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Bacterial communities in a Neotropical full-scale drinking water system including intermittent piped water supply, from sources to taps

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WATER IMPACT STATEMENT:

Intermittent water supply (IWS) is a substandard piped water service that is common around the world. Understanding how IWS influences microbial communities in drinking water can provide insight to better manage IWS and protect water quality. This study assessed bacterial

communities from source to tap in a full-scale drinking water system with IWS in Arraiján, Panama.

ABSTRACT

Understanding the microbial ecology of drinking water systems is crucial to delivering safe water. Intermittent water supply is a substandard water service that is common around the world; however, we do not yet understand how this service influences the communities of microorganisms in distribution systems. We used rRNA gene metabarcoding alongside traditional measurements of water quality to assess bacterial diversity and structure in a Neotropical drinking water system with continuous and intermittent supply. We sampled from source and treated water before distribution at three drinking water treatment plants, household taps across the distribution network, and in an intermittent supply zone immediately after supply restarted and 24 hours after. Each treatment plant had a diverse microbiome, dominated by Proteobacteria; treatment and distribution changed the diversity and composition of bacterial communities. Communities differed at the start of IWS supply and 24 hours after, and several taxa were only found in first flush samples, suggesting intrusion of new bacteria into pipes or regrowth of bacteria between supply cycles. *Pseudomonas* was found to be more common in IWS samples than elsewhere in the network. Unique taxa found only at the restart of IWS service have the potential to be used as indicators of intrusion in future research.

1. INTRODUCTION

In low- and middle-income countries (LMICs), the consistent delivery of microbiologically safe water remains a challenge for many utilities that face resource limitations and rapid population growth. Despite the increase in access to safe drinking water reported between 2000 and 2017 in

Latin América and Caribbean countries (1), many social, economic, and environmental factors continue to induce drinking water to be treated insufficiently and supplied intermittently. Such factors include scarcity of water resources, inadequate infrastructure, unplanned expansions in the drinking water distribution system (DWDS), excessive water losses, increasing consumer demands, or a combination of these factors (2–5). Intermittent water supply (IWS) is a common practice in LMICs and refers to water supply to consumers for less than 24 hours per day on average (6). IWS impacts over one billion people around the world, causing public health risks, such as diarrheal infections, and difficulties to consumers and water utilities (7–12); yet, due to water scarcity and climate change, this practice is expected to increase (8,13).

Several characteristics of IWS have the potential to increase the deterioration of drinking water quality compared to continuous supply systems, including: 1) microbial growth in bulk water, pipe-wall biofilm and loose deposits while water is drained or stagnant between supply, and subsequent flushing and detachment of microorganisms when supply restarts (known as first flush); 2) intrusion and backflow of contaminants via pipe leaks, loose fittings and/or customer connections during periods of low or negative pressure; and 3) recontamination and microbial growth during household storage (5,14,15). In first flush samples, higher bacterial indicator concentrations, low to no chlorine residual and higher turbidities have been documented (15–18); a deeper understanding of specific regrowth and intrusion mechanisms could provide a basis for mitigation efforts. While low pressures and pressure transients are components of IWS cycles, their frequency and severity in IWS systems and subsequent impacts on infrastructure and health risk are poorly understood and are difficult to study (15,19,20). These pressure events can result

in intrusion of contaminants if there is an external source of contamination and there is a pathway for that contaminant to enter the distribution (21–23).

Culture-based techniques using indicator organisms have been widely used to assess microbial water quality in IWS systems and have shown that water quality can deteriorate during distribution and after household storage (15,17,24–27). However, indicator organisms do not represent the behavior of all pathogens nor the entire drinking water microbial community. In addition, the monitoring of chlorine residual and fecal indicators may not be sufficient to characterize water quality microbial risks in such dynamic systems.

Molecular techniques are useful tools to characterize the whole microbial community in drinking water, but most research has been conducted on water systems in industrialized countries (28–35) or in laboratory settings (36). Thus, most of our understanding of microbiomes in drinking water comes from drinking water systems with continuous water supply (CWS). Previous microbial community studies have shed light on the links between microbial communities and water source, treatment and distribution. From these systems, we know that drinking water has a relatively diverse bacterial profile dominated by Proteobacteria, Firmicutes, and Actinobacteria (28,37). As source water is treated, a general downward trajectory in bacterial diversity is expected (38); a reduction in diversity has also been found to be coupled with increases of certain bacterial taxa after the filtration step (39,40). In the distribution system, factors such as stagnation, pipe material, disinfectant residuals, and treatment processes can drive significant differences in microbial communities (33,37,41–45). Despite this growing body of literature, microbial communities in drinking water systems in LMIC with IWS are underexplored and they

have been studied to a limited extent using DNA high-throughput sequencing approaches. Although limited, these studies have also found Proteobacteria and Firmicutes within the most abundant phyla and that physico-chemical water parameters play important roles in shaping the microbial community. In addition, genera such as *Cyanothece* (Cyanobacteria –cytotoxin producer), *Acinetobacter* (Proteobacteria – potential pathogen)*, and Methylobacterium* (Proteobacteria – methylotrophic organism) have been found in higher abundances in IWS (36,46). Nonetheless, these existing studies have not shed light on how the main phases of IWS (draining or stagnant water followed by filling, followed by stable or unstable supply) impact water quality and microbial communities in full-scale systems (36,46–48). Furthermore, limited research has been conducted to investigate microbial communities in drinking water in neotropical environments, where IWS is prevalent (5).

To address this knowledge gap, we studied the bacterial communities in a large, full-scale drinking water system, in Panama (Latin América), that includes IWS in a portion of the network as well as other features that are commonly encountered in LMIC (i.e., source water variability, operational, and infrastructure deficiencies) over a 15-month period. By sampling from source to tap, the influence of drinking water treatment and distribution was characterized, including impacts resulting from IWS cycles experienced in a section of the distribution system. We used 16S rRNA gene metabarcoding combined with traditional culture-based methods and water quality metrics to characterize microbial communities. The specific objectives of this study were to: 1) obtain an overview of bacterial diversity and community structure from water sources and along surface water treatment process; 2) compare bacterial communities across multiple locations in the drinking water distribution system; and 3) assess the impacts of IWS on the

bacterial community by comparing first flush water and water after the service had stabilized (24 hour after supply restart). The results provide insights into the unique impacts of IWS on the bacterial community in drinking water, and ultimately can contribute to developing effective strategies to improve the design and management of water quality and public health.

2. MATERIALS AND METHODS

2.1 Study location

This study was conducted in Arraiján, Panama, a rapidly growing peri-urban area west of the Panama Canal and Panama City, Panama (**Figure 1.a**). Arraiján's drinking water system served approximately 283,500 inhabitants and was supplied by three drinking water treatment plants (DWTP A, B and C, **Figure 1.b,c**) (12,17,49). These treatment plants sourced their water from the Panama Canal Watershed, primarily from Lake Gatún, which is recharged by the Chagres River, and lake Miraflores (both artificial lakes used in the operations of the Panama Canal). Conventional surface water treatment was practiced, with slight differences between plants (**Supplemental Table 1**; at DWTP A and B, free chlorine was applied upstream of filtration). While most of the DWDS in Arraiján provided continuous water supply, several zones operated intermittently (12,17). In addition, the DWDS suffers from around 40% water loss due to pipe failures, illegal connections, and other factors (e.g., loose fittings) (15).

To assess the effects of treatment, piped distribution, and IWS, the drinking water system of Arraiján was sampled between December 2016 and February 2018 (**Supplemental Table 2)**. A total of 118 samples were collected. Locations in the distribution system were divided into three regions (A-C) that corresponded to the DWTP from which the water supply originated (**Figure**

1.c). All three DWTPs drew their source water from different locations (**Figure 1.c, Supplemental Table 1**). Seven different types of samples were collected, following the flow of water from source to tap: i) source water samples at each DWTP intake (labeled Source); ii) filter effluent from within each DWTP (Filtration); iii) finished, disinfected drinking water before distribution (Disinfection); and iv) locations in the distribution system which are commonly used to monitor water quality in the network (Distribution) (**Figure 1.b,c**). Three sample types were collected to assess the effect of IWS on bacterial communities: v) upstream samples were taken at a pump station near the entrance of the IWS zone where the water supply was continuous (Entrance); vi) downstream, first-flush samples were taken immediately after supply restarted (First Flush); and vii) at the same downstream location, samples were taken 24 hours after restart when supply had stabilized (Stable) (**Figure 1.d**). The number of samples collected during the wet and dry seasons was insufficient to discern the effects of seasonality; in addition, precipitation events occurred during the dry season.

This IWS zone within the Arraiján distribution network has been previously described in Erickson et al. (2017) and is located within our sampling Region B. Due to insufficient supply capacity, this zone received water intermittently and supply was managed by the utility with a valve located at the entrance to the zone (17). The zone had approximately 232 connections, which were primarily residential, and was scheduled to receive water for three of every six days (i.e. alternating three days with water supply and three days without). This schedule was carried out by manually opening and closing the control valve at the entrance of the area. Sanitation consisted of flush toilets connected to septic systems or pit latrines, which were typically located in the back of the houses. Because the drinking water supply pipes were typically located in

front of the houses, it was unlikely that sewage could enter the drinking water pipes. Samples were collected from two monitoring stations that were previously installed in this zone (17); an upstream location at the pump station supplying the IWS zone, and the IWS location which was approximately 1.5 kilometers downstream of this entrance/pump station (**Figure 1.d**).

2.2 Sample collection

Water samples were collected between the hours of 6 a.m. and 3 p.m. on 89 unique sampling days. Because of the long travel times between laboratory and sample sites, and the time required to process samples in the field and in laboratory, only one or two sites could be visited on each sampling day. Samples from within a DWTP were always collected on the same day. In the DWTPs, grab samples were collected directly from sampling ports. For Filtration samples, a composite sample was generated by combining aliquots from all working filters (excluding those in maintenance or that were being cleaned). Samples taken at the 18 locations in the DWDS were collected after 3-5 minute flushing of the sampling line (except first-flush samples). For the collection of first-flush samples, we arrived at the sampling location when the supply was off and waited until supply began. Once supply started, the line was flushed for only 1 minute, to allow stagnant water in the sampling line to flush out. Then, water was collected during the first 1.5 - 2 hours of supply; the collection time (volume) was larger than used in previous studies (8,32) so that sufficient biomass could be concentrated for 16S rRNA amplicon sequencing (see below).

2.3 Water quality parameters

Several parameters were analyzed at the time of sampling at all locations: free and total chlorine (Hach Portable Colorimeter II), turbidity (Hach Portable Turbidimeter 2100Q), pH, temperature,

conductivity (EC), total dissolved solids (TDS) (HANNA Instruments pH/EC/TDS tester or Extech ExStik II pH/EC meter) and pressure (Eastman 45169 Water Pressure Test Gauge, adapted to fit sampling tap).

In the IWS zone, some water quality parameters were also measured continuously using the monitoring stations at the upstream and downstream locations (Points 1 and 2 in **Figure 1.d**). Each monitoring station was equipped with Q46/76 turbidity sensors, Q45H/62 chlorine sensors (Analytical Technology Inc., Collegeville, PA), ECO-3 RTU or LPR-3li pressure monitors (AQUAS Inc., Taipei, Taiwan and Telog Instruments Inc. Victor, NY, respectively).

2.4 Culture-based indicator bacteria

Samples for bacterial enumeration were collected from all sampling locations in sterile 100-mL bottles with sodium thiosulfate to neutralize residual chlorine and transported on ice to the research institute INDICASAT-AIP in Panama City, Panama. These samples were processed within 3 hours of collection to measure heterotrophic plate count (HPC) by the most probable number (MPN) method using Colilert Quanti-tray 2000 (IDEXX laboratories Inc, Westbrook, ME, USA). Samples were incubated at 37°C and counted after 38-44 hours. Field and laboratory blanks were collected every five samples. Total coliform bacteria and *E. coli* indicators were not measured in this study as the utility conducted routine monitoring of these indicators at the sample locations and a prior study found that they were below the detection limit in the vast majority of samples (17).

2.5 16S rRNA gene metabarcoding

To characterize the bacterial communities, large bulk water volumes were collected to concentrate the microbial biomass for DNA extraction and 16S rRNA gene sequencing. Sample volumes ranged from 15 - 20 L for source water samples, 100 L for filtration samples, 170 - 200 L for first flush samples, and 450 - 500 L for samples after disinfection and in the continuous supply portions of the DWDS. These volumes were chosen after a preliminary analysis using16S rRNA gene sequencing was carried out, testing different water volumes for the different sample types. For volumes greater than 100 L, the biomass was concentrated on-site from each water sample by dead-end ultrafiltration (DEUF) using REXEED 25S ultrafiltration membrane cartridges (Asahi Kasei, Tokyo, Japan) as described by Smith and Hill (50). Ultrafilters were transported at 4°C to the Smithsonian Tropical Research Institute (STRI) molecular laboratory and processed within five hours of collection. Backflushing of ultrafilters was performed as described in Smith and Hill, 2009, and backflush sample volumes ranged between 600 – 800 mL. After backflushing, further concentration of bacterial cells was carried out by vacuum filtering 200 mL of the backflush sample sequentially through 5-µm and 0.22-µm pore size mixed cellulose filters (EDM Millipore, Burlington, MA, USA). Final filter samples were stored at -80 degrees C until DNA isolation, which was performed using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) with the following adjustments: The 5-µm and 0.22-µm mixed cellulose filters were cut into pieces under sterile conditions and combined in 5-mL low-bind tubes with bead-beat solution and Solution C1. Bead-beating and subsequent steps in the DNA extraction protocol were performed according to the manufacturer's recommendations. The isolated DNA was eluted in a final volume of 60 µL. DNA extractions were stored at -20 degrees C prior to the preparation of sequencing libraries. Amplification of the V4 variable region of the

16S rRNA gene and subsequent sequencing was performed following the protocols described in Chavarria et al. (2021) (51).

2.6 Data analysis

Bacterial detection by HPC and analysis of water quality parameters was performed as described in Kumpel & Nelson (2014) (52). One half of the lower detection limit was substituted for values below the detection limit and the upper detection limit was substituted for values above the upper detection limit. Statistical tests for HPC data were performed on their rank values using Kruskal-Wallis to avoid assumptions about the distribution of the data. Untransformed data were used for water quality parameters. Statistical analysis and plotting were performed using the statistical software R (R Core Team, 2012). Values were considered significant at $p < 0.05$.

Quantitative Insights Into Microbial Ecology 2 (QIIME2) platform v2020.11 and the statistical software R were used for data analysis of 16S rRNA gene data analysis of paired-end sequence reads (53) following Chavarria et al. (2021). Alpha- and beta- diversity were calculated using the diversity core-metrics function in QIIME2 from the resulting ASV table, standardized to a sequencing depth of 2200 sequences per sample. Differences in alpha diversity (Shannon diversity) by categorical metadata variables were tested by Kruskal-Wallis, with pairwise differences between types of sample also tested in QIIME2 using pairwise Wilcox tests with Benjamini-Hochberg corrections. Statistical significance in beta diversity of the different types of supply was determined by permutation-based ANOVA (PERMANOVA) tests with 999 permutations on weighted UniFrac distances (54,55). To explore the structure of microbial communities and visualize beta-diversity dissimilarity, an ordination approach was adopted

using principal coordinate analysis (PCoA) using weighted Unifrac distances generated in R with the package Phyloseq (56). Multibar taxonomy plots were also generated in R. The QIIME2 plugin ANCOM and linear discriminant analysis Effect Size (LEfSe) were used to detect differentially abundant taxa accounting for compositional differences (57,58). Differences were considered to be significant if p-value < 0.05. To understand further the relationships between water characteristics and microbial composition, Spearman's correlation tests and canonical correspondence analysis (CCA) were performed in R with the Vegan package (59).

3. RESULTS AND DISCUSSION

3.1 Water quality from source to tap - continuous distribution and influences of IWS

Significant differences in water quality were observed at the three DWTPs that serve Arraiján, Panama (**Supplemental Figure 1, Supplemental Table 3**). Average turbidity, conductivity, and TDS of source water in DWTP A were significantly higher than in source water for DWTP B and C (Kruskal-Wallis, $p < 0.01$) **(Supplemental Figure 1A-C).** These differences in turbidity, conductivity and TDS are likely due to the proximity of DWTP A intakes to the shipping channel of the Panama Canal and the influence of its dynamic operations (60,61) (**Figure 1**).

Treatment at the three DWTPs did not significantly change conductivity, pH, temperature, or TDS across sample types (Kruskal-Wallis, $p > 0.05$); temperature and pH were also similar across all regions (**Supplemental Figure 1D-E)**. In the distribution system, chlorine was the only parameter measured that was found to vary between samples, with chlorine levels decreasing with physical distance from the respective DWTP (**Supplemental Figure 1F,G**). Pressure differences were found across sampling locations in all three regions. These pressures

varied between 5 and 60 PSI and were found to be significantly different in some instances (Kruskal-Wallis, $p < 0.05$) (**Supplemental Figure 1H)**. After water treatment and before distribution, all samples met the Panamanian drinking water standards at the time of this study, which required a chlorine concentration between 0.8 mg/L and 1.5 mg/L, with the type of chlorine species unspecified (62,63). Since the time of this study, the Panamanian drinking water standards for chlorine residual have undergone several revisions (62,63). Thus, further research is warranted to characterize the implications of these changes on chlorine residual and microbial water quality.

As expected, HPC concentrations in source samples for all three regions were significantly higher than all other samples (except first flush samples), surpassing 1.0×10^4 MPN/100mL (**Supplemental Figure 2)**. Treatment significantly decreased these concentrations (Kruskal-Wallis, $p < 0.05$), and finished water had concentrations of HPC well under 100 MPN/mL for all DWTPs.

In the IWS zone, continuous monitoring (through the monitoring station) and grab sample measurements of free chlorine residual, pressure, and turbidity were generally in agreement, except for a few occasions when the free chlorine sensor measured lower values than grab sample measurements (lower by 0.3 mg/L on average) **(Figure 2 and Supplemental Table 3).** Based on grab samples, significantly lower chlorine concentrations (both free chlorine residual and total chlorine) were observed in first flush samples compared to other sample types (Kruskal-Wallis, $p < 0.05$) (**Figure 3a,b**). Average turbidity was below the water quality standard of 1 NTU (0.5 ± 0.1 NTU) but tended to be higher in the first-flush samples (**Figure 3c**). The continuous monitoring data captured turbidity measurements above 1 NTU during and

well past the first 24 hours of supply cycle (**Figure 2**). Water temperatures during the first flush were significantly higher than stable IWS and at the entrance where supply is continuous (**Figure 3d,** Kruskal-Wallis, $p < 0.05$). EC, pH and TDS were not statistically different (Kruskal-Wallis, $p > 0.05$, respectively) (**Figure 3e, f, g**). Average pressure in the IWS zone ranged between 4 and 59 PSI and varied significantly between the entrance, first flush and stable supply sampling locations at the moment of sampling (Kruskal-Wallis, $p < 0.05$). The average pressure for first flush samples was 7.5 psi (7.5 ± 2.5 PSI, measured 1.5-2 h after supply started) while for stable supply it was 21 psi (21 ± 8.2 PSI). At the entrance, pressure averaged 59 psi (59 ± 10.4 PSI) (**Figure 3h and Supplemental Table 3**). Concentrations of HPC at the entrance to the IWS zone averaged 22 MPN/100 mL (22 ± 14 MPN/100 mL) and remained close to or below the lower detection limit (**Figure 3i)**. In contrast, first flush samples averaged an HPC concentration of 4.3 x 10^4 MPN/100 mL (4.3 ± 2.8 x 10^4 MPN/100 mL) and were significantly higher than concentrations from stable IWS (Kruskal-Wallis, $p < 0.001$) which averaged 70 ± 109 MPN/100 mL and ranged from 5 to 300 MPN/100 mL. Other studies have also found a deterioration of water quality during first flush events when supply is restarted regardless of the duration of the stoppage (15–17,64–66); although differences in water quality between first flush and stable supply may not be significant in all cases (16).

From our HPC results it is not possible to determine whether the high concentrations in first flush samples are the result of intrusion or re-growth in the stagnant water, biofilm, and loose deposits. *E. coli* data from an earlier study at the same site also did not provide insight, as most first flush samples were negative (17). It should be noted, however, that at a different study site in another IWS system it was concluded that high concentrations of *E. coli* in first flush samples

could not reasonably be attributed to regrowth; thus, intrusion was likely (15). It is more straightforward to implicate intrusion, for example, when fecal indicator organisms are detected in the distribution system but were absent immediately after treatment. However, when there is no external fecal pollution source, measurements of indicator bacteria may be inadequate to distinguish intrusion from re-growth.

3.2 Bacterial community diversity and composition from source to tap during continuous and IWS

A total of 113 samples were successfully sequenced (113/118 of total samples), including samples from each of three locations in the DWTPs, 18 locations in the continuous DWDS, first-flush samples (location 3B, **Figure 1c**) and samples for IWS during stable supply (**Supplementary Table 2**). We obtained a total of 2,695,623 sequence reads (range 36,219 - 76,898 reads per sample). After quality filtering, merging reads, chimera removal, and filtering low abundance ASVs, we obtained between 2,235 and 53,117 reads per sample and 4,771 ASVs (737 genera). Rarefaction curves demonstrated that the sequence efforts could capture the majority of the bacterial diversity in the dataset (not shown).

3.2.1 Diversity changes from source to DWDS

From source to tap, bacterial diversity decreased across the treatment and distribution systems (**Supplemental Figure 3, 4**, Shannon, $p < 0.05$). Source samples had the highest diversity and water samples from the distribution network had lower diversity in all three regions (**Supplemental Figure 3**). In the DWTPs, diversity declined as source water was filtered. Although disinfection tended to reduce diversity further, Shannon indices were not significantly different from the filtration step (**Supplemental Figure 3**, **4a)**. This finding could potentially be

due to the persistence of DNA from microorganisms that have been inactivated(67) as water samples were taken immediately after disinfection and before any water storage. Overall, Disinfection sample at DWTP B had the lowest diversity which corresponded to higher overall chlorine (total and residual) compares to Disinfection samples from the other two DWTPs. In the DWDS, samples with higher mean diversity corresponded to sampling locations with the lowest chlorine concentrations (locations 4C and 5C) (**Supplemental Figure 4b**).

Using PCoA based on Weighted UniFrac distance, clustering by sample type revealed differences in community structure between source water samples and the DWDS (**Figure 4**). There was a general transition in microbial community structure from untreated source water to the DWDS; samples from the DWTP that contained some chlorine (chlorinated Source samples and Filtration samples) clustering between unchlorinated Source samples and samples from the DWDS. Disinfection samples from DWTP B and C exhibited much larger shifts in community structure than those from DWTP A. In the DWDS, bacterial community structure was found to be significantly different across regions ; however R values were low, likely due to outlier samples driving these differences (ANOSIM, A/B: $R = 0.29$ $p < 0.01$; A/C: $R = 0.29$ $p < 0.01$, B/C: $R = 0.13$ $p < 0.05$) and this significance could be overestimated due to dispersion within sample types (PERMDISP, F-value = 0.10, $p > 0.05$). The bacterial community composition found in certain locations was very different than in the rest of the DWDS. In particular, the bacterial community found in location 5C, which is the location with the lowest chlorine concentrations, was significantly different from all other locations in this study and more similar to water still undergoing treatment than other sites in the distribution network (**Figure 4**).

Bacterial communities found across all samples were classified into 43 bacterial phyla, 433 families, and 780 genera. Fifteen bacterial phyla comprised over 98% of the bacterial community (except in IWS samples) (**Supplemental Table 4**). Four phyla represented over 95% of the total ASVs for all samples combined: Proteobacteria (70.6% ± 6.36%), followed by Cyanobacteria (12.6% ± 7.81%), Bacteroidetes (5.4 % ± 3.18%) and Actinobacteria (3.6% ± 1.62%). These results are generally consistent with studies that have investigated bacterial communities in drinking water systems in temperate climates (28,32,68,69). When looking at each DWTP independently, we observed several differences between bacterial phyla (**Supplemental Table 4**). As previously mentioned, a general downward trajectory in bacterial diversity is expected as water moves along the treatment train. This is the general pattern that we observed with a decrease in diversity with treatment while the relative abundance of some phyla increased with treatment (**Supplemental Table 4**). While the sample numbers are too small to draw conclusions about the impact of specific treatment steps on specific phyla, differences in filtration practices (media, backwashing, chlorination) and chlorination practices (chlorination dosage and contact times) are expected to influence the bacteria that are present in the finished treated water that enters the distribution system (33,45,70).

Within the DWDS, differences in relative abundances of top phyla were also observed. In region A, a decrease (although not significant) in Proteobacteria was observed between samples from Disinfection and samples in the distribution system. This decrease in Proteobacteria was coupled with a significant decrease of Firmicutes and an increase of Cyanobacteria (Kruskal-Wallis, $p < 0.05$), suggesting sunlight exposure during storage. In regions B and C, Proteobacteria represented over 84 % of relative abundance with minor differences between other phyla (**Supplemental Table 4**). Top bacterial families according to sampling location were observed to

have differences even when supplied by the same DWTP (**Figure 5**). Among locations in region A, the family Obscuribacteraceae (Cyanobacteria) and specifically the genus *Obscuribacter* was particularly abundant. The families Silvanigrellaceae (Proteobacteria) and Pseudomonadaceae (Proteobacteria) were abundant across region B, and Oxalobacteraceae (Proteobacteria) was abundant across region C (**Figure 5**). The genus *Phreatobacter* (Proteobacteria) was common across all regions, indicating that although significant differences existed in bacterial communities after treatment and distribution, some bacteria (i.e., *Phreatobacter, Undibacterium*, *Pedobacter*) can be prevalent across the network. Although a recent discovery, the genus *Phreatobacter* has now been identified in several DWDSs (32,71–74), and has been found in distribution systems with chlorine residual. In Arraiján's DWDS, chlorine residual was well above 0.2 mg/L for most samples; however, it is still unclear what role *Phreatobacter* plays in the drinking water microbiome of these systems. Other enriched genera in the DWDS included: *Methylobacterium, Obscuribacter, Acinetobacter* in region A*; Pseudomonas*, *Nevskia*, and *Silvanigrella* in region B and C.

3.2.2 Influence of IWS on drinking water bacterial communities

Our analysis of the IWS zone showed high variability in bacterial community composition and structure as water flow initiated and stabilized over time. First flush samples tended to have higher diversity compared to samples taken from the same location after supply had stabilized and samples from the entrance where supply was continuous, although these differences were not significant (Shannon, Kruskal-Wallis, $p > 0.05$; **Supplemental Figure 5**). Community analysis using weighted UniFrac also did not reveal a strong pattern of clustering of community structure by sample type but samples from the entrance (continuous supply) differed from IWS samples

(**Supplemental Figure 6**). While there was high dispersion within each group (PERMDISP, F-value = 0.522, $p > 0.05$), ANOSIM tests showed significant differences between the communities of first flush, entrance and stable supply samples (ANOSIM, $R = 0.29$, $p < 0.05$). Overall, our sampling revealed a highly variable bacterial community. It is likely that the dynamic conditions created by IWS contribute to this variability, as the bacterial taxa found at the entrance of the IWS zone were similar to other locations in region B of the DWDS (**Figure 5**).

However, interpretation of these results is limited by differing sample sizes, as we had more first flush samples (n=14) compared to stable supply (n=7) and entrance (n=4) samples. Thus, an additional analysis was conducted comparing only the first flush and stable IWS samples that were sampled within 24 hours of each other (n=6 pairs, **Figure 6a,b**). Paired first flush and stable supply samples also showed high variability in bacterial composition and community structure, with some sample pairs showing similar communities within pairs (e.g. pairs 2 and 6) and others showing large shifts in community composition. All sample pairs were unique suggesting that each IWS cycle impacted the water microbiome. However, pairs collected during consecutive weeks (e.g. pairs 2, 3, and 4) were more similar to each other than those collected at other times in the year (**Figure 6 a,b**). Although the water quality metrics during first flush events differed from other supply samples, the high degree of variability among first flush samples (and among stable supply samples) was not evident from analysis of traditional water quality metrics (e.g. free chlorine residual in stable supply was consistent) (**Supplemental Table 3)**. In addition to the unique features of intermittent supply, other factors likely influenced the bacterial communities in these samples, including seasonal effects on the water source and

conditions in the distribution system, as well as operational changes at the treatment plant and in the distribution system. Seasonal differences are likely to significantly affect microbial communities in IWS systems, specifically in regions with drastically different dry and rainy seasons and where water sources are limited (5,75–77). Some systems supply only a few hours per day during the dry season and switch to continuous supply during the wet season (5). Several studies have looked at water source seasonality and enteric pathogens and infections in water systems known to have deficiencies, including likely intermittency (75,78–80); however, the specific relationship between IWS features such as first flush and stable IWS, seasonality, and microorganisms is not well documented, and likely system-dependent. It is also important to note that although our sampling strategy was designed to capture differences between the first flush samples and their corresponding stable supply, sensor monitoring data revealed that water quality could vary even after 24 hours of continuous supply, which was not captured by our grab sampling (**Figure 2**, **Supplemental Figure 7).**

Bacterial communities in the IWS zone clearly differed from those found at the entrance of the IWS zone where the bacterial communities were similar to other locations in region B of the DWDS (**Supplemental Table 5, Supplemental Figure 6**). Four taxa, *Silvanigrella* (Oligoflexia)*, Undibacterium* (Proteobacteria) and an uncultured Obscuribacter (Cyanobacteria) and *Burkholderia* (Proteobacteria) were the most common taxa (with > 4.5% relative abundance) at the entrance. Downstream, the most abundant genera among first-flush samples were an unclassified species of *Sphingomonas* (22.1%) followed by *Pseudomonas* (Proteobacteria, 12.9%), *Rheinheimera* (Proteobacteria, 11.9%), and *Undibacterium* (Proteobacteria, 7.0%). Although stable IWS supply samples contained similar phyla as first-flush samples

((Proteobacteria (90.5%), followed by Cyanobacteria (5.46%), Bacteroidetes (2.64%), Firmicutes (1.24%)), the most abundant genera were *Pseudomonas* (19.1%), followed by *Silvanigrella* (15.0%), and *Candidatus Megaira* (Proteobacteria, 9.4 %). Thus, clear distinctions in community composition were observed between first flush and stable supply samples, although these differences were not consistent (**Figure 6a**). However, using PCoA based on Weighted UniFrac distance, we saw consistent shifts in the orientation of paired first flush and stable supply samples across PCoA axis 2 (**Figure 6b**) although we were not able to establish what drives this pattern. Community structure among paired samples was not significantly different (ANOSIM, $R = 0.18$, $p > 0.05$).

From our results, taxa that are unique to the IWS zone were identified (**Supplemental Table 5**). One hundred thirty-three genera were found only in first flush samples, 12 only in Stable IWS, and 28 were found in both first flush and stable supply that were not found in Continuous samples (**Supplemental Table 5**), suggesting that these bacteria may have been introduced into the DWDS by mechanisms found only in this IWS setting. Previous research in this IWS sampling location detected prolonged low and negative pressures and our sampling also showed lower pressure at this location than elsewhere in the Region B network (**Supplemental Figure 1**, location 3B). Low pressures could drive intrusion and backflow into this section of the DWDS (17,19) resulting in changes to the composition of the bacterial community at this location.

Two genera that were found in IWS samples are of particular interest: identified, such as *Rheinheimera* and *Pseudomonas*(32,81,82). Interestingly, *Rheinheimera* has been found primarily in biofilm drinking water samples and thus could be an indicator of biofilm detachment during first flush and/or microbial growth inside pipes between IWS cycles (32,81). While also

493 found elsewhere in the network, *Pseudomonas* was most prevalent in samples from IWS, both in
494 our first flush and stable sampling. *Pseudomonas* can be pathogenic and can survive in many
495 different environments due in part to its minimal nutritional requirements (83). In addition,
496 *Pseudomonas* spp. Have a tremendous ability to form biofilms which makes them a model for
497 biofilm-forming organisms (83). Prior research has shown that their ability to grow on a wide
498 range of organic compounds and their resistance to chlorine allows them to colonize distribution
499 networks, including premise plumbing (84,85). Our results suggest that IWS provides conditions
500 for the proliferation of *Pseudomonas* in this distribution system. The high relative abundance of
501 *Pseudomonas* in downstream samples could be due to detachment of biofilm during supply
502 cycles in IWS (86). In addition to detachment as pipes fill, the high relative abundance in stable
503 supply samples suggest that detachment or migration from the biofilm could be occurring during
504 the entire supply cycle, possibly due to fluctuations in pressure (**Figure 2 and Supplemental**
505 **Figure 7**) (36,87,88).

506 The origin of unique ASVs found only in first-flush samples remains a key question for future
507 research. While it is possible that these taxa are present elsewhere in the network but at low
508 enough frequencies that we were not able to detect them, further investigation as to whether any
509 taxa could be used to determine the source of organisms would be of value, in particular to
510 distinguish between intrusion versus in-situ growth between supply cycles (e.g., in stagnant
511 water, biofilms, or loose deposits). Further, based on the higher number of observed ASVs in
512 some first-flush samples, ASV number may also hold promise as a signal for intrusion and
513 growth between supplies (**Supplemental Figure 8**). However, more studies are needed to
514 determine if higher ASVs are consistently observed during first flush compared to stable supply.
515

3.3 Relationship between water quality parameters and bacterial community composition

Pearson correlations were used to determine water quality parameters that were associated with microbial community structure and were plotted using a canonical correspondence analysis plot (CCA; **Figure 7a**). Among all water quality parameters measured, chlorine (Pearson's R = 0.24, $p < 0.01$), turbidity (Pearson's R = 0.16, $p < 0.05$), conductivity (Pearson's R = 0.20, $p < 0.05$), TDS (Pearson's R = 0.26, $p < 0.01$) and pressure (Pearson's R = 0.11, $p < 0.05$) were significantly associated with differences in microbial community composition.

In IWS, significant correlations were also found between community structure and free chlorine residual (Spearman's R=0.28, $p < 0.01$), total chlorine (Spearman's R=0.23, $p < 0.01$), temperature (Spearman's R=0.24, $p < 0.05$), and pressure (Spearman's R=0.35, $p < 0.01$), whereas turbidity, pH, conductivity, and TDS were not statistically correlated. CCA also revealed relationships between these water quality metrics and bacterial community structure (**Figure 7b)**. Although these correlations were weak, they demonstrated the importance of water quality parameters on microbial community structure. Similar results have been found between water quality parameters and bacterial structure in CWS systems (89–91). It is important to note that collinearity exists between water quality parameters which can confound these results. Nonetheless, factors such as chlorine concentration, turbidity, and pressure are of particular importance when assessing microbiomes in drinking water and water quality in general.

3.4 Limitations and future research

This study exemplifies a number of limitations related to the difficulty investigating a large, full-scale, dynamic, intermittent water supply system. Although we selected an IWS sampling

location that was supposed to operate on a schedule (three days on and three days off, managed by a manual valve), the actual supply was often unpredictable (mainly due to upstream pump failures). In addition, in several instances, the supply stopped a few hours after starting, which prevented the collection of a sample after 24 hours of supply restart (and was a significant burden for consumers). We chose to collect samples from a large number of sites and sample large volumes, and as a result could only collect a relatively small number of samples for each sampling location. While this approach provided a descriptive picture of the different sources of variability found in this large full-scale system with IWS, it reduced the statistical power of our findings. With the limited resources and logistical constraints in this low-resource setting, we were able to collect 118 samples, of which 113 of those were successfully sequenced and analyzed. Overall, the sequencing samples represent a total volume of 2,590 L of first flush water and 36,100 L of water sampled across the whole network. (For comparison, this volume is equivalent to that of 361,000 grab samples of 100 mL each). Building on the findings from this study, future research can be designed to better understand and isolate specific factors that influence the water microbiome.

For example, in future studies it would be insightful to collect pore water and soil samples from areas surrounding IWS drinking water pipes to identify microbial taxa that could be used to distinguish intrusion from other mechanisms that cause changes in the microbial community (14). This approach has been used in continuous water systems to a limited extent to assess intrusion contamination (22). Combining sequencing data with a measure of total bacterial cells, such as by flow cytometry or qPCR, would allow comparison of absolute abundance and provide more insight into whether differences between entrance, first flush, and stable supply samples

were due to growth or intrusion of cells. Future studies could also focus on smaller portions of a single distribution system and aim to collect even numbers of samples from each location. To complement studies in dynamic real-world systems that contain IWS, it may be necessary to develop a model IWS system that can be operated under more consistent conditions (e.g., buried pipe that is operated on a supply schedule with consistent pressure and chlorine residuals). Furthermore, household water storage is a key feature of IWS that can change the microbial composition of drinking water; yet little is known about the microbial composition of stored water in IWS settings (92,93). Future research should also consider incorporating other types of sequencing and sequencing approaches that can distinguish nucleic acids from viable and non-viable cells, provide higher resolution to detect pathogens of concern, or to elucidate function (70,90). Overall, to further our understanding of IWS microbiomes and translate this knowledge into strategies to manage health risks, more laboratory and field studies are necessary that incorporate a range of scenarios and methodological approaches.

4. CONCLUSIONS

The water system in Arraiján is dynamic and composed of three DWTPs and a distribution network with both continuous and intermittent supply. Here, we used DNA metabarcoding alongside traditional methods to characterize water quality and investigate bacterial abundance, diversity and taxonomy throughout the three centralized surface DWTPs and distribution network and highlight the influence of IWS. A unique but diverse microbiome existed within each of the three DWTPs, dominated by Proteobacteria but with differences at the family and genus level. Treatment processes were found to have varied effects on bacterial diversity and structure at the different DWTPs, likely driven by DWTP operations and different water sources.

In addition, similar treatment processes enriched different bacterial taxa at different DWTPs. Diversity and bacterial community composition did not vary between Filtration and Disinfection samples for regions A and B but did for Region C. In addition, bacterial communities in the DWDS varied significantly from those in the finished water at the DWTPs. The abundance of the genera *Obscuribacter* (Cyanobacteria) and *Phreatobacter* (Proteobacteria) and the increase in the number of unique ASVs in the DWDS across all regions suggests a strong influence from the pipe network on bacterial communities in drinking water. In the IWS zone, several taxa were detected in first flush samples that were not present upstream or after 24 hour of stable supply, which could indicate intrusion and/or growth of microorganisms between supply cycles. *Pseudomonas* was among the most abundant taxa in IWS; yet, further research is needed to assess how IWS conditions may be conducive to the proliferation of *Pseudomona*s and other potential opportunistic pathogens and which factors are key for this proliferation to occur.

Our results provide useful context for assessing the impacts of IWS alongside other factors that shape the water microbiome. While the study is primarily descriptive, the results demonstrate important changes in the bacterial communities found across different stages in the water delivery chain and specifically in IWS, and we provide recommendations for future research that aim to describe the impacts of IWS. Ultimately, a deeper understanding of the microbial communities in piped, centralized drinking water systems in low- and middle-income countries, and how the unique features of IWS impact their water microbiomes, will provide a better characterization of the potential risks of IWS. This knowledge can be used to improve the design and management of drinking water infrastructure to protect water quality and public health.

DATA AVAILABILITY

Raw sequence files and metadata, are available on Figshare at: https://figshare.com/s/841335a0a1bcb5cee7a2

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