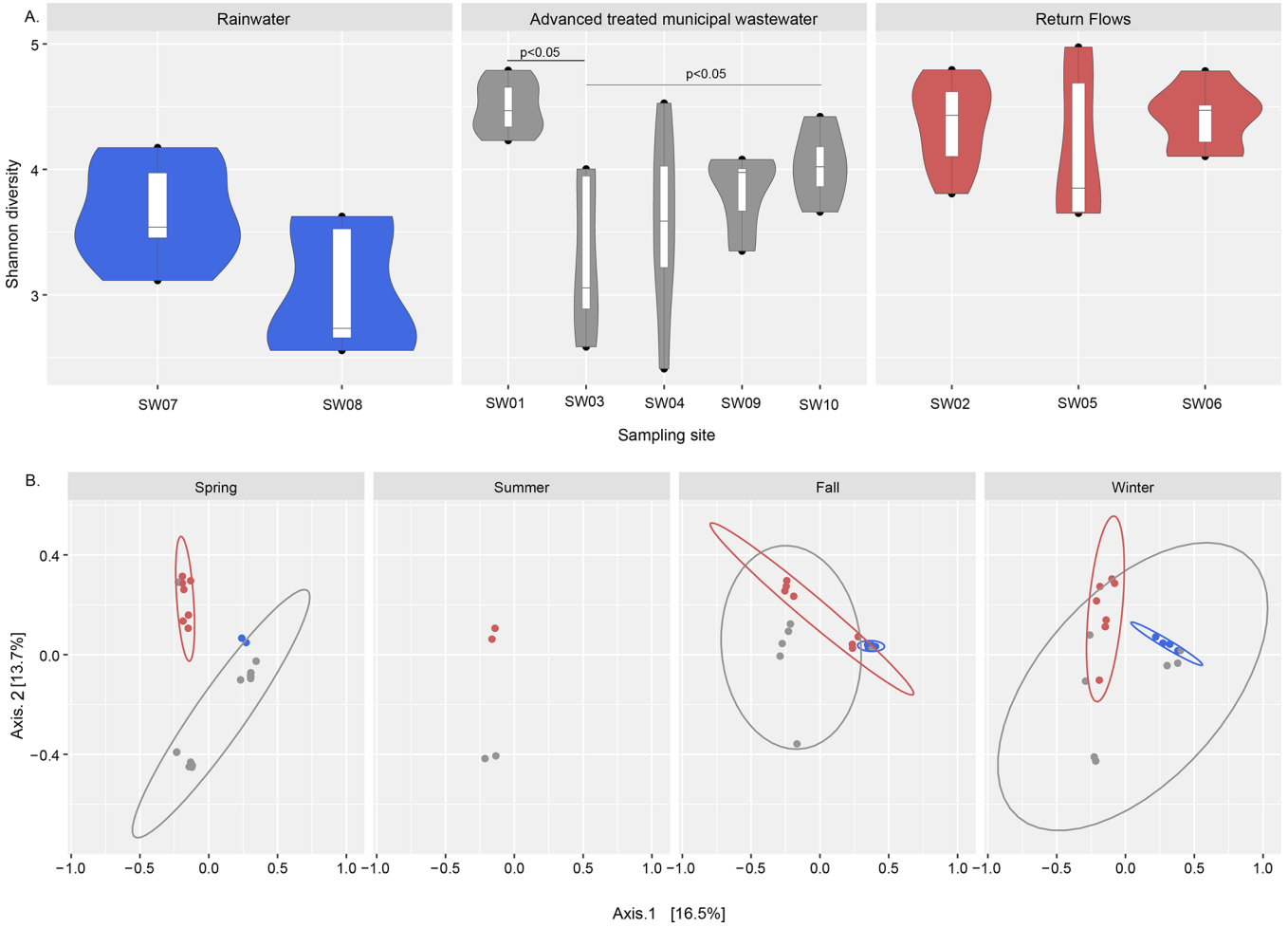

Figure 2. Bacterial diversity within and between water types in the Southwest region. (A) Violin box plots representing alpha diversity metrics (Shannon diversity) across rarefied data, colored by water types: rainwater (blue), advanced treated municipal wastewater (dark gray), and return flows (salmon pink). (B) PCoA plots of Bray−Curtis computed distances between the seasons among the different water types.

nontidal freshwater sites in the summer and fall, while *Flavobacterium* spp. and *Rhodoferax* spp. seemed to be more dominant across these sites in the winter. A similar observation was also made for the two on-farm pond water sites. Meanwhile, Uncl. *Rhizobiales* and Uncl. TM7−1 (an unclassified *Saccharibacteria*) were dominant during the summer and fall in water samples recovered from the vegetable processing plant. The three tertiary treated municipal wastewater sites had a high abundance of *Flavobacterium* spp. in the spring and Uncl. *Actinomycetales* in the summer. Tidal brackish water had a high abundance of *Flavobacterium* spp. in the winter and *Synechococcus* spp. in the summer. In the Southwest region, *Limnohabitans* spp. was the predominant bacterial genus across all seasons among the return flows and a single rainwater collection site (SW08), while *Novosphingobium* and *Rhodobacter* species were predominant in the SW07 rainwater collection site (Figure S5). The Family *Procabacteriaceae* (Candidatus family of *Betaproteobacteria*) was the predominant bacterial taxa among the SW04 site (secondary treated municipal wastewater) during all seasons, while this bacterial taxon was observed at the SW10 site (reverse osmosis concentrate) only during the spring.

Our Lefse analysis identified 56 bacterial genera and 13 bacterial species with significantly different ($p < 0.05$) relative abundances between the different water types irrespective of regions (Figures S6 and S7). Among these, 18 bacterial genera were at significantly higher relative abundances in the vegetable processing water, while the nontidal freshwater samples had only one bacterial genus that was at a significantly higher abundance compared to the other sample types. The pond water and rainwater had 12 bacterial genera that were at a significantly higher relative abundance, while the tidal brackish water and the return flows had four and two bacterial genera, respectively, at significantly higher abundances compared to the other sample types (Figure S6). At the species level, both the nontidal freshwater and pond water samples were characterized by significantly higher relative abundances of *Limnohabitans curvus*, which were observed to be the least abundant in rainwater. *Enterobacter cloacae*, *Arcobacter cryaerophilus*, *Sulfuricurvum kujiense*, *Acinetobacter johnsonii*, and *Propionispira arboris* were at a significantly higher relative abundance in the vegetable processing water compared to other sample types. *Polynucleobacter cosmopolitanus* and *Blastomonas natatoria* were observed to be at significantly higher relative abundances in the advanced treated municipal wastewater and return flow samples and at the lowest relative abundances in the rainwater. The rainwater had higher relative abundances of *Methylotenera mobiliz*, *Methylobacterium adhaesivum*, and *Janthinobacterium lividum* (Figure S7).

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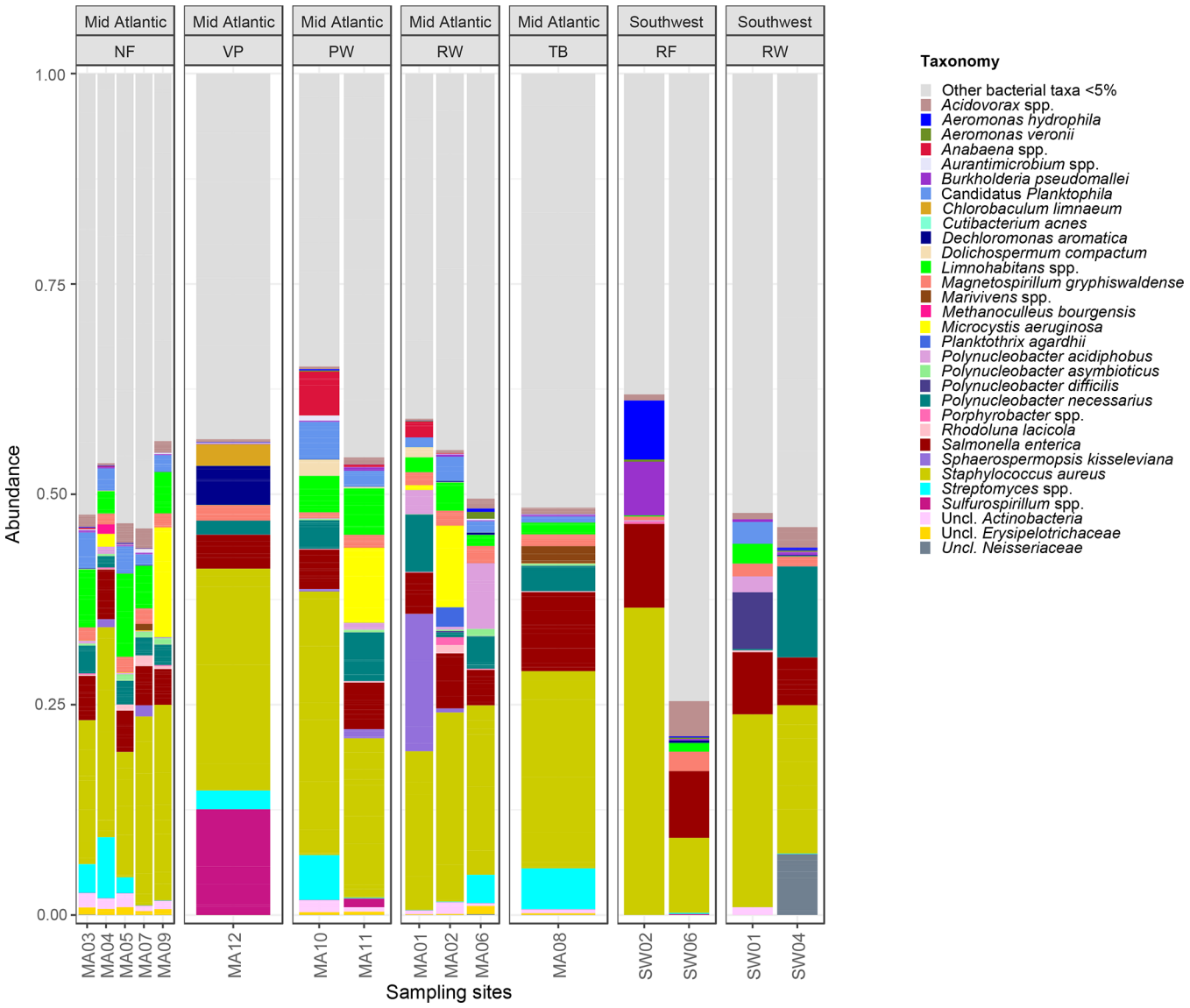

Figure 3. Metagenomic taxonomic profiles of bacterial microbiota across different sampling sites and water types in the Mid-Atlantic and Southwest regions. NF = nontidal freshwater; VP = vegetable processing water; PW = pond water; RW = advanced treated municipal wastewater; TB = tidal brackish water; RF = return flows.

Bacterial Composition Assessed via Metagenomics and Comparison to 16S rRNA Data. Metagenomic sequencing revealed that *Staphylococcus aureus* was the predominant bacterial species across all water types. In addition, all of the water types harbored *Salmonella enterica*, as well as a low relative abundance of *Acidovorax* spp. and *Magnetospirillum gryphiswaldense*, irrespective of the region. *Polynucleobacter necessarius* was observed in all water types except for the return flows from the Southwest region. Similarly, *Limnohabitans* spp. was present in all water types except for the vegetable processing water recovered from the Mid-Atlantic region and two sampling sites (SW02 (return flows) and SW04 (secondary treated municipal water)) in the Southwest region. *Microcystis aeruginosa* was observed in four of the sampling sites (tertiary treated municipal water (MA02), nontidal freshwater (MA04 and MA09), and pond water (MA11)), while *Streptomyces* spp. was observed in seven of the sampling sites in the Mid-Atlantic region (nontidal freshwater (MA03, MA04, and MA05), tertiary treated municipal water (MA06), tidal brackish water (MA08), pond water (MA10), and vegetable processing water (MA12)). *Sulfurospirillum* spp. and *Dechloromonas aromatica* were unique bacterial taxa to vegetable processing water (MA12), while *Burkholderia pseudomallei* was unique to one of the return flows (SW02) collected in the Southwest region (Figure 3).

When comparing our 16S rRNA gene sequencing data with our metagenomic sequencing data from the subset of 110 water samples, we observed differing patterns due to the finer resolution of the metagenomic data. For example, more bacterial genera and fewer species-level identifications were achieved with our 16S rRNA data when compared to our metagenomic data. Our 16S rRNA sequencing data revealed the presence of the *Actinobacteria* phylum and *Synechococcus* spp. in all water types except for the vegetable processing water and the return flows when compared to our metagenomic data. *Limnohabitans* spp. (*Proteobacteria*) were observed across all of the nontidal freshwater sampling sites according to both our 16S rRNA and metagenomic sequencing data. In addition,

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while *Aeromonas* spp. (*A. media* and *A. hydrophila*) were observed in the return flows (SW02) via our metagenomic data, we could only identify the family *Aeromonadaceae* via our 16S rRNA data (Figure S8).

Antibiotic Resistance Genes. Diverse ARGs were present across all sampling sites and regions (Figure 4). The most prominent ARGs identified coded against MLS (macrolide, lincosamide, and steptogramin) antibiotics, aminoglycosides, aminocoumarins, fluoroquinolones, cationic antimicrobial peptides, elfamycins, rifampin, and tetracyclines (Figure 4). *Mycobacterium tuberculosis*-specific drug resistance (MTSB) was observed in all of the Mid-Atlantic sampling sites and only one Southwest sampling site (secondary treated municipal wastewater, SW04). Similarly, fusidic acid, phenicol, and fosfomycin resistance were observed only in one (nontidal freshwater (MA03)), two (advanced treated municipal wastewater (MA01) and pond water (MA11)), and three (tertiary treated municipal wastewater (MA01), pond water (MA11), and vegetable processing water (MA12)) sampling sites, respectively, in the Mid-Atlantic region (Figure 4). Interestingly, a peak in ARG counts was observed during the warmer months (July and August), while lower counts were observed during the colder months (October and November) across the different sampling sites in the Mid-Atlantic region (Figure 5).

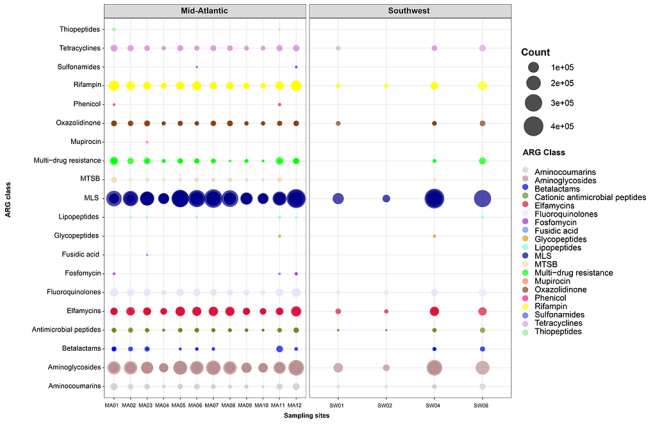

Figure 4. Dot plot of antibiotic resistance genes observed in different water types in the Mid-Atlantic and Southwest regions. The sizes of the dots represent the raw counts/number of reads. MLS refers to macrolide, lincosamide, and streptogramin antibiotics, and MTSB refers to *Mycobacterium* tuberculosis-specific drug resistance.

The CARD database revealed that the majority of the ARGs (except rifampin) were detected at one Mid-Atlantic tertiary treated municipal wastewater site (MA06) and were predicted to be associated with a high number of identified bacteria (Figure 6). *Photobacterium damselae* and *Vibrio fluvialis*, that were predicted to carry MLS and sulfonamide resistance genes, were identified in the other two Mid-Atlantic tertiary treated municipal wastewater sites (MA01 and MA02), respectively. *Vibrio cholerae*, that was predicted to carry sulfonamide resistance genes, was only observed in the Southwest return flows (SW02) and the secondary treated municipal wastewater samples (SW04).

Biocide and Metal Resistance Genes. The AMR++ database also helped in identifying resistance gene encoding for multibiocide resistance which were predominant in both the Mid-Atlantic and Southwest regions, except for two Mid-Atlantic tertiary treated municipal wastewater sites (MA01 and SW01) and a Mid-Atlantic pond water site (MA10) (Figure S9). The next most predominant biocide resistance genes encoded for phenolic compound resistance, which was observed across all water types except for three sites in the Mid-Atlantic region (MA08 (tidal brackish water), MA09 (nontidal freshwater), and MA10 (pond water)) and two sites in the Southwest region (SW01 (secondary treated municipal wastewater) and SW02 (return flows)). Gene encodings for peroxide resistance were observed across all water types in both regions, while gene encodings for biguanide resistance were present in two of the municipal wastewater sites (MA06 (tertiary treated wastewater) and SW04 (secondary treated wastewater)), one pond water site (MA11), and the vegetable processing water site (MA12). Gene encodings for acid resistance were identified at three sampling sites (nontidal freshwater (MA03), pond water (MA11), and secondary treated municipal wastewater (SW04)) (Figure S9).

Similar to the biocide resistance genes, gene encodings for multimetal resistance were predominant in 10 of the 12 sampling sites in the Mid-Atlantic region and three of the four sampling sites in the Southwest region. Iron resistance genes were observed across all of the sampling sites in the Mid-Atlantic region, except for sampling sites MA02 (tertiary treated municipal wastewater) and MA12 (vegetable processing water) and were completely absent in the Southwest region. Copper resistance genes were observed in all of the sampling sites in the Mid-Atlantic region and three of the four sampling sites in the Southwest region, of which one site was a secondary treated municipal wastewater site (SW04), one site a tertiary treated municipal wastewater site (SW01), and one site a return flow site (SW06) (Figure S10).

DISCUSSION

Agricultural irrigation water sources such as recycled water and surface water can be excellent substitutes for traditional groundwater sources, given increasing groundwater shortages in key food production areas. However, our data suggest that some of these sources will require additional treatment before use. Our findings show that diverse bacterial communities (including bacterial pathogens), as well as antibiotic, metal, and biocide resistance genes, are present across multiple alternative irrigation water types in two U.S. regions. Previous cross-sectional studies have evaluated these water quality parameters in select surface and recycled waters. Yet, to our knowledge, this is the most comprehensive longitudinal study to date of U.S. recycled water and surface water used for irrigation, comparing diverse sources across seasons and multiple sites in the Mid-Atlantic and Southwest regions of the U.S. Our findings improve understanding of the potential differences in the risk of exposure to bacterial pathogens and antibiotic-resistant bacteria originating from diverse irrigation water sources across seasons and U.S. regions.

Overall, our study revealed the presence of diverse bacterial communities in untreated surface waters and advanced treated municipal wastewaters. In addition, we observed that bacterial diversity was significantly influenced by water type and season, especially in the Mid-Atlantic region. For example, our 16S rRNA gene sequencing data revealed a higher relative abundance of *Limnohabitans* (Figure 3) in nontidal freshwater samples (Mid-Atlantic region) in the summer and fall. A metagenomic profiling of the James River (a large tributary of the Chesapeake Bay) that was completed during the summer of 2012 also revealed a higher relative abundance of

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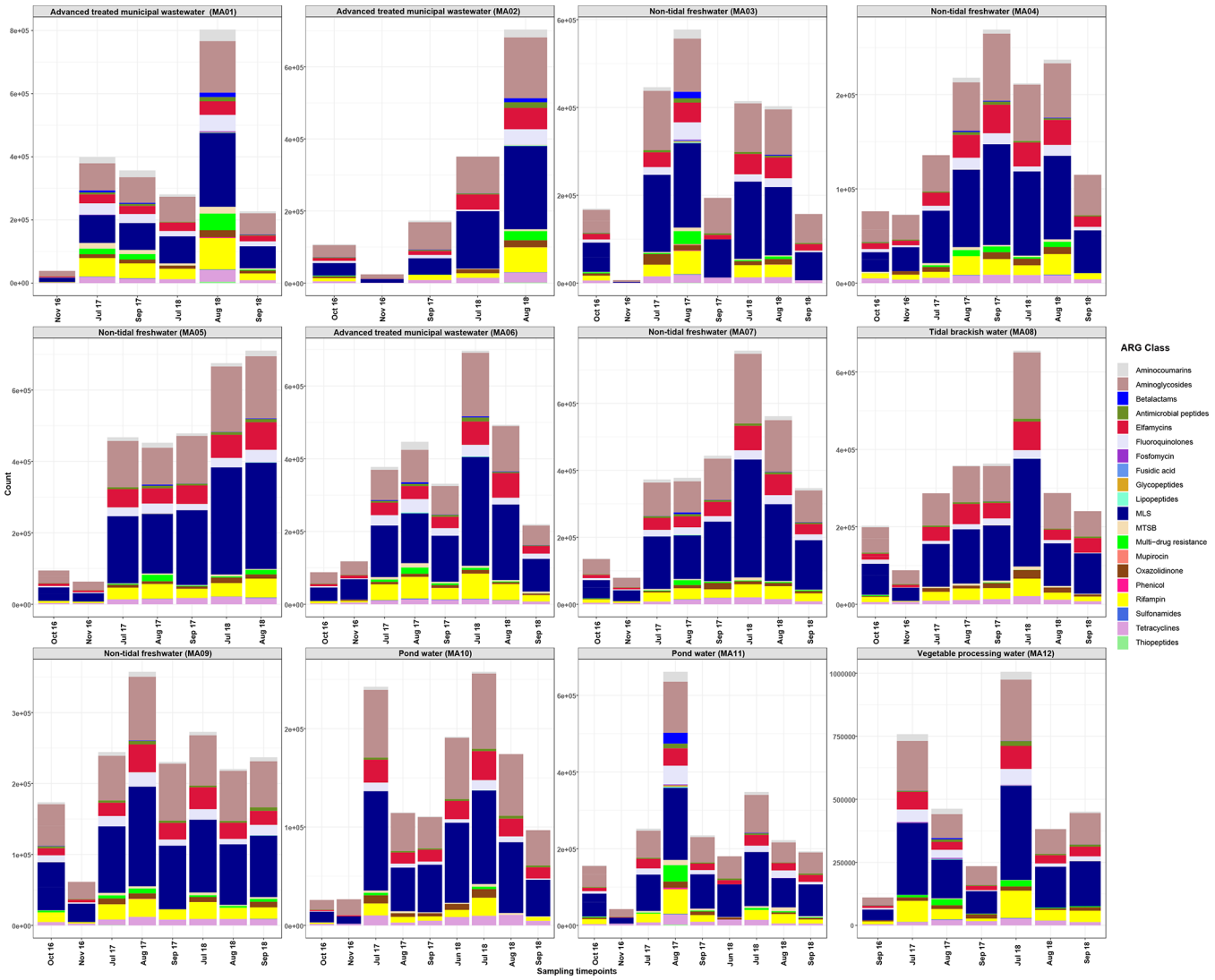

Figure 5. Bar plot demonstrating seasonality of antibiotic resistance genes observed in different sampling sites in the Mid-Atlantic region over the 2-year sampling period. MLS refers to macrolide, lincosamide, and streptogramin antibiotics, and MTSB refers to *Mycobacterium* tuberculosis-specific drug resistance.

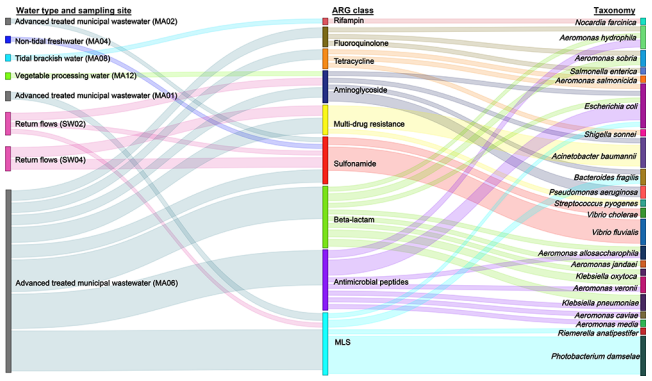

Figure 6. Sankey plot of taxonomic profiles associated with the antibiotic resistance genes identified across the different water types.

Limnohabitans during a summer sampling period.[59] Another study had contrasting results, revealing that the highest relative abundance of *Limnohabitans* occurred during spring and the lowest relative abundance occurred during summer in freshwater from the Římov reservoir (Czech Republic).[60] In addition, most of the surface waters in the Mid-Atlantic region harbored *Limnohabitans*, *Polynucleobacter*, and *Rhodoferax*, which have been commonly observed in other surface waters.[61] *Novosphingobium* spp. was another predominant bacterium present in one of the harvested rainwater sites (SW07) in our study, and other studies corroborate the high prevalence of this bacterium in harvested rainwater.[62,63]

In addition to diverse bacterial communities, we also observed (via culture-independent, metagenomic sequencing) some pathogenic bacterial species across different water types, including *Staphylococcus aureus*, *Salmonella enterica*, *Aeromonas hydrophilia*, and *A. veronii* (Figure 3). *S. aureus*, for example, was predominantly found in all water types using our metagenomic sequencing data (Figure 3). *S. aureus* is a Gram-positive commensal and opportunistic pathogen commonly found in the upper respiratory tract and skin. It is often associated with health care acquired infections and is known to be resistant to methicillin. While our metagenomic data cannot speak to the viability of *S. aureus* detected in the water samples tested here, our group previously cultured methicillin-resistant *S. aureus* (MRSA) in 83% of influent samples and only 8% of

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effluent samples (samples that were not chlorinated) from four wastewater treatment facilities in the U.S.[64] These findings demonstrate that tertiary treatments (including chlorine) are likely necessary for wastewater intended for reuse in food crop irrigation to reduce the potential risk of MRSA and other bacterial pathogens contaminating irrigated crops.

Beyond *S. aureus*, *Salmonella enterica* was detected (via culture-independent, metagenomic sequencing) in all water samples in the present study. *Salmonella* causes an estimated 1.34 million infections, 26,500 hospitalizations, and 420 deaths in the U.S. every year.[65] *Salmonella* infections have often been attributed to under-cooked poultry products; however, in recent years, salmonellosis has also been attributed to the consumption of contaminated fresh produce such as tomatoes and lettuce.[66] *Salmonella* contamination of fresh produce may occur at any point from farm to table.[67] These bacteria have the ability to attach to and internalize within fresh produce,[68] which is of considerable concern if irrigation water sources are contaminated with *Salmonella* species. However, irrigation method, climate, and irrigated plant type can all play roles in risk. For example, a recent study by our group tested radishes and kale drip-irrigated with *Salmonella*-contaminated river water and observed no transfer of *Salmonella* to the crops.[28]

Both in our previously published culture data derived from the same longitudinal study[14,16] and our metagenomic data reported here (Figure 3), *Salmonella* species were identified, predominantly occurring in surface waters, especially tidal brackish water (MA08) samples and to a lesser extent in recycled waters. Wang et al. reviewed pathogen removal from wastewater treatment facilities and reported that 63.38%−99.99% *Salmonella* removal efficiencies can be achieved,[69] which may explain why we observed less *Salmonella* in the recycled waters compared to surface waters. However, interestingly, we were able to detect *Salmonella* in all tested water types through our metagenomic approach using a lower volume of water (500 mL) compared to our culture-based study[14,16] that frequently required testing 10 L of these same water samples to detect *Salmonella*. *Salmonella* can enter a viable but nonculturable state, which may be one explanation why we were able to detect these bacteria more effectively through metagenomic sequencing. Another reason why we were able to detect *Salmonella* in all tested water types using metagenomics and smaller sampling volumes could be that metagenomic sequencing might be detecting persistent relic DNA that is not associated with live organisms. Similar to *S. aureus*, *Salmonella* is also commonly resistant to antibiotics, and our metagenomic data predicted that fluoroquinolone resistance genes were carried by *Salmonella enterica* in recycled water samples from one of our advanced treated municipal wastewater sites (Figure 6).

Other Gram-negative opportunistic pathogens observed in our study, including *A. hydrophilia*, *A. veronii*, and *A. cryaerophilus*, have been occasionally isolated from fresh fruits and vegetables and have been responsible for foodborne illnesses.[70−73] *Aeromonas* spp. are ubiquitous in nature and have been detected globally in a broad range of foods and water sources (surface, ground, and mineral bottle water).[70,71] To date, there have been no foodborne outbreaks related to *Aeromonas* in the U.S., but a study by Zhang et al. found that *A. hydrophilia* was the causative agent for a foodborne outbreak (associated with salads) in a college in China.[72] Interestingly, it was reported that the salads had been washed with contaminated water.

In our previously published[15,74] culture-based studies, where the samples were enriched, *A. hydrophilia*, *A. veronii* ,and *A. jandaei* were detected in all of our tested water types in the Mid-Atlantic region, while our metagenomic data (Figure 3) revealed *A. hydrophilia* and *A. veronii* predominantly in only one of the pond water sites (MA10) and one tertiary treated municipal wastewater site (MA06). Solaiman and Micallef also reported that *A. hydrophilia* and *A. jandaei* persisted in sampled water for 24 h, while *A. veronii* could associate with irrigated lettuce leaves for up to 120 h.[74] Additionally, metabolically active *Aeromonas* spp., especially *A. hydrophilia* and *A. media*, have also been detected at relatively high abundances in the tested recycled water (MA06) from the Mid-Atlantic region in a separate study published by our group.[36]

Besides bacterial pathogens in the tested water sources, we also observed resistance gene codings against antibiotics (Figure 4), biocides (Figure S9), and metals (Figure S10). The widespread use and misuse of antibiotics in both human and veterinary medicine has resulted in the ubiquitous presence of antibiotics and ARGs in our water sources.[8,75,76] However, a knowledge gap exists in terms of understanding the relationship between environmental concentrations of the antibiotics themselves and the prevalence of ARGs.[25] In the present study, we observed the presence of diverse ARGs (Figure 4), biocide resistance genes (Figure S9), and metal resistance genes (Figure S10) across nearly all sampling sites. Interestingly, Panthi et al. previously reported on the presence of azithromycin (a macrolide-type antibiotic), ciprofloxacin (a fluoroquinolone), and sulfamethoxazole (a sulfonamide) in all of our tested water types from the Mid-Atlantic region.[77] Specifically, azithromycin was found to be more predominant in recycled water sources. This is in line with our present finding of resistance gene codings for MLS antibiotics, particularly in recycled water sources (Figure 4). Similarly, counts of resistance gene codings for fluoroquinolones were observed to be higher in vegetable processing water (MA12), and levels of ciprofloxacin were also observed to be higher in this same water source.[32] Moreover, sulfamethoxazole was found at elevated levels in the vegetable processing water and recycled waters of the Mid-Atlantic region.[77] Likewise, resistance gens codings for sulfonamides were also found in these water sources (Figure 4). In addition, we also observed the presence of *A. hydrophila* and *A. veronii* in one of the tertiary treated municipal wastewater sites (MA06) that was predicted to carry beta lactam resistance genes (Figure 6), which has been documented in multiple studies.[78−80] Previous studies have also described the presence of *Vibrio* species (in wastewater effluent and surface waters) carrying sulfonamide resistance genes.[81−83] Similarly, our study identified *Vibrio* species (*V. fluvialis* and *V. cholerae*) in Southwest return flows that were predicted to carry sulfonamide resistance genes (Figure 6).

Beyond documenting the presence of multiple ARGs across diverse recycled irrigation water sources, our findings are among the first to demonstrate seasonality in the levels of ARGs detected in these sources (Figure 5). For example, our data revealed high counts of ARGs during the summer (Figure 5), especially in one of the tertiary treated municipal wastewater sites (MA06) in the Mid-Atlantic region (Figure 5). These findings are consistent with observations made by Garner et al. and Czatzkowska et al. when wastewaters were tested for multiple ARGs.[84,85]

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These data demonstrating seasonality in levels of AMR across diverse recycled and surface water sites in two U.S. regions could inform the development of a new environmental monitoring arm of the U.S. National Antimicrobial Resistance Monitoring System (NARMS) that is being explored by the U.S. Centers for Disease Control and Prevention, the U.S. Food and Drug Administration, the U.S. Department of Agriculture, and the U.S. Environmental Protection Agency.[86] Since 1996, the NARMS program has been monitoring antibiotic-resistant enteric bacteria recovered from humans, animals, and retail meats. However, since 2017, NARMS has been focused on transitioning the program to a One Health surveillance model that includes monitoring of surface waters in both urban and rural areas for levels of AMR pathogens. Our longitudinal Mid-Atlantic and Southwest data, demonstrating seasonality in the relative abundance of antibiotic resistance genes, can help inform the frequency, timing, and location of sampling efforts as this new environmental monitoring arm of NARMS develops.

In addition to ARGs, we identified diverse biocide and metal resistance genes across different recycled water types. A systematic and quantitative data analysis of completely sequenced bacterial genomes from 565 different genera deposited in NCBI revealed the co-occurrence of ARGs, metal resistance genes, and biocide resistance genes.[87] Similar results were also observed in surface waters of the Duhaney River in Jamaica (an urban waterway)[88] and contaminated soils recovered from Savannah River sites in Georgia, U.S.[89]

Overall, our comprehensive longitudinal water quality analysis reported here provides valuable data in terms of the presence of diverse bacterial communities and ARGs in multiple recycled irrigation water sources and surface water sources across two U.S. regions. However, the study also had several limitations. As described above, we extensively relied on culture-independent methods (16S rRNA gene sequencing and metagenomic sequencing). Hence, the viability of the diverse bacteria detected cannot be determined. In addition, like all marker-based sequencing methods there could have been inherent biases introduced during PCR amplification. Moreover, in many cases, particularly in terms of our 16S rRNA gene sequencing data, we were unable to assign species-level classifications, which was in part due to limitations in existing databases, an inherent issue with massive parallel sequencing studies. Finally, due to financial constraints, we were unable to submit a greater number of samples (especially those from the Southwest region) for metagenomic sequencing.

Nevertheless, our culture-independent study revealed the presence of diverse bacterial communities, ARGs, metal resistance genes, and biocide resistance genes in a wide array of irrigation water sources in the Mid-Atlantic and Southwest U.S., as well as fluctuations in the levels of these targets across seasons. To our knowledge, these are the most comprehensive longitudinal data of their kind and indicate that most of these recycled water and surface water sources (particularly during the summer months) may require additional treatments to diminish antibiotic resistance dissemination, ensure the safety of irrigated food crops, and protect public health. However, it is important to note that future work is still needed to better understand the utility of using metagenomic data versus culture-based data in assessing and preventing microbial and AMR risks associated with irrigation water from recycled and surface water sources. Finally, as noted above, the extensive data set generated in this study can be used to help inform the development of a new water sampling arm of the U.S. NARMS program which will ultimately improve the assessment and prevention of risks associated with the presence of antibiotic-resistant enteric pathogens in multiple water types, including recycled and surface waters used for agricultural irrigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.est.2c02281.

Table 1: Description of Mid-Atlantic and Southwest sampling sites and water types included in this study (XLSX)

Supporting figures (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Amy R. Sapkota − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States;* orcid.org/0000-0001-8666-6978; Phone: 301-405-1772; Email: ars@umd.edu; Fax: 301-314-1012

Authors

Leena Malayil − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States*

Padmini Ramachandran − *Office of Regulatory Science, Division of Microbiology, United States Food and Drug Administration, College Park, Maryland 20740, United States*

Suhana Chattopadhyay − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States*

Sarah M. Allard − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States;* Present Address: Sarah M. Allard: Department of Pediatrics, University of California San Diego, San Diego, California 92161, United States

Anthony Bui − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States*

Jicell Butron − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States*

Mary Theresa Callahan − *Department of Plant Science and Landscape Agriculture, University of Maryland, College Park, Maryland 20740, United States*

Hillary A. Craddock − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States;* Present Address: Hillary A. Craddock: MAGICAL Group, Department of Health Systems Management, School of Public Health, Faculty of Health Sciences, Ben-Gurion University of the Negev, Beer-Sheva 8410501, Israel.

Rianna Murray − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States*

Cheryl East − *Northeast Area, Beltsville Agriculture Research Center, Environmental Microbiology and Food Safety Laboratory, Agriculture Research Service, United States*

Department of Agriculture, Beltsville, Maryland 20705, United States

Manan Sharma − *Northeast Area, Beltsville Agriculture Research Center, Environmental Microbiology and Food Safety Laboratory, Agriculture Research Service, United States Department of Agriculture, Beltsville, Maryland 20705, United States*

Kalmia Kniel − *Department of Animal and Food Sciences, University of Delaware, Newark, Delaware 19716, United States*

Shirley Micallef − *Department of Plant Science and Landscape Agriculture, University of Maryland, College Park, Maryland 20740, United States;* Present Address: Shirley Micallef: Centre for Food Safety and Security Systems, University of Maryland, College Park, Maryland 20742, United States.

Fawzy Hashem − *Department of Agriculture and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, Maryland 21853, United States*

Charles P. Gerba − *Department of Environmental Science, University of Arizona, Tucson, Arizona 85719, United States*

Sadhana Ravishankar − *School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, Arizona 85721, United States*

Salina Parveen − *Department of Agriculture and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, Maryland 21853, United States*

Eric May − *Department of Agriculture and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, Maryland 21853, United States*

Eric Handy − *Northeast Area, Beltsville Agriculture Research Center, Environmental Microbiology and Food Safety Laboratory, Agriculture Research Service, United States Department of Agriculture, Beltsville, Maryland 20705, United States*

Prachi Kulkarni − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States*

Brienna Anderson-Coughlin − *Department of Animal and Food Sciences, University of Delaware, Newark, Delaware 19716, United States*

Shani Craighead − *Department of Animal and Food Sciences, University of Delaware, Newark, Delaware 19716, United States*

Samantha Gartley − *Department of Animal and Food Sciences, University of Delaware, Newark, Delaware 19716, United States*

Adam Vanore − *Department of Animal and Food Sciences, University of Delaware, Newark, Delaware 19716, United States*

Rico Duncan − *Department of Agriculture and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, Maryland 21853, United States*

Derek Foust − *Department of Agriculture and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, Maryland 21853, United States*

Joseph Haymaker − *Department of Agriculture and Resource Sciences, University of Maryland Eastern Shore, Princess Anne, Maryland 21853, United States*

Walter Betancourt − *Department of Environmental Science, University of Arizona, Tucson, Arizona 85719, United States*

Libin Zhu − *School of Animal and Comparative Biomedical Sciences, University of Arizona, Tucson, Arizona 85721, United States*

Emmanuel F. Mongodin − *Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, Maryland 21201, United States;* Present Address: Emmanuel F. Mongodin: National Heart, Lung and Blood Institute (NHLBI), Division of Lung Diseases, National Institutes of Health (NIH), Bethesda, Maryland 20824, United States.

Amir Sapkota − *Maryland Institute for Applied Environmental Health, University of Maryland School of Public Health, College Park, Maryland 20740, United States*

Mihai Pop − *Department of Computer Science and Center for Bioinformatics and Computational Biology, University of Maryland, College Park, Maryland 20742, United States*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.est.2c02281

Author Contributions

L.M. performed the lab experiments and bioinformatic analyses and wrote and edited the manuscript. P.R. performed the bioinformatic analyses and contributed to data interpretation. S.C., S.M.A., A.B., and H.A.C. performed the laboratory experiments. J.B. performed data cleaning and bioinformatic analyses. M.T.C., R.M., C.E., E.M., E.H., P.K., B.A.-C., S.C., S.G., A.V., R.D., D.F., and J.H. collected water samples and contributed to laboratory experiments. M.S., K.K., S.M., F.H., S.P., and M.P. contributed to study design and site selection, supervised laboratory experiments, and contributed to data interpretation. L.Z. and W.B. collected and processed water samples from the southwestern sites. L.Z. shipped the samples to MD for further analyses. C.P.G. and S.R. coordinated the sample collection and shipping. L.Z., W.B., C.P.G., and S.R. contributed toward data interpretation. E.F.M. contributed to the data analysis and interpretation, reviewed and edited the manuscript, and approved the final version. A.R.S. acquired funding, contributed to the lab experiments and data interpretation, and wrote portions of the manuscript. All authors reviewed and edited the manuscript and approved the final version.

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Notes

Dr. Mongodin contributed to this article as an employee of the University of Maryland School of Medicine. The views expressed are his own and do not necessarily represent the views of the National Institutes of Health or the United States Government.

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