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Spatiotemporal trends in particle-associated microbial communities in a chlorinated drinking water distribution system

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Abstract

Various spatiotemporal, hydraulic, and water quality parameters can affect the microbial community composition of water within drinking water distribution systems (DWDSs). Although some relationships between various paravmeters and microbial growth are known, the effects of spatial and temporal trends on particle-associated microbial communities in chlorinated DWDSs remain poorly understood. The objectives of this study were to characterize the microbial community composition of both particle-associated bacteria (PAB) and total bacteria (TB) within a full-scale chlorinated DWDS, and assess relationships between microbiavvl community and various spatiotemporal, hydraulic, and water quality parameters. Bulk water samples were collected from the treatment plant, a storage tank, and 12 other sites in a rural chlorinated DWDS at varying distances from the treatment plant on four sampling dates spanning six months. Amplicon sequencing targeting the 16S rRNA gene was performed to characterize the microbial community. Gammaproteobacteria dominated the DWDS, and hydraulic parameters were well-correlated with differences in microbial communities between sites. Results indicate that hydraulic changes may have led to the detachment of biofilms and loose deposits, subsequently affecting the microbial community composition at each site. Spatial variations in microbial community were stronger than temporal variations, differing from similar studies and indicating that the highly varied hydraulic conditions within this system may intensify spatial variations. Genera containing pathogenic species were detected, with *Legionella* and *Pseudomonas* detected at every site at least once and *Mycobacterium* detected at most sites. However, only one sample had quantifiable *Pseudomonas aeruginosa* through quantitative polymerase chain reaction (qPCR), and no samples had quantifiable *Legionella pneumophila* or *Mycobacterium avium*, indicating a low human health risk. This study establishes spatial variations in PAB associated with varied hydraulic conditions as an important factor driving microbial community within a chlorinated DWDS.

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1. Introduction

In the United States, the quality of drinking water is regulated by the Safe Drinking Water Act, along with state and local guidelines. The majority of regulations focus on drinking water leaving the treatment plant; however, the quality of drinking water may change as it travels from the treatment plant through the drinking water distribution system (DWDS) to consumers' taps. Many factors can influence water quality throughout DWDSs, including the presence of disinfectant residual [1–4], microbial growth and biofilm formation [5–7], the accumulation and transport of sediments and loose deposits [8–10], hydraulics and stagnation [10–12], pipe size and material [13, 14], and various spatial and temporal influences [8, 10, 15–18].

Bacteria can survive treatment and enter DWDSs or enter through intrusion or maintenance work on the system. To prevent the presence of bacteria, treatment facilities often maintain a disinfectant residual, such as chlorine or chloramine, within DWDSs. In the United States, a disinfectant residual is required [19]. However, biofilms that grow on pipe walls and sediments can create habitats for bacteria to grow, protected from disinfectants. Bacteria and biofilms exert a demand on disinfectant residual, further creating an environment suitable for bacterial growth. Other compounds present in DWDSs, such as corrosion products, can also react with disinfectants and decrease disinfectant residual [19], allowing for more bacterial growth. Just as the absence of disinfectant residual can create health concerns, so can its presence. Disinfectants can react with organic materials within DWDSs to create disinfection byproducts, such as trihalomethanes and haloacetic acids [20], some of which have been linked to cancer and reproductive effects [21].

Previous studies have attempted to draw relationships between various parameters and microbial community composition within DWDSs. Potgieter et al. [18] found that spatial dynamics were stronger than temporal when considering a DWDS using multiple disinfection strategies throughout the system, but temporal dynamics were stronger within each disinfection section. Other studies have also found strong temporal variations in microbial community throughout DWDSs [16, 17]. Sekar et al. [22] found that cells attach to DWDS pipes during low flow periods and mobilize during active flow periods, influencing daily and weekly patterns of bacterial abundance based on periods of high and low demand. Douterelo et al. [11] found that hydraulic conditions can cause changes to the composition and structure of microbial communities within biofilms and bulk water. Relationships between microbial community and various water quality parameters, such as chlorine residual, temperature, pH, and metal concentrations [16, 18, 23], have been studied in the past, but considerable variation between systems has been documented. These variations between systems indicate that the factors affecting microbial community in DWDSs are complex and vary based on multiple system-specific characteristics.

While the effect of biofilms growing on pipe walls on microbial community has been extensively studied, the effect of biofilms growing on sediments and other suspended particles is largely unknown [24, 25]. Liu et al. [6] found that over 98% of total bacteria within an unchlorinated DWDS were found within the pipe wall biofilm and loose deposits, with loose deposits contributing more biomass to the system than biofilm. Van der Wielen and Lut [26] found that sediment-associated biofilms in DWDSs are an important contributor to microbial community. To prevent the buildup of sediments within DWDSs, some utilities apply a flushing routine, where high-flow velocities are applied throughout the DWDS to disturb, resuspend, and remove particles from the system [27]. However, these practices have been found to sometimes increase the quantity of bacteria within the system. Osborne et al. [10] found that after flushing, the concentration of total suspended solids (TSS) decreased in a storage tank but the concentration of particle-associated bacteria (PAB) increased throughout the DWDS,

suggesting that bacteria associated with sediments from the tank may have been resuspended and redistributed as a result of flushing. El-Chakhtoura et al. [9] also found that flushing increases biomass within DWDSs due to the resuspension of pipe biofilm and loose deposits.

Chen et al. [8] recently published a paper in which differences between planktonic bacteria and PAB were studied in an unchlorinated DWDS. The study found planktonic bacteria and PAB from produced water were the main contributors to bacteria within the DWDS, and that the influence of biofilm and loose deposits to the planktonic bacteria and PAB was the highest during daily demand peaks. However, this study did not capture variations over multiple sampling events and included limited sampling points to capture spatial variations. Additionally, the study was on an unchlorinated system and the authors expressed the need for future study of chlorinated systems to better understand particle-associated microbiology within DWDSs. Osborne et al. [10] demonstrated that biofilms formed on particles represented a substantial portion of overall microbial loading within a DWDS. The current study expands upon this research to establish which bacteria are present and associated with particles, and to determine any potential human health risk.

Although many bacteria that grow within DWDSs are not harmful to human health and may actually help select against pathogens [28], opportunistic pathogens (OPs), such as *Legionella pneumophila*, *Pseudomonas aeruginosa*, and *Mycobacterium avium*, can inhabit DWDSs and premise plumbing, causing health effects such as Legionnaires' disease, pneumonia, and nontuberculous Mycobacteria infections [29]. These OPs can thrive even in the presence of disinfectant residual [30, 31] and are the leading cause of waterborne disease outbreaks in developed countries [32]. An estimated 7.15 million waterborne illnesses occur annually in the United States, causing 118,000 hospitalizations, 6,630 deaths, and approximately $3.33 billion in healthcare costs [33]. The majority of hospitalizations and deaths are caused by biofilm-associated OPs (*L. pneumophila*, *P. aeruginosa*, nontuberculous mycobacteria) [33]. It is therefore important to understand relationships between microbial community and various spatiotemporal, hydraulic, and water quality factors to prevent the growth of harmful organisms and ensure the safety of consumers.

While numerous studies have attempted to establish relationships between various parameters and microbial growth, as summarized above, the effects of spatial and temporal trends on particle-associated microbial communities in chlorinated DWDSs remain poorly understood. The objectives of this study were to 1) examine the microbial community composition within a full-scale chlorinated DWDS, 2) explore relationships between microbial community and various spatiotemporal, hydraulic, and water quality parameters, 3) investigate how sediment may be a driver of these relationships through examining both particle-associated bacteria (PAB) and total bacteria (TB), and 4) establish potential human health implications of bacteria within the DWDS. Amplicon sequencing targeting the 16S rRNA gene was performed to characterize the microbial community of bulk water from the treatment plant, a storage tank, and 12 other sites throughout a chlorinated drinking water distribution system. Analyses were separated into both PAB and TB for each sample, and results were compared to various physicochemical and hydraulic parameters. The results from this study will inform treatment plant operators on the effects particles can have on microbial community, helping shape treatment and management techniques to improve the biological water quality of drinking water throughout DWDSs.

2. Materials and methods

2.1 Study site and sample collection

The study site chosen for this project was a small DWDS in West Virginia, which serves 1,443 residential and 126 commercial/industrial/public customers (total population served of ~3,750

people). The study site is located in the U.S. Climate Region of Ohio Valley, and the CONUS Climate Division of North Central West Virginia, with an average annual temperature of approximately 54˚F (12.2˚C) and an average annual rainfall of approximately 50 inches (127 cm) [34]. The DWDS includes 65.2 miles of distribution main piping, primarily consisting of polyvinyl chloride (PVC) pipes ranging from 2 to 12 inches in diameter. The water treatment plant is supplied by surface water, which is treated by chemical coagulation, flocculation, sedimentation, filtration, and disinfection with free chlorine.

The chosen water treatment plant performs biannual maintenance flushing of the DWDS to resuspend and remove sediment. Water chemistry as well as the contribution of PAB to overall microbial loading in this distribution system has been previously studied by Osborne et al. [10].

Drinking water was sampled at 14 different locations throughout the DWDS (Fig 1). Sample sites included the treatment plant (site 1), a storage tank (site 2), and 12 sites at varying locations throughout the DWDS (sites 3–14). All samples were collected with the approval of the drinking water utility with utility personnel present to facilitate site access. Sampling events were organized before and after a week-long flushing campaign that took place in March 2021 to investigate the effects of flushing on microbial community. The first sampling event was in March 2021, two weeks before the flushing campaign started. Then sampling occurred on three dates following the flushing campaign: April 2021 (1 week after the conclusion of the flushing campaign), June 2021 (9 weeks after), and August 2021 (16 weeks after). On each sampling date, bulk water samples were collected at each site from cold water taps within buildings, fire hydrants, pump stations, or outside spigots, depending on the site. Sampling ports were thoroughly flushed until stabilization of water temperature to ensure samples consisted of water from the distribution system and not building plumbing. Sampling occurred at approximately the same time of day on each sampling date.

Bulk drinking water samples were collected for molecular analysis (3 L autoclaved polypropylene bottle dosed with 48 mg/L sodium thiosulfate to quench chlorine), water chemistry analysis (1 L acid-washed bottle), and organic carbon analysis (250–500 mL amber glass bottle baked at 550˚C) on each sampling date. Grab samples were used for sample collection to align with the sampling procedures used for regulatory compliance sampling associated with the Revised Total Coliform Rule [35]. Samples were kept on ice during transport. Within 4 hours of sample collection, samples for molecular analysis from each site were filtered in parallel through both a 2 μm pore size polycarbonate membrane filter (Millipore-Sigma) to capture PAB and a 0.45 μm pore size mixed cellulose ester membrane filter (Fisher) to capture TB. Up to 1250 mL of water was filtered for each sample through each type of filter. Some exceptions were made for samples with a large amount of sediment, clogging the filter before the full volume could be filtered. Filters were stored at −20˚C until DNA extraction.

2.2 Physicochemical water quality

Physicochemical water quality parameters, including pH, temperature, conductivity, total chlorine, turbidity, TSS, total organic carbon (TOC), iron, magnesium, manganese, and heterotrophic plate count (HPC) were analyzed, as described in Osborne et al. [10].

2.3 DNA extraction

Filters were torn into approximately 1 cm^2 pieces using sterile forceps and transferred into DNA extraction lysing matrix tubes. DNA extraction was performed using the Fast DNA SPIN Kit (MP Biomedicals) according to manufacturer instructions. One DNA extraction was

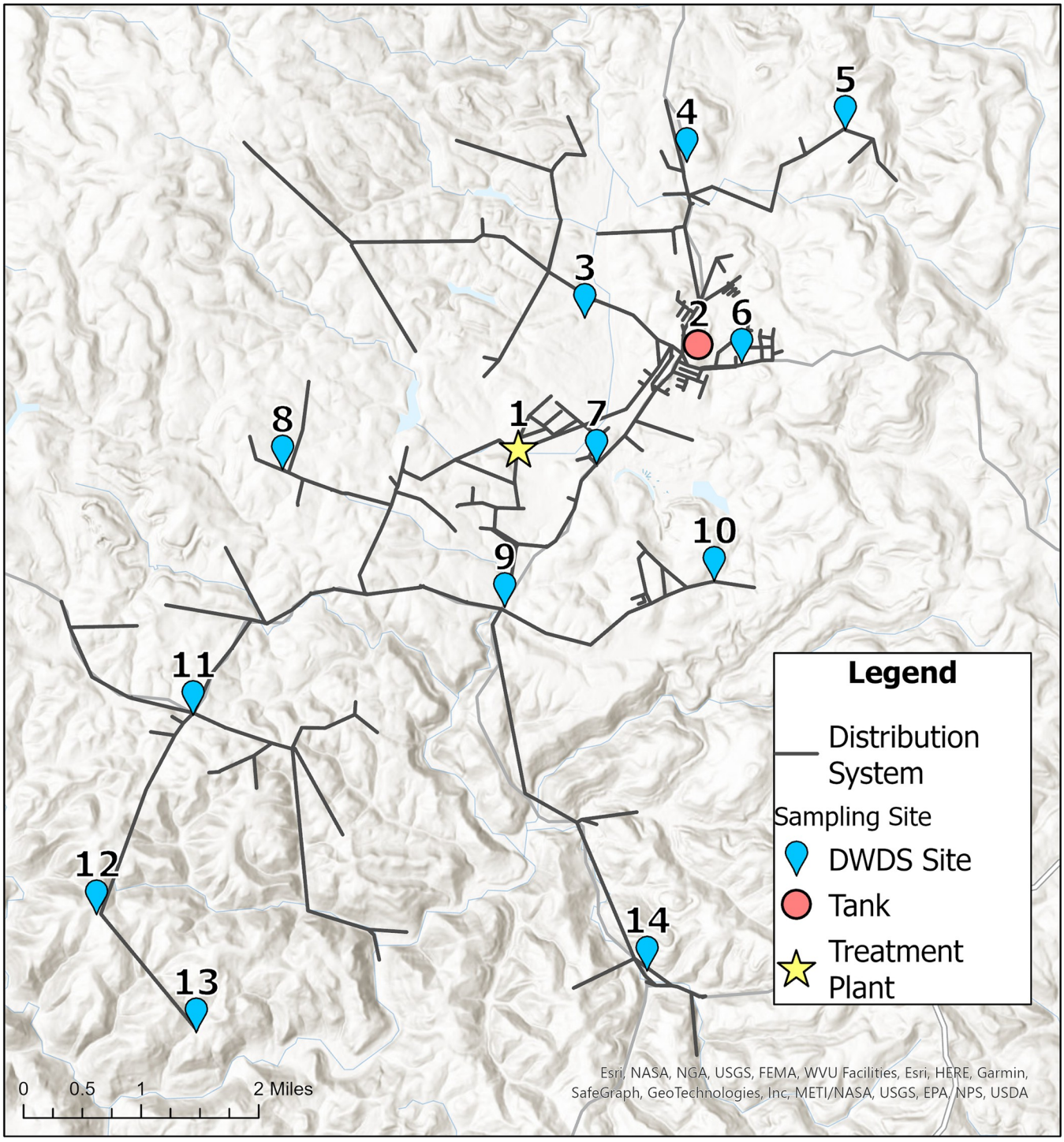

Fig 1. Sampling site locations. The basemap used in this figure was developed by Esri (https://www.arcgis.com/home/item.html?id=2ef1306b93c9459ca7c7b4f872c070b9#:~:text=This%20map%20features%20shaded%20relief,access%20token%20to%20access%20them).

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performed with no filter as a DNA extraction blank, as well as one blank filter of each pore size (2 μm and 0.45 μm) as filter blanks. DNA extraction product was stored at– 20˚C for use in molecular analyses.

2.4 Preparation of 16S rRNA gene amplicon libraries

Amplicon libraries were prepared using a two-step nested-polymerase chain reaction (PCR) method, adapted from Shaw et al. [36]. This approach was selected because adequate DNA mass could not be obtained through one round of amplification. The first round of PCR was performed using universal 16S rRNA primers 8F (`5′- AGAGTTTGATYMTGGCTCAG -3′`) [37] and 1492R (`5′- GGWTACCTTGTTACGACTT -3′`) [38]. Reactions consisted of a final concentration of 0.8X Platinum Hot Start PCR Master Mix, 200 nM of forward and reverse primers, 1 μl of template DNA extract, and water to a final volume of 25 μl. One negative control was included per batch of 24 samples processed, using molecular grade water in place of template DNA extract. PCR was performed in triplicate, and the thermocycler conditions included 3 minutes of initial denaturation at 94˚C, 25 cycles of denaturation (94˚C for 45 seconds), annealing (50˚C for 60 seconds), and extension (72˚C for 90 seconds), followed by final extension at 72˚C for 10 minutes and a hold at 4˚C after completion of the run. Triplicates were then combined to a total volume of 75 μl per sample and stored at -20˚C.

The second round of PCR was performed using barcoded 515F forward primers [39] and reverse primer 926R [40] targeting the V4 and V5 regions of the 16S rRNA gene. Reactions consisted of a final concentration of 1X Platinum Hot Start PCR Master Mix, 200 nM of forward and reverse primers, 1 μl of template DNA (i.e., PCR product from the first round of PCR), and water to a final volume of 25 μl. One negative control was prepared per barcoded primer, with 1 μl of molecular grade water in place of the template. PCR was performed in triplicate, and the thermocycler conditions included 3 minutes of initial denaturation at 94˚C, 35 cycles of denaturation (94˚C for 45 seconds), annealing (50˚C for 60 seconds), and extension (72˚C for 90 seconds), followed by final extension at 72˚C for 10 minutes and a hold at 4˚C after completion of the run. Triplicates were then combined to a total volume of 75 μl per sample and stored at -20˚C.

Gel electrophoresis was performed to confirm amplification of the target amplicon size. Gel made of 1% agarose was prepared with 10 μl of SYBR Safe Gel Stain (Life Technologies) per 100 mL. The gel was covered in 1X TAE buffer solution and wells were loaded with a mixture of 1 μl PCR product and 1 μl 6X DNA loading dye (Life Technologies). At least one low mass ladder was prepared per gel. The gel was run at a voltage of 200 V for 40 minutes, then the resulting gel was examined under UV light. The expected band size of ~450–500 base pairs was confirmed for each sample, and negatives were confirmed.

Amplicons were quantified using a Qubit Fluorometer 2.0 and a dsDNA HS assay kit (Life Technologies). An equal mass of amplicon from each sample (240 ng of DNA) were combined into a single, sterile tube. The amplicon pool was purified using an Invitrogen PureLink PCR Purification Kit.

2.5 Illumina sequencing and microbial community profiling

Sequencing was conducted by the Genomics Core Facility at West Virginia University (Morgantown, WV) on the prepared libraries using an Illumina MiSeq and a 250-cycle paired-end protocol. QIIME2 (v.2021.4) [41] software was accessed through the West Virginia University High Performance Computing facility for microbial community profiling. Only forward reads were used in the analysis due to the low quality of reverse reads (expected errors >2 for 98.6% of reads). First, quality filtering was performed on the forward reads using the DADA2 plugin to remove low quality sequences and chimeras. After quality filtering, 6,144 unique features were counted with lengths ranging from 248 to 251 base pairs. Samples contained between 8,512 to 72,345 reads, with an average of ~39,400 reads. Operational taxonomic units (OTUs) were clustered with >99% similarity and classified using a trained classifier and the Silva

rRNA database (v.132) [42]. Nontarget sequences, such as sequences related to mitochondria and chloroplasts, were removed. For alpha diversity, Shannon's diversity index was calculated using the *alpha* function, and for beta diversity, Bray-Curtis dissimilarity was calculated using the *core-metrics-phylogenetic* function with a sampling depth equal to the minimum number of reads (8,356) included from any individual sample. Reads have been deposited in the National Center for Biotechnology Information's Sequence Read Archive under BioProject PRJNA961195.

2.6 Quantification of pathogenic species

Quantitative polymerase chain reaction (qPCR) was performed for detection of DNA associated with three pathogenic species: *L. pneumophila*, *M. avium*, and *P. aeruginosa*, using a QuantStudio 3 real-time PCR system (Applied Biosystems, Thermo Fisher, USA). Samples positive for the genus level of each organism via Illumina sequencing were tested for the species (i.e., only samples positive for the genus *Legionella* via Illumina sequencing were tested for the species *L. pneumophila*). All samples were diluted 1:5 before qPCR, as determined by a trial with a subset of samples to minimize inhibition. All reactions were conducted in triplicate, and each 96-well plate included a triplicate negative control and a standard curve consisting of seven serially diluted triplicate target DNA standards. Primers and double-stranded DNA standards were purchased from Integrated DNA Technologies (IDT), Inc.

For detection of *L. pneumophila*, qPCR was conducted targeting the *L. pneumophila mip* gene with previously published primers [43]. Triplicate reactions were prepared to a total concentration of 1X TaqPath ProAmp Mastermix (Life Technologies), 250 nM of forward and reverse primers, 187.5 nM of probe, 1 μl of template, and water to a total volume of 10 μl. Thermocycler conditions were as follows: 95˚C for 2 minutes, followed by 40 cycles of 95˚C for 5 seconds and 65˚C for 10 seconds. The limit of quantification (LOQ) for *L. pneumophila* was determined to be 100 gene copies / ml sample.

For detection of *M. avium*, previously published primers were used [44]. Triplicate reactions were prepared to a total concentration of 1X PowerUp SYBR Green Mastermix (Life Technologies), 400 nM of forward and reverse primers, 1 μl of template, and water to a total volume of 10 μl. Thermocycler conditions were as follows: 94˚C for 1 minute, 30 cycles of 94˚C for 1 minute, 62˚C for 2 minutes, and 72˚C for 1 minute, followed by a melt curve of 95˚C for 15 seconds and 60˚C increasing at a rate of 0.15˚C/s up to 95˚C. The LOQ for *M. avium* was 10,000 gene copies / ml sample.

For detection of *P. aeruginosa*, a multiplex qPCR assay was utilized with the previously published *ecf*X and *gyr*B primers and probes [45]. Triplicate reactions were prepared to a total concentration of 1X TaqMan Mastermix, 250 nM of *ecf*X forward and reverse primers, 250 nM of *gyr*B forward and reverse primers, 187.5 nM of both *ecf*X and *gyr*B probes, 1 μl of template, and water to a total volume of 10 μl. Thermocycler conditions were as follows: 95˚C for 10 minutes followed by 50 cycles of 95˚C for 15 seconds and 60˚C for 1 minute. The LOQ was 1,000 gene copies / ml sample for *gyr*B and 100 gene copies / ml sample for *ecf*X.

Sequences of forward and reverse primers and probes used in this analysis are in S1 Table.

2.7 Distribution system modeling

Modeling of the DWDS was performed using KYPIPE Pipe2022 v.11.002. Data was compiled from the West Virginia Water Development Authority's Geographic Information System database and paper records provided by the utility. The final model was reviewed and confirmed by the utility staff. A typical diurnal profile for residential water demand was assumed [46] to model demand within KYPIPE via the automatic distribution demand function. Minimum,

maximum, and average values of pressure, velocity, and flow per site were determined through an extended period simulation conducted over 24 hours, and "Δ flow" for each site was calculated by subtracting the minimum flow from the maximum flow.

2.8 Statistical analyses

2.8.1 Analysis of similarities (ANOSIM). Analysis of similarities (ANOSIM) was performed to test if there was a statistical difference between the microbial communities of categorical groups of samples. The *anosim* function in the vegan package [47] and code adapted from Zorz [48] was used for this analysis in RStudio v.4.2.2.

2.8.2 Correlation tests. To assess Spearman's rank correlations between water quality and hydraulic parameters and alpha diversity, analysis was performed in RStudio with the *rcorr* function from the Hmisc package [49]. To find Spearman's rank correlations between most abundant taxa (OTUs collapsed at the species level) and various water quality and hydraulic parameters, analysis was performed in RStudio with the *taxa.env.correlation* function from the microbiomeSeq package [50, 51]. Mantel tests were conducted using the *mantel* function from the vegan package [47] in RStudio to determine correlations between water quality and hydraulic parameters and microbial community. Mantel tests were run with 9,999 permutations using Bray-Curtis dissimilarity on OTUs collapsed at the species level, and Euclidean dissimilarity on water quality and hydraulic parameters. To find the best subset of water quality and hydraulic parameters with maximum rank correlation with microbial community dissimilarities, the *bioenv* function from the vegan package [47] was used in RStudio using Bray-Curtis dissimilarity and Spearman's rank correlations.

2.8.3 Hypothesis tests. Hypothesis tests for differences in alpha diversity between groups of samples were conducted using the *kruskal.test* and *wilcox.test* functions from RStudio [52]. Post-hoc Wilcoxon tests were performed using the *pairwise.wilcox.test* function from RStudio [52].

2.8.4 Determination of biomarkers. Linear discriminant analysis effect size (LEfSe) was performed to determine biomarkers, (e.g., the bacteria most likely to explain differences between different categorical classes). This analysis was performed using the Huttenhower Galaxy Server LDA Effect Size tool [53] with an alpha of 0.05 for the factorial Kruskal-Wallis test among classes and a 2.5 threshold on the logarithmic linear discriminant analysis (LDA) score. The following classes were analyzed individually: sampling date, sampling site, and TB versus PAB.

2.8.5 Figures. Figures were created using the ggplot2 package [54] in RStudio. Maps were made using ArcGIS Pro v.3.0.3 and a base map developed by Esri [55].

3. Results

3.1 Microbial community composition

3.1.1 Abundant phyla and classes. A total of 40 phyla of bacteria were detected across all samples. The TB samples included 32 different phyla, with the most abundant being Proteobacteria (average relative abundance = 88.1%), Bacteroidetes (4.2%), and Firmicutes (3.8%). The Proteobacteria phylum was made of the following classes: Gammaproteobacteria (average relative abundance = 53.4%), Alphaproteobacteria (32.8%), and Deltaproteobacteria (1.9%). All the sites had either Gammaproteobacteria (sites 1, 2, 3, 5, 7, 8, 9, 10, 11, 13, 14) or Alphaproteobacteria (sites 4, 6, 12) as the most abundant phylum or class (Fig 2).

The PAB samples included 37 different phyla, with the most common phyla being Proteobacteria (average relative abundance = 80.4%), Firmicutes (10.0%), and Bacteroidetes (5.8%). The Proteobacteria phylum was made of mostly bacteria from the Gammaproteobacteria class (average relative abundance = 56.1%), along with some bacteria from the Alphaproteobacteria

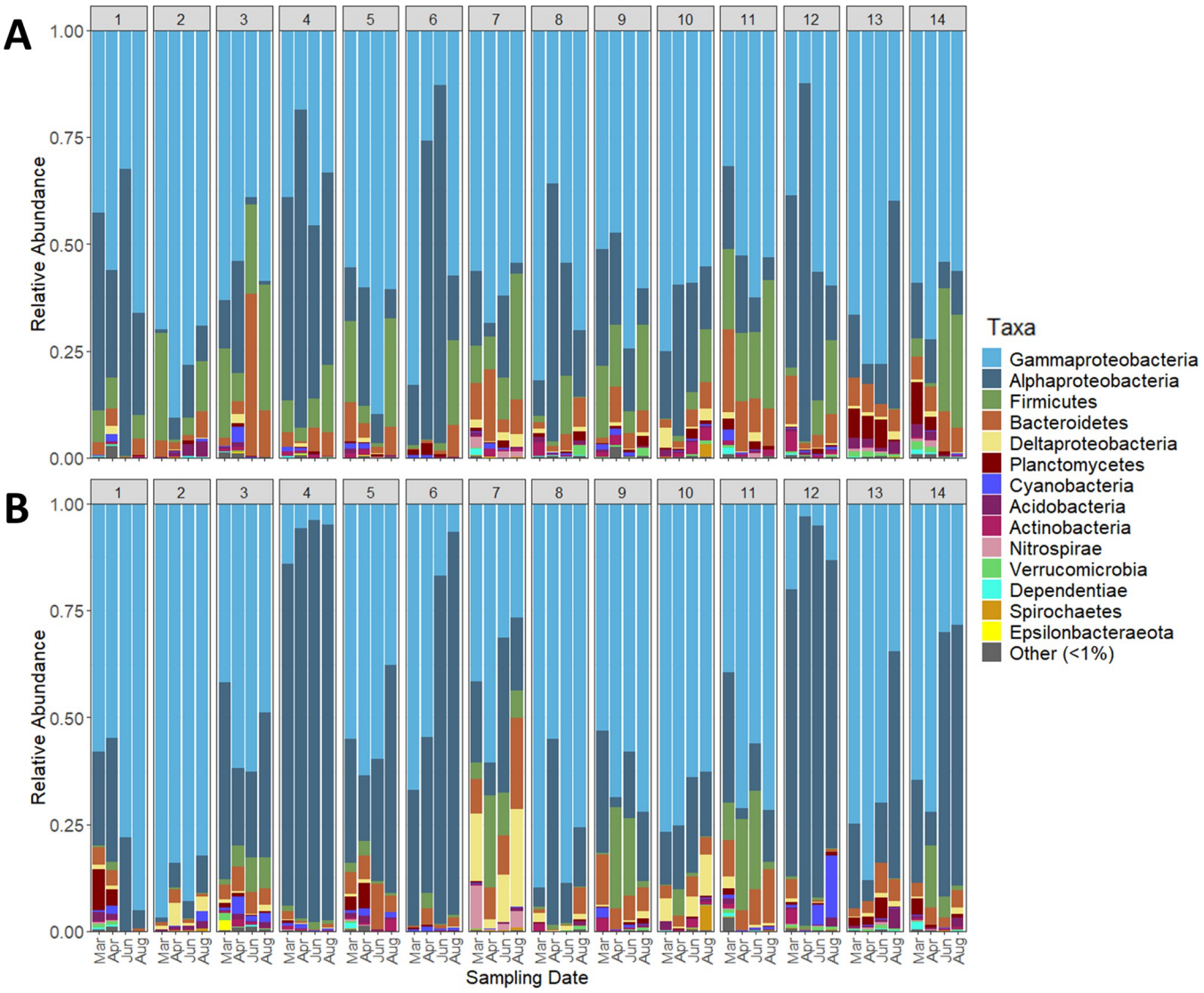

Fig 2. Relative abundance of taxa within (A) TB samples and (B) PAB samples. Any phylum or class with less than 1% relative abundance in every sample was classified as "other".

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(32.8%) and Deltaproteobacteria (1.9%) classes. Gammaproteobacteria was the most abundant phylum or class among PAB samples for every site except for sites 4 and 6, which were dominated by Alphaproteobacteria (Fig 2).

3.1.2 Alpha diversity. Alpha diversity (i.e., within-sample diversity) was calculated using the Shannon diversity index. The differences in alpha diversity between sampling dates (Kruskal-Wallis: $\chi^2 = 3.7107$, df = 3, p = 0.2944) and between TB versus PAB (Paired Wilcoxon: V = 1018, p = 0.0734) were not significant. However, the differences in alpha diversity between sampling sites were statistically significant (Kruskal-Wallis: $\chi^2 = 44.738$, df = 13, p = 2.314e-05). A post-hoc pairwise Wilcoxon test concluded that 8 pairs of sampling sites had significant differences ($p < 0.05$) in alpha diversity (sites 2 & 7, 2 & 10, 4 & 10, 6 & 7, 6 & 10, 6 & 11, 6 & 14, and 8 & 10). Spatiotemporal variation in alpha diversity throughout the system is presented in Fig 3.

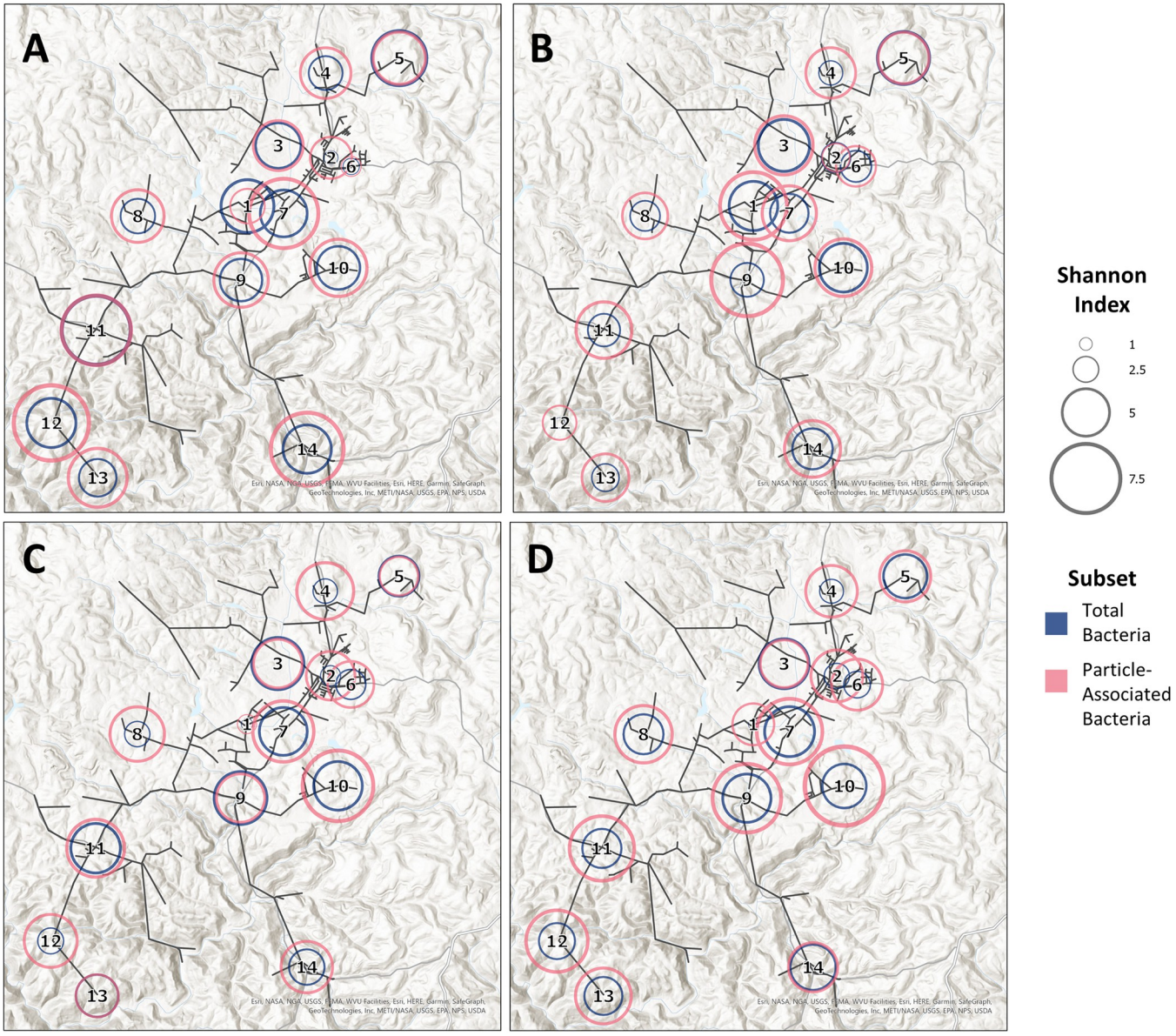

Fig 3. Alpha diversity of samples taken on four sampling dates: (A) March, (B) April, (C) June, (D) August. The size of the circles represents the Shannon index (larger circles represent a higher Shannon index and therefore higher alpha diversity). The darker, blue circles represent TB, and the lighter, pink circles represent PAB. The basemap used in this figure was developed by Esri (https://www.arcgis.com/home/item.html?id=2ef1306b93c9459ca7c7b4f872c070b9#:~:text=This%20map%20features%20shaded%20relief,access%20token%20to%20access%20them).

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Spearman's rank correlations were assessed between water quality and hydraulic parameters and alpha diversity. The following parameters were significantly ($p < 0.05$) correlated with alpha diversity: HPC ($\rho = -0.216$), minimum site velocity ($\rho = 0.265$), maximum site velocity ($\rho = 0.300$), maximum site flow ($\rho = 0.256$), and site Δ flow ($\rho = 0.256$).

3.1.3 Beta diversity. Beta diversity (i.e., between-sample diversity) was calculated using a Bray-Curtis dissimilarity matrix. There was generally lower Bray-Curtis dissimilarity (i.e., more similar microbial communities) between sites within the PAB samples (average dissimilarity = 0.75 ± 0.17) (Fig 4) than within the TB samples (average dissimilarity = 0.86 ± 0.17) (Fig 5).

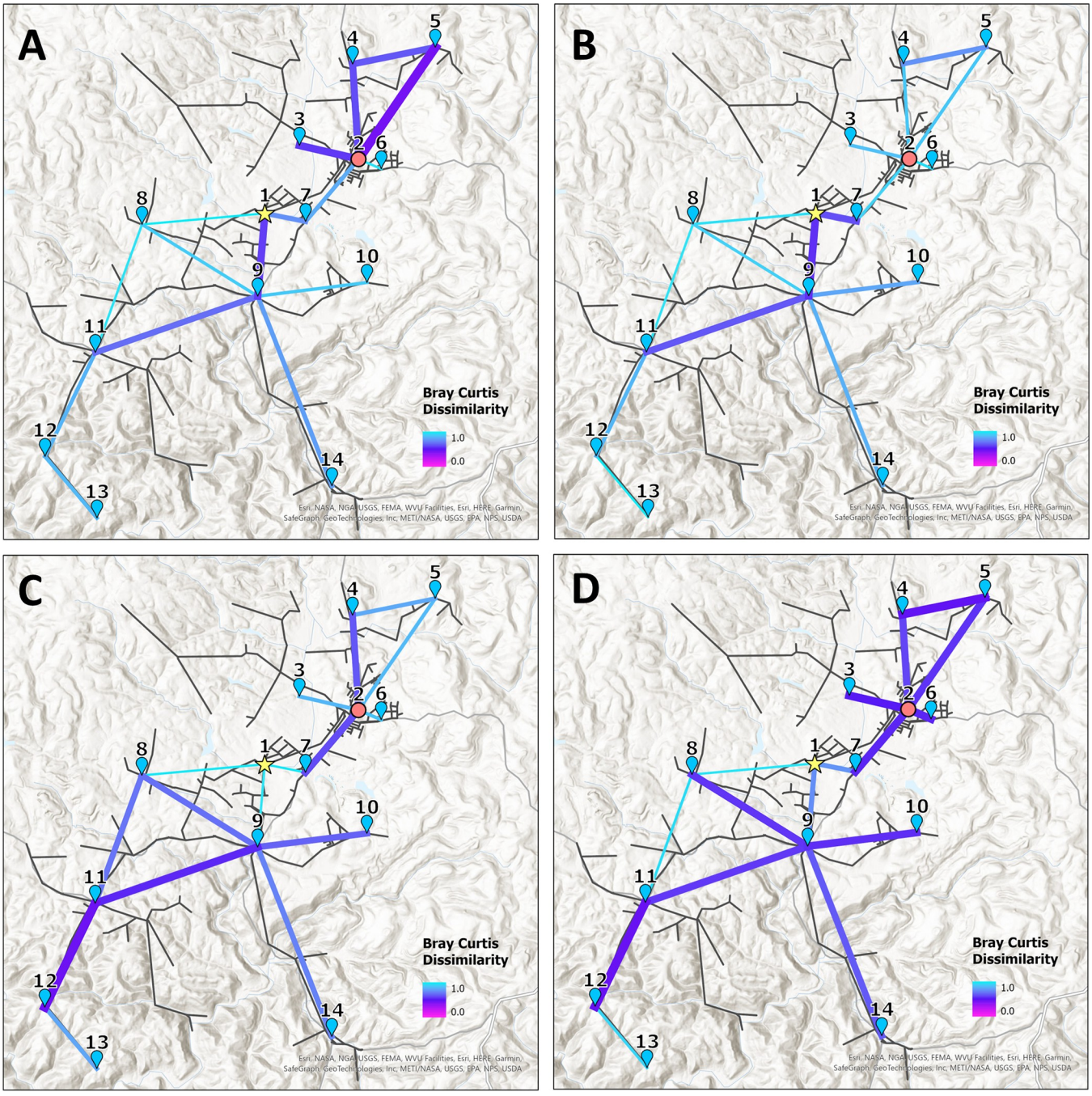

Fig 4. Bray-Curtis dissimilarity between nearby sites for PAB samples on four sampling dates: (A) March, (B) April, (C) June, (D) August. The color and thickness of the lines represent the Bray-Curtis dissimilarity index between the connected sites, with thicker lines representing a lower dissimilarity (i.e., more similar microbial communities between the sites). The basemap used in this figure was developed by Esri (https://www.arcgis.com/home/item.html?id=2ef1306b93c9459ca7c7b4f872c070b9#:~:text=This%20map%20features%20shaded%20relief,access%20token%20to%20access%20them).

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3.2 Differences in microbial communities between groups

There was a significant, but weak difference between the microbial communities of the samples when grouped by sampling date (ANOSIM; R = 0.042, p = 0.006) and when grouped by TB versus PAB (ANOSIM; R = 0.088, p = 0.0001). However, the strongest significant

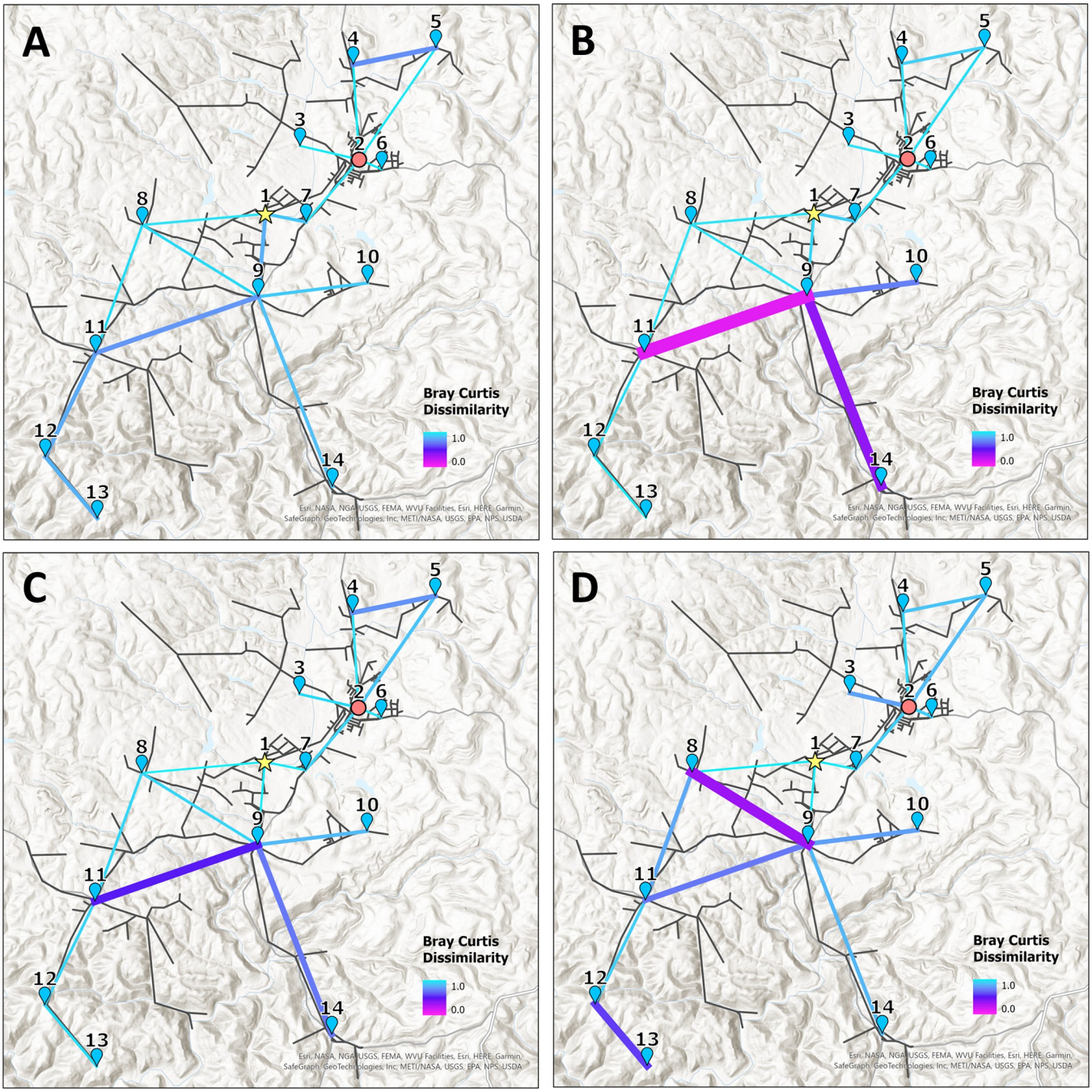

Fig 5. Bray-Curtis dissimilarity between nearby sites for TB samples on four sampling dates: (A) March, (B) April, (C) June, (D) August. The color and thickness of the lines represent the Bray-Curtis dissimilarity index between the connected sites, with thicker lines representing a lower dissimilarity (i.e., more similar microbial communities between the sites). The basemap used in this figure was developed by Esri (https://www.arcgis.com/home/item.html?id=2ef1306b93c9459ca7c7b4f872c070b9#:~:text=This%20map%20features%20shaded%20relief,access%20token%20to%20access%20them).

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difference between groups was observed when the samples were grouped by site (ANOSIM; $R = 0.581$, $p = 0.0001$).

Samples were divided into TB and PAB subsets and the ANOSIM analyses were repeated. The differences between bacterial communities when grouped by sampling date were stronger

and more significant when only considering the TB subset (ANOSIM; R = 0.130, p = 0.0004) compared to the PAB subset (ANOSIM; R = 0.051, p = 0.044). PAB samples had stronger differences between site (ANOSIM; R = 0.662, p = 0.0001) than TB samples (ANOSIM; R = 0.456, p = 0.0001).

3.3 Drivers of microbial community composition

3.3.1 Biomarkers. Linear discriminant analysis effect size (LEfSe) was used to determine the biomarkers, or taxa that are most likely to explain differences, between groups of samples. Biomarkers classified for each site are shown in Fig 6. Some genera that contain pathogenic species were identified as biomarkers: *Stenotrophomonas* (site 5), *Pseudomonas* (site 5), *Acinetobacter* (site 5), *Sphingomonas* (site 12), and *Legionella* (site 14). Biomarkers that contain bacteria involved in corrosion (e.g., sulfate-reducing bacteria (SRB), sulfur-oxidizing bacteria (SOB), iron-reducing bacteria (IRB), and iron-oxidizing bacteria (IOB)) were also biomarkers: *Sulfuricella* (SOB–site 2), *Gallionella* (IOB–site 10), *Geothrix* (IRB–site 2), *Pseudomonas* (IRB–site 5), *Geobacter* (IRB–site 7), *Desulfosporosinus* (IRB–site 7), and *Bacillus* (IRB–site 11). Some genera that contain pathogenic species were identified as biomarkers when the data were classified as TB versus PAB (Fig 7): *Stenotrophomonas* and *Pseudomonas* for PAB and *Legionella* for TB. Biomarkers related to corrosion were also found: *Desulfovibrio* (SRB) for TB, and *Pseudomonas* (IRB) and *Bacillus* (IRB) for PAB. When classified by sampling date, only April had biomarkers with LDA $>$ 2.5 and $p < 0.05$, which all were a part of the Sericytochromatia class of the Cyanobacteria phylum.

3.3.2 Correlations of water quality and hydraulic parameters with microbial community. Physicochemical water quality parameters are discussed in detail in Osborne et al. 2022 [10]. In short, the average temperature of the water samples was 16.73±5.22˚C, the average total chlorine was 1.34±0.44 mg/L, the average pH was 7.55±0.26, the average conductivity was 136.84 ±17.93 μS/cm, the average turbidity was 1.97±4.95 NTU, the average TSS was 0.94±1.04 mg/L, and the average TOC was 8.36±4.21 mg/L across all samples on all sampling dates. The effects of various water quality and hydraulic parameters on microbial community were analyzed through the *mantel* function from the vegan package in R. The Euclidean dissimilarity matrices of water quality parameters (total chlorine, turbidity, HPC, iron concentration), geospatial parameters (pipe miles from treatment plant and elevation), and hydraulic parameters (pipe diameter, minimum site pressure, average site pressure, maximum site pressure) were associated with significant differences in Bray-Curtis dissimilarity between samples (Table 1). The *bioenv* function from the vegan package in RStudio was used to determine that total chlorine, turbidity, HPC, and iron concentration was the subset of water quality variables with the best correlation (r = 0.334) to microbial community data. Conductivity was excluded from this analysis due to missing data points. Repeating this analysis with only sites within the distribution system (sites 3–14) to include hydraulic parameters, the subset of water quality and hydraulic parameters with the best correlation (r = 0.465) to microbial community data was total chlorine, HPC, iron concentration, manganese concentration, pipe miles from treatment plant, and pipe diameter.

3.3.3 Correlations of water quality and hydraulic parameters with genera containing pathogenic species. The microbiomeSeq package in RStudio was used to find Spearman's rank correlations between microbial community and various water quality and hydraulic parameters. Correlations between relative abundance of genera containing pathogenic species (*Legionella*, *Pseudomonas*, and *Mycobacterium*) and water quality and hydraulic parameters were assessed. Significant correlations to several water quality and hydraulic parameters were found for *Pseudomonas* (Table 2), but no significant correlations were found for *Legionella* or *Mycobacterium*.

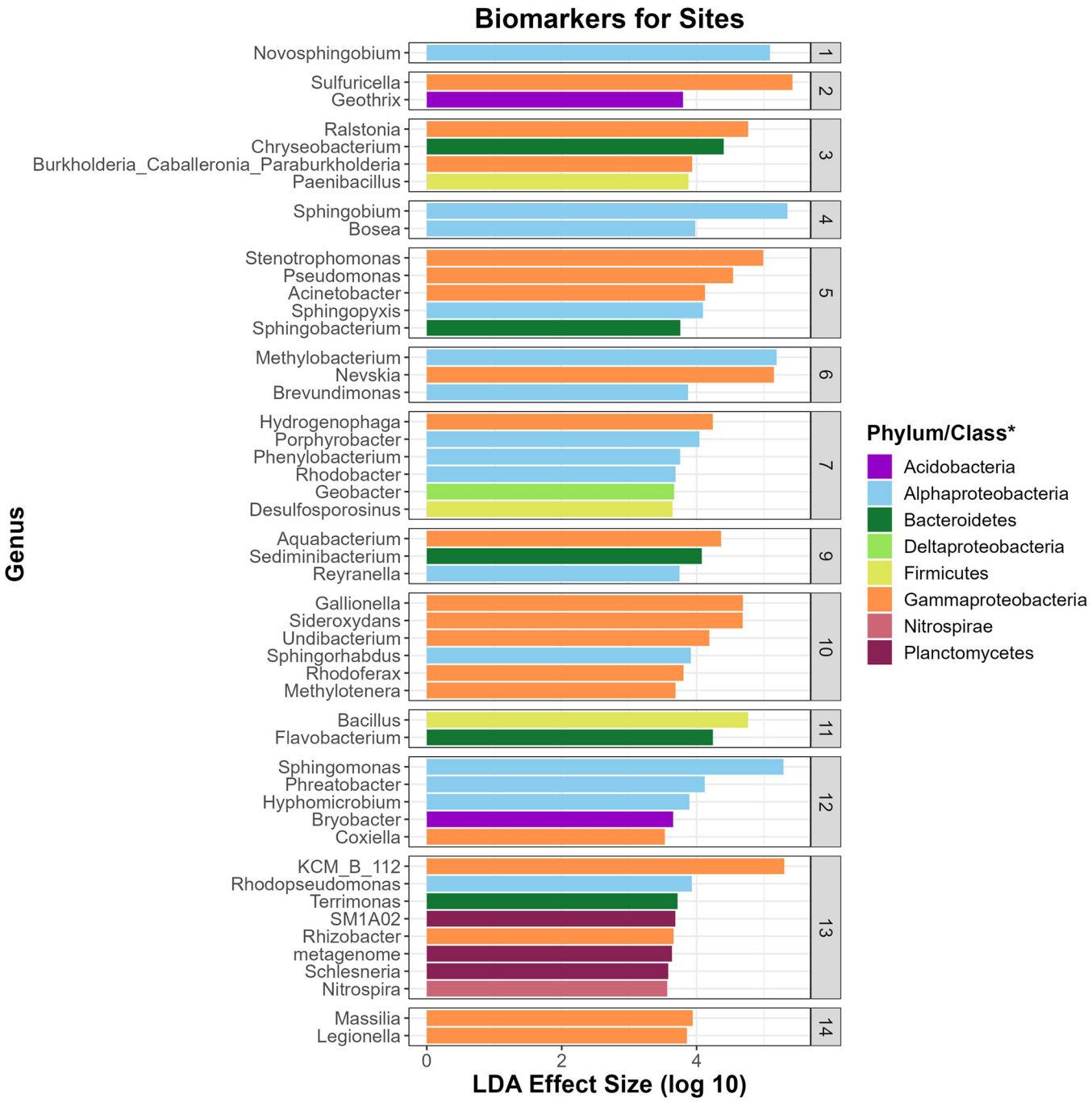

Fig 6. Linear discriminant (LDA) effect size for genera between sites. Results shown are LDA > 2.5 and $p < 0.05$. Biomarkers with unknown or uncultured genera were excluded from the figure. *Proteobacteria were split into class while all other taxa are at the phylum level.

https://doi.org/10.1371/journal.pwat.0000183.g006

3.4 Pathogenic taxa

3.4.1 Detection of genera containing pathogenic species. Using the sequencing results, the genera *Legionella* and *Pseudomonas* were detected at every site at least once, within both PAB and TB samples (Fig 8). *Mycobacterium* were detected at 12 of the 14 sites for PAB, and 8 of the 14 sites for TB.

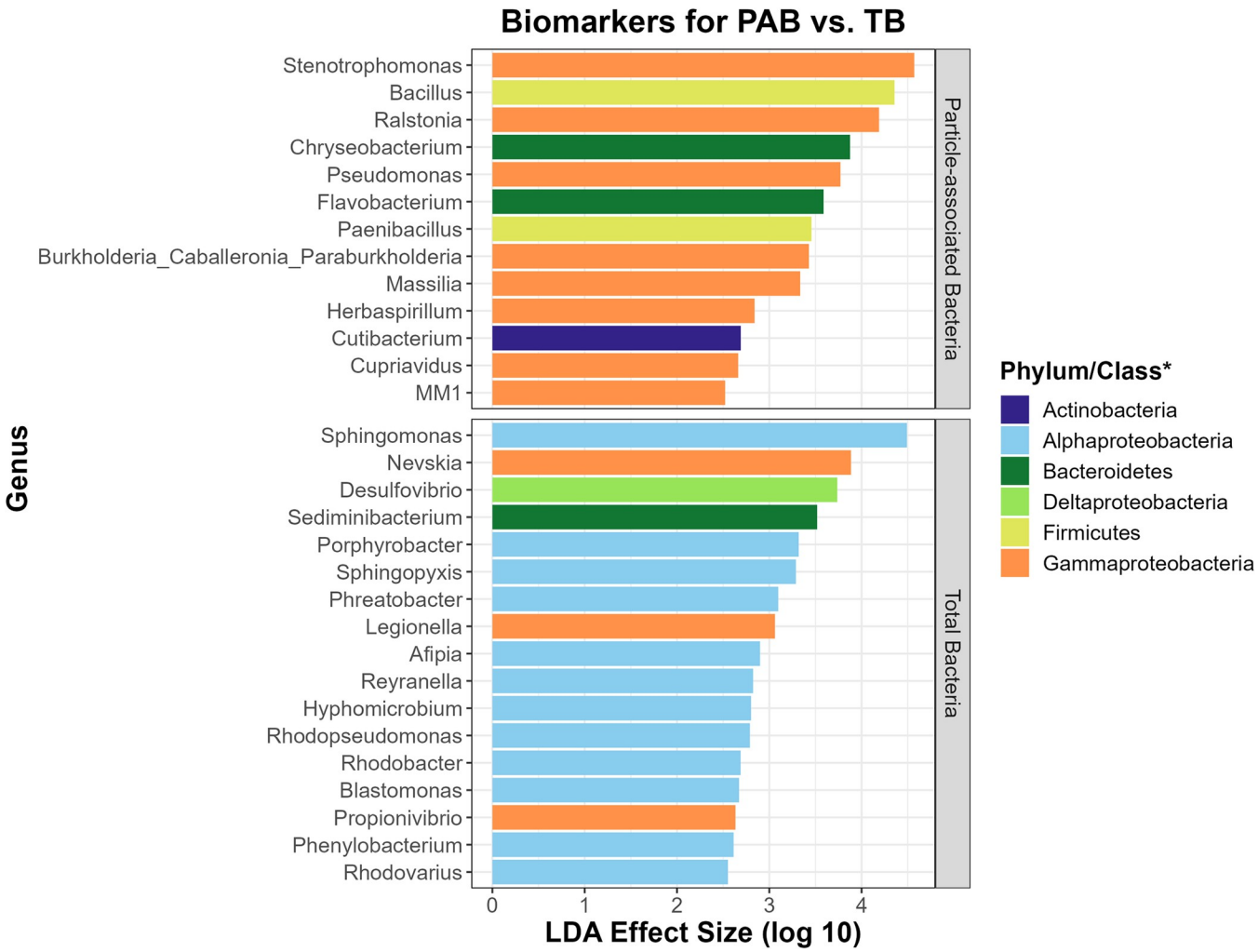

Fig 7. Linear discriminant (LDA) effect size for genera between TB and PAB. Results shown are LDA > 2.5 and $p < 0.05$. Biomarkers with unknown or uncultured genera were excluded from the figure. *Proteobacteria were split into class while all other taxa are at the phylum level.

https://doi.org/10.1371/journal.pwat.0000183.g007

3.4.2 Quantification of pathogenic species. No samples had quantifiable *L. pneumophila* or *M. avium* above the limit of quantification, detected via qPCR. Only one sample had quantifiable *P. aeruginosa*: the sample from site 10 collected in March (log gene copies / ml = 202.4).

4. Discussion

4.1 Gammaproteobacteria and Alphaproteobacteria dominated the DWDS

Similar to other studies looking at drinking water microbial composition in chlorinated systems [3, 4, 14], Proteobacteria was the most dominant phylum within both the TB and PAB samples. Previous studies show that the dominant classes of Proteobacteria within DWDSs vary between systems, with Alphaproteobacteria usually dominant in the bulk water of chlorinated systems [1, 3, 11, 16, 56, 57]. However, within this study, most sites were dominated by Gammaproteobacteria, with only 3 of 14 sites for TB and 2 of 14 sites for PAB being dominated by Alphaproteobacteria, on average across sampling dates. Gammaproteobacteria has

Table 1. Mantel test results of correlations between microbial community and water quality and hydraulic parameters.

	Parameter	Correlation coefficient, r	p-value	n
Water Quality Parameters	Total Chlorine	0.259	0.0001***	112
	Fe	0.236	0.0001***	112
	Turbidity	0.219	0.0001***	112
	HPC	0.182	0.0001***	112
	Temperature	0.050	0.0190*	112
	TSS	0.053	0.1023	112
	Mn	0.034	0.1930	112
	TOC	0.020	0.2788	112
	Conductivity	0.004	0.4539	108
	pH	-0.006	0.5387	112
	Mg	-0.009	0.5738	112
Geospatial Parameters	Pipe miles from treatment plant	0.177	0.0001***	112
	Elevation	0.134	0.0006***	112
Hydraulic Parameters	Pipe diameter	0.319	0.0001***	96
	Average site pressure	0.119	0.0049**	96
	Maximum site pressure	0.119	0.0059**	96
	Minimum site pressure	0.116	0.0062**	96
	Maximum site velocity	0.024	0.2900	96
	Average site flow	0.020	0.3083	96
	Maximum site flow	0.020	0.3112	96
	Site Δ flow	0.021	0.3127	96
	Minimum site flow	0.005	0.4348	96
	Average site velocity	-0.010	0.5707	96
	Minimum site velocity	-0.088	0.9695	96

Asterisks denote significance:

*** $p \leq 0.001$

** $p \leq 0.01$

* $p \leq 0.05$.

https://doi.org/10.1371/journal.pwat.0000183.t001

been abundant in biofilms and loose deposits within previous studies of DWDSs [6, 11, 58], suggesting the potential effects pipe- and particle-associated biofilms and loose deposits may have over bulk water on the studied DWDS. A study conducted by El-Chakhtoura et al. [9] also found DWDS samples dominated by Gammaproteobacteria, and justified the dominance by an increase in biomass within the systems, especially during flushing. Garner et al. [59] similarly found a DWDS dominated by Gammaproteobacteria. Most of the known fecal and opportunistic waterborne pathogens are within the Gammaproteobacteria class (e.g., *Escherichia coli*, *Klebsiella pneumoniae*, *L. pneumophila*, *P. aeruginosa*, *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, etc.) [11], indicating a potential human health impact from the abundance of this class. Differing from similar studies [3, 4, 14], Betaproteobacteria was not detected in this study. Other dominant phyla within the samples include Bacteroidetes and Firmicutes, which have also been reported in similar studies [3, 4, 14, 60–62]. It is important to recognize that previous studies have used differing primer sets to amplify the 16S rRNA gene, potentially causing differences in the quantification of taxa. For example, the 515F/806R primer set has been shown to overestimate Gammaproteobacteria in comparison to the primer set used in this study, 515F/926R [39].

Table 2. Water quality and hydraulic parameter Spearman's rank correlations with relative abundance of *Pseudomonas*.

Subset	Parameter	Correlation coefficient, ρ	p-value
Total Bacteria	Maximum site velocity	0.49	0.003**
	Turbidity	-0.47	0.004**
	Fe concentration	-0.48	0.005**
	Average site velocity	0.47	0.008**
	Maximum site flow	0.44	0.017*
	Site Δ flow	0.44	0.017*
	Minimum site velocity	0.41	0.037*
Particle-Associated Bacteria	Maximum site velocity	0.53	0.001***
	Maximum site flow	0.45	0.001***
	Average site velocity	0.45	0.011*
	Site Δ flow	0.45	0.012*

Asterisks denote significance:

*** $p \leq 0.001$

** $p \leq 0.01$

* $p \leq 0.05$. Only significant results are shown.

https://doi.org/10.1371/journal.pwat.0000183.t002

4.2 Spatial variations are stronger drivers of microbial community than temporal variations

4.2.1 Overall spatial and temporal variations. Differences in alpha diversity (i.e., within-sample diversity) were not statistically significant between sampling dates, but they were

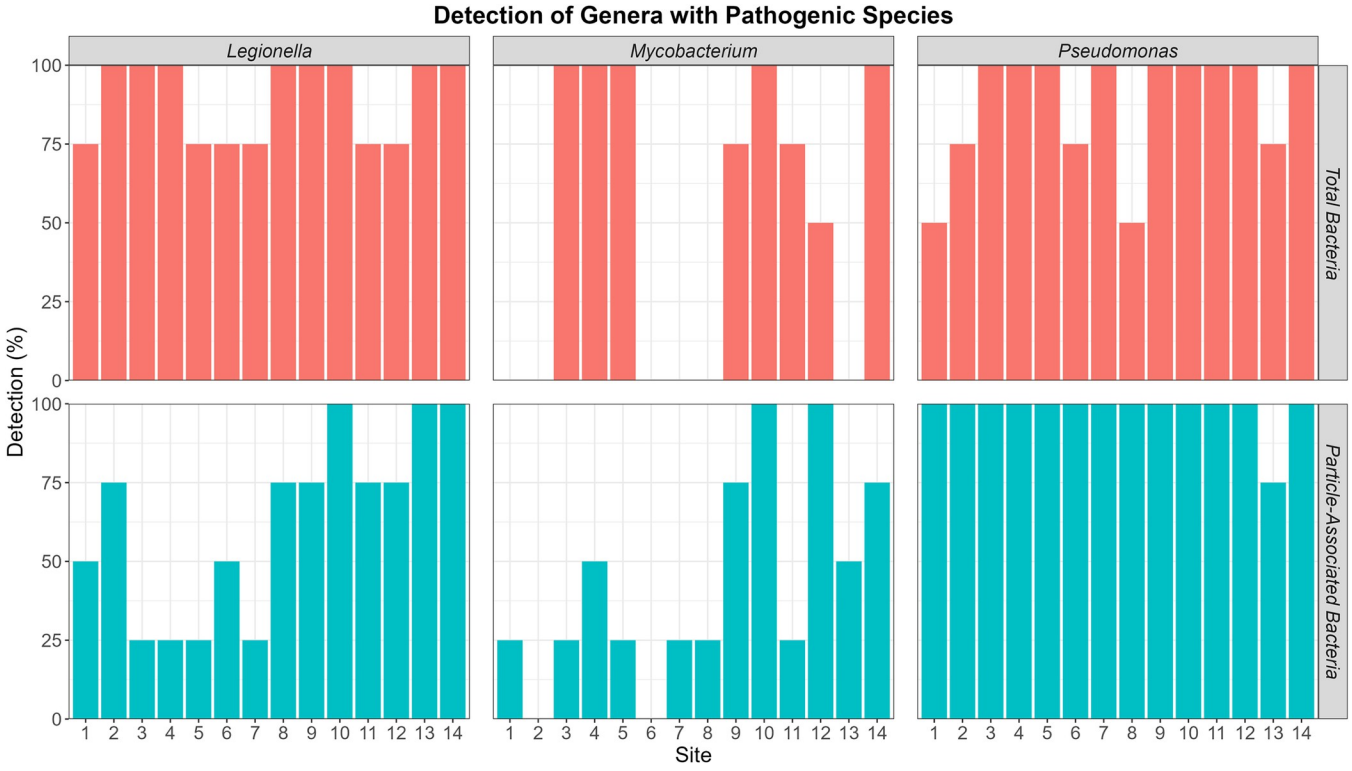

Fig 8. Detectvvvion of genera containing pathogenic species.

https://doi.org/10.1371/journal.pwat.0000183.g008

between sampling sites. Other studies have found the opposite, where temporal variability was more apparent for alpha diversity than spatial variability, with increases in alpha diversity observed either in the summer and autumn [16] or during the winter [18, 63]. Douterelo et al. [58] found that biofilms have more stable microbial communities over time than planktonic samples in chlorinated DWDSs, therefore the stability over time in this study may be an indication of the role of growth in biofilms seeding the bulk water rather than planktonic growth within the DWDS. It is also important to recognize the potential limitation of this study to see long-term temporal variations, given that sampling took place on only 4 sampling dates over a span of 6 months. When looking at beta diversity (i.e., between-sample diversity), the current study found statistically significant differences between microbial communities when grouped both spatially and temporally, although the relationship was much stronger among spatial differences (ANOSIM: $R = 0.581$, $p = 0.0001$) than temporal (ANOSIM: $R = 0.042$, $p = 0.006$). Conversely, Pinto et al. [17] found that temporal variations have more influence on beta diversity than spatial variations. The *bioenv* test determined that pipe miles from treatment plant was one of the variables with best correlation to microbial community data, further reinforcing the importance of spatial variations in this study. Discrepancies between similar studies demonstrate the complexity of DWDSs and suggest that the interaction of multiple intricate parameters are likely drivers of microbial community.

4.2.2 Temporal variations were stronger within TB than PAB. When dividing the samples into TB and PAB and repeating ANOSIM analyses, temporal variations were stronger within TB than PAB. Other studies have been conducted to compare short-term temporal variations in TB and PAB over 24-hour periods and have found increases in particle and microbe concentrations during water demand peaks, demonstrating the release of biofilm and loose deposits, or particles that settle and accumulate on the bottom of pipes, during periods of high water demand [8, 64]. However, this is the first study to look at temporal trends in the microbial community of TB versus PAB in a chlorinated DWDS over multiple sampling dates.

4.2.3 Spatial variations were stronger within PAB than TB. Spatial variations in the current study were stronger within PAB than TB. A study on an unchlorinated system by Chen et al. [8] found similar results, with highly similar microbial communities between locations among planktonic bacteria, but more disparate communities between locations among PAB. This finding is the opposite of what was examined in another unchlorinated system, where PAB had more similar microbial communities across sites than TB [65]. It is hypothesized, however, that PAB may have a larger function in bacterial community development in chlorinated systems than in unchlorinated systems, because of the ability for particles to protect bacteria from disinfection and cause the rapid decay of disinfectant residual [6]. This explains why the current study on a chlorinated system may have had different results.

4.3 Microbial community varies between PAB and TB

4.3.1 Alpha diversity. Alpha diversity was higher for PAB than for TB for 62.5% of the samples in the study, coinciding with results from other studies that show that alpha diversity is higher within biofilms than within bulk water samples [11, 66]. However, the differences in alpha diversity between TB and PAB were not statistically significant. When looking at beta diversity, the differences between TB and PAB were statistically significant, although weak (ANOSIM $R = 0.088$). Douterelo et al. [11] also found statistically significant differences in between bulk water and biofilm samples using beta diversity, although the relationship within that study was much stronger than the one found in this study and was conducted at the genus and class level instead of the species level.

4.3.2 Biomarkers. More biomarkers were found for comparisons of microbial community between sites and TB versus PAB than between sampling dates, again reinforcing the importance of spatial variations in this study. When grouped by site, most of the biomarkers (71.4%) were part of the Proteobacteria phylum, within the Gammaproteobacteria (38.8%), Alphaproteobacteria (30.6%), or Deltaproteobacteria (2.0%) classes. Gammaproteobacteria has been common in biofilms and loose deposits in previous studies [6, 11, 58], indicating the likely importance of biofilm-laden sediments in this study as an influence on microbial community differences between sites. When grouped by TB and PAB, most of the PAB biomarkers were part of the Gammaproteobacteria class (65.1%), while most of the TB biomarkers were part of the Alphaproteobacteria class (70.6%). This result further enforces that Gammaproteobacteria is associated with sediments and Alphaproteobacteria is associated with bulk water within this study.

4.4 Hydraulic parameters are stronger drivers of microbial community than water quality parameters

4.4.1 Hydraulic parameters. Spearman's correlations indicated that many hydraulic parameters, including minimum site velocity, maximum site velocity, maximum site flow, and site Δ flow, were all significantly positively correlated with alpha diversity. Douterelo et al. [11] previously found that alpha diversity was higher in biofilms formed under conditions of highly varied flow. Sloughing from biofilms formed under these conditions may explain why site Δ flow was correlated with alpha diversity within this study. Mantel's test revealed hydraulic parameters as significant influences on microbial community, with pipe diameter, minimum site pressure, average site pressure, and maximum site pressure all having positive correlations with beta diversity, although the relationships are weak. The strongest relationship was between pipe diameter and beta diversity ($r = 0.319$). The relationship between hydraulic parameters and alpha diversity may point to changes in pressure, velocity, or flow causing biofilms and loose deposits to detach from the pipe wall and contribute to an increased diversity within the system. The relevance of hydraulic parameters for impacting alpha diversity may also help explain the role of spatial variations as drivers of the overall microbial community because hydraulic factors vary widely between sites in the distribution system. The branched layout of the DWDS and low density of consumers may have a role in the importance of hydraulic parameters in this study compared to other studies that have grid layouts and a higher density of consumers. Additionally, elevation was found to be correlated with microbial community similar to site pressure parameters. In regions with substantial variation in elevation within the system, elevation may serve as a valuable proxy for site selection to assess hydraulic impacts on microbial community without prerequisite hydraulic modeling. More information is needed on how the elevation profile, layout, and consumer distribution of DWDSs impacts hydraulic variations and microbial communities, particularly in rural systems.

4.4.2 Water quality parameters. In this study, the only water quality parameter that had a significant Spearman's rank correlation with alpha diversity was HPC, which had a weak negative correlation ($\rho = -0.216$). Other studies have found significant negative correlations between chlorine residual and alpha diversity [18, 23], and positive correlations between water temperature and alpha diversity [16]. For beta diversity, Mantel's test revealed temperature, total chlorine, turbidity, HPC, and iron concentration as weakly positively correlated with beta diversity. Perrin et al. [16] found differing results that beta diversity (weighted UniFrac distances) in a chlorinated DWDS was most influenced by temperature, conductivity, and pH, and did not find significant impacts from residual chlorine concentration. Using the *bioenv* function from the vegan package in R, the subset of water quality parameters with the best

correlation to microbial community data was found to be total chlorine, turbidity, HPC, and iron concentration.

4.5 Several genera that could impact human health or infrastructure integrity were detected

4.5.1 Pathogenic biomarkers. Three sites (sites 5, 12, and 14) had biomarkers that were genera that contain pathogenic species of bacteria. These sites were 3 of the 5 furthest sites from the treatment plant (> 5 mi), with distances of 5.2 mi, 6.8 mi, and 7.6 mi, respectively, from the treatment plant. The other two sites (sites 10 and 13) from the farthest reaches of the distribution system did not include any potentially pathogenic biomarkers.

Site 5 had three genera that contain pathogenic species as biomarkers: *Stenotrophomonas*, *Pseudomonas*, and *Acinetobacter*. Other studies have detected opportunistic pathogens or their genetic signatures from these genera, including *Stenotrophomonas maltophilia* [59], *P. aeruginosa* [3, 59], and *Acinetobacter baumannii* [59] within drinking water, which can all cause various multi-drug resistant infections [67, 68]. Site 12 had *Sphingomonas* as a biomarker. *Sphingomonas* are chlorine resistant and play an important role in biofilm formation [69]. The genus contains some pathogenic species, including the opportunistic pathogen *Sphingomonas paucimobilis* which can cause bloodstream and peritoneal infections [70]. Dias et al. [3] also found indicator species within the *Sphingomonas* genus for a chlorinated distribution system. Site 14 had *Legionella* as a biomarker, which contains *L. pneumophila*, an opportunistic pathogen that causes Legionnaires' disease and is a common inhabitant of DWDSs [71]. *Legionella spp*. was previously noted as an indicator genera of a chlorinated systems studied by Ji et al. [14].

Two genera containing pathogenic species were biomarkers for PAB: *Stenotrophomonas* and *Pseudomonas*. The presence of *Stenotrophomonas* and *Pseudomonas* as biomarkers for both PAB and site 5 indicates that site 5 may be strongly influenced by particles, while the presence of *Legionella* as a biomarker of both TB and site 14 indicates that site 14 may be strongly influenced by bulk water.

4.5.2 Detection of pathogenic genera and species. *Pseudomonas* was the most prevalent OP among the three examined (*Pseudomonas*, *Mycobacterium*, and *Legionella*) in the samples, being detected in 98.2% of the PAB samples and 87.5% of the TB samples. The average relative abundance of *Pseudomonas* was also the highest among the examined OPs and had a higher average relative abundance in PAB samples (3.87%) than in TB samples (2.65%). *Mycobacterium* and *Legionella* both had higher average relative abundances in TB (0.21% and 0.57%, respectively) than PAB (0.13% and 0.34%, respectively).

The relative abundance of *Pseudomonas* decreased after the flushing event, while the relative abundance of *Legionella* and *Mycobacterium* increased post-flush, potentially indicating the resuspension and distribution of particle-associated *Pseudomonas* during the flushing event. Within the tank (site 2), the relative abundance of *Pseudomonas* in PAB decreased from 15.6% pre-flush (March) to 0.37% post-flush (April). This supports that there may have been redistribution of *Pseudomonas* from the tank to the rest of the DWDS because of flushing.

Through Spearman's rank correlations, many significant positive correlations were found between *Pseudomonas* in TB samples and hydraulic parameters (maximum site flow, site Δ flow, minimum site velocity, average site velocity, and maximum site velocity). Significant positive correlations were also found between these same hydraulic parameters, besides minimum site velocity, and *Pseudomonas* in PAB samples. *Pseudomonas* in TB samples was negatively correlated with iron concentration and turbidity, although these trends were not found within PAB. *Legionella* and *Mycobacterium* had no significant correlations with hydraulic or water quality parameters.

Although genera containing pathogenic species (*Pseudomonas*, *Mycobacterium*, and *Legionella*) were detected, the quantification of pathogenic species via qPCR was performed to assess human health risk. No samples had quantifiable *L*. *pneumophila* or *M*. *avium* above the limit of quantification, and only one sample (site 10 from March) had quantifiable *P*. *aeruginosa*. These results indicate that even though genera containing pathogenic species may be present within the DWDS, the actual quantity of pathogenic species is low or undetectable, indicating a low human health risk to consumers of the drinking water.

4.5.3 Corrosion biomarkers. Genera containing bacteria involved in corrosion of DWDS materials were found to be biomarkers for sites, TB, and PAB. The tank (site 2) had an SOB (*Sulfuricella*) as a biomarker, site 10 had an IOB (*Gallionella*) as a biomarker, and various sites had IRB as biomarkers (sites 2, 5, 7, and 11). When grouped by TB versus PAB, TB had an SRB (*Desulfovibrio*) as a biomarker, and PAB had two different IRB as biomarkers (*Bacillus* and *Pseudomonas*). These corrosion-related bacteria have been found within DWDSs [72] and tanks [73] in previous studies, and can lead to the corrosion of cast iron pipes. Corrosion can deteriorate water quality through the release of corrosion by-products and can form scale that reduces the water transportation capacity and offers protection for pathogenic and opportunistic bacteria to grow [72, 74, 75].

5. Conclusions

The studied DWDS was dominated by Gammaproteobacteria. The prevalence of Gammaproteobacteria in biofilms and loose deposits in previous studies of DWDSs indicates that biofilms and loose deposits may have a stronger effect on microbial community than bulk water in the studied DWDS. The DWDS was influenced to a greater extent by spatial variations than temporal variations, especially within PAB, further indicating that biofilms, which have a more stable composition over time, are seeding the bulk water and having a stronger effect on microbial community than planktonic growth. Hydraulic conditions were associated with increases in alpha diversity to a greater extent than water quality, demonstrating that changes in pressure, velocity, or flow may cause biofilms and loose deposits to detach from pipe walls and contribute to an increased diversity within the system. Genera containing pathogenic species were detected throughout the DWDS, with *Pseudomonas* and *Legionella* detected at every site at least once within both PAB and TB samples and *Mycobacterium* detected at 12 of 14 sites for PAB and 8 of 14 sites for TB. The relative abundance of *Pseudomonas* was correlated with hydraulic parameters, particularly within TB, indicating that hydraulic conditions within DWDSs may potentially cause consequences to human health. Although genera containing pathogenic species were detected, only one sample contained quantifiable levels of *P*. *aeruginosa* genetic material, indicating a low human health risk to consumers. This study establishes spatial variations in PAB associated with varied hydraulic conditions as an important factor driving microbial community within a chlorinated DWDS. Future work is needed to assess how the layout and consumer distribution of DWDSs impacts hydraulic variations and subsequently microbial communities, particularly in rural systems, to better understand why particles had such an impactful role in this study.

Supporting information

S1 Table. Forward and reverse primers and probes for quantification of pathogenic species.
(XLSX)

Author Contributions

Conceptualization: Emily Garner.

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Supervision: Emily Garner.

Writing – original draft: Madison Ferrebee.

Writing – review & editing: Emily Garner.

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