Title

Towards a better understanding of water microbiomes from source to tap in a rapidly urbanizing area with a tropical climate

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Towards a better understanding of water microbiomes from source to tap in a rapidly urbanizing area with a tropical climate

By

Karina Chavarria

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of the

University of California, Berkeley

Committee in Charge:

Professor Kara L. Nelson, Chair

Professor Ashok J. Gadgil

Professor John D. Coates

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Abstract

Towards a better understanding of water microbiomes from source to tap in a rapidly urbanizing area with a tropical climate

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Karina Chavarria

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Advisor: Kara L. Nelson, Chair

Access to safe drinking water remains a key concern across the globe. From the water source to the tap, many factors can influence microbial communities in water. This dissertation explores bacterial communities at various stages in the drinking water system serving the peri-urban region of Arraiján, Panama. First, the influence of land use and reforestation efforts on stream water bacterial communities was evaluated. Bacterial communities in the water column of four streams in the Panama Canal Watershed were assessed, each influenced by a different land use common in the Neotropics: mature forest, secondary forest, silvopasture and traditional cattle pasture. Land use was a key factor in microbial diversity and community composition. Streams influenced by forested areas exhibited diversity and community structure significantly different than streams influenced by lands with cattle. Reforested areas and agroforestry efforts such as silvopastures have the potential to protect water quality and significantly influence nearby water microbiomes.

Moving downstream in the drinking water system, the effects of water treatment and distribution were evaluated in the region of Arraiján, Panama, as well as the effects of intermittent water supply in a section of Arraiján's drinking water distribution system. Treatment processes were determined to have varied effects on bacterial diversity and structure at the three different drinking water treatment plants surveyed. Water quality parameters such as chlorine concentration and temperature correlated with bacterial composition in treated drinking water. In the distribution system, bacterial diversity and structure varied spatially. Intermittent water supply significantly influenced microbial communities in drinking water, driving the proliferation of *Pseudomonas* and exhibiting higher number of unique bacteria that could result from intrusion or regrowth between supply cycles. Two key features of intermittent water supply were assessed in controlled laboratory bench-scale experiments: stagnation and drainage. Stagnation led to higher bacterial concentrations and significantly influenced bacterial diversity and structure. The effect of drainage was evident only in biofilm bacterial communities. The application of DNA sequencing to study source water and treated drinking water demonstrated the ability to characterize changes to the microbiome that would not have been evident through the use of traditional water quality parameters. This work provides helpful insights into drinking water bacterial communities in a tropical setting that could be useful for better managing water systems in similar settings.

I dedicate this thesis to:

My mother, Irma

My father, Daniel

My brother, David

My partner, Christopher

My beloved country Guatemala

Para todos los migrantes alrededor del mundo en busca de una vida mejor
(To all migrants around the world searching for a better life)

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*

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Chapter 1

Introduction

1.1 Motivation

In many parts of the world, including Latin America and the Caribbean (LAC), forests create critical areas that provide protection to water resources (Aide et al., 2013). Land use changes in a watershed can significantly impact water quality downstream. Conversion of natural environments into anthropogenic landscapes to cater to the increasing human demand for resources is one of the main factors behind the degradation of water quality (Barten and Ernst, 2004). Increases in agricultural and urban development have been described as one of the greatest contributors to the increase of nutrients and sediments in freshwater ecosystems worldwide (Mello et al., 2018; Morton et al., 2006). In the tropics, land use has changed drastically with significant conversion of forests to subsistence farms and cattle pastures (ACP, 2010, 2006; Endreny, 2005; Wohl et al., 2012). Non-point source pollution such as agricultural runoff, which can contain fertilizers, pesticides, microorganisms and nutrients from livestock and manure, can cause a number of water quality problems, including algae blooms, fish kills due to low dissolved oxygen concentrations in the water, and microbial risks in drinking water. Non-point source pollution, however, is difficult to assess due to the complex and diffuse nature of interactions between hydrologic and landscape patterns (Barten and Ernst, 2004; Ivey et al., 2006). It is well accepted that watersheds with high natural forest cover provide better water quality than other land uses (Mello et al., 2018). Wang et al. 2013 associated forestland with good water quality by comparing forests to farms and urban lands (R. Wang et al., 2013). Streams in protected forests have been found to have better overall water quality (Huang et al., 2016; Knee and Encalada, 2014; Mello et al., 2018). However, many tropical watersheds are today a mix of forests in varying degrees of conservation, with patches of agriculture and other land uses (Mello et al., 2018; Ribeiro et al., 2009; Singh and Mishra, 2014). Thus, the effects of land use on water quality in tropical watersheds with a mixed forest-agricultural landscape need further research.

Recent international initiatives have brought forth efforts to halt deforestation, increase forest restoration and improved agricultural practices. Forest recovery through tropical secondary forest has provided improved forest health and ecosystem services. In LAC, over 35 million hectares of

woody vegetation began recovery between 2001-2010 (Aide et al., 2013). Tropical secondary forests have become an important source for timber, environmental services such as protection from erosion and atmospheric carbon fixation, and forest rehabilitation. However, such forests are not, in many aspects, equivalent to faunally intact-old primary forest.

In recent years, agroforestry has also demonstrated to provide environmental accountability and enhanced production. Within agroforestry, silvopastures, which combine trees and livestock with forage to form an agricultural system, have gained popularity as an alternative land use system that is economical, feasible, and ecofriendly (Jose and Dollinger, 2019). Most studies on silvopasture systems have focused on forage production, livestock performance and environmental services (e.g., carbon storage, efficient nutrient cycling, shade and food for livestock) (Jose et al., 2019; Jose and Dollinger, 2019). However, no study, to my knowledge, has assessed (in LAC) how these forests restoration efforts and improved agricultural practices influence microbial water quality and microbiomes in water and how these microbiomes compare to water microbiomes found near traditional grazing pastures and old forest at the same time. With increasing changes in tropical watersheds, it is important to understand the impact of land-use on our natural resources. There is a need to further our understanding of the role of forest areas as indicators of water quality in the current landscape in tropical watersheds. In addition, further understanding is needed on the influence of common land uses such as secondary forests and silvopastures.

Once a water resource becomes the source of drinking water for a particular population, it is typically the responsibility of the water utility to provide water in adequate quantities that is safe for consumption. Drinking water treatment relies on several physico-chemical processes to ensure the provision of safe water. Among conventional treatment processes, rapid sand filtration and disinfection are recognized as causing the greatest changes in water microbial composition (E. I. Prest et al., 2016; Zeng et al., 2013). Biofiltration, via sand filtration, is one of the oldest methods for water treatment and is traditionally designed for particle removal, including pathogens. Although the role of biological filtration in shaping microbial communities of finished water is not well understood, it has been suggested that the filtration process itself can exert environmental pressures and select for different communities that develop in the filtration media, triggering differences in effluent communities and affecting the overall quality of the drinking water (Pinto et al., 2012; Vignola et al., 2018). Disinfection, the final step in treatment, is critical to inactivate pathogens and inhibit microbial growth during distribution (Xu et al., 2017). However, disinfection is not effective at inactivating the entire microbiome of drinking water; rather, it exerts a selective pressure on microbial communities (Gomez-Alvarez et al., 2014; Holinger et al., 2014; Wang et al., 2014a). Previous studies on the effects of chlorine disinfection on microbial composition have yielded contrasting results, demonstrating either significant influence on microbial composition or no significant changes after filtration in water samples taken at drinking water treatment plants (DWTPs) (Bruno et al., 2018; C. Li et al., 2017). These findings indicate that the microbiome of the finished treated water that is later supplied to the drinking water distribution system (DWDS) may depend on how each treatment process is carried out and operated at DWTPs. Thus, understanding the influence of water sources and treatment on microbial communities can help guide strategies for controlling microbial communities (including pathogens) in DWTPs and the finished water. Once water is treated, it is supplied by a complex distribution network.

DWDSs are built and managed with the purpose of delivering safe water from the DWTP to consumers' taps. While piped water (DWDS) supply on household premises is considered the highest water service level, not all such supplies provide safe water and the quality of service can vary significantly. DWDSs can suffer from operational deficiencies and high rates of water loss that can lead to inadequate service, water quality problems, and supply becoming intermittent in resource-constrained settings. As water demand continues to increase due to rapid urbanization and water supplies become more vulnerable due to factors such as land use and climate change, DWDSs operating under substandard conditions will continue to pose many challenges to the provision of safe water and to the goal of achieving water security. Intermittent water supply (IWS), a common practice in low- and middle-income countries, is defined as piped water supply service available to consumers for less than 24 hours per day on average (Simukonda et al., 2018). IWS is estimated to affect at least one billion people worldwide (Bivins et al., 2017). In LAC, 60% of the population is served water intermittently (PAHO and WHO, 2001). One-third and one-half of urban supplies in Africa and Asia, respectively, operate intermittently (WHO and UNICEF, 2000).

While prevalent, IWS is a substandard water service. Insufficient water resources, poor infrastructure, unplanned expansions in the DWDS, excessive water losses, increasing consumer demands, or a combination of these factors lead to a drinking water system supplying water intermittently (Klingel, 2012; Kumpel and Nelson, 2014; Rosenberga et al., 2008). IWS is an inconvenience for users, requires consumers to store water, can lead to pipe damage, and can make it difficult for utilities to deliver an equitable supply to all consumers (Klingel, 2012; Lee and Schwab, 2005; Vairavamoorthy et al., 2007, 2008). IWS can also pose serious health risks. Bivins et al., 2017, estimated that IWS might account for 17.2 million infections leading to millions of cases of diarrhea and 100,000 diarrheal Disability Adjusted Life Years (DALYs).

In IWS, there are key features that may impact drinking water microbiomes. In particular, pipes can experience drained periods and water stagnation between supply cycles. Biofilms and loose deposits in drained pipes may experience dry periods (depending on the duration of the supply cycles) and subsequent re-wetting periods when supply is restarted. Biofilm-associated microbial communities are likely affected by dry/re-wetting periods, depending on the biofilm's susceptibility to desiccation, leading to potential changes in diversity and community structure and drive the survival of desiccation-resistant microbial communities (Sabater et al., 2016; Timoner et al., 2014, 2012). Water stagnation in IWS can occur in sections of the DWDS that cannot to drain, such as low-lying pipes. Changes in microbial community structure due to stagnation in IWS systems are likely to occur. They will depend on the duration of the stagnation periods, the persistence of disinfectant residual and the composition of the microbial community (Bautista-de los Santos et al., 2019). To my knowledge, no study has been carried out to assess microbial communities in water from an entire drinking water system (from source to tap) with IWS.

Cultured-based techniques have been widely used to assess microbial water quality in water resources, DWTPs, DWDSs and IWS systems to determine water quality. The use of indicator organisms and other traditional water quality parameters is the standard approach to assessing water quality deterioration across many water environments (Baxter-Potter and Gilliland, 1988; Davies-Colley et al., 2004; Hazen and Toranzos, 1990; Kumpel and Nelson, 2014). However, indicator organisms do not represent the behavior of all pathogens nor the entire water microbial

community. Through culture-based approaches, only a minor fraction of the microbial profiles of water can be detected and microbial communities need to be considered as a whole to better predict water and infrastructure quality (Bautista-De los Santos et al., 2016). Microbial communities in IWS have been researched to a limited extent using DNA sequencing approaches, but existing studies have not shed light on how key features of IWS impact microbial communities (Montoya-Pachongo et al., 2018; Tokajian and Hashwa, 2004). Furthermore, limited research has been conducted to investigate microbial communities in water in tropical environments; Importantly, many countries where land conversion is impacting water resources, and IWS is prevalent are located in the tropics (Guariguata and Ostertag, 2001; Kozovits and Bustamante, 2013; Kumpel and Nelson, 2016). Understanding how unique features of IWS impact water microbiomes in a full-scale drinking water system and through laboratory-scale control experiments may help to better characterize risks of IWS and provide insight into strategies to protect water quality.

1.2 Dissertation research objectives

This dissertation explores water bacterial communities at various stages of the drinking water cycle in Panama, Central America, through 16S rDNA metabarcoding in combination with traditional measures of water quality. Four sub-watersheds, part of the Panama Canal Watershed (PWC), were examined to assess the effects of land use on water quality and stream bacterial communities (**Figure 1.1**). These streams eventually end at Gatún lake, one of the main sources of drinking water for Panama City and adjacent regions (including Arraiján). To further our understanding of drinking water systems and IWS, the drinking water system of Arraiján, Panama, was investigated (**Figure 1.1**). Arraiján is a rapidly growing peri-urban area located west of the Panama Canal and part of the PCW. This research aimed to characterize bacterial communities in source waters and how the bacterial community in drinking water is influenced by treatment, distribution and IWS. In addition to extensive field surveys conducted, a laboratory experiment was carried out to further investigate the effects of two key features (stagnation and drainage) of IWS on bacterial communities in a controlled setting. I hope this research work can aid in developing recommendations to current water management strategies and future research to protect water quality in resource-constrained regions.

The main objectives were:

1. *Characterize bacterial diversity of water resources in Panama and assess the effects of land use on microbial water quality.*

2. *Explore bacterial diversity of drinking water and assess the effects of treatment processes and distribution bacterial communities.*

3. *Assess the impact of IWS cycles on bacterial communities in drinking water in a full-scale DWDS.*

4. *Characterize bacterial communities in biofilm and bulk water before and after drainage and stagnation, common features of IWS.*

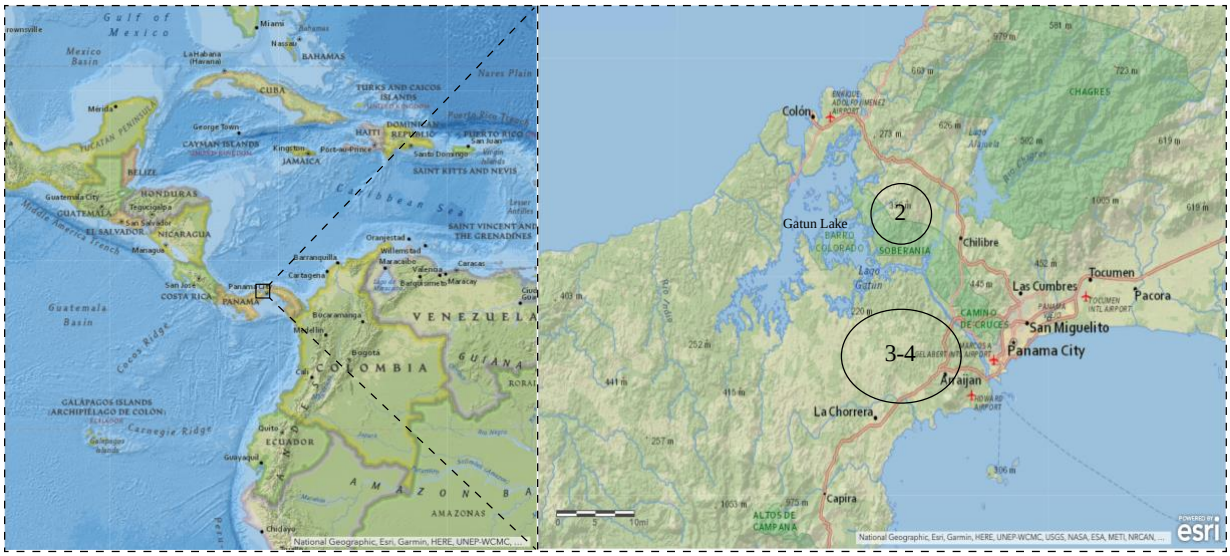

Figure 1.1. Map of study sites. **Study sites are described in detail in Chapters 2-4. Field work was carried out in the Panama Canal Watershed, Panama, Central America, in an area that is part of the Agua Salud Project from the Smithsonian Tropical Research Institute (Chapter 2) and in the peri-urban region of Arraiján, Panama (Chapters 3 and 4). All study sites are part of the same watershed.**

1.3 Dissertation overview

The chapters in this dissertation describe the use of molecular methods combined with traditional approaches to assess water quality and water microbial communities from sources to taps in a rapidly growing area in Panama, Central America. **Chapter two** focuses on drinking water resources, specifically on the influence of land use changes due to deforestation, reforestation, and agricultural practices on bacterial communities in nearby streams. This study was carried out through a two-year field survey that consisted of sampling (weekly) four distinct streams, that were adjacent to four different land uses in the Panama Canal Watershed (PWC) and that are common in the Neotropics: mature forest, secondary forest, silvopasture and traditional cattle pasture.

In **Chapter three**, three DWTPs serving Arraiján, Panama, were investigated to assess how source water and treatment processes affect the drinking water microbial communities. Sampling was carried out during three sampling excursions at the source water intake pipes before any treatment, after sand filtration, and after disinfection processes. In addition, eighteen locations were selected throughout the DWDS serving Arraiján to characterize temporal and spatial variations of microbial communities in DWDSs with IWS. Sampling excursions to the DWDS were conducted one to three times a week for one year.

Chapter four focuses specifically on a section of the Arraiján DWDS with IWS to explore the effects of IWS on bacterial communities in drinking water. One neighborhood that was part of Arraiján's distribution system was chosen to further examine the effects of intermittency. Supply to this neighborhood was rationed, by the water utility, with a valve at the entrance to the neighborhood. Due to insufficient distribution capacity in the area, the operational schedule was

Three-day supply cycles (three days on, three days off). However, actual supply during the study period often deviated from this schedule, with longer periods between supply cycles. Microbial water quality was compared between samples taken during the first flush of water from the tap after supply restarted and 24 hours after supply had stabilized. Sampling was carried out one year.

Chapter five examines key features of IWS, specifically stagnation and drainage, in simplified laboratory reactors. To investigate the effects of stagnation and drainage on biofilm and bulk water microbial composition, an experiment was conducted using three biofilm rotating annular reactors (ARs) to simulate hydraulic conditions found in DWDSs, including biofilm formation in PVC pipes. The ARs were operated differently to simulate three conditions: i) Continuous supply; ii) IWS where pipes do not drain causing water stagnation, and; iii) IWS where pipes drain completely. The stagnation and drainage periods were designed to simulate IWS conditions in the DWDS in Arraiján and lasted three days, with four days of CWS in between each stagnation/drainage event. This bench-scale laboratory experiment was carried out for nine months.

Lastly, **Chapter six** provides a summary of the contributions of this research and a discussion of their significance. Research limitations, lessons learned, and future research needs are also discussed.

Chapter 2

Land use drives stream bacterial community composition in lowland tropical watersheds: study case in the Panama Canal Watershed

2.1 Introduction

Microbial communities in freshwater environments play a significant role in global energy fluxes and diverse biogeochemical (e.g., C, N, P, S, among others) processes. Freshwater ecosystems host distinct microbial communities associated with water and sediment (Hutchins and Fu, 2017) and that can be influenced by stream nutritional status, hydrological regimes, and climate change (Hayden and Beman, 2016; He et al., 2015). In the temperate zone, stream microbial communities have been shown to shift with the conversion of natural ecosystems to agriculture (Ogden et al., 2013; Ramankutty et al., 2018; Rhodes et al., 2001). Given the ever-accelerating pace of global change across tropical landscapes and watersheds (Aide et al., 2013; Ribeiro et al., 2009; Romero et al., 1999), it is imperative that we understand drivers of stream microbial community diversity and composition in order (as a precursor) to ensure long-term stability of key biogeochemical ecosystem processes as well as water quality.

Tropical watersheds are critical to the provision of ecosystem services that sustain livelihoods and ensure the well-being of nearly three billion people (Christian et al., 2019; Hall, J.S.; Moss, D.; Stallard, R.F.; Raes, L.; Balvanera, P.; Asbjornsen, H.; Murgueitio, E.; Calle, Z.; Slusser, J.; Kirn, 2015; Pan et al., 2011; Vörösmarty et al., 2000). Watershed residents and their downstream neighbors rely on freshwater ecosystems for the provision of abundant, clean water for drinking and irrigation, protein, energy, as well as recreation and other cultural ecosystem services (Hall, J.S.; Moss, D.; Stallard, R.F.; Raes, L.; Balvanera, P.; Asbjornsen, H.; Murgueitio, E.; Calle, Z.; Slusser, J.; Kirn, 2015). In addition, freshwater streams exchange water, energy, materials, and nutrients with the surrounding environment such that water quality, sediment characteristics, and biological communities reflect characteristics from upstream and even downstream environments. Thus diversity and community structure of stream biota are associated with water and sediment (Hutchins and Fu, 2017) and can be influenced by stream nutritional status, hydrological regimes, and environmental change (Cavaco et al., 2019; De et al., 2020; Hayden and Beman, 2016; He et al., 2015; Shabarova et al., 2021) and may reflect levels of disturbance and degradation (Hilderbrand et al., 2020).

Freshwater streams receive water from the adjacent land, making them especially vulnerable to the impacts of land-cover change, such as conversion of tropical forest to cattle pasture. Studies have shown that a range of stream properties can change with a changing landscape, including water temperature, total dissolved solids, and water flow paths (Barten and Ernst, 2004; Bramley and Roth, 2002; Butler et al., 2013; Gregory Martin, Chansotheary Dang, Ember Morrissey, Jason Hubbart, Elliot Kellner, Charlene Kelly, Kirsten Stephan, 2020). Further questions regard the extent to which stream microbial community diversity and structure is maintained or can recover as forests are allowed to regenerate in the surrounding land and the tradeoffs made when managing land for livelihoods and other ecosystem services.

Recent studies have highlighted the potential of naturally regenerating tropical secondary forests to accrue biomass rapidly (Poorter et al., 2016) and sequester vast quantities of carbon such that facilitating and incentivizing these second growth forests can be a valuable tool in combating climate change (Chazdon et al., 2016; Hall, J.S., Plisinski, J.S., Mladinich, S.K., van Breugel, M., Lai, H.R., Asner, G.P., Thompson, 2021). Given that these forests have been shown to recover 80% of their tree species diversity in 50 years (Rozendaal et al., 2019) and can recover a significant portion of both saturated hydraulic conductivity (Hassler et al., 2011) and flow paths in the bio-perturbation zone (Birch et al., 2021b, 2021a) in less than a decade post cattle removal, promoting the growth of secondary forests represents an apparent win-win solution for restoring ecosystem services. However, to date, little is known about how tropical secondary forests influence water quality and water microbiomes.

Animal husbandry in the form of raising cattle, goats, sheep, horses, and other domesticated species cover over one-quarter of the land area of LAC, with clearing forest for cattle production being a major driver of deforestation (FAO, 2011; Hall, J.S.; Moss, D.; Stallard, R.F.; Raes, L.; Balvanera, P.; Asbjornsen, H.; Murgueitio, E.; Calle, Z.; Slusser, J.; Kirn, 2015). Given the cultural context of cattle ranching and the human preference for beef and milk, it is unrealistic to expect this to stop. The need to find solutions where some tropical forest ecosystem services can be restored or maintained while the land can also be used for food production (Knoke et al., 2016) has sparked intense interest in silvopasture systems. Compared to traditional cattle production, silvopasture systems have several environmental benefits, including carbon sequestration by trees and restoration and protection of riparian zones by living fences and gallery forests that serve as corridors connecting forest patches across the landscape (Dibala, 2019; Jose, 2009; Jose and Dollinger, 2019). While protecting and planting riparian corridors, a key component of silvopastures, have been shown to promote better water quality (Hilary et al., 2021; Jose, 2009), little is known as to how these practices impact nearby water microbial communities.

This study assessed how secondary forest regrowth and improved agricultural practices influence water quality and stream bacterial communities by comparing stream water from sites where adjacent lands are managed for known ecosystem service tradeoffs. These streams have no known point sources of pollution and are adjacent to four land use types that are common throughout the Neotropics: a) Mature forest (MF), b) Young secondary forest (SF), c) Silvopasture (SP), and d) Traditional cattle pasture (CP). The main research questions asked: 1) How does land use influence headwater stream bacterial communities? 2) Which bacterial taxa (i.e., indicators) are associated with the different land uses? and 3) How do traditional water quality metrics correlate with microbial community structure in this tropical landscape? The results are discussed in the context

of informing management decisions, including maintaining and restoring riparian corridors to protect freshwater microbial community diversity and structure in rapidly changing tropical landscapes that are under intense pressure to provide for local livelihoods and ecosystem services.

2.2 Materials and methods

2.2.1 Study Site

For over two years, from June 2017 to August 2019, weekly samples (2L) were collected at four experimental watersheds within the Agua Salud facility of the Smithsonian Tropical Research Institute (STRI), located in the Panama Canal Watershed (PWC) of central Panama (9°13'N, 79°47'W, 330 m above sea level) (**Figure 2.1**). The Agua Salud Project (Stallard et al., 2010) seeks to understand the ecosystem services produced by tropical forests in a seasonal climate and how they change with land use and climate change. The study area is characterized by a dense network of streams in a landscape of ridge-like hills with steep slopes – a tropical steepland (van Breugel et al., 2013). Mean annual rainfall at the principal meteorological tower is 2700 mm (Ogden et al., 2013), with annual precipitation during this study of 2,188, 3,055, and 2,339 mm for the years 2017, 2018 and 2019, respectively (Paton, 2019).

Four streams were selected, located in paired experimental watersheds at Agua Salud where stream flow is continually monitored (Stallard et al., 2010), and other upstream uses do not confound land use. These four stream catchments have similar morphologies, soils, underlying geology, and rainfall (Ogden et al., 2013). The streams all show strong seasonal variation in base-streamflow, with reduced flow during the dry season (Ogden et al., 2013). Each stream is embedded in a different land use: MF, SF, SP and CP. The 9.5 ha MF catchment has had continuous forest cover for over 80 years, the earliest dates of STRI's aerial photos. Cattle were removed from the 6.1 ha SF catchment in 2007, one year before land acquisition by the Agua Salud Foundation and transfer of management oversight to STRI. The catchment was a distinct management unit or paddock where cattle entered the stream to drink and for shade. Riparian forest covered the entire length of the stream at the time of cattle removal (14% of the SF area) and secondary forest has been allowed to regenerate naturally throughout the remaining catchment since 2007. The 42.4 ha CP has been under traditional management since before 1980 (Ogden et al., 2013), with cattle rotated monthly between this and another pasture 5 km distant. While the 9.4 ha SP management was not established until 2015, the pasture area had been maintained as traditional pasture for at least 40 years prior to planting of improved pasture and fencing of the riparian zone. Both the CP and SP systems maintain significant tree cover (25 % in CP and 32 % in SP), but riparian forest makes up less than 10 % of CP and all 32 % of the SP system. Cattle have complete access to the streams along their length in the CP system, whereas stream channels in the SP were fenced in 2015 to limit cattle entrance to a narrow access point for drinking. Both cattle systems (CP and SP) maintain similar cattle densities at 0.64 and 0.85 head per ha, for CP and SP, respectively. However, while the cattle are in the SP system year-round, in the CP system they are rotated every 30 days between our research catchment and a different farm outside of our research system.

To assess the potential importance of forest buffers, we divided each stream into sections with and without riparian forest buffer on either side of streams during the duration of our study. Stream length was measured from the recorded natural spring where water flow begins to the stream weir.

Only the CP system had deforested lengths, which amounted to 50% of the cumulative length of the principal stream and higher-order tributaries (**Figure 2.1**).

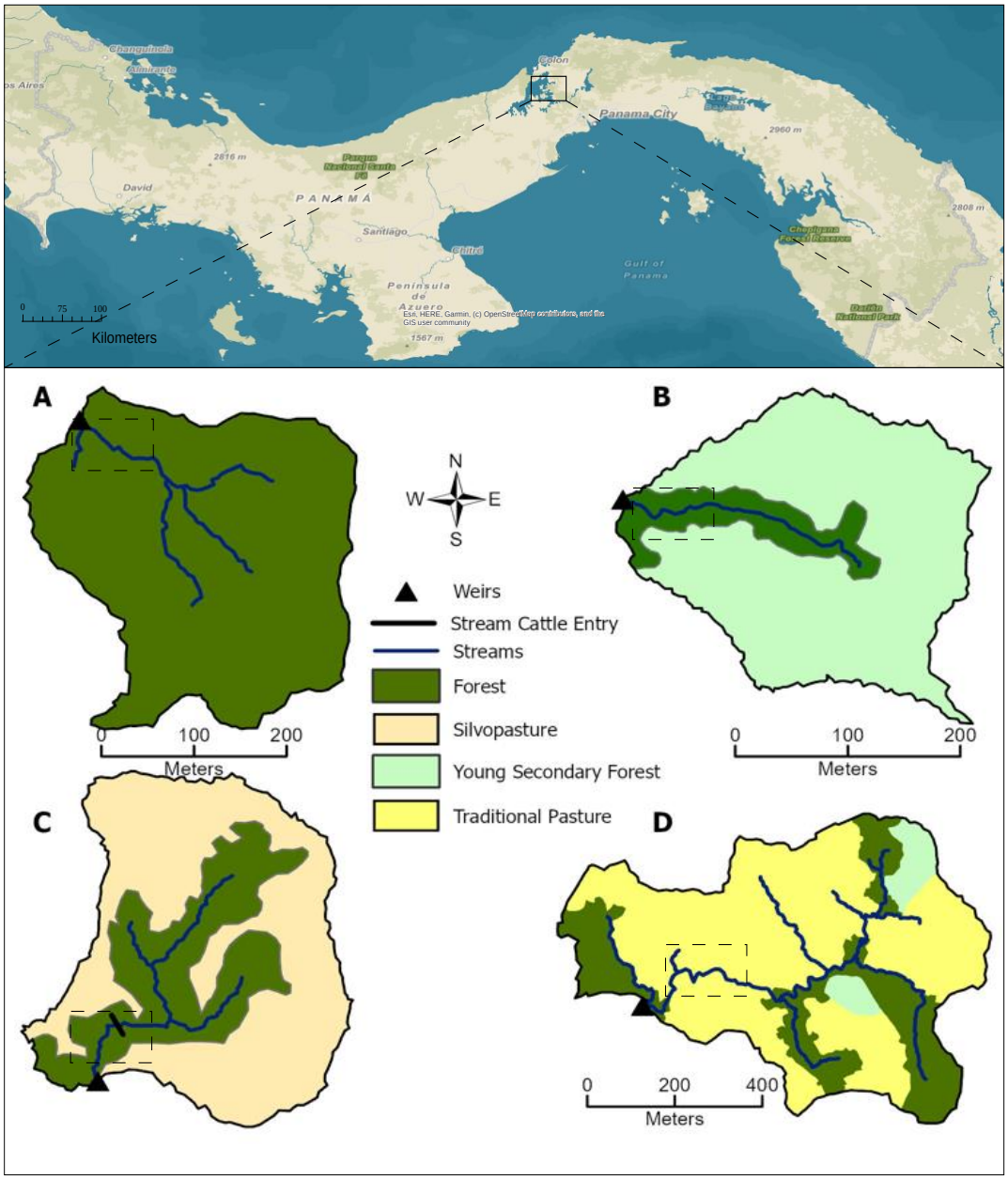

Figure 2.1. Sampling sites A) Map of Panama indicating where Agua Salud is located; B) Schematics of four experimental watersheds in the Agua Salud site showing catchment areas and land use cover. Dark green indicates older and riparian forest, light green indicates young secondary forest (approximately10 years old), tan indicates silvopasture, and yellow indicates traditional cattle pasture. Dash squares indicate weirs where samples were collected.

2.2.2 Sampling, water quality and nutrient measurements.

Weekly water samples were collected from the MF, SF, SP and CP streams following relevant protocols as described in Murphy, S.F., and Stallard, 2012 and the USGS Fact Sheet 2010–3121: Water-Quality Sampling by the U.S. Geological Survey) (n = 451). Most samples were collected between 8:00 A.M. and 12:00 P.M. during baseflow conditions and were transported at 4° C to STRI's laboratory for processing within three hours of collection.

Turbidity, pH, temperature, conductivity, total dissolved solids and dissolved oxygen were measured and recorded on site during each sample collection. Turbidity was measured with a field-portable turbidimeter (MicroTPW, HF Scientific Inc., Fort Myers, FL, USA), pH, temperature, conductivity and total dissolved solids were measured with a HANNA portable multiparameter meter (HANNA Instruments, Smithfield, RI, USA) and dissolved oxygen was measured using a portable dissolved oxygen meter (HANNA Instruments, Smithfield, RI, USA). Measurements of nitrite, nitrate, phosphate, sulfate, iron and ammonia concentrations were measured in the laboratory using the HACH DR 900 Multiparameter Colorimeter (HACH, Vernon Hills, IL, USA). All previous measures were performed following the manufacture's recommendations.

Total coliform and *E. coli* (One hundred mL water samples) were analyzed using Colilert reagents (IDEXX Laboratories, Inc., Westbrook, ME, USA). Bacterial concentrations were quantified using the most probable number method with Quanti-tray 2000 trays (IDEXX Laboratories, Inc.). Samples were incubated at approximately 35° C for 24 hours. Microbial detection limits for total coliform and *E. coli* had a lower bound of < 1 MPN/100mL and an upper bound of > 2419.6 MPN/100mL.

2.2.3 DNA extraction, PCR amplification and Illumina sequencing.

Water samples for DNA isolation were stored at 4° C upon arrival at STRI's laboratory and filtered within 24 hours of collection. Each sample was filtered through stacked 1.2 µm and 0.22 µm 47 mm mixed cellulose filters (EDM Millipore, Burlington, MA, USA) using a vacuum pump. Sample filters were then were stored at -20°C until DNA was extracted. Isolation of DNA was performed using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with the following adjustments. To maximize DNA recovery, filters were cut into smaller fragments under sterile conditions prior to the addition of the PowerSoil beads and bead solution, as described in (Liao et al., 2015). Isolated DNA was eluted in a final volume of 60 µL. After DNA extraction, all samples were stored at -20°C until sequencing libraries were prepared.

Library preparation for amplicon sequencing of the V4 variable region of the 16S rRNA gene was conducted using a two-stage PCR protocol. First, the V4 region was amplified using the primer pair 515F (Parada) and 806R (Apprill) (Apprill et al., 2015; Caporaso et al., 2011; Walters et al., 2016), modified to include the Illumina sequencing primer on their 5' end and phased to improve sequencing run quality (Wu et al., 2015). PCR reactions included 5 µL Platinum 2X Mastermix (ThermoFisher), 1 µL of each primer (10 mM) and 2.5 µL of genomic DNA as template (12.5 µL total PCR volume). Thermocycler settings for PCR1 were: initial denaturation at 94°C for 3 min,

28 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s and a final elongation at 72°C for 10 min. PCR reactions were performed in triplicate, verified on a 1.5% agarose gel and pooled after amplification. PCR2 amplifications to add index and remaining Illumina adaptors were carried out with 2.5 μL of pooled PCR product in 12.5 uL reactions with the following program for amplification: 94°C for 3 min, eight cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s and a final elongation at 72°C for 10 min. Products were cleaned and normalized to equal concentrations using Just-a-Plate 96 PCR Purification and Normalization Kit following the manufacture's recommendations (Charm Biotech, San Diego, CA, USA). The resulting library was further cleaned with AMPure beads and quantified by fluorometric quantification with the HS (High Sensitivity) dsDNA Assay for Qubit (Invitrogen, Carlsbad, CA, USA) and Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA). Paired-end sequencing was performed on a 2 x 250 bp paired-end MiSeq run (Illumina, San Diego, CA, USA).

2.2.4 Data analysis

Quantitative Insights Into Microbial Ecology 2 (QIIME2) platform v2020.6 and R v.3.6.0 were used for the majority of sequencing data analyses (Bolyen et al., 2019). Primer trimming, sequencing quality control, and forward and reverse read merging were performed using QIIME2's Cutadapt and DADA2 plugins to produce amplicon sequence variants (ASVs) (Bolyen et al., 2019; Callahan et al., 2016; Martin, 2011). Taxonomy was assigned using a naïve Bayesian classifier trained on the SILVA 99% sequence similarity database (v32) (Pruesse et al., 2007; Wang et al., 2007). Sequences were further filtered using BLAST with a 97% confidence. ASVs with low abundance (0.005%) were filtered from the final working ASV table to avoid potential sequencing error (Bokulich et al., 2013). ASVs classified as mitochondria or chloroplast were also removed from further analysis.

Alpha diversity (Faith's Phylogenetic diversity (PD)), beta diversity metrics (Bray and weighted UniFrac), and principal coordinates analysis (PCoA) were estimated using QIIME2's q2-diversity after samples were rarefied to 2100 sequences per sample. Dry season – wet season comparisons were made by grouping samples based on their week of collection and following seasonal standards (Paton; 2019); dry seasons included Jan – April from 2018 and 2019, while wet seasons included May – Dec. of 2017, 2018, and 2019. To assess bacterial community structure and differences among land-use types, a network was constructed based on the weighted UniFrac distance matrix and a rarefied ASV abundance table developed in QIIME2 and was visualized using the R packages Phyloseq and igraph (Csárdi and Nepusz, 2006). Representative taxa for each land use were identified via indicator species analysis (permutation tests assessing the statistical significance of taxa-site group associations based on phi coefficient of association and the indicator value index (IndVal)) using the R package Indicspecies (De Cáceres and Legendre, 2009). This analysis determines a parameter ranging from 0 to 1 to assess the quality of the indicators based on the relative abundance and relative frequency across different habitat environments. A genus was considered an indicator taxon if the indicator value for a particular group is > 0.3 and if the taxa have a significant p-value ($p < 0.05$).

Non-parametric pairwise Kruskal-Wallis tests were performed to assess differences in ASV abundance and taxa at different ranks in different land-use types and between seasons. To evaluate differences between bacterial communities, complementary non-parametric analyses for

multivariate data were used: permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2017) and analysis of similarity (ANOSIM) (Clarke, 1993) using weighted UniFrac distance. Permutational analysis of multivariate dispersions (PERMDISP) was used to ensure significant differences were not due to differences in dispersion. Analysis of Composition (ANCOM) and Linear Discriminant Analysis Effect Size (LefSe) were used to identify differentially abundant bacterial ASVs between different land uses (Mandal et al., 2015; Segata et al., 2011). A significance threshold was established as $p < 0.05$ with multiple comparisons using 999 permutations. These statistical tests were done in QIIME2 with corresponding q2-diversity and q2-composition plugins. LefSe was computed in R with the microbiomeMarker package.

Correlations between relative abundances of taxa in each sample from each land-use type and all measured environmental factors were calculated using Mantel tests with 999 permutations. Benjamini-Hochberg corrections were performed to control for false discovery rates of *P* values. Canonical correspondence analysis (CCA) was performed to visualized associations between water parameters and community structure. For water quality comparisons, statistical tests for indicator bacteria were performed on their rank values of their log transformations. Untransformed data were used for turbidity, pH, temperature, conductivity, total dissolved solids, dissolved oxygen, nitrite, nitrate, phosphate, sulfate, iron, and ammonia. Tests for significance were performed using non-parametric Kruskal-Wallis and pairwise Wilcox tests with Benjamini-Hochberg corrections.

2.3 Results

2.3.1 General characteristics of sampled streams

Water quality parameters and nutrient concentrations were measured on site and in the laboratory. Concentrations of nitrate, nitrite, ammonia, phosphate, and sulfate and pH were similar across sites and seasons, while temperature, conductivity, total dissolved solids (TDS), dissolved oxygen (DO), turbidity, total hardness, and total iron concentrations varied (Kruskal-Wallis, $p < 0.05$) (**Table 2.1).** Temperature measures ranged between 23 and 28 °C. Temperatures from MF and SF were comparable throughout the study, while samples from SP and CF were significantly higher (Kruskal-Wallis, $p < 0.01$). Mean turbidity measures were below 20 NTU for all sites. The highest turbidity measures were found in samples from CF during the wet seasons. The lowest turbidities were found in samples from SF. Overall turbidity showed significant differences between all land uses (Kruskal-Wallis, $p < 0.0001$) (**Table 2.1)**. Conductivity measures ranged between 0.01 and 0.6 mS/cm and were highest among MF (Mean ± SD, 0.23 ± 0.08) samples followed by CF (Mean ± SD, 0.19 ± 0.08). Similar to conductivity, TDS ranged between 50 and 250 mg/L and was highest among MF samples, followed by CF. Conductivity and TDS were significantly different between sites (Kruskal-Wallis, $p < 0.01$) except between SF and SP (Kruskal-Wallis, $p > 0.05$).

The highest concentrations of dissolved oxygen (DO) in water were found in samples from MF and SF (**Table 2.1**). DO decreased in all streams during the dry seasons, but CP and SP had significantly lower concentrations (Kruskal-Wallis, $p < 0.001$). Stream water total hardness was highest in MF during the dry season and the lowest in CP during the wet seasons.

Nutrient concentrations were found to be comparable between samples taken at the different sites, except for total iron levels; total iron concentrations were particularly high in the CP (**Table 2.1**). However, it is important to note that our inability to detect differences in many nutrient concentrations could be linked to the field meters used which most likely were not sensitive enough to accurately measure their concentrations in these oligotrophic streams.

Table 2.1. **Description of sites and mean and standard deviation of water quality and nutrients metrics measured in four streams surrounded by different land uses in the Agua Salud research site, Panama. [a-d] Within a row, means without a common superscript are differ statistically ($p < 0.05$) by non-parametric Kruskal-Wallis and pairwise Wilcox (with Benjamini-Hochberg correction) tests. * Within each site, statistical differences were found between dry and wet seasons.**

Site Location	Mature Forest (MF)	Secondary Forest (SF)	Silvopasture (SP)	Cattle Pasture (CP)
Area (ha)	9.49	6.08	9.36	42.39
	n = 113	**n** = 111	**n** = 114	**n** = 113
	Mean ± SD	**Mean ± SD**	**Mean ± SD**	**Mean ± SD**
Runoff rate (mm/hr)	4.75 ± 13.04^{c}	4.36 ± 12.20^{d}	5.88 ± 26.66^{b}	6.11 ± 29.45^{a}
pH	6.89 ± 0.38^{a*}	6.97 ± 0.36 ^{a*}	6.86 ± 0.38 ^{a}	6.80 ± 0.45^{a*}
Conductivity (mS/cm)	0.23 ± 0.08^{a*}	0.14 ± 0.06^{c}	0.15 ±0.09^{c}	0.19 ± 0.08^{b*}
TDS (mg/L)	141.0 ± 31.9^{a*}	85.8 ± 18.4^{c}	82.8 ± 19.9^{c}	127.9 ± 53.2^{b*}
Temperature (˚C)	25.3 ± 0.8^{b*}	25.4 ± 0.8^{ab*}	25.7 ± 0.9^{a*}	25.6 ± 0.8^{a*}
Dissolved O2 (mg/L)	6.82 ± 1.04^{a*}	6.36 ± 0.78^{b*}	5.59 ± 1.19^{c*}	5.30 ± 1.40^{c*}
Turbidity (NTU)	7.83 ± 18.56^{b*}	3.46 ± 11.09^{c}	3.26 ± 3.50^{d}	19.66 ± 48.86^{a*}
Hardness (mg/L)	87.6 ± 45.4^{a}	52.1 ± 25.5^{b}	45.2 ± 9.8 ^{b}	44.2 ± 14.5 ^{b}
Iron (μM/L)	3.34 ± 1.87 ^{b*}	4.62 ± 4.37 ^{b}	5.80 ± 6.64 ^{b*}	24.08 ± 11.74^{a}
Nitrite (μM/L)	0.12 ± 0.49 ^{a}	0.20 ± 0.83 ^{a}	0.13 ± 0.47 ^{a}	0.12 ± 0.27 ^{a}
Nitrate (μM/L)	7.31 ± 5.85 ^{a}	9.12 ± 10.57 ^{a}	9.69 ± 13.64 ^{a}	8.71 ± 7.63 ^{a}
Phosphate (μM/L)	5.26 ± 5.40^{a}	3.85 ± 3.97^{b}	3.58 ± 4.38^{ab}	3.53 ± 2.78^{b}
Sulfate (μM/L)	2.48 ± 8.31^{a}	5.78 ± 10.44^{a}	4.58 ± 8.95^{a}	4.10 ± 7.64^{a}
Ammonia (μM TN/L)	2.32 ± 3.24^{a}	2.23 ± 0.73^{a}	2.14 ± 1.81^{a}	6.84 ± 12.99^{a}
Total coliforms (MPNx10⁴/100mL)	2.08 ± 1.40^{a*}	1.67 ± 1.33 ^{a*}	1.71 ± 1.29 ^{a*}	2.43 ± 1.62 ^{a*}
E. coli (MPNx10²/100mL)	4.91 ± 3.57^{b*}	3.32 ± 5.76^{b}	5.21 ± 6.14^{a}	7.57 ± 8.48^{a*}

2.3.2 Stream bacterial diversity and composition

Illumina sequencing produced a total of 3,682,634 high-quality reads that were assigned to 16,985 ASVs, corresponding to 1,210 genera, across 451 water samples from the different land-uses (range 2187 – 23444 sequences per sample). Sampling across streams was even with 111-114 samples for each land use. Rarefaction curves indicated that sequencing efforts were sufficient to capture the diversity of these communities (**See Appendix A, Figure 1A**). Forested streams had more unique ASVs (those not shared with other sites) (MF = 1894 unique ASVs corresponding to 97 genera and SF = 1815 unique ASVs corresponding to 96 genera) and CP the fewest (1442 unique ASVs corresponding to 46 genera). Alpha diversity of bacterial communities differed among streams (Kruskal-Wallis, $p < 0.05$**)**, with streams in forested catchments (MF and SF) exhibiting the highest diversity while CP had the lowest (**Figure 2.2**). Seasonal differences were also observed within each land-use type, with both CP and SP showing seasonal drops in overall diversity during the dry season months of both 2018 and 2019 that were much less pronounced in the forested catchments in 2018. All streams showed a drop in diversity towards the end of the dry season and the beginning of the wet season in 2019 (**See Appendix A, Figures A2-A5, A)**.

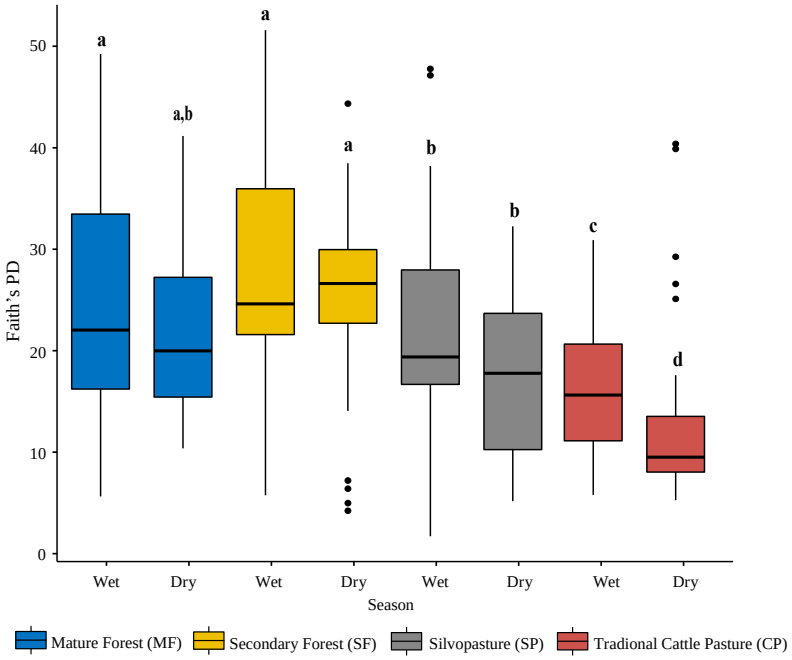

Figure 2.2. Faith's Phylogenetic Diversity (alpha diversity) of stream water in each land use by season. Letters indicate significant differences between land uses ($p < 0.05$) based on Kruskal-Wallis and pairwise Wilcox (with Benjamini-Hochberg correction) tests. Boxplot center lines show the medians, the upper and lower limits indicate the 25th and 75th percentiles, and the whiskers extend to 150% of the interquartile range. Outlier points are shown by dots.

Principal Coordinates Analysis (PCoA) using weighted UniFrac distances revealed that communities in CF samples were clearly different from those in the MF, SF and SP catchments (**Figure 2.3**). The first three PCoA axes accounted for ~ 54.6% of the variation, with the CF samples separating from the other land types mainly along the first axis (PCoA 1 = 29.83%). This difference was statistically significant and not due to dispersion of bacterial communities within land-use type (PERMANOVA, P < 0.001; ANOSIM, P < 0.0001; PERMDISP, *P* > 0.05). Distances based on compositional relationship of community members (i.e., Bray-Curtis) yielded similar results. PCoA using weighted UniFrac distances also revealed seasonal community patterns between samples within each land-use type (**See Appendix A, Figures A2-A5, B).**

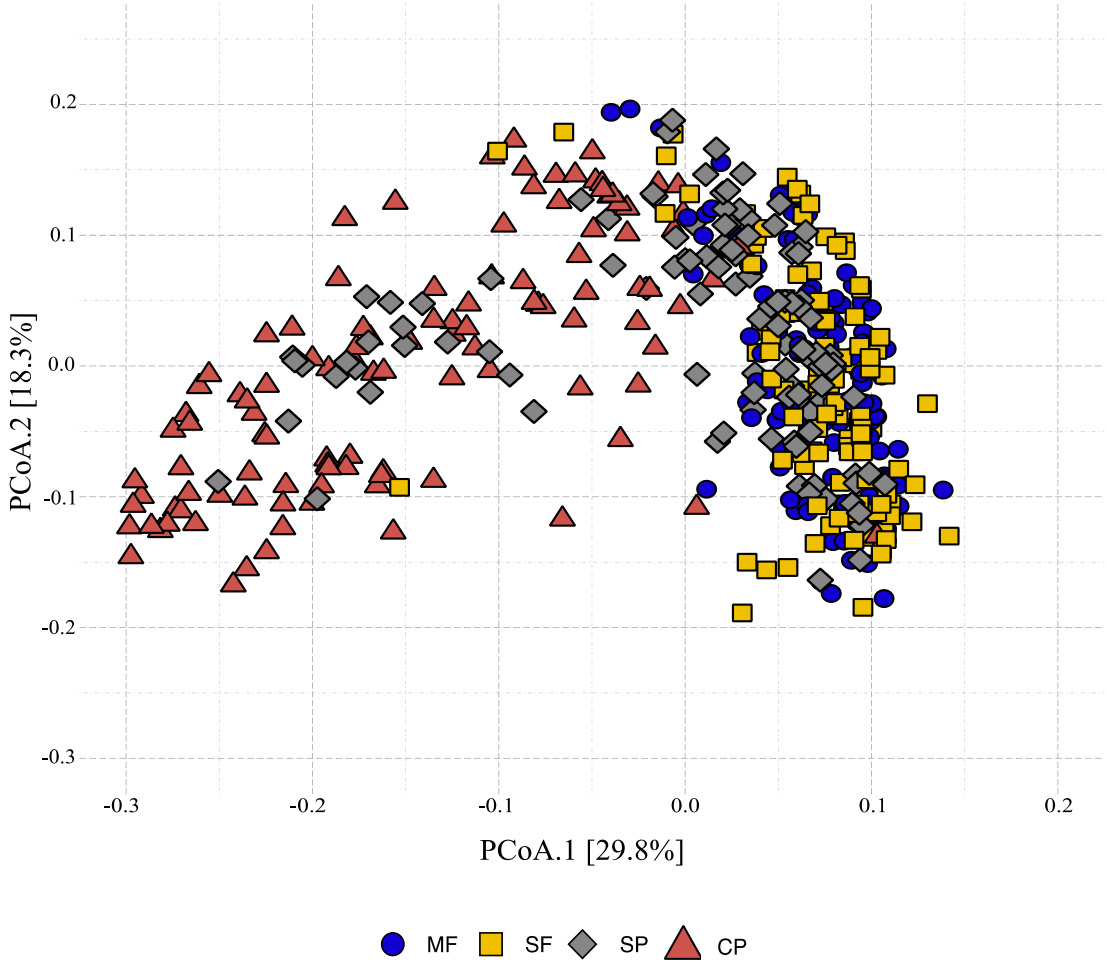

Figure 2.3. Principal coordinates analysis (PCoA) based on weighted UniFrac distances of stream water bacterial communities surrounded by different land uses. Community composition was determined from samples rarefied to 1200 sequences/sample. MF = Mature Forest, SF = Secondary Forest, SP = Silvopasture, CP = Cattle Pasture

To explore the effect of land use on bacterial interactions, we constructed an inferred bacterial interaction network using weighted UniFrac distances (**Figure 2.4**). Communities in the forested streams (MF and SF) clearly clustered together and were distinct from those in the CP, which showed the most variability over the course of our study. The SP stream showed the most dramatic seasonality, where dry season samples clustered with those from CP while wet season samples were more similar to MF and SF. The network had 357 nodes (MF = 87, SF = 79, SP = 85, CP = 106), each representing a distinct sample, 4387 edges connecting these nodes (showing potential similarities), an average undirected connectivity of 13 and an average clustering coefficient of 0.61. The resulting network clustered samples into groups representing two distinct communities: one associated with land-use sites and a second one associated with the dry and wet seasons (**Figure 2.4**). While samples from MF, SF and CF showed close connectivity within each site for both dry and wet season samples, respectively, SP samples showed greater seasonal divergence. SP dry season samples were similar to those from CF, while wet season samples are more similar to MF and SF (**Figure 2.4**). SP seasonal variations were highest among all sites, with dry and wet seasonal samples showing less than 40% relationship. Clear seasonal clusters developed for each site; however, their differences are less noticeable as compared to seasonal clusters for SP.

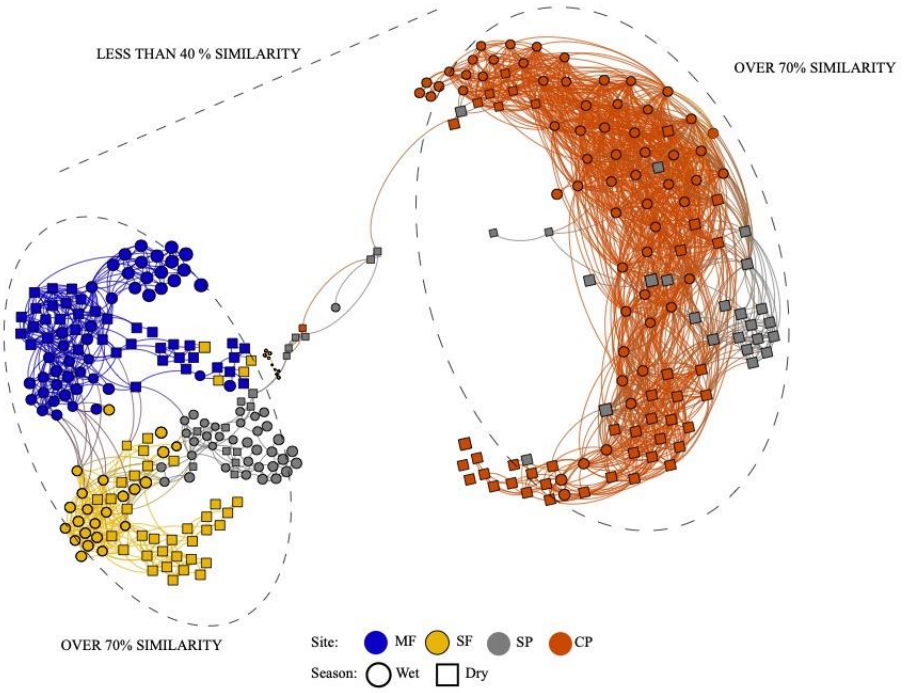

Figure 2.4. **Inferred bacterial network of bacterial community structure similarity between stream water samples surrounded by different land use types. The network was built based on co-ocurrence of dominant ASVs in these stream bacterial communities. MF = Mature Forest, SF = Secondary Forest, SP = Silvopasture, CP = Cattle Pasture.**

2.3.3 Associations between bacterial taxa and land use

There were 19 phyla represented in the dataset; however, over 90% of the total community could be attributed to four phyla: Proteobacteria (68.1 ± 1.3%), Bacteroidetes (15.2 ± 1.9%), Epsilonbacteraeota (5.1 ± 4.4%), and Actinobacteria (3.6 ± 2.6%). Proteobacteria, particularly Alpha- and Gamma-proteobacteria, were ubiquitous across streams, although their high relative abundance varied across sites and seasons (**Table 2.2**). With the lowest relative abundance of Proteobacteria among land-use types, CP showed a sharp decline in Alphaproteobacteria during the dry seasons and a corresponding increase in the abundance of Bacteroidetes. MF, SF and SP had lower relative abundances of Actinobacteria than CP and higher abundances of Epsilonbacteraeota during all seasons. Throughout our two-year weekly monitoring, the relative abundance of different top taxa among sites and seasonal variability within sites were noticeable (**See Appendix A, Figures A2-A5, C).**

Table 2.2. Seasonal mean relative abundance ± standard deviation of bacterial phyla (>1% of all ASVs) found in samples from the four streams surrounded by different land uses that were sampled in this study. Within each row, letters indicate significant differences between land uses ($p < 0.05$) based on Kruskal-Wallis and pairwise Wilcox (with Benjamini-Hochberg correction) tests.

Site	Mature Forest (MF)		Secondary Forest (SF)		Silvopasture (SP)		Cattle Pasture (CP)	
Season	Wet	Dry	Wet	Dry	Wet	Dry	Wet	Dry
Phylum - Class	Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD	
Proteobacteria	**73.1 ± 0.4** [a]	**74.1 ± 0.5** [a]	**75.0 ± 0.4** [a]	**75.2 ± 0.4** [a]	**74.7 ± 0.5** [a]	**74.4 ± 0.6** [a]	**69.5 ± 0.6** [b]	**59.2 ± 0.7** [c]
- Gammaproteobacteria	54.56 ± 0.4	56.3 ± 0.5	54.9 ± 0.4	54.5 ± 0.5	57.9 ± 0.5	58.2 ± 0.7	57.2 ± 0.7	49.6 ± 0.8
- Alphaproteobacteria	12.3 ± 0.2	12.4 ± 0.2	12.2 ± 0.2	12.4 ± 0.2	11.2 ± 0.3	13.1 ± 0.4	9.2 ± 0.2	1.7 ± 0.3
- Deltaproteobacteria	6.2 ± 0.2	5.5 ± 0.2	8.0 ± 0.2	8.3 ± 0.2	5.6 ± 0.2	3.2 ± 0.2	3.1 ± 0.2	1.9 ± 0.2
Bacteroidetes	**10.4 ± 0.2** [c]	**10.7** [c] **± 0.2**	**8.9 ± 0.2** [d]	**10.9 ± 0.2** [c]	**9.3 ± 0.3** [c]	**12.6 ± 0.8** [b]	**13.8 ± 0.6** [b]	**21.3 ± 0.9** [a]
- Bacteroidia	10.4 ± 0.2	10.7 ± 0.2	8.8 ± 0.1	10.8 ± 0.2	9.3 ± 0.3	12.6 ± 0.8	13.7 ± 0.6	21.3 ± 0.9
Actinobacteria	**0.6 ± 0.1** [d]	**0.6 ± 0.1** [d]	**0.7 ± 0.4** [d]	**1.0 ± 0.3** [d]	**1.3 ± 0.5** [d]	**4.3 ± 0.7** [c]	**9.2 ± 0.8** [b]	**13.4 ± 0.8** [a]
- Actinobacteria	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.5	0.8 ± 0.3	1.1 ± 0.5	4.3 ± 0.7	9.1 ± 0.8	13.3 ± 0.8
- Thermoleophilia	0.2 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	< 0.1	0.1 ± 0.1	< 0.1	< 0.1	< 0.1
- Acidimicrobiia	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.2 ± 0.1	< 0.1	< 0.1	0.1 ± 0.0	< 0.1
Epsilonbacteraeota	**3.3 ± 2.0** [a]	**4.3 ± 1.2** [a]	**2.0 ± 1.0** [a]	**1.9 ± 1.4** [a]	**2.9 ± 0.9** [a]	**2.3 ± 0.7** [a]	**0.7 ± 0.3** [b]	**0.6 ± 0.3** [b]
- Campylobacteria	3.3 ± 2.0	4.3 ± 1.2	2.0 ± 1.0	1.9 ± 1.4	2.9 ± 0.9	2.3 ± 0.7	0.7 ± 0.3	0.6 ± 0.3
Verrucomicrobia	**2.8 ± 0.2** [a]	**2.6 ± 0.2** [a]	**2.6 ± 0.2** [a]	**2.8 ± 0.2** [a]	**2.3 ± 0.3** [a]	**1.2 ± 0.2** [b]	**1.7 ± 0.2** [a]	**2.6 ± 0.4** [a]
Firmicutes	**2.2 ± 0.2**	**1.7 ± 0.3**	**1.5 ± 0.1**	**1.0 ± 0.1**	**2.9 ± 0.2**	**1.4 ± 0.1**	**1.3 ± 0.2**	**0.5 ± 0.1**
- Clostridia	1.7 ± 0.2	1.2 ± 0.3	0.9 ± 0.1	0.6 ± 0.1	2.0 ± 0.2	0.9 ± 0.1	0.8 ± 0.2	0.3 ± 0.1
Planctomycetes	**1.3 ± 0.1** [a]	**1.4 ± 0.1** [a]	**1.1 ± 0.1** [a]	**0.2 ± 0.1** [b]	**0.6 ± 0.2** [b]	**0.4 ± 0.1** [b]	**0.2 ± 0.1** [b]	**0.2 ± 0.1** [b]
- Planctomycetacia	0.5 ± 0.1	0.8 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	< 0.1
Acidobacteria	**1.3 ± 0.2**	**0.8 ± 0.1**	**1.5 ± 0.2**	**0.9 ± 0.2**	**0.8 ± 0.2**	**0.3 ± 0.1**	**0.7 ± 0.1**	**0.2 ± 0.1**
Patescibacteria	**0.6 ± 0.1**	**0.7 ± 0.1**	**1.4 ± 0.1**	**1.4 ± 0.1**	**1.3 ± 0.2**	**1.3 ± 0.2**	**0.5 ± 0.2**	**0.5 ± 0.1**
Omnitrophicaeota	**0.6 ± 0.1**	**0.5 ± 0.2**	**1.2 ± 0.2**	**0.9 ± 0.1**	**0.4 ± 0.2**	**0.2 ± 0.1**	**0.6 ± 0.2**	**0.2 ± 0.1**

Indicator taxon analyses using the R package indicspecies identified 48 indicator genera: 29 for samples collected in forests (MF and SF) and 19 for pastures (CP and SP). When looking only at indicator taxa with relative abundance above 5%, six were observed for forested areas and nine for pastures (**Figure 2.5**). While present at all sites, seasonal patterns of increase and decline of Forest indicator taxa, such as *Hydrogenophaga* (Betaproteobacteria), *Pseudomonas* (Gammaproteobacteria), and *Cellvibrio* (Gammaproteobacteria) were particularly strong in MF, SF and SP, and they all but disappeared in the CP during the dry seasons. Indicator taxa of streams influenced by cattle pastures showed low relative abundance in the MF and SF, while seasonality was strong in the CP and SP, particularly for *Pseudarcicella* (Bacteroidetes), *Sediminibacterium* (Bacteroidetes), *Curvibacter* (Betaproteobacteria), and *Rhodobacter* (Alphaproteobacteria) (Kruskal-Wallis, $p < 0.05$; **Figure 2.5**). Linear discriminant analysis (LefSE) was also used to determine the taxa that described the most variance among samples from the different land-use types (**See Appendix A, Figure A6**). The two forested streams (MF and SF) had many more ASVs showing differential abundance than SP and CP (25 and 26 genera vs. 7 and 5 genera, respectively). While many of these were only identified to the family or order level, when ASVs sequences were further analyzed through the Basic Local Alignment Search Tool (BLAST), we found their closest relatives to be associated with taxa commonly found either in soils or water environments and were also in accordance with our indicator taxon analysis (**Figure 2.5**).

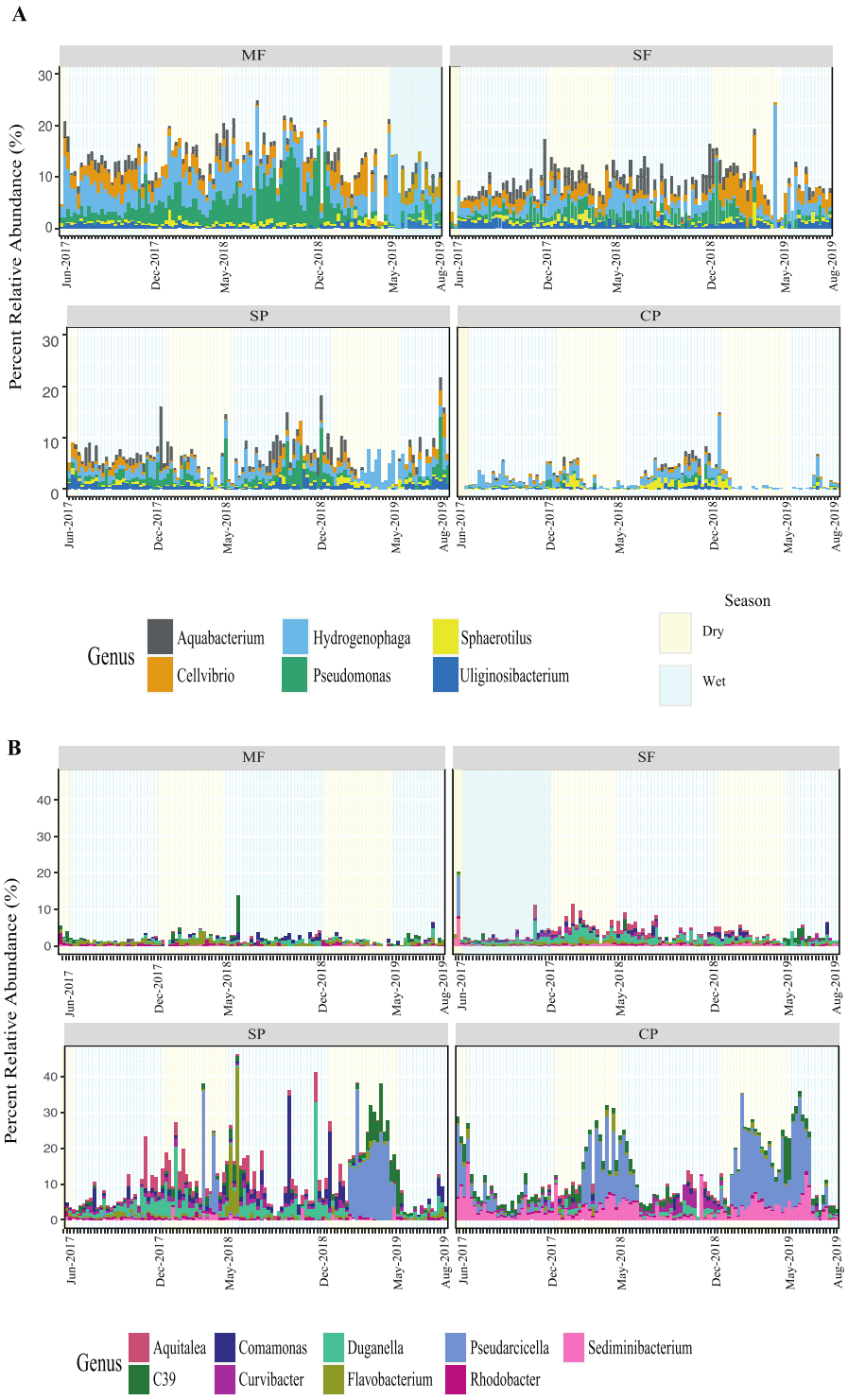

Figure. 2.5. Relative abundance of indicator genera for A) forests and B) pastures across the four land uses based on indicator taxa analysis (Indicspecie). Indicator genera with an overall relative abundance higher than 5% are shown. Colored bars in the plot backgrounds represent seasons. MF = Mature Forest, SF = Secondary Forest, SP = Silvopasture, CP = Cattle Pasture.

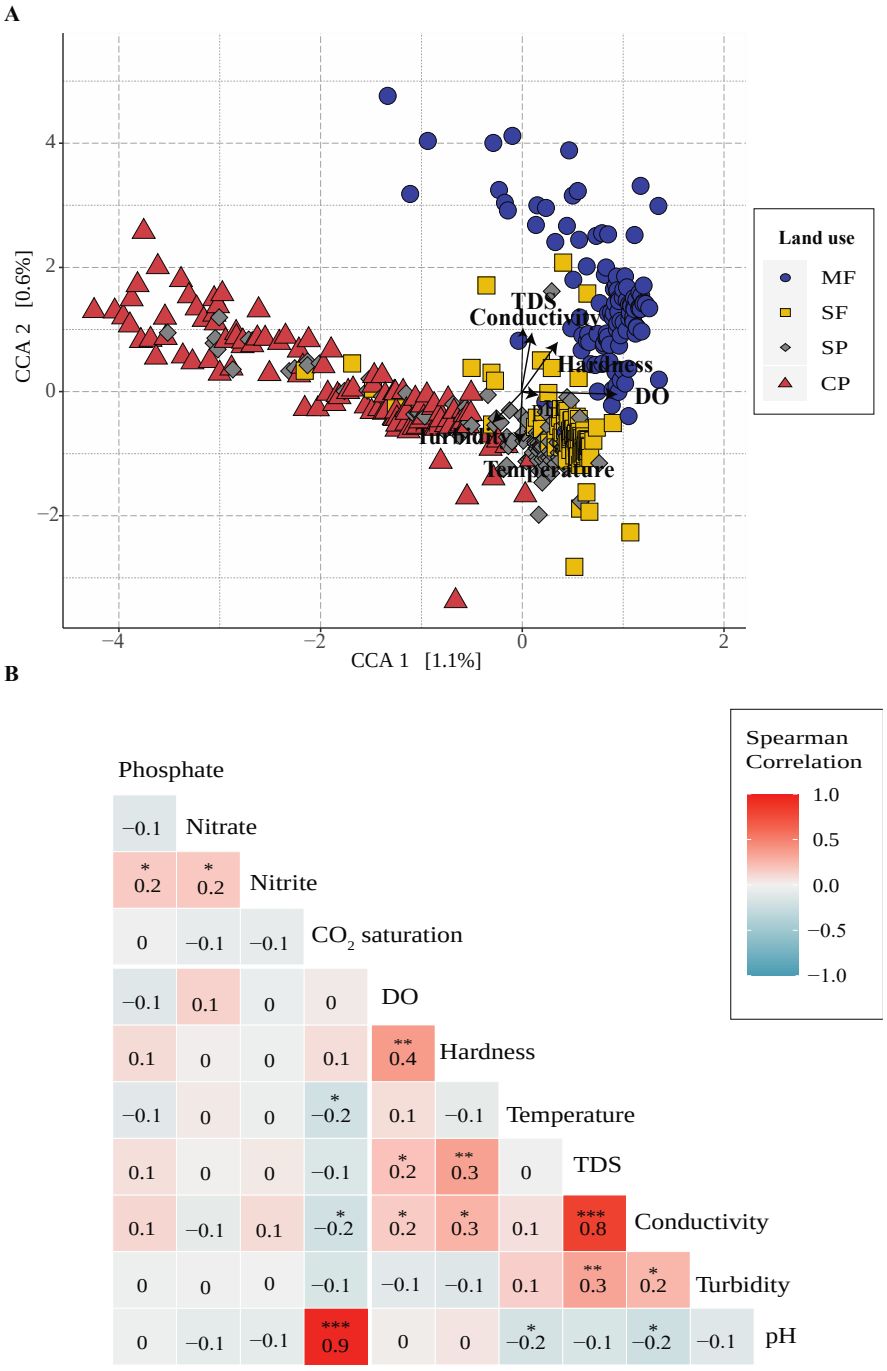

Figure 2.6. A) Canonical correspondence analysis plot (CCA) of community composition between samples across land-use type and water quality parameters, and (B) collinearity heatmap of water quality parameters across all catchments. Arrows refer to environmental conditions. Mature Forest, SF = Secondary Forest, SP = Silvopasture, CP = Cattle Pasture.

2.3.4 Associations between bacterial communities and environmental factors

Mantel tests (with 999 permutations) were conducted to look for relationships between beta diversity (using weighted UniFrac distance) and environment (Physicochemical factors), and canonical correspondence analysis was used to illustrate the results (**Figure 2.6A**). Indices of bacterial community structure were positively correlated with pH, dissolved oxygen, turbidity, TDS, total iron and ammonia; however, all showed "weak" strength of association (r < + 0.39). The strongest correlation found among all factors measured was between beta diversity and DO (p < 0.001, r = + 0.38). Water temperature, nitrite, nitrate, sulfate and phosphate did not correlate with community structure (p > 0.05). We used Spearman's correlation coefficients to assess collinearity and the strength of the correlations between microbial communities and water parameters (**Figure 2.6B**). pH was found to be significantly and negatively correlated with several parameters, including conductivity and temperature (p < 0.05). As expected, conductivity and TDS were significant and positively correlated parameters and also correlated with Turbidity (p < 0.001). Conductivity and TDS were also positively correlated with DO (p < 0.01).

2.3.5 Culture-dependent indicator bacteria

We assessed common indicator bacteria (total coliform and *Escherichia coli*) using the standard culture-based Colilert test by IDEXX. Total coliforms were ubiquitous across all sites, and there were no significant differences in their concentrations between the different land-use types when aggregating all measurements (Kruskal-Wallis, p > 0.05); however, some seasonal differences can be observed (**Figure 2.7A)**. *Escherichia coli* was also detected in samples from all sites, but concentrations varied among land use and seasons (**Figure 2.7B)**. The lowest concentrations were found during the dry season; whereas, the highest concentrations were found in samples from SP and CP during the wet seasons and were significantly different from those found in forested sites (Kruskal-Wallis, p < 0.05).

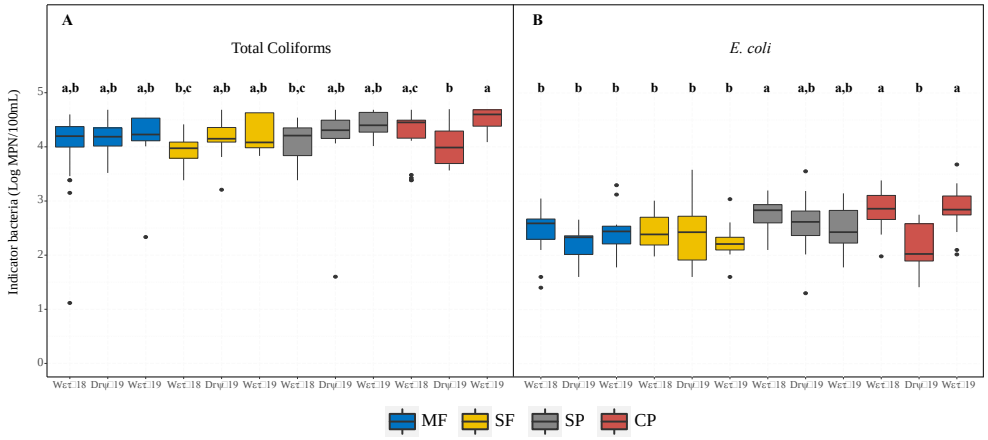

Figure 2.7. Culture-dependent indicator bacteria. Seasonal differences between total coliforms (A) and *E.coli* (B) concentrations for each land use type. MF = Mature Forest, SF = Secondary Forest, SP = Silvopasture, CP = Cattle Pasture.

2.4 Discussion

This study demonstrated that streams adjacent to young naturally regenerated forest growing on abandoned pastures could largely recover bacterial species diversity and community composition as well as measures of water quality after only a decade of cattle removal (**Figures. 2.2-2.5; Tables 2.1,2.2**). This study further shows that maintaining streamside forest buffers in silvopasture systems helps protect and restore water quality and stream bacterial communities from the effects of cattle on the landscape, particularly in the wet season. Seasonal differences in bacterial diversity, community structure, and water quality measures in samples from SP and CP point to the detrimental effects of allowing cattle into streams during the dry seasons as bacterial community composition in the SP shifted to resemble that of our traditional cattle pasture during the dry season (**Figures 2.4 and 2.5; Table 2.1**).

2.4.1 Recovery of stream bacterial communities in secondary forests

Streams influenced by forested sites showed overall higher diversity and more consistent community structure across seasons (**Figures 2.2, 2.3**), suggesting that these communities are more resilient to temporal and environmental changes. The higher diversity in forested streams also reflects a more robust community that is able to maintain itself throughout the seasons. Samples from SF showed similar levels of diversity as MF (**Figure 2.2**), suggesting that these bacterial communities can recover in as little as a decade of forest regrowth in the surrounding landscape, at least in cases such as this where riverine forest was maintained in the past when cattle were present on the landscape but had full access to the stream channel. The communities in SF and MF were also closely related (**Figures 2.3, 2.4),** yet can be distinguished from each other by several taxa that are common in oligotrophic stream systems worldwide (**Figure A6**). Many of these taxa have been found in both soils and various aquatic habitats and have been demonstrated to cope well with stressful conditions (Biebl and Pfennig, 1981; Derlet et al., 2004; Nakai et al., 2014; Poindexter, 2015). While seasonal variation in the bacterial communities can be seen in both of these catchments, their overall diversity remains constant year-round (**Figure 2.2**), suggesting that the communities can adapt quickly to conditions, such as changes in stream flow and decreases in DO, associated with seasonality. Our data suggest rapid recovery of dominant forest stream water taxa and the likely ability of the community to adjust to changing conditions when land management permits natural successional processes to occur on the landscape.

2.4.2 Silvopasture systems can improve water quality

Silvopasture systems are popular land management systems for their environmental and economic benefits (Chizmar et al., 2020; González, 2013). Planting trees in pastures can improve animal health by reducing heat stress (Murgueitio et al., 2011) and riverine forests and living fences associated with silvopasture systems further enhance biodiversity values by serving as perches for birds and biological corridors (Harvey et al., 2008; Murgueitio et al., 2011). Planting nitrogen-fixing and other tree species can enhance nutrient cycling (Batterman et al., 2013; Epihov et al., 2021), carbon storage in the soil (Pan et al., 2011) and sequester carbon in trees (Chazdon et al., 2016; Hall, J.S., Plisinski, J.S., Mladinich, S.K., van Breugel, M., Lai, H.R., Asner, G.P., Thompson, 2021); however, silvopasture systems may not improve the overall carbon balance as increased stocking can increase methane emissions, particularly in systems where trees and shrub species planted do not directly target improved digestion by cattle. Riparian buffers filter runoff

and have been shown to improve stream water quality by reducing the velocity of runoff and promoting infiltration, sediment deposition and nutrient retention (Anderson et al., 2009; Atangana et al., 2014; Ewel, 1999; Harvey et al., 2006; Harvey and González Villalobos, 2007; Jose, 2009; Mayer et al., 2007; Nair, 1997; Nair et al., 2007; Nichols, 2005; Young, 1997).

Bacterial community diversity and structure for samples from SP showed the most variability, with seasonal associations linking these communities to streams in the other land uses (**Figures 2.2-2.4**). During the dry season months, stream bacterial communities in the SP catchment resembled those of samples taken from CP, with members of the Spirosomaceae family (Bacteroidetes) having a high relative abundance (**Figures A4, A5**), while during the rainy seasons the communities were more similar to forested areas (MF and SF; **Figure 2.4**). Other important taxa driving these differences included *Aquitalea* (Betaproteobacteria), *Pseudarcicella* (Bacteroidetes), and *C39* (Betaproteobacteria) appearing to drive these links to the CP during the dry seasons, while *Aquabacterium* (Betaproteobacteria) and *Pseudomonas* (Gammaproteobacteria) linked the SP community to the forested catchments during the wet seasons (**Figure 2.5, A2-A5, C**). *Aquitalea*, *Pseudarcicella*, and *C39* are bacteria that have been found in eutrophic water environments (Kopprio et al., 2020; Lau et al., 2006; Zwirglmaier et al., 2015), suggesting a seasonal influence of cattle in the SP catchment.

Runoff in areas influenced by livestock can influence stream water quality in various ways, including significant sediment and nutrient deposition, fecal contamination inputs and even changes in stream channel morphology (Belsky et al., 1999; Webber et al., 2010). Although several of our measured water quality parameters did not vary between catchments, the results show that samples from CP had higher turbidity and some nutrients (ammonia and total iron) but lower DO and higher water temperatures during the dry season compared to forested sites (**Table 2.1**). The higher relative abundance of Bacteroidetes and Actinobacteria in samples from CP (**Table 2.2, Figure A6**) combined with lower DO, higher temperatures and lower overall diversity suggest that current cattle management practices in this landscape negatively influencing water quality and stream bacterial communities (Badgley et al., 2019; Liao et al., 2015). Samples from both the CP and SP had higher abundance of bacteria found in fecal matter, such as *Flavobacterium* (Bacteroidetes) and *Sediminibacetrium* (Bacteroidetes) (De et al., 2020; Hagey et al., 2019). These genera showed clear seasonal trends, with higher abundance during the dry seasons. The higher total iron concentrations in the CP could also contribute to the differences in microbial community composition seen in this catchment. We found ASVs assigned to genera known for iron (II) oxidation like *Gallionella* (Betaproteobacteria) and *Leptothrix* (Betaproteobacteria) as well as iron (III) reduction such as *Geothrix* (Acidobacteria)and *Geobacter* (Deltaproteobacteria; data not shown); however, their relative abundances were low across the dataset and not statistically different between sites. Interestingly, *Rhodobacter* (Alphaproteobacteria), an anaerobic photosynthetic purple bacteria that oxidizes iron (II) when exposed to light, was found to be an indicator for sites influenced by cattle (**Figure 2.5**). This genus was enriched in both the CP and SP in the wet seasons and had very low relative abundances in the forested catchments. However, total iron concentrations were lower in the SP year-round so we do not attribute the higher abundance of this taxa in the CP to higher iron in the water column. Thus, although iron concentrations are much higher in the CP stream and there is some signal in the microbial community, total iron is not driving the significant differences in community composition between the CP site and the other sites in this study.

While seasonal shifts in bacterial communities can be seen in all catchments, the SP and CP show the most variation. Whereas cattle in our CP catchment have full access along all stream channels in the catchment (**Figure 2.1d**), the stream channel is fenced in the SP catchment and cattle may only access it at a single point (**Figure 2.1c**). During the wet season, cattle visit the stream to drink and return upslope to graze, spending little time near the stream channel. The riverine forest buffer in the SP catchment acts as an effective filter, ameliorating the impact of the cattle on the stream channel and helping to preserve water quality (Hilary et al., 2021) despite nearly two-thirds of the catchment surface are being managed as cattle pasture year round. In contrast, during dry season months, the cattle tend to congregate at both the CP and SP streams, where there is more shade to avoid the heat and drink. We believe that increased congregation leads to more defecation and disturbance directly in the stream and, combined with the reduced dry-season flow, drives the reductions in dissolved oxygen, higher water temperatures (**Table 2.1**), and dramatic changes in bacterial community composition (**Figure 2.4**) that was observed in the SP stream. This highlights the benefits of maintaining a riparian forest buffer with fencing but also suggests that installing pumping or other water management systems that keep cattle out of the stream entirely would enhance water quality. Providing shade through tree planting or shelters away from stream channels may also reduce cattle congregation in streams in both traditional cattle pasture and silvopasture systems, with corresponding water quality benefits.

2.4.2 Culture-based indicator bacteria and whole bacterial communities

Diffuse sources of fecal pollution like grazing livestock are an important source of contamination in freshwater sources. Traditionally, indicator bacteria such as coliforms and *E.coli* have been widely used to monitor water quality (Hazen, 1988); however, there is a concern that the presence of these organisms in tropical freshwater environments may not indicate contamination or the presence of other pathogens (Viau et al., 2011). In this study, measurable concentrations of both total coliforms and *E.coli* in the water column were found in samples from all sites using a traditional culture-based protocol. There were no significant differences between coliform concentrations and the different land use types; however, there were significant differences between *E.coli* concentrations. Although we were able to discern the seasonal influence of livestock on the water quality of streams at the SP and CF sites, the high concentrations measured of both total coliforms and *E.coli* at all sites demonstrate the need for improved indicators of microbial contamination. Through metabarcoding, we were able to determine that several genera present in the dataset can result in false positives using the common test Colilert Quanti-tray 2000 (**See Appendix A, Figures A7-A8**). Here we found members of the phylum Bacteroidetes as an alternative indicator of fecal contamination, detecting them predominately in samples from CF and SP and identifying seasonal trends that can help guide land use management and the protection of water quality. Animal fecal markers with *Bacteroidale*s targets, like livestock-associated BacCow and canine-associated BacCan, have been identified and shown to be successful at identifying non-human fecal sources of pollution (Holcomb and Stewart, 2020; Teixeira et al., 2020). In microbial community assessments, taxa such as the order *Bacteroidale*s can be used to determine fecal pollution in watershed and other water systems.

2.5 Conclusions

the results of this study demonstrate that land use and forest cover can significantly affect tropical freshwater bacterial communities. Natural regeneration of tropical forests can rapidly enhance water quality and stream bacterial diversity and community composition over short time periods. Similarly, the maintenance of riparian forest buffers in silvopastoral systems can positively impact the health and resilience of aquatic microbiomes. These impacts are most apparent during the wet season when the base flow is higher and cattle are less likely to congregate in stream access points, suggesting that preventing cattle from accessing streams may have important effects on stream health and water quality. It is recognized that this study is limited to a single example of each land use and that additional study is needed to understand the functional implications of the observed changes in these bacterial communities. However, this dataset encompasses two years of measurements, spanning three wet seasons and two dry seasons, suggesting that the observed patterns are robust. Management efforts, such as maintaining riparian corridors, limiting livestock access to streams, and permitting forest regrowth, can protect streams from the negative effects of human activities and will likely have cumulative effects, promoting healthier communities and ecosystems downstream and strengthening the resilience of freshwater microbial communities to seasonal and environmental changes in tropical watersheds.

Chapter 3

Effects of treatment and distribution on bacterial communities in drinking water in the peri-urban region of Arraiján, Panama

3.1 Introduction

Much of the research to characterize microbial communities in drinking water has been conducted in industrialized countries (Bautista-De los Santos et al., 2016; Bruno et al., 2018; Douterelo et al., 2020; Neu and Hammes, 2020; Perrin et al., 2019; Potgieter et al., 2019; E. I. Prest et al., 2016; H. Wang et al., 2013; Wang et al., 2014). From these studies, an understanding of the effects of treatment and piped distribution on microbial communities in drinking water is growing. There is evidence that microbial communities are mainly introduced by the source water (Chao et al., 2013; Lautenschlager et al., 2014; Yang et al., 2016) and the first three treatments generally applied (coagulation, flocculation and clarification/sedimentation) have little or no significant change in the microbial community structure in water (Bruno et al., 2018; Lin et al., 2014; Potgieter et al., 2019). On the other hand, biofiltration (e.g., slow sand, rapid sand and granular activated carbon filtration) has been found to harbor specific microbial communities and can cause significant changes in the drinking water microbiome. There is growing evidence that biofiltration can influence the water microbiome downstream, including in the distribution network (Q. Li et al., 2017a; Pandit and Kumar, 2019; Pinto et al., 2014, 2012). Although there is a growing body of literature regarding drinking water microbiomes, microbial communities in drinking water systems in developing countries are vastly unexplored.

In developing countries, the consistent delivery of microbiologically safe water is still a challenge for many utilities that face resource limitations and rapid population growth. Waterborne illness remains a major concern, especially in tropical countries with poor water supply infrastructure (Nichols et al., 2018; Pandit and Kumar, 2015; Sweileh et al., 2016). Many developing countries are located where droughts and seasonal changes in precipitation bring about challenges to water sources at different times of the year. In addition, energy insecurity can affect the production of drinking water. While the production and delivery of safe drinking water in developing countries continues to be a challenge, microbial assessments using molecular technologies, similar to those now popularly used in developed countries to assess drinking water, could fill in gaps that currently culture-based methods for the detection of indicator bacteria have (Field and Samadpour, 2007;

Straub and Chandler, 2003). Monitoring for chlorine residual and fecal indicators may be insufficient to characterize water quality microbial risks in such dynamic systems. A deeper understanding of the microbial communities in centralized drinking water systems in developing countries could provide useful insights for improving the design and management of drinking water infrastructure.

The goal of this study was to investigate the influence of drinking water treatment (including granular media filtration and disinfection) and distribution on bacterial communities in drinking water from a system in a tropical environment and with system limitations that are commonly encountered in developing countries (i.e., source water variability, operational, and infrastructure deficiencies). Bacterial diversity, taxonomy and abundance were evaluated along the surface water treatment process trains of three DWTPs serving a peri-urban region in Panama (Central America) and its corresponding DWDS. High-throughput sequencing of bacterial 16S rDNA was used in combination with traditional water quality metrics to characterize bacterial communities in bulk water.

3.2 Materials and methods

3.2.1 Sampling sites

This study was conducted in Arraiján, Panama, a rapidly growing peri-urban area west of the Panama Canal and Panama City, Panama. 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 3.1**) (Erickson et al., 2020; 2017; INEC, 2019). These treatment plants sourced their water from the Panama Canal Watershed, primarily from Lake Gatún, which is recharged by the Chagres River and the Miraflores Lake (an artificial lake used in the operations of the Panama Canal). Conventional surface water treatment was practiced, with slight differences between plants (**Table 3.1**). DWTP A is managed by the governmental agency Panama Canal Authority, DWTP B is privately managed by the corporation Aguas de Panama, and DWTP C together with Arraijan's DWDS is managed and operated by Panama's Institute of National Aqueducts and Sewers (known as IDAAN for its initials in Spanish). While most of the DWDS in Arraiján provides continuous water supply, several zones operate intermittently (Erickson et al., 2020; 2017).

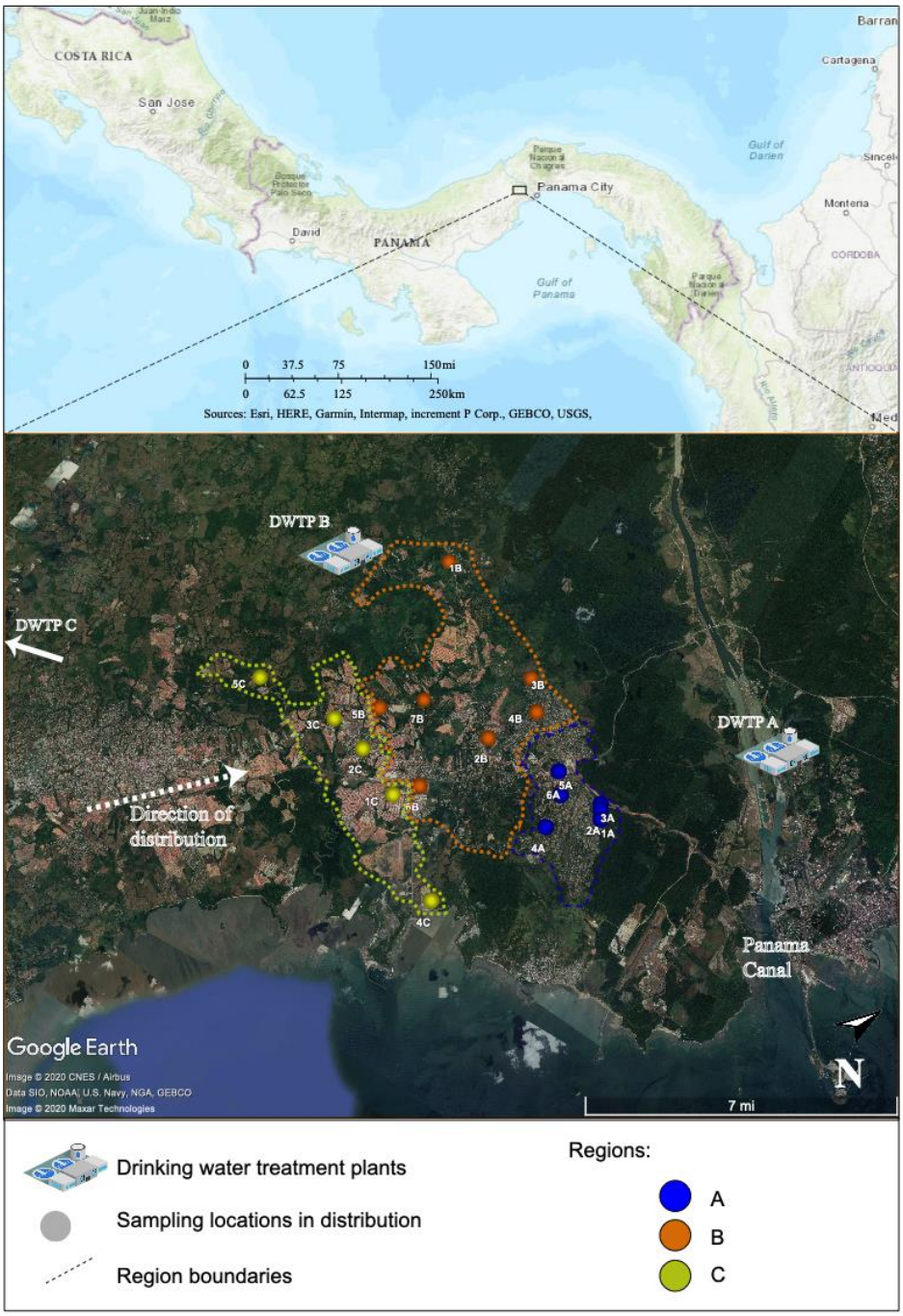

Figure 3.1. A) Map of Panama indicating where Arraiján is located; B) Map of sampling locations in the drinking water system of Arraiján, Panama. Sampling sites include three treatments plants and 18 locations in the distribution systems.

To assess the effects of treatment and piped distribution on bacterial communities, the drinking water system was sampled for a period of approximately 11 months. Four types of samples were collected: i) source water samples at each intake (labeled Source); ii) filter effluent (labeled Filtration); iii) finished, disinfected drinking water before distribution (labeled Disinfection), and iv) 18 locations in the distribution system commonly used by IDAAN to monitor water quality in the network (excluding premise plumbing) (**Figure 3.1 and 3.2**). Locations in the distribution system were divided into three regions (A-C) that corresponded to the treatment plant from where the water supply originated (**Figure 3.1**).

Table 3.1. General parameters for the three DWTPs in this study. ACP = Panama Canal Authority. IDAAN = Panama's Institute of National Aqueducts and Sewers. ACP and IDAAN are governmental agencies. Aguas Panama is a private entity that sells water to IDAAN for its distribution. * Miraflores lake is a small man-made lake that separates the main three locks in the Panama Canal and is located near the Pacific Ocean entrance of the Panama Canal (Source: Planning Department of IDAAN, 2019). Gatun lake is a large artificial lake (425 km^2) that forms a major part of the Panama Canal.

	DWTP A	DWTP B	DWTP C
Year of construction	1915	2002	2014
Regions served	Arraijan & La Chorrera	Arraijan	Panama City & Arraijan
Elevation (m)	60	123	122
Administered by	ACP	Aguas Panama	IDAAN
Population served	199,777	278,157	128,091
Source water	Miraflores lake*	Gatun Lake	Gatun Lake
Capacity (MGDs)	31.6	20.1	13.4
Treatment	Conventional	Conventional	Conventional
Coagulant and additives	potassium aluminium sulfate, sodium silicofluoride, calcium hydroxide	potassium aluminium sulfate, sodium silicofluoride, calcium hydroxide	potassium aluminium sulfate, activated carbon, calcium hydroxide
Filtration media	Gravel, sand, anthracite media	Gravel, sand, anthracite	Gravel, sand, anthracite
Filtration cleaning cycle	52 - 60 hours/turbidity check	52 - 60 hours/turbidity check	60 -72 hours/turbidity check
Number of filters	20	10	18
Disinfectant	Chlorine gas	Chlorine gas	Chlorine gas

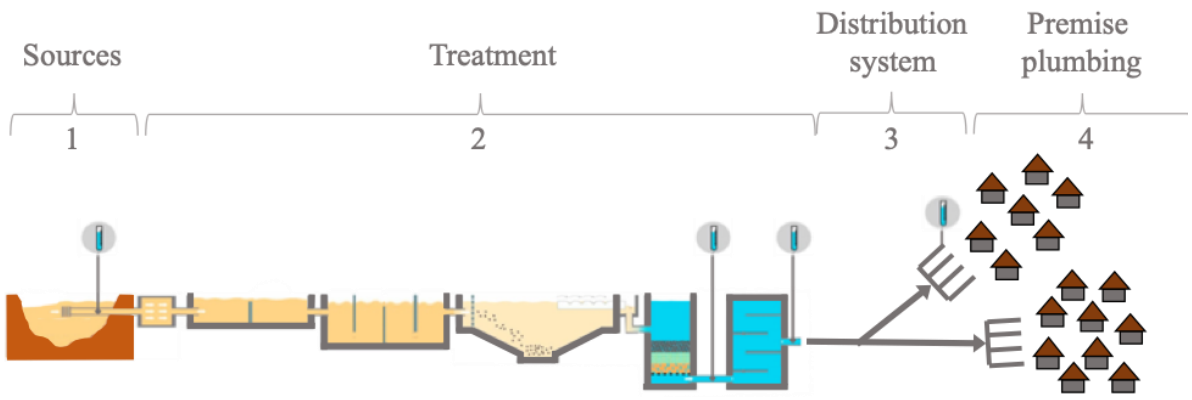

Figure 3.2. Schematic representation of the main components of a drinking water system for surface water as source water. Conventional treatment depicted consists of coagulation, flocculation, sedimentation, filtration and disinfection. Sampling locations shown in schematic.

3.2.2 Sampling and water quality parameters

Sampling took place between 6 a.m. and 3 p.m. during three sampling excursions: One during the dry season (December 2016 – April 2017) and two during the wet season (May – October, 2017). The following parameters were analyzed immediately on site: free and total chlorine (Hach Portable Colorimeter II), turbidity (Hach Portable Turbidimeter 2100Q), pH, temperature, conductivity, total dissolved solids (HANNA Instruments pH/conductivity/TDS tester or Extech ExStik II pH/conductivity meter) and pressure (Eastman 45169 Water Pressure Test Gauge, adapted to fit sampling tap). In the DWTPs, water samples were collected at each location immediately after having access (**Figure 3.2**). For Filtration samples, a composite sample was collected with water from all working filters (excluding those in maintenance or that were being cleaned). Samples taken at the various locations in the DWDS were collected after 3-5 minute flushing.

3.2.3 Culture-based indicator bacteria

Samples for bacterial enumeration were collected in sterile 100-mL bottles with sodium thiosulfate to neutralize residual chlorine and transported to STRI's laboratory on ice. These samples were tested within 3 hours of collection for 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 conducts 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 (J. J. Erickson et al., 2017).

3.2.4 16S rDNA sequencing

To characterize the bacterial communities, large bulk water volumes were collected to concentrate the microbial biomass for DNA extraction and 16S rDNA sequencing. Sample volumes ranged from 15 - 20 L for source water samples, 100 L for filtration samples, and 450 - 500 L for samples after disinfection and in the DWDS. These volumes were chosen after a preliminary analysis (16S rDNA 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 (Smith and Hill, 2009) and in the Center for Disease Control Standard Operating Procedure for dead-end ultrafiltration for pressurized water sources and backflushing (CDC, Doc. No. WDPB.DR.C.003, unpublish SOP). Ultrafilters were transported at 4°C to the metagenomics laboratory at STRI and processed within 5 hours of collection. Backflushing of ultrafilters was performed as described in Smith and Hill, 2009. 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°C until DNA extraction.

Isolation of DNA was performed using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The PowerSoil Kit was used following the manufacturer's instructions with the following adjustments. The 5-µm and 0.22-µm mixed cellulose filters were cut into pieces in sterile conditions and combined in 5-mL low-bind tubes before extractions. Bead-beat solution and Solution C1 were added to the 5-mL low-bind tubes, and bead-beating was performed. The isolated DNA was eluted in a final volume of 60 uL. After DNA extraction, all samples were stored at -20°C prior to the preparation of sequencing libraries. Concentrations of DNA were measured by NanoDrop (Thermo Fisher Scientific).

Similar to the amplification process described in Chapter 2, the V4 variable region of the 16S rRNA gene was amplified in triplicate 12.5 uL reactions using a two-step PCR protocol. Briefly, the V4 region was amplified using phased primers [515F (Parada) GTGYCAGCMGCCGCGGTAA and 806R (Apprill) GGACTACNVGGGTWTCTAAT] with Platinum 2X Mastermix (ThermoFisher) and 2.5 uL of genomic DNA as template for the first PCR step. PCR1 cycling conditions had an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 90 s and a final elongation at 72°C for 10 min. The resulting PCR1 products were pooled and 2.5 uL were used as template for PCR2 with eight cycles to add unique barcodes and Illumina sequencing adaptors. Negative and positive controls containing nuclease-free water and previously PCR-positive samples were included in all PCR amplification steps. PCR2 products were cleaned and normalized with PCR Purification and Normalization Plates (Charm Biotech, San Diego, CA, United States) and pooled into a single sequencing library. The library was further concentrated using KAPA Pure Beads as per manufacturer specifications (Kapa BioSystems), quantified by Qubit High Sensitivity dsDNA Assay (Thermo Fisher Scientific), and quality checked by a Bioanalyzer dsDNA High Sensitivity assay. The resulting library was sequenced on an Illumina MiSeq with V3 chemistry on a 2 x 250 paired-end run at the STRI metagenomics laboratory.

3.2.5 Data analysis

Bacterial detection by HPC and analysis of water quality parameters were performed as described in Kumpel and Nelson, 2014. 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. Untransformed data were used for water quality parameters. Statistical analysis was performed using the statistical software R v3.6.0 and Coin package (R Core Team, 2012), performing tests for significance based on non-parametric Kruskal-Wallis test with Benjamini-Hochberg corrections. Values were considered significant at $p < 0.05$.

For 16S rDNA data, raw FASTQ sequencing reads were demultiplexed and mapped to 10 % PhiX. The QIIME2 platform v2019.10 was used for the majority of the analysis of paired-end sequence reads. Preprocessing consisting of primer trimming, sequencing quality control, forward and reverse read merging were also performed using QIIME2's Cutadapt and DADA2 plugins to produce amplicon sequence variants (ASVs) (Bolyen et al., 2019; Callahan et al., 2016; Martin, 2011). Taxonomy was assigned using a naïve Bayesian classifier trained on the SILVA 99% sequence similarity database (v32) (Pruesse et al., 2007; Wang et al., 2007), and sequences were further filtered using BLAST with a 97% confidence. ASVs with low abundance (0.005%) and those classified as mitochondria or chloroplast were also removed from further analysis.

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 2000 sequences per sample. Differences in alpha diversity (Observed ASVs, Shannon and phylogenetic diversity indices) by categorical metadata variables were tested by Kruskal-Wallis, with pairwise differences between types of samples. Statistical significance between beta diversity was determined by analysis of similarity (ANOSIM) (Clarke, 1993) with 999 permutations on weighted UniFrac distances (Anderson, 2017; Lozupone et al., 2011). 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. Multibar taxonomy plots were also generated in R v3.6.0. The QIIME2 plugin ANCOM was used to detect differentially abundant taxa accounting for compositional constraints along with linear discriminant analysis effect size (LEfSe) (Mandal et al., 2015; Segata et al., 2011). Differences were considered to be significant if p-value < 0.05. Spearman's correlation tests and canonical correspondence analysis (CCA) were performed in R with the Vegan package to further understand the relationships between water characteristics and microbial composition.

3.3 Results and Discussion

3.3.1 Differences in water quality in Arraiján's drinking water system

Water quality was determined by measuring several physico-chemical parameters (total and free chlorine, pH, conductivity, temperature, TDS, turbidity and pressure) for each water sample (**Figure 3.3**). Differences were observed between water quality across samples taken at the three DWTPs assessed in this study. DWTP A consisted of two source intakes, one where the source

water was chlorinated and one without chlorination. DWTP B did not chlorinate at the intake but did practice pre-chlorination (0.8 mg/L ± 0.3 mg/L). On the other hand, DWTP C practiced post-chlorination (**Figure 3.3A-B)**. The average turbidity of source water in DWTP A was well above 30 NTU and significantly different (Kruskal-Wallis, $p < 0.01$) than source water for DWTP B and C, where turbidity averaged 2.2 NTU and 3.6 NTU, respectively **(Figure 3.3C).** Similar to turbidity**,** conductivity and TDS were much higher and significantly different (Kruskal-Wallis, $p < 0.01$) in samples from DWTP A compared to DWTP B and C (**Figure 3.3E-F**). Differences in turbidity, conductivity and TDS in samples from the source at DWTP A are likely due to its proximity to the Panama Canal (and the influence of its dynamic operations). Freshwater from the Gatun and Miraflores lakes (where drinking water is sourced) is used for filing the navigation locks during ship transit. Saltwater is introduced to the lakes by density currents. The salinity of the Canal also undergoes regional and seasonal variability due to varying precipitation, evaporation and shipping intensity (Salgado et al., 2020). The Panama Canal Commission estimated a net loss of freshwater of about 52 million gallons per lockage operation (Parchure et al., 2000). This estimate was published before the Panama Canal expansion in 2016, which has been suggested to have increased salinity concentrations along the Panama Canal to a greater extent (Salgado et al., 2020, Schreiber et al. *in prep*). Other water quality parameters such as temperature and pH were consistent across all samples. pH measurements were similar across sample locations and sample types, and there were no significant differences (**Figure 3.3D)**. Temperatures averaged around 28°C and were not different across locations or sampling times (Kruskal-Wallis, $p > 0.05$) (**Figure 3.3E)**. Treatment at the three DWTPs did not significantly change conductivity, pH, temperature, and TDS (Kruskal-Wallis, $p > 0.05$).

In the DWDS, turbidity measurements we not statistically different between sampling points (Kruskal-Wallis, $p > 0.05$) **(Figure 3.3C)**. Conductivity and TDS were significantly different between samples in region A and samples from the other two regions (Kruskal-Wallis, $p < 0.05$, respectively) due to higher conductivity and TDS in the source water in region A as described above. Pressure differences were found across sampling locations in the distribution system. These pressures varied between 5 – 60 PSI and were found to be significantly different in some instances (Kruskal-Wallis, $p < 0.05$) (**Figure 3.3H)**. Except for chlorine, water quality measures were found to be consistent within each region. Some water quality metrics at regions B and C were more congruent, likely due to having similar source water because of the proximity of their source water intakes located near one another and away from the Panama Canal transit route.

After water treatment and before distribution, all samples met the Panamanian drinking water standards at the time of this study (COPANIT, 1999) (**Figure 3.3**). Since then, the drinking water standards for chlorine residual in Panama have changed and now require that drinking water free chlorine residual be in the of range 0.05 mg/L to 0.8 mg/L (COPANIT, 2019), in contrast to the previous chlorine regulations which required a chlorine concentration between 0.8 mg/L and 1.5 mg/L, although it did not specify the type of chlorine measured. Overall, a range of chlorine concentrations was observed in the DWDS that seemed to correspond to the distance each sampling location was to a DWTP. The majority of samples had chlorine residual above 0.2 mg/L, although some locations have very low to no detectable chlorine residual (locations 4C and 5C) (**Figure 3.3A-B**). DWDS locations in regions A and B had the highest chlorine residual concentrations found in Arraiján's DWDS. However, now that the drinking water regulations have changed, further research is necessary to understand the implications of such drinking water

regulation changes that require lower chlorine residual in the system. It is important to note that this particular DWDS suffers from around 40% of water loss due to pipe failures, illegal connections, and other factors (e.g., loose fittings) (Erickson et al., 2020). Drinking water standards that lower the range of acceptable chlorine residual in the network could potentially lead to further loss of chlorine residual at distal parts of the DWDS and higher microbial risk.

As expected, HPC concentrations in source samples surpassed 1.0 x 10^4 MPN/100mL (**Figure 3.4)**. During treatment, these concentrations significantly decrease, and finished water had concentrations of HPC well under 100 MPN/mL. In the DWDS, we found an inverse relationship between HPC and chlorine concentrations; HPC concentrations increased with lower chlorine residual, but this correlation between HPC and chlorine concentrations is not consistent. Other factors like water age can also influence these results (Kennedy et al., 2020). Pressure differences, on the other hand, were not associated with chlorine or HPC concentrations (**Figures 3.3. and 3.4**). This is contrary to other studies that have found an inverse relationship between Chlorine, HPC and Pressure (Lee and Schwab, 2005; Shamsaei et al., 2013). We found that pressure at sampling locations was related to the proximity of a nearby pump in the DWDS.

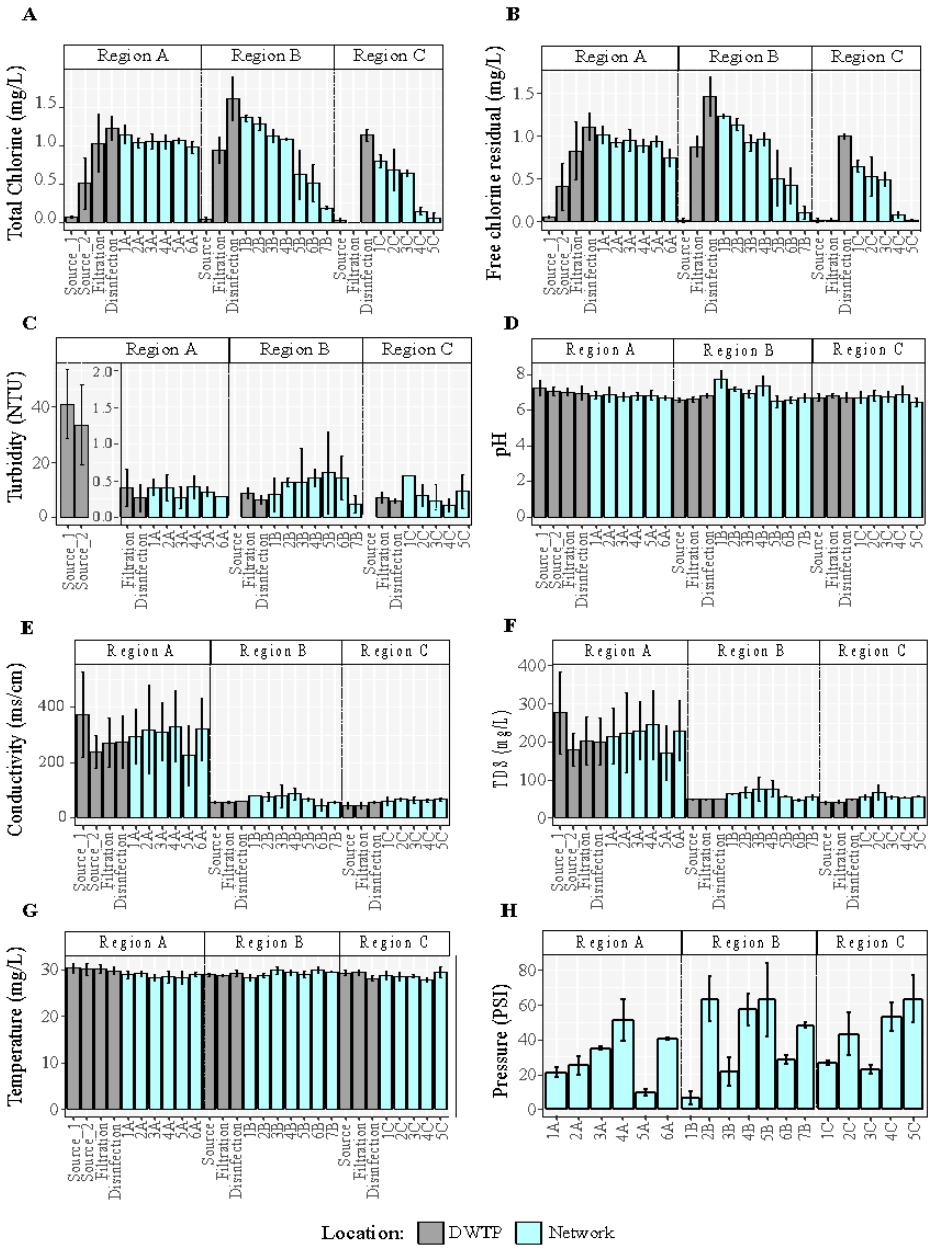

Figure 3.3 Mean and standard deviation of water quality metrics measured in this study (on site). Each location is represented by three to four samples taken on site. Measurements are divided in three regions that correspond to the three DWTPs that served Arraiján, Panama. TDS = Total dissolved solids.

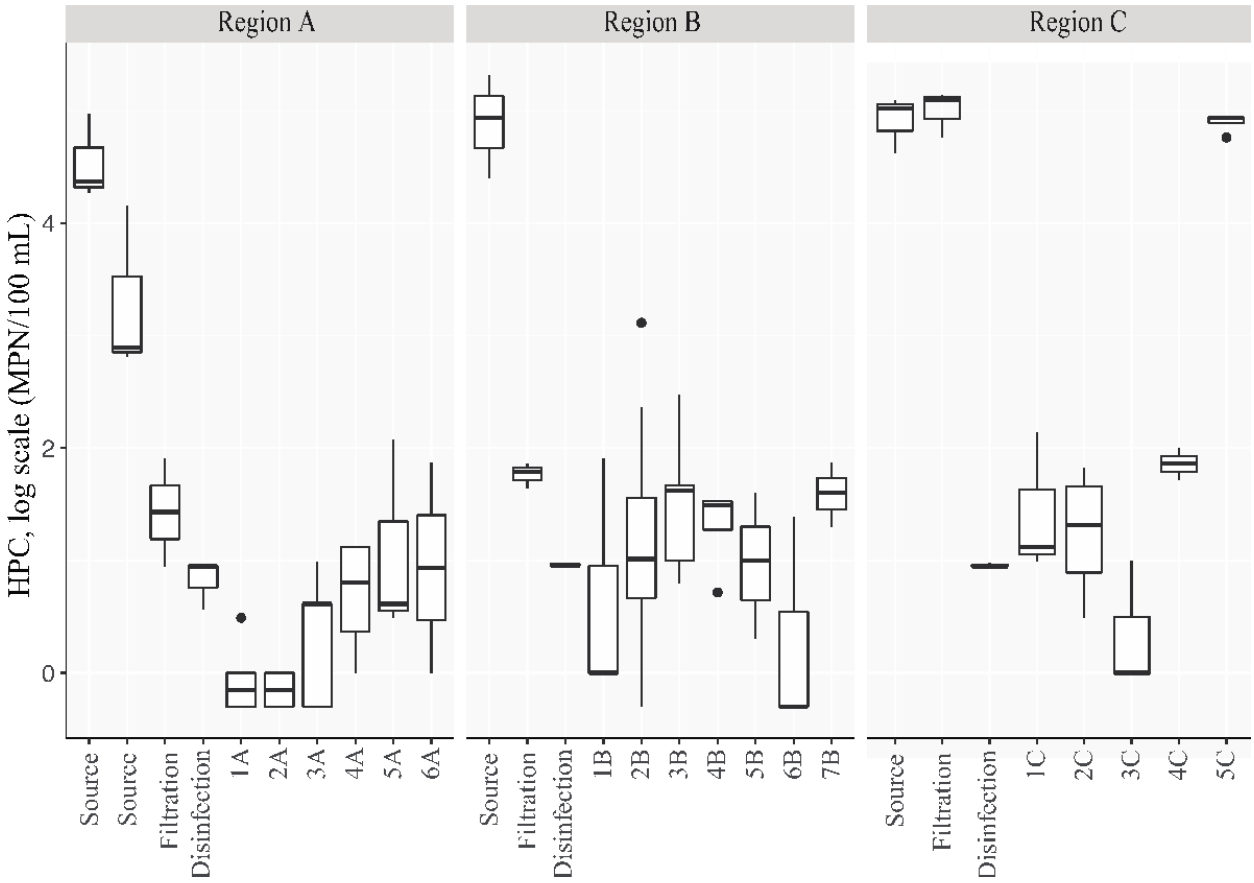

Figure 3.4. Heterotrophic plate count (HPC) concentrations found across sample types and sampling locations. Measurements are divided in three regions that correspond to the three DWTPs that served Arraiján, Panama. MPN = Most probable number.

3.3.2 Bacterial community diversity and composition by 16S rDNA sequencing reveals a diverse community

In total, 90 samples (three to four samples of 28 locations over 11 months) were collected from the drinking water system serving Arraiján, Panama, and successfully sequenced. We obtained a total of 2,512,566 sequence reads; between 36,219 and 76,898 reads were obtained per sample. After quality filtering, merging reads, chimera removal, 97% quality control by BLAST, and filtering low abundance ASVs, we obtained between 5,235 and 53,117 reads per sample and 4,811 ASVs (737 genera). Rarefaction curves demonstrated that the sequence efforts could capture the majority of the bacterial diversity in the dataset (**See Appendix A, Figure A9**).

Alpha diversity metrics at each treatment step tested (combined effects for the three different systems) showed significant differences (**Figure 3.5**). Diversity (both in the number of ASVs,

Shannon and Phylogeny diversity indices) decreased as water undergoes treatment and distribution. Although differences were found between the different diversity indices, all showed significant differences between the source waters and water after disinfection and distribution (Kruskal-Wallis, $p < 0.05$). Similarly, all alpha diversity indices showed significant differences between water after filtration and distribution (Kruskal-Wallis, $p < 0.05$). On the other hand, samples taken after filtration and disinfection were not found to be statistically different (Kruskal-Wallis, $p < 0.05$). The lack of difference between diversity in Filtration and Disinfection samples could potentially be due to the persistence of DNA from microorganisms that have been inactivated (Acharya et al., 2020) as water samples were taken immediately after disinfection and before any water storage. Similar diversity trends are seen when looking at each DWTP individually. Alpha diversity outliers (with high diversity) in the DWDS correspond to sampling locations with the lowest chlorine concentrations (locations 4C and 5C).

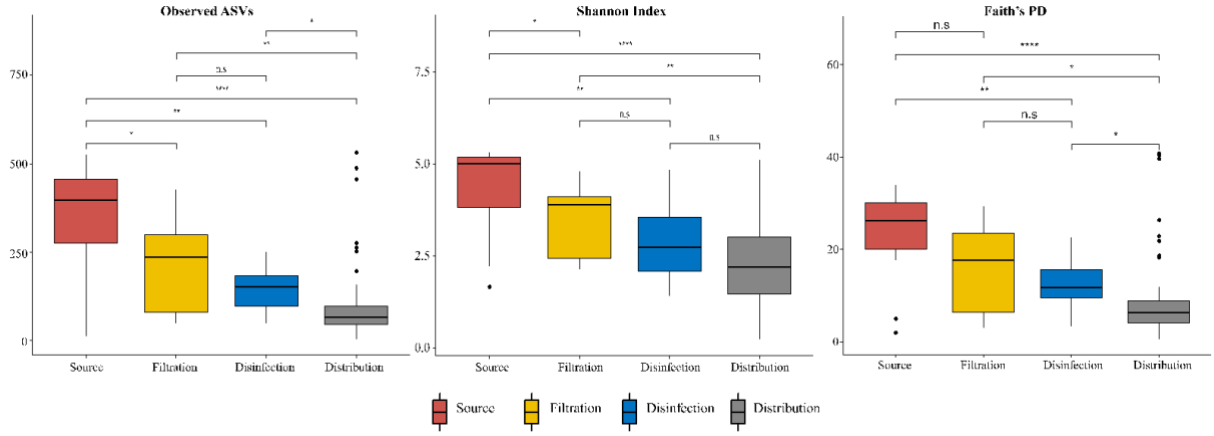

Figure 3.5. Alpha diversity metrics for the different sample types in this study. Asterix represent statistical significance (Kruskal-Wallis tests with bonferroni corrections) * = ($p < 0.05$), ** = ($p < 0.01$), * = ($p < 0.001$).**

Weighted UniFrac-based PCoA did not revealed a strong pattern of clustering of community structure by sample type, although a clear separation between source water samples (without chlorine residual) and DWDS is evident. There is a general trend in **Figure 3.6** with a transition in microbial community structure from untreated source water to the DWDS, with samples collected during treatment (i.e., immediately after filtration and disinfection) located in between source water samples and samples from the DWDS. Significant differences in microbial communities between sample types, except between Filtration and Disinfection samples were found (ANOSIM, Source/Filtration: R = 0.347, $p < 0.01$; Source/Disinfection: R = 0.60, $p < 0.001$; Source/Distribution: R = 0.438, $p < 0.001$; Filtration/Disinfection: R = 0.035, $p > 0.05$; Filtration/Distribution: R = 0.206, $p < 0.05$; Disinfection/Distribution: R = 0.202, $p < 0.05$). However, this significance could be due to some dispersion between sample types (PERMDISP, F-value = 0.10, $p > 0.05$) Alpha and beta diversity measures suggest strong differences along drinking water treatment and distribution, except between Filtration and Disinfection. Weighted UniFrac-based PCoA also show differences in community composition at each DWTP (**See Appendix A, Figures 10,A,C,E**).

Bacterial community structure in the DWDS was found to be significantly different across regions (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 the bacterial community composition found in certain locations was very different than in the rest of the DWDS (**See Appendix A, Table A2**). In particular, the bacterial community found in location 5C, which is the location with the lowest chlorine concentrations, is significantly different from all other locations in this study.

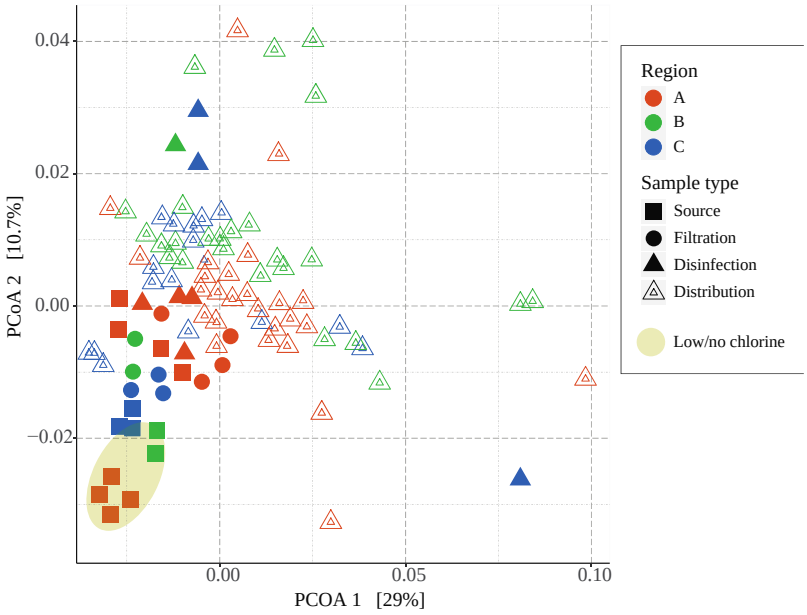

Figure 3.6. Beta diversity metrics for the different sample types in this study using principal coordinate analysis (PCoA) with weighted UniFrac distances.

3.3.3 Taxonomic classification of bacterial communities along drinking water treatment and distribution

Fifteen bacterial phyla and 71 classes were identified as making the majority of bacterial communities in the dataset (**See Appendix A, Table A2**). Four phyla represented over 95% of the total ASVs for all samples combined: Proteobacteria (Mean ± standard deviation (Mean ± SD, 70.6% ± 6.36%), followed by Cyanobacteria (Mean ± SD, 12.6% ± 7.81%), Bacteroidetes (Mean ± SD, 5.4 % ± 3.18%) and Actinobacteria (Mean ± SD 3.6% ± 1.62%). Other studies have also found Proteobacteria, Bacteroidetes, Actinobacteria, Cyanobacteria and Planctomycetes as top phyla in drinking water systems (Bautista-De los Santos et al., 2016; Q. Li et al., 2017; R. Li et al., 2017; Lin et al., 2014; Ma et al., 2017; Pinto et al., 2012, 2014; Caitlin R Proctor and Hammes, 2015). At taxonomic level of Class, the most abundant were Alpha- (Mean ± SD 34.7% ± 5.67%) and Gamma-proteobacteria (Mean ± SD 29.4% ± 7.25%), Vampirivibrionia (Mean ± SD 11.5% ± 9.05%), Oligoflexia (Mean ± SD 6.03% ± 1.03%), Bacteroidia (Mean ± SD 5.1% ± 0.03%), Actinobacteria (Mean ± SD 3.0% ± 1.90%), Bacilli (Mean ± SD 1.49% ± 1.0%) and Planctomycetes (Mean ± SD 1.25% ± 1.0%). Despite the dominance of Alphaproteobacteria and

Gammaproteobacteria, which is consistent with previous studies of drinking water systems (Q. Li et al., 2017; Ma et al., 2017; Pinto et al., 2012), the drinking water system of Arraiján contained a vast diversity of bacterial groups and differences in bacterial composition between each DWTP and DWDS.

When looking at each DWTP independently, we observed several differences between bacterial phyla (**See Appendix A, Table A2**). Source water in region A had higher Proteobacteria (relative abundance) than source water from regions B and C. In addition, the relative abundance of Proteobacteria was somewhat consistent as the water was treated and distributed in region A. In contrast, in DWTP B and C, the relative abundance of Proteobacteria significantly increased (Kruskal-Wallis, $p < 0.05$) as the water moved through the treatment train from source to disinfection and distribution (**See Appendix A, Table A2**). Actinobacteria and Bacteroidetes significantly decreased as water was treated and distributed; however, Bacteroidetes were significantly higher in Filtration samples in DWTP C compared to the other DWTPs. Another significant change was the increase in Firmicutes (relative abundance) in Disinfection samples in DWTP A (Kruskal-Wallis, $p < 0.05$) (**See Appendix A, Table A2**). Phyla that commonly have high relative abundances in some water sources such as Bacteroidetes and Actinobacteria have been found to have preferential removal during water treatment (Mukherjee et al., 2016; Pfannes et al., 2015; Xu et al., 2017). This preferential removal has been suggested to be due to preferential grazing by protists and the inability of anaerobic members to survive the aerobic conditions found in biosand filters (Fierer et al., 2010; Xu et al., 2017). Here, we found results from DWTP A and B in accordance with previous publications, including the increase of Firmicutes after filtration in DWTP A (Q. Li et al., 2017). However, we also found an increase in the relative abundance of phyla Bacteroidetes (the most abundant and enriched genera was *Parasediminibacterium,* a strict aerobe) after Filtration in DWTP C, which is contrary to these previous publications and our results.

Within the DWDS, differences in relative abundances of top phyla were also observed. In region A, a decrease (although not significant) in Proteobacteria (relative abundance) was observed between Disinfection samples and samples in the distribution. This decrease in Proteobacteria was coupled with a significant decrease of Firmicutes and an increase of Cyanobacteria (the most abundant genera was *Obscuribacter*) between Disinfection and Distribution samples (Kruskal-Wallis, $p < 0.05$). In regions B and C, Proteobacteria represented over 84 % of relative abundance with minor differences between other phyla (**See Appendix A, Table A2**). Among locations in region A, the family Obscuribacteraceae was a particularly abundant family with the relatively new genera *Obscuribacter.* Silvanigrellaceae and Pseudomonadaceae were abundant across regions B, and Oxalobacteraceae was abundant across regions C (**Figure 3.7**).

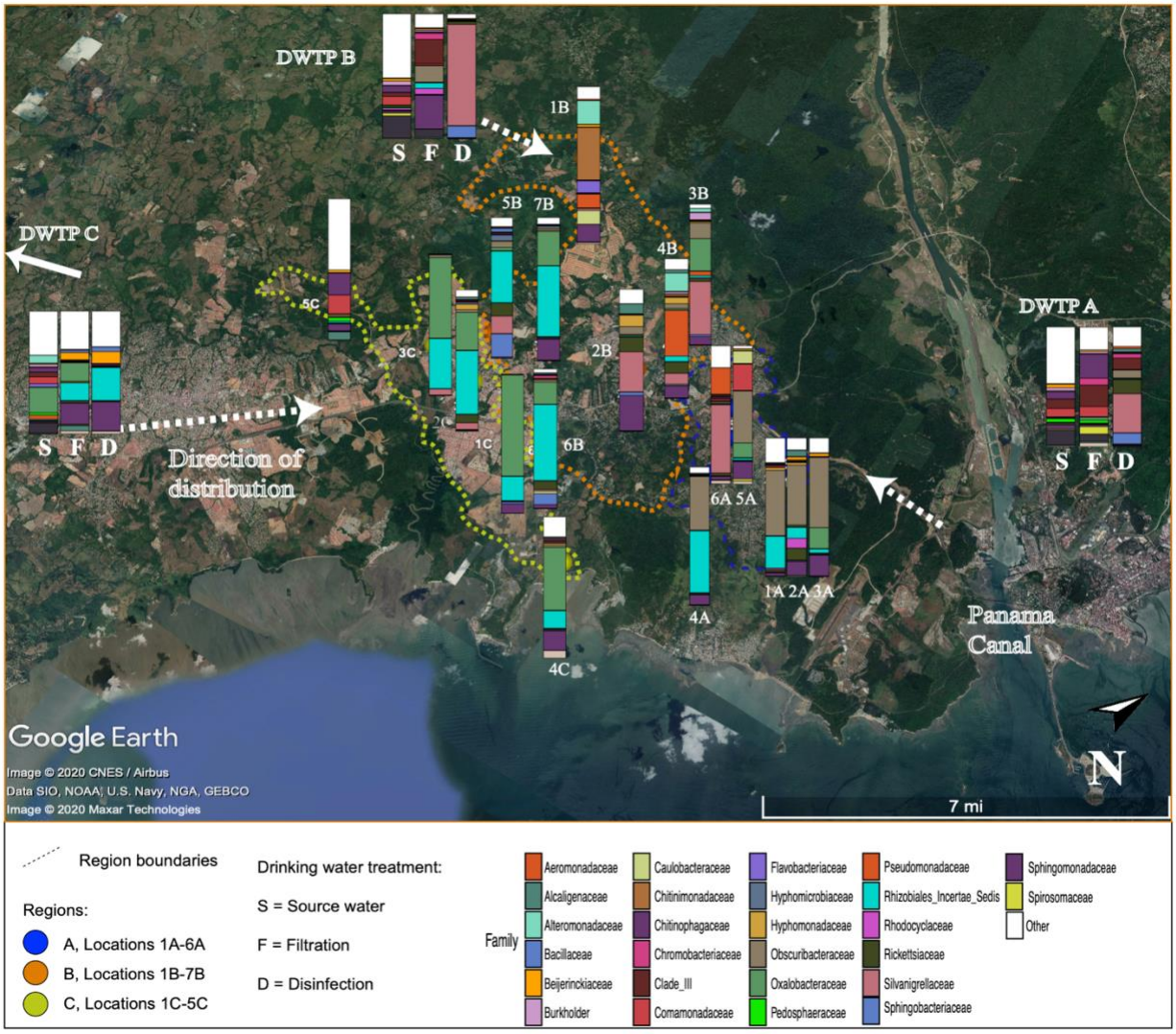

Figure 3.7. Sampling site map with top (most abundant) families in samples according to location.

To investigate the seeding effect of source water to disinfected and distributed water, Venn diagrams were generated for the treatment processes and distribution (**Appendix A, Figures A10B, A10D, A10F**). Different numbers of ASVs across sample types and regions were observed. As expected, many ASVs were found to be unique to samples taken at the source intake. The number of unique ASVs decreased as the water moved through the treatment train for all DWTPs. Among ASVs found in source and treated water, 114 ASVs (66%) were shared between all three DWTPs. It was shown that 65, 12, and 60 ASVs were universally present from source to distribution in regions A, B and C, respectively. A significantly higher number (Kruskal-Wallis, $p < 0.05$) of unique ASVs were found in DWDS samples for all regions. (**Appendix A, Figures A10B, A10D, A10F**).

LEfSe and ANCOM were used to determine taxa enriched across the drinking water system (**See Appendix A, Table A3**). Many of these genera were unclassified; however, as expected, several genera were found to be enriched at the sources, including genera belonging to Bacteroidetes and Actinobacteria. *Mycobacterium* was found to be enriched in Filtration samples in DWTPA as well as *Methylobacterium*. *Mycobacterium* has been found in other DWDS to persist after filtration processes (Hilborn et al., 2006; Thomson et al., 2013). Low chlorine residual and the protection provided by biofilm formation could contribute to the enrichment of *Mycobacterium* and other microorganisms during the filtration process (Hilborn et al. 2006, Haig et al. 2018). *Obscuribacter*, *DSSF69.* and *Polynucleobacter* were among the genera enriched in Filtration samples in DWTP B. Filtration samples from DWTP C had several enriched bacteria, including *Curvibacter*, *Polynucleobacter*, *Bdellovibrio*, *Cyanobium*, *Parasediminbacterium.* In this study, filtration enriched different genera at different DWTPs. Differences in enriched bacteria after filtration can be due to factors including source water, operational differences (e.g., the time between filter cleaning operations), pre-chlorination, and temperature (Oh et al., 2018; Pinto et al., 2012). *Methylobacterium* continued to be enriched in Disinfection samples in DWTP A and Distribution samples in region A.

In the distribution system, *Phreatobacter* (Proteobacteria) was enriched across all regions (**See Appendix A, Table A3**), indicating that although significant differences exist in bacterial communities along the DWDS, some bacteria are abundant across the network. Although information about these genera is limited, they have been found in DWS and household water purifiers (Lin et al., 2020; Ling et al., 2018; Van Assche et al., 2019). Further research is needed to understand further spatial and temporal effects on bacterial communities in highly dynamic DWDSs. Other enriched genera in the DWDS included: *Methylobacterium, Obscuribacter, Acinetobacter* in region A*; Pseudomonas*, *Nevskia*, and *Silvanigrella* in region B; and C. and *Sediminibacterium* in region C. These genera have been found in drinking water from different drinking water systems (Carvalheira et al., 2021; Haig et al., 2018; Ling et al., 2018; Liu et al., 2014; Tsagkari and Sloan, 2019; Wu et al., 2020). Of special attention is the enrichment of genera known as having pathogenic species (*Pseudomonas*, *Mycobacterium*, *Acinetobacter*) (Bertelli et al., 2018; Carvalheira et al., 2021; Kelly et al., 2014; Wang et al., 2012).

3.3.4 Bacterial community composition is weakly correlated to water quality parameters measured

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 3.8**). 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 significant to microbial community composition. Although these correlations were somewhat weak, they demonstrated the importance of water quality parameters on microbial community structure. It is important to note that collinearity exists between water quality parameters which can confound these results. Nonetheless, factors such as chlorine concentration, turbidity, conductivity, TDS, and pressure are of particular importance when assessing microbiomes in drinking water and water quality in general.

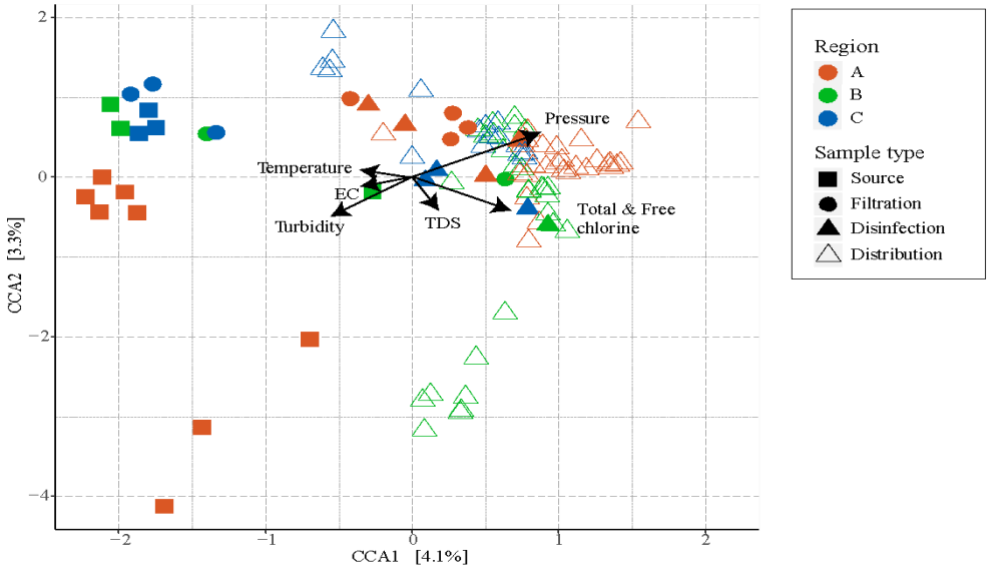

Figure 3.8. Canonical correspondence analysis (CCA) plot of association between water quality metrics measured and microbial community composition

Water treatment is critical to public health. Despite advances in drinking water technologies, safe water production remains a challenge, especially in resource-constrained regions. Challenges in providing safe water include source water contamination, treatment process failures, failures in the distribution system, and emergent contaminants and pathogens. Drinking water treatment processes are designed to meet particular drinking water standards according to water (source) characteristics and regional/local regulations. In Panama, the standard conventional treatment includes multi-step processes, which include rapid sand filtration and disinfection. However, in drinking water systems, drinking water treatment processes can pose selective pressures on bacterial communities, and some bacteria can survive those stresses, reaching the DWDS (Lin et al. 2014; Pinto et al., 2012; Proctor and Hammes 2015). In this study, treatment processes in three DWTPs were evaluated. All three DWTPs operated according to Panamanian regulations and finished water before distribution met drinking water standards from Panama. We found a diverse bacterial community in the drinking water system of Arraiján, Panama. The results show that source water and similar treatment processes at different DWTPs can enrich different bacteria and exhibit different bacterial community structures. Here, it is hypothesized that different DWTP configurations and operations could result in significant variation of microbial communities in drinking water. Although apparent clustering (beta diversity analysis) was not observed between different sample types, there were significant differences in bacterial diversity and community structure across the different treatment processes and distribution as well as across regions in Arraiján's drinking water system. Filtration and disinfection exerted changes in drinking water bacterial communities found in source water; however, bacterial communities in the DWDS were strongly shaped by the DWDS, differences that were associated spatially across the DWDS. Water quality parameters such as chlorine residual, turbidity, and conductivity were also important in determining bacterial community composition. Although collinearity exists between water quality

parameters (making it difficult to disentangle the effects of each parameter), characteristics of water remain critical at determining bacterial diversity.

It is important to note that although each sample represents a large volume of water collected, we worked with a limited number of samples, which decreased the statistical analysis's power. More robust assessments of microbiomes in DWSs in developing countries are needed in order to understand how operations, environmental factors, and common deficiencies found in resourced-constrained systems influence water microbiomes. During this study, two major electrical outages occurred in the region that left two of the DWTPs unable to operate for several hours after each incident. Although we were unable to test the DWTPs or the DWDS immediately after these incidents (we were unable to acquire the proper permissions, and we did not know where supply was being delivered after supply restart), such deficiencies are common and likely to influence water quality and microbiomes in the DWTPs as well as in the DWDS.

3.4 Conclusion

Arraiján's DWS is a dynamic system composed of three DWTPs and a distribution network with different types of supply. Here, we applied molecular approaches alongside traditional methods to determine water quality and investigate bacterial abundance, diversity and taxonomy throughout the three centralized surface DWTPs and distribution network. We found very diverse bacterial communities in drinking water, and that water quality and bacterial communities vary significantly across space. This is particularly important as focusing on portions of a drinking water system may not capture the dynamics of a system. These results suggest that when assessing drinking water quality and microbial communities in water, the whole system needs to be put into perspective to bring forth more accurate representations of microbiomes in DWSs. Although we were unable to determine temporal differences, it is important to note that temporal effects on microbial communities can be significant in such dynamic systems.

The main findings of this study are:

- A substantial, diverse microbiome exists at each of the three DWTPs, dominated by Proteobacteria but with differences at the genus level along the treatment train.

- Treatment processes were found to have varied effects on bacterial diversity and structure at the different DWTPs, perhaps driven by DWTP operations. 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; however, bacterial communities in the DWDS vary significantly from those in the finished water at the DWTPs.

- The abundance of the relatively new 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.

Chapter 4

Bacterial communities in an intermittent piped water supply in Arraiján, Panama

4.1 Introduction

In low- and middle-income countries, Intermittent water supply (IWS) is a common practice and can be defined as piped water supply service that is available for less than 24 hours per day on average (IWA, 2016) and is estimated to affect at least one billion people worldwide (Bivins et al., 2017). Insufficient water resources, inadequate infrastructure, unplanned expansions in the drinking water distributions system, excessive water losses, increasing consumer demands, or a combination of these factors can lead to water being supplied intermittently (Galaitsi et al., 2016; Klingel, 2012; Kumpel and Nelson, 2016; Rosenberga et al., 2008). IWS is an inconvenience for users, requires consumers to store water, imposes coping costs on households, can cause pipe damage, and can make it difficult for utilities to deliver an equitable water supply (Burt et al., 2018; Erickson et al., 2020; Fontanazza et al., 2007; Guragai et al., 2017; Lee and Schwab, 2005). IWS can also pose health risks (Bivins et al., 2017), including millions of infections annually, causing millions of cases of diarrhea and 100,000 diarrheal Disability Adjusted Life Years (DALYs).

Several characteristics of IWS have the potential to increase the deterioration of drinking water quality compared to continuous flow systems: 1) intrusion and backflow of contaminants via pipe leaks, loose fittings, and customer connections during periods of low or negative pressure; 2) microbial growth in bulk water, pipe-wall biofilm, and loose deposits during the time water is drained or left stagnant, and after supply restarts, flushing and detachment of microorganisms; and 3) recontamination and microbial growth during household storage (Bautista-de los Santos et al., 2019; Kumpel and Nelson, 2013, 2016).

Culture-based techniques have been widely used to assess microbial water quality in IWS systems with the objective of determining whether water is safe for consumption. The use of indicator organisms and other traditional water quality parameters has shown that water quality can deteriorate during distribution and after household storage (Erickson et al., 2017; Falconi et al., 2017; Gonzalez et al., 2020; John et al., 2014; Kumpel and Nelson, 2013; Shields et al., 2015). However, indicator organisms do not represent the behavior of all pathogens nor the entire drinking water microbial community. Using culture-based approaches, only a minor fraction of the microorganisms in drinking water can be detected. Characterizing whole microbial communities

may provide insights into the impacts of infrastructure deficiencies on water quality. Microbial communities in IWS have been studied to a limited extent using DNA sequencing approaches, but existing studies have not shed light on how key features of IWS impact microbial communities and have not directly assessed microbial communities in IWS cycles (Montoya-Pachongo et al., 2018; Tokajian et al., 2005). Furthermore, limited research has been conducted to investigate microbial communities in drinking water in tropical environments. This is particularly important as many countries where IWS is prevalent are located in the tropics (Kumpel and Nelson, 2016).

The overall goal of this study was to investigate how IWS influenced bacterial communities in a portion of the full-scale drinking water distribution system in Arraiján, Panama. To characterize bacterial communities associated with IWS, we used high-throughput amplicon sequencing of 16S rDNA combined with traditional culture-based methods and traditional water quality metrics. The specific objectives of this study were to: 1) compare bacterial community composition at a location upstream from the area with IWS (with continuous supply) to a downstream location with IWS; 2) assess the bacterial diversity and structure associated with IWS at the start of supply cycles (first flush) and after supply was stabilized; and 3) quantify indicator bacteria concentrations and traditional water quality metrics associated with the drinking water supply at the start of supply cycles and during stable supply in IWS. The results provide insights into how IWS shapes microbiomes in piped drinking water and further illustrate the complexities of IWS. Understanding how the unique features of IWS impact water microbiomes provide a better characterization of the risks of IWS, which can be used to develop strategies to protect water quality.

4.2 Background

This study was also conducted in the DWDS in Arraiján, Panama (Same DWDS described in Chapter three). For approximately 11 months, we sampled one section of the network that provided water intermittently. To assess IWS effects on bacterial communities, three types of samples were collected: 1) upstream, near the entrance of the IWS zone, samples were taken at a pump station and the supply was continuous (labeled Entrance); 2) downstream, first-flush samples were taken immediately after supply restarted (labeled First Flush); and 3) at the exact downstream location, samples were taken 24 hours after supply restart and supply had stabilized (labeled Stable).

One zone within the Arraiján DWDS, previously described in J. J. Erickson et al., 2017 (previously labeled Zone 3), was chosen to examine the effects of IWS on bacterial communities in drinking water. This zone received water intermittently due to insufficient supply capacity. The supply was managed by the utility with a valve located at the entrance to this zone. This zone had approximately 232 connections and was scheduled to receive water for three days every three days (i.e., alternating three days with water supply and three days without). Sanitation in this zone consisted of flush toilets connected to septic systems or pit latrines, typically located in the back of the houses, whereas the drinking water supply pipes were typically located in the front of the house. We used two previously installed continuous monitoring stations in this zone as sampling locations (Erickson et al., 2020), upstream near the entrance and at one downstream location (**Figure 4.1**).

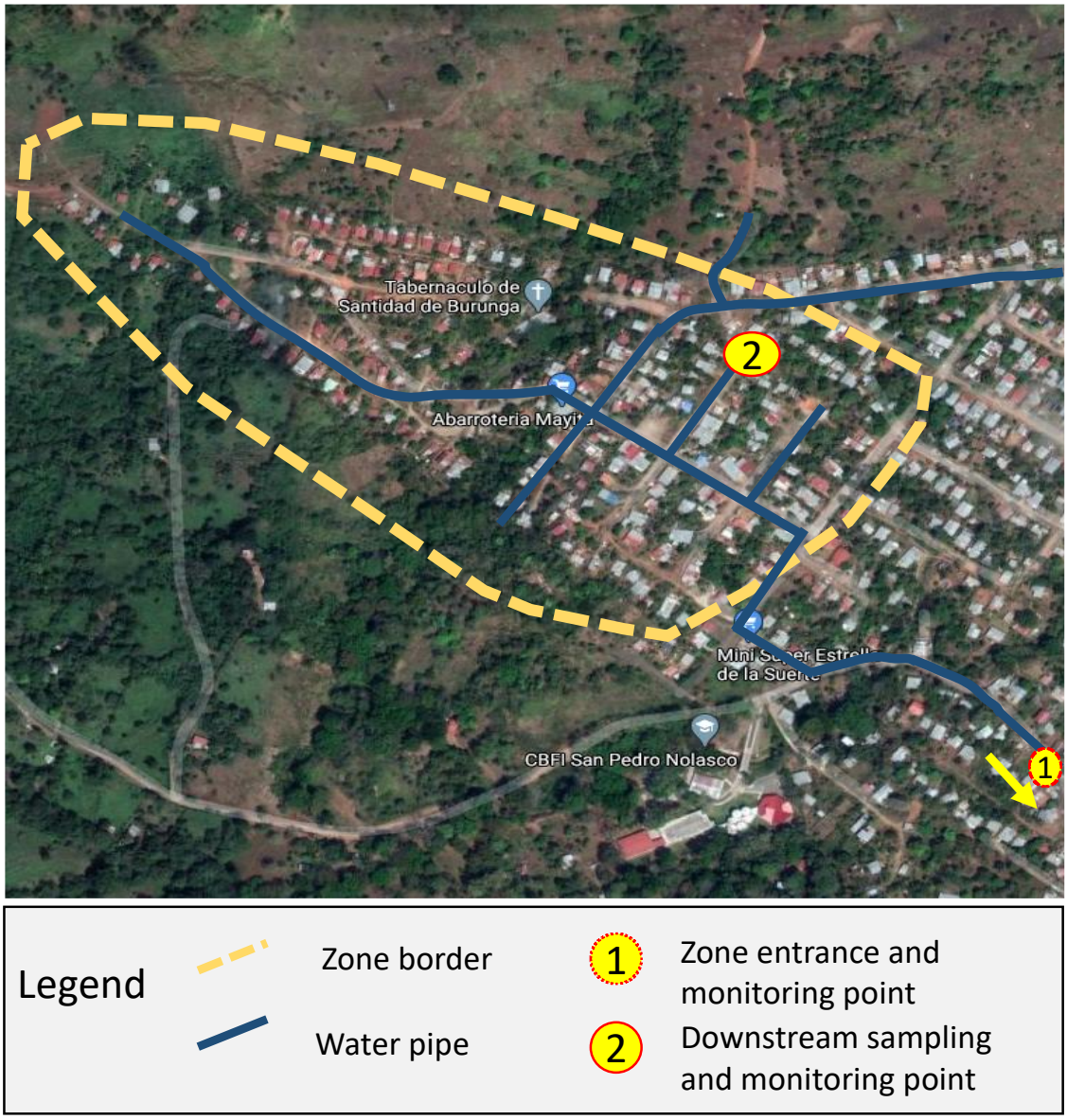

Figure 4.1: Schematic of the IWS zone located in Arraiján, Panama. This zone, with 232 buildings, was supplied water by a pump station managed by IDAAN with a supply schedule that consisted of the provision of water for 3 days followed by 3 days with no supply. This schedule was carried out by IDAAN by manually opening and closing a control valve at the entrance of the area. Because the zone's entrance monitoring station (with online sensors) experienced frequent negative pressures, grab samples for water quality analysis were collected at the nearby pump station (not shown).

4.3 Material and methods

4.3.1 Water quality parameters

Water samples were collected between December 2016 through October 2017 and between 6 a.m. and 3 p.m. For the collection of first-flush samples, we arrived at the sampling location when the supply was off and waited until the supply began. Once supply started, water was collected during the first 1.5 - 2 hours of supply. The following water quality parameters were analyzed immediately on site: free and total chlorine (Hach Portable Colorimeter II), turbidity (Hach Portable Turbidimeter 2100Q), pH, temperature, conductivity, total dissolved solids (HANNA Instruments pH/conductivity/TDS tester or Extech ExStik II pH/conductivity meter) and pressure (Eastman 45169 Water Pressure Test Gauge, adapted to fit sampling tap).

Some water quality parameters were also measured continuously using previously installed monitoring stations at the upstream and downstream locations. 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). The equipment in the monitoring stations was powered by a 12-volt battery charge with a solar panel installed at the top of the monitoring station box and was calibrated and maintained as described by Erickson et al., 2017. The entrance of the IWS zone sustained negative pressures previously and in this study. Thus, water samples that represented continuous supply in this area were taken upstream at a nearby pump station that pumps water to this zone and the surrounding areas.

4.3.2 Culture-based indicator bacteria detection

Similar to the process described in **Chapter 3**, grab samples for bacterial enumeration were collected in sterile 100-mL bottles and transported to STRI laboratory on ice. These samples were tested within 3 hours of collection for 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. Total coliform bacteria and *E. coli* indicators were not measured as the utility conducts routine monitoring of these indicators at these locations, and Erickson et al., 2017 found that they were below the detection limit in the majority of samples.

4.3.3 16S rDNA sequencing

Biomass was concentrated from each water sample on-site by dead-end ultrafiltration (DEUF) using REXEED 25S ultrafiltration membrane cartridges (Asahi Kasei, Tokyo, Japan) as previously described in **Chapter 3** (Smith and Hill, 2009). Briefly, Ultrafilters were transported at 4°C to the metagenomics laboratory at STRI and processed within 5 hours of collection. Isolation of DNA was performed using the DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany). The PowerSoil Kit was used following the manufacturer's instructions with previously described adjustments (Materials and Methods in Chapter 3). Amplification of the V4 variable region of the 16S rRNA

gene was conducted in triplicate 12.5 μL reactions using a two-step PCR protocol (Materials and Methods in Chapter 3). The V4 region of the 16S rRNA gene was amplified using phased primers [515F (Parada) GTGYCAGCMGCCGCGGTAA and 806R (Apprill) GGACTACNVGGGTWTCTAAT] with Platinum 2X Mastermix (ThermoFisher) and 2.5 μL of genomic DNA as template for the first PCR step. The resulting PCR1 products were pooled and 2.5 μL of PCR product were used as template for PCR2 with eight cycles to add on unique barcodes and Illumina sequencing adaptors. Negative and positive controls containing nuclease-free water and previously PCR positive samples were included in all PCR amplification steps. PCR2 products were cleaned and normalized with PCR Purification and Normalization Plates (Charm Biotech, San Diego, CA, United States) and pooled into a single sequencing library. The library was further concentrated, quantified by Qubit High Sensitivity dsDNA assay (Thermo Fisher Scientific), and quality checked by a Bioanalyzer dsDNA High Sensitivity assay. The resulting library was sequenced on an Illumina MiSeq with V3 chemistry on a 2 x 250 paired-end run at STRI's Sequencing Facility (STRI in Naos, Panama).

4.3.4 Data analysis

Data analysis was performed as described in Chapter 3. Briefly, for bacterial detection by HPC, 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. Untransformed data was used for other water quality parameters. Statistical analysis and plotting were performed using the statistical software R v.3.6.0, the Coin and ggplot2 package (R Core Team, 2012). Significance tests were performed by using non-parametric pairwise Kruskal-Wallis test with Benjamini-Hochberg corrections. Values were considered significant at $p < 0.05$.

The QIIME2 platform v2019.10 and R v.3.6.0 were used for the majority of the analysis of paired-end sequence reads in a similar fashion as described in Chapter 3. Briefly, Preprocessing was performed using QIIME2's Cutadapt and DADA2 plugins to produce amplicon sequence variants (ASVs) (Bolyen et al., 2019; Callahan et al., 2016; Martin, 2011), and taxonomy was assigned using a naïve Bayesian classifier trained on the SILVA 99% sequence similarity database (Pruesse et al., 2007; Wang et al., 2007). Sequences were also filtered and removed based on BLAST (97% confidence), low abundance (0.005%), and ASVs classified as mitochondria or chloroplast.

Alpha- and beta- diversity were calculated using QIIME2 from the resulting ASV table standardized to a sequencing depth of 2000 sequences per sample. Differences in alpha diversity (Observed ASVs, Shannon, and Simpson indices) by categorical metadata variables were tested by Kruskal-Wallis, with pairwise differences between sample types. Statistical significance between beta diversity of the different types of supply (Entrance vs. First Flush vs. Stable) was determined by ADONIS permutation-based ANOVA (PERMANOVA) tests with 999 permutations on weighted UniFrac distances (Anderson, 2017; Lozupone et al., 2011). Beta diversity was explored using weighted UniFrac distances and an ordination approach using principal coordinate analysis (PCoA) generated in R with the package Phyloseq. Heatmap visualizations were generated using R packages Phyloseq (Foster et al., 2017; McMurdie and Holmes, 2013). 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 constraints (Mandal et al., 2015; Segata et al., 2011).

Spearman's correlation tests and canonical correspondence analysis (CCA) were performed (using the Vegan package in R) to further understand the relationships between microbial communities and water quality parameters.

4.4 Results

A total of 27 grab samples were successfully collected from the IWS zone (upstream and downstream) for water quality analysis and sequencing: four samples at the upstream pump station, 14 first flush and seven stable supply samples from the downstream monitoring station. Unfortunately, it was not possible to collect more samples at the pump station on all sampling dates due to inaccessibility (i.e., the door lock to the pumping station was broken and operators could not access it for an extended period of time) or due to a pump failure that lasted over four weeks. In addition, we could not collect more samples during stable supply due to either the lack of water supply 24 hours later or logistical and safety issues. It is important to note and recognize that the asymmetry in sample collection between the three sites is a limitation, and note that challenges with implementing an experimental design are common when conducting research on dynamic IWS systems.

4.4.1 Water Quality during IWS

Continuous monitoring 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 4.2).** Except for first-flush samples, grab samples had an average free and total chlorine concentration equal to or above 0.9 mg/L (0.9 ± 0.1 mg/L). 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 4.3a,b**). Average turbidity levels in grab samples were below the water quality standard of 1 NTU (0.5 ± 0.1 NTU) but tended to be higher in the first-flush samples (**Figure 4.3c**). The continuous monitoring data captured turbidity measurements above 1 NTU during and well past the first 24 hours of supply cycle (**Figure 4.2**). Water temperatures during the First Flush ranged from 29.7 - 33.3°C and were significantly higher than Stable IWS and at the Entrance (Kruskal-Wallis, $p < 0.05$). Water temperatures during stable supply (Stable samples) in IWS and at the entrance (Entrance samples) ranged between 28.3 - 30°C and were not statistically different (Kruskal-Wallis, $p > 0.05$) (**Figure 4.3d**). EC, pH, and TDS were not statistically different between Entrance and downstream First Flush and Stable supply samples (Kruskal-Wallis, $p > 0.05$, respectively) (**Figure 4.3e, f, g**). The average pressure in the distribution system at sampling locations ranged between 4 and 59 PSI and varied significantly between the Entrance of the IWS zone, the First Flush, and Stable supply sampling events (Kruskal-Wallis, $p < 0.05$). The average pressure for First Flush samples was only 7.5 psi (7.5 ± 2.5 PSI, measured 1.5-2 h after supply started), while for Stable supply, it was 21 psi. At the entrance, pressure averaged 59 psi (59 ± 10.4 PSI) (**Figure 4.3h**).

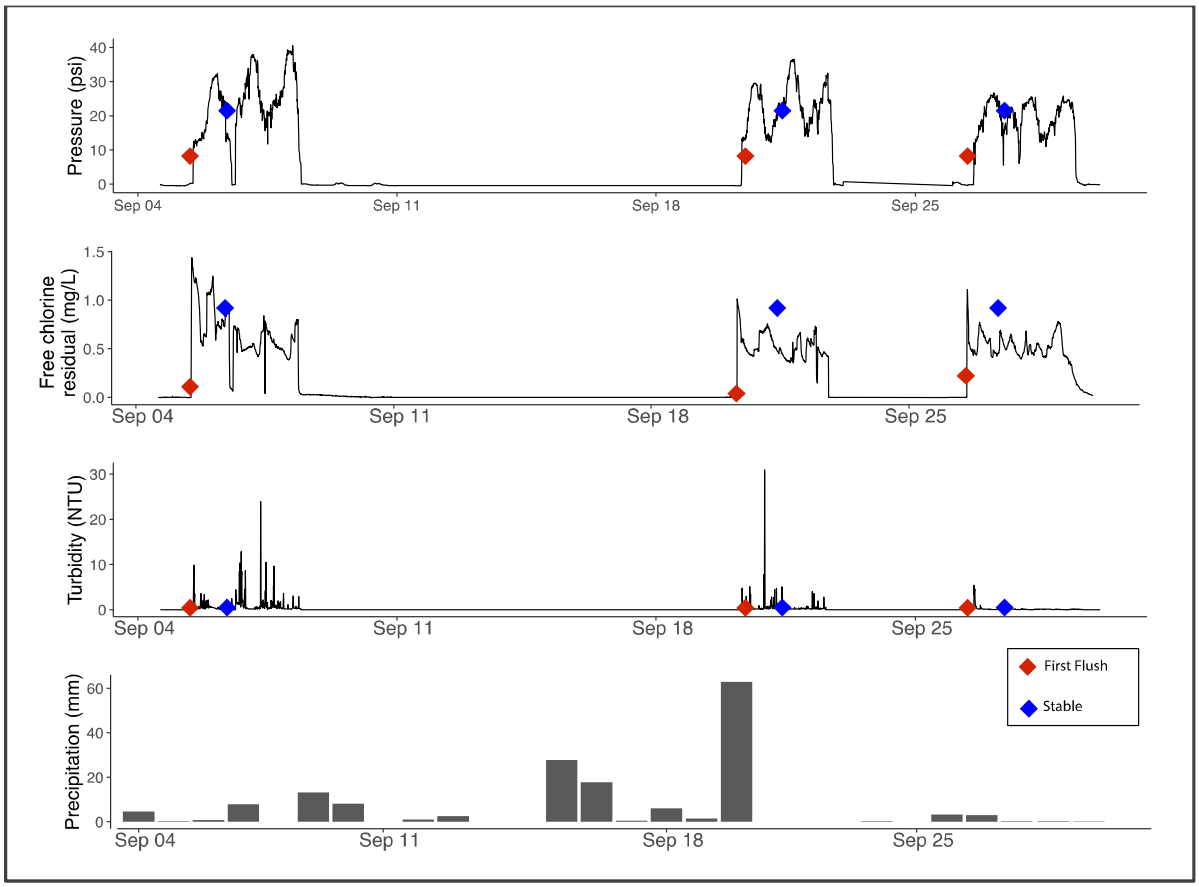

Figure 4.2 Water quality parameters measured in grab samples and with on-line sensors at the downstream monitoring station for three consecutive IWS cycles in September 2017. The grab sample measurements also indicate the time at which water was collected and concentrated for sequencing for first flush (red diamond) and stable supply (blue diamond) conditions. Precipitation events are also reported.

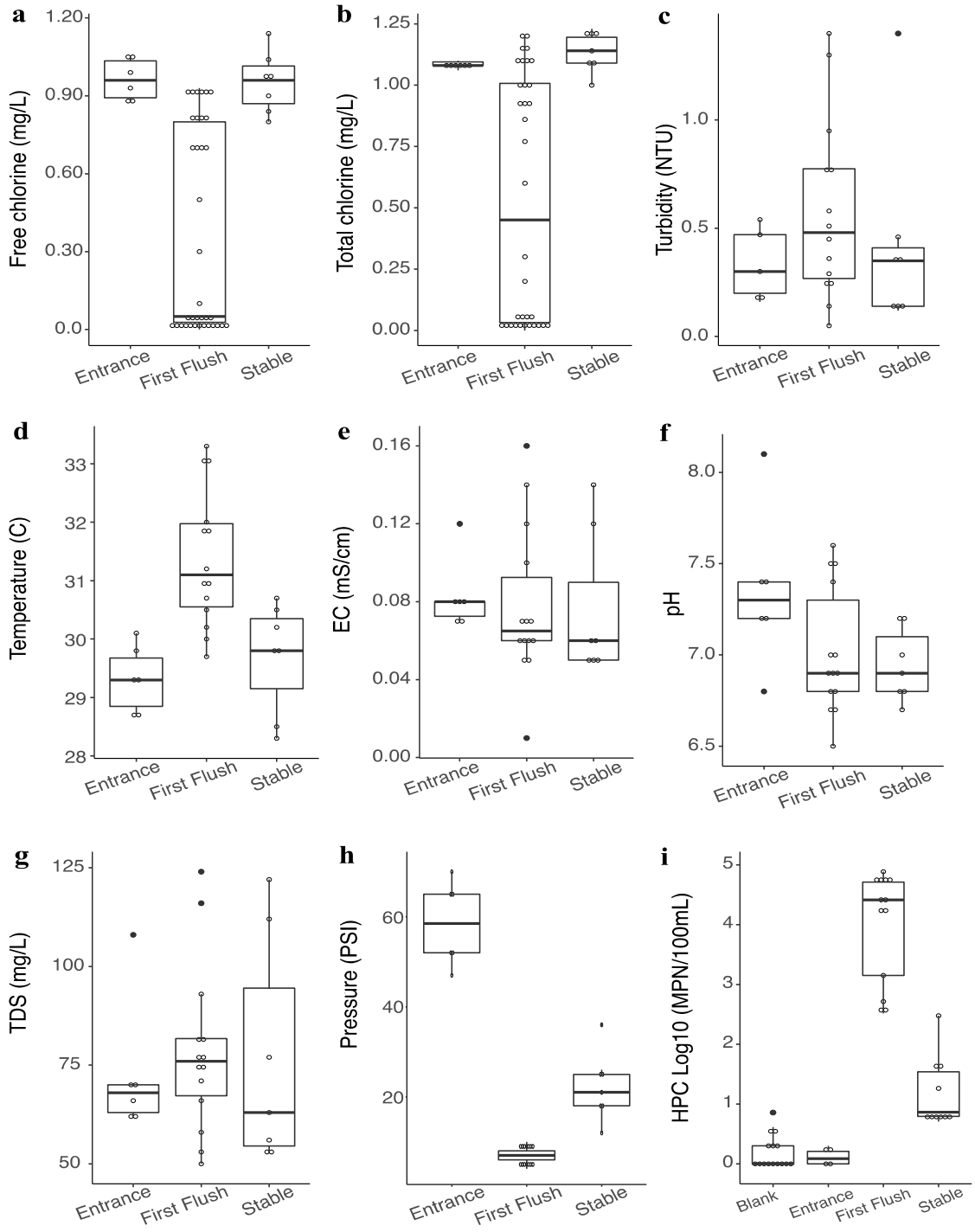

Figure 4.3: Comparison of water quality parameters, measured in grab samples, at the study zone entrance, and at the downstream location in both first flush and stable supply samples. EC = electroconductivity, TDS = Total dissolved solids, HPC = Heterotrophic plate count.

HPC indicator bacteria concentrations differed by sample type **(Figure 4.3i).** Concentrations of HPC at the Entrance to the IWS zone averaged 22 MPN/mL (22 ± 14 MPN/100mL) and remained close to or below the lower detection limit. First Flush samples averaged an HPC concentration of 4.3 x 10^4 MPN/mL (4.3 ± 2.8 x 10^4 MPN/100mL) and ranged between 326.5 and 7.7 x 10^4 MPN/100mL. Concentrations of HPC were significantly higher during First Flush events than Stable IWS (Kruskal-Wallis, $p < 0.001$). In IWS Stable supply samples, HPC concentrations were lower, with an average of 70 MPN/100mL (70 ± 109 MPN/100mL) and ranging from 5.1 and 299.7 MPN/mL.

4.4.2 Bacterial diversity and community composition in IWS

Illumina MiSeq sequencing generated between 39,555 to 88,929 reads per sample. After sequencing processing and filtering, between 19,133 and 47,691 reads per sample were obtained, representing 1275 ASVs (229 bacterial taxa after taxa agglomeration at the genus level) (**Table 4.1**).

Table 4.1: 16S rRNA gene amplicon sequence reads results after data preprocessing and filtering

Sample	Date	input	filtered	% of input passed filter	denoised	merged	% of input merged	non-chimeric	% of input non-chimeric	97% QC & Final filtering
ENTRANCE	04.11.17	61357	56540	92.15	55324	50239	81.88	8767	14.29	3434
ENTRANCE	08.18.17	43335	38100	87.92	37753	34239	79.01	4306	9.94	3815
ENTRANCE	09.29.17	88929	83790	94.22	82648	72582	81.62	9069	10.2	6663
ENTRANCE	10.11.17	60531	57226	94.54	56259	50467	83.37	5002	8.26	1799
FIRST_FLUSH	02.02.17	57627	50634	87.87	50273	48725	84.55	3089	5.36	2888
FIRST_FLUSH	02.07.17	51482	49044	95.26	48229	42796	83.13	6217	12.08	3095
FIRST_FLUSH	03.09.17	59374	51620	86.94	51261	46571	78.44	2719	4.58	2335
FIRST_FLUSH	04.22.17	57080	54236	95.02	53426	46390	81.27	6940	12.16	2543
FIRST_FLUSH	04.28.17	52765	47208	89.47	46347	40688	77.11	5198	9.85	2961
FIRST_FLUSH	05.06.17	50148	47681	95.08	46553	40842	81.44	7591	15.14	3846
FIRST_FLUSH	05.12.17	62280	56127	90.12	54986	50155	80.53	6617	10.62	4776
FIRST_FLUSH	07.20.17	53453	49575	92.75	49037	44220	82.73	6187	11.57	4874
FIRST_FLUSH	07.25.17	43314	40280	93	39611	35770	82.58	5378	12.42	4408
FIRST_FLUSH	08.01.17	81048	69099	85.26	68406	64849	80.01	5458	6.73	5295
FIRST_FLUSH	08.07.17	64084	55208	86.15	54676	52149	81.38	4571	7.13	4323
FIRST_FLUSH	08.15.17	47130	42643	90.48	42391	39611	84.05	4862	10.32	4620
FIRST_FLUSH	09.05.17	54374	49475	90.99	46820	38890	71.52	7834	14.41	5177
FIRST_FLUSH	09.20.17	60806	57455	94.49	53923	44358	72.95	7252	11.93	4734
FIRST_FLUSH	09.26.17	39555	37284	94.26	34935	29543	74.69	7398	18.7	4946
STABLE	04.24.17	63361	59107	93.29	58210	52285	82.52	6321	9.98	2589
STABLE	07.06.17	48470	46405	95.74	45556	40381	83.31	4880	10.07	2010
STABLE	07.26.17	60608	52109	85.98	51497	46468	76.67	5067	8.36	4682
STABLE	08.02.17	51379	49030	95.43	48592	44015	85.67	8652	16.84	6108
STABLE	08.08.17	55357	52554	94.94	52015	47073	85.04	7555	13.65	5489
STABLE	09.06.17	64458	58489	90.74	57412	49879	77.38	8430	13.08	4176
STABLE	09.27.17	44044	41888	95.1	41014	36548	82.98	6075	13.79	3707

4.4.2.1 Taxonomy based variation in community structure

Proteobacteria was the dominant phylum (90.7 % relative abundance) for all locations, followed by Cyanobacteria (4.7%), Bacteroidetes (2.22%), and Firmicutes (0.81%) (**Table 4.2**). Among Proteobacteria, Gammaproteobacteria was the most abundant class (45.8%), followed by Alpha-

and Deltaproteobacteria (40.1% and 13.9%, respectively). Cyanobacteria were mainly composed of an unclassified bacterium in the order Obscuribacterales (79.7% of all Cyanobacteria). Within the Bacterioidetes, Sphingobacteriales, Cytophagales, and Flavobacteriales were the top orders (42.3%, 17.3% and 11.7%, respectively). *Bacillus* spp. was the dominant genus (30.2%) within the phylum Firmicutes. Only one taxon, *Sphingomonas spp.,* was found present in most samples (over 80% of samples with over 0.5% relative abundance).

At the Entrance of the IWS zone, Proteobacteria represented 87.4% of the total community with Gamma- and Deltaproteobacteria as the dominant classes (36.1% and 33.4%, respectively). Cyanobacteria and Bacteroidetes phyla represented 10.1% and 1.3% relative abundance, respectively (**Figure 4.4A** and **Table 4.2**). *Silvanigrella* (Oligoflexia, 32%), *Undibacterium* (Betaproteobacteria, 22%), *DSSF69* (Alphaproteobacteria, 3.8%), and *Pseudomonas* (Gammaproteobacteria, 3.7%) were among the most abundant genera found in samples taken at the Entrance. Four taxa, *Silvanigrella* (Oligoflexia), *Pedobacter* (Bacteroidetes), an uncultured Obscuribacterales (Cyanobacteria), and *Sphingomonas* (Proteobacteria), were the most persistent taxa (with > 1% relative abundance).

Table 4.2. **Taxa mean relative abundance and standard deviation for entire data set and each sample type.**

	Overall	Entrance (Loc1)	First Flush (Loc2)	Stable (Loc2)
Phylum	mean ± sd	mean ± sd	mean ± sd	mean ± sd
Proteobacteria	85.83 ± 3.4	82.37 ± 4.51	88.77 ± 3.08	81.08 ± 3.6
Cyanobacteria	6.99 ± 1.65	12.94 ± 2.27	3.41 ± 0.8	11.77 ± 1.72
Bacteroidetes	3.12 ± 0.9	2.81 ± 0.57	2.84 ± 0.73	3.95 ± 1.26
Firmicutes	1.58 ± 0.72	0.62 ± 0.4	1.97 ± 0.75	1.24 ± 0.59
Planctomycetes	0.82 ± 0.83	0.24 ± 0.13	0.91 ± 0.81	0.96 ± 0.98
Actinobacteria	0.57 ± 0.75	0.54 ± 0.98	0.78 ± 0.77	0.1 ± 0
Chlamydiae	0.33 ± 0.54	0.15 ± 0.04	0.49 ± 0.55	0.07 ±
Dependentiae	0.29 ± 0.37	-	0.39 ± 0.37	0.25 ± 0.51
Fusobacteria	0.2 ± 1.03	0.32 ± 0.54	-	0.57 ± 1
Acidobacteria	0.09 ± 0.34	-	0.15 ± 0.34	-
Nitrospirae	0.09 ± 0.46	-	0.15 ± 0.46	-
Verrucomicrobia	0.08 ± 0.61	-	0.14 ± 0.61	-
Gemmatimonadetes	0.01 ± 0	-	0.01 ± 0	-

Downstream, Proteobacteria was also the most abundant phylum, yet Gamma- and Alphaproteobacteria were the dominant classes among samples. In First Flush samples, Proteobacteria abundance averaged 93.1%, followed by Bacteroidetes (2.1%), Cyanobacteria (1.82%) and Firmicutes (1.4%). Among Proteobacteria, Alphaproteobacteria represented the highest abundance (47.8%), closely followed by Gammaproteobacteria (46.4%). The most abundant genera among First Flush samples were *Sphingomonas* (19.1% of total sequences) followed by *Rheinheimera* (13.8%), *Undibacterium* (7.6%), and *Pseudomonas* (7.2%). In First Flush samples, three taxa were also found most persistent (over 50% of samples): *Sphingomonas*, *Undibacterium*, and *Pseudomonas*. Although Stable 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 genus was *Pseudomonas* (26.4%), followed by *Rheinheimera* (10.8%), and *Candidatus Megaira* (6.6 %). *Sphingomonas* and *Obcuribacter* were present in over 60% of Stable samples and were found to be enriched (by ANCOM and LefSe).

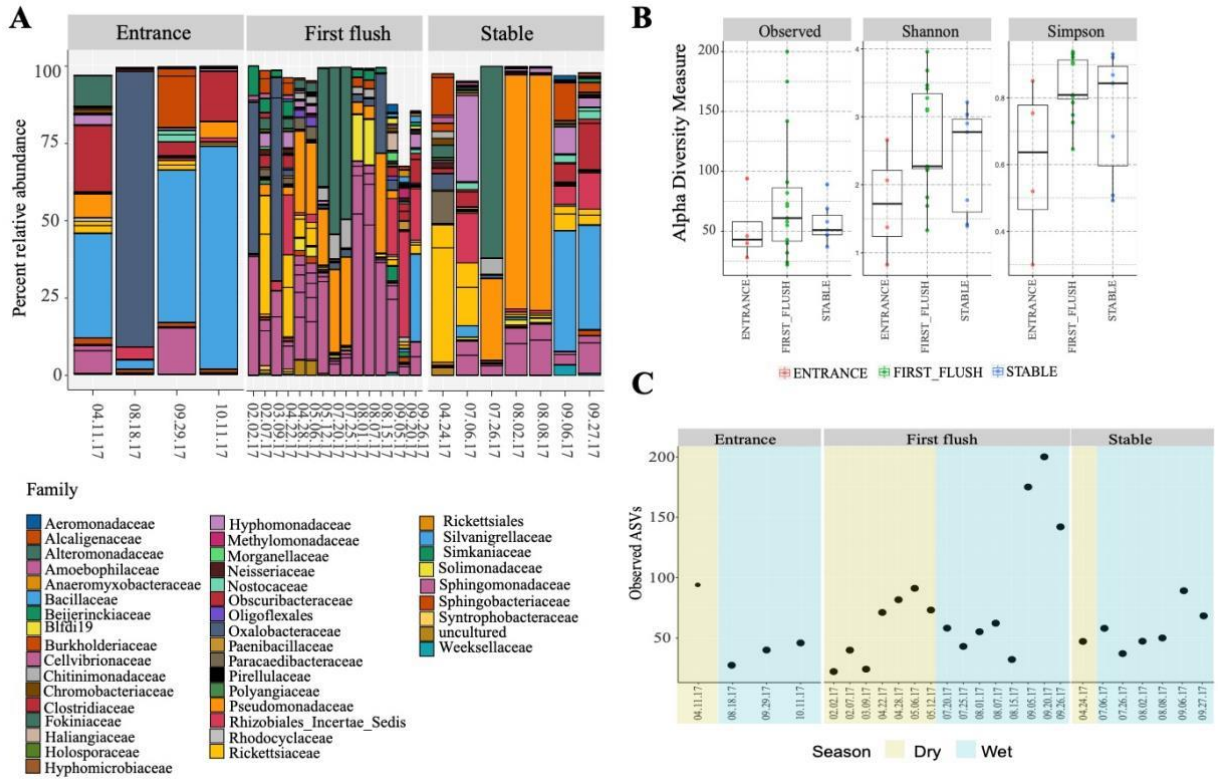

Figure 4.4 Microbial community diversity in stable and intermittent water supply. A) Family level classification of samples taken at each location and time (> 5% relative abundance shown); B) Alpha diversity indices between first flush and stable supply; C) Observed ASVs throughout time and seasons.

Among all samples, a "core" bacterial community was determined with at least 50 % presence for each sample type (i.e., 11 taxa were present in at least 50% of samples from each sample type with an abundance of > 0.5 %) (**Figure 4.5A**). Samples from the entrance and stable supply shared about the same number of ASVs as ASVs that were unique to each of these two types of samples. In contrast, First Flush samples had more unique ASVs (132 ASVs, representing 57.6% of first flush ASVs) than shared ASVs with the other sample types. (**Figure 4.5B**). Among First Flush samples, the phyla Acidobacteria, Nitrospirae, Verrucomicrobia, and Gemmatimonadetes were present in low abundances (less than 0.20 %) but were not present at all in samples from the Stable supply or Entrance. Similarly, the candidate phylum Dependentiae was only present in First Flush and Stable supply samples, and Fusobacteria was only present in Stable supply IWs and at the Entrance where the supply was continuous (**Table 4.2**). However, a limitation to interpreting these results is the larger number of First Flush samples (15) compared to Stable supply (7) and Entrance (4). As evident in Figure 4.4C, the number of observed ASVs was particularly high in three first flush samples, which may bias the comparison to the other sample types. Thus, an additional

analysis was conducted only comparing the First Flush and Stable IWS samples that were sampled within 24 hours of each other. Within this set of six paired samples, 77 ASVs were shared among both First Flush and Stable supply samples, while First Flush samples had 99 unique ASVs and Stable IWS had only eight unique ASVs. The number of unique and shared ASVs between the six sample pairs was highly variable.

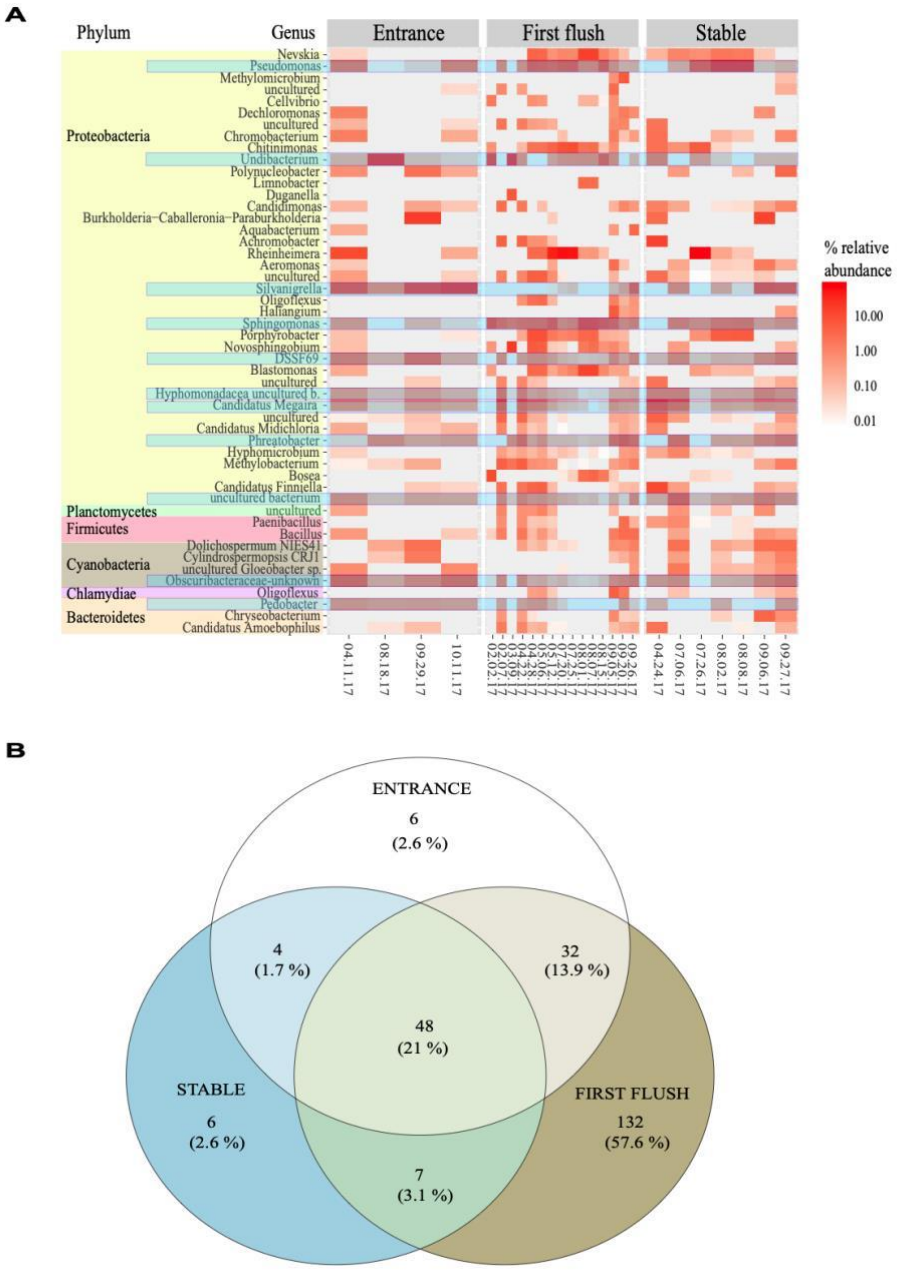

Figure 4.5: Microbial diversity in IWS. A) Heatmap of top 50 taxa with the 11 members of the core community highlighted in blue; B) Venn Diagram of shared ASVs by sample type.

4.4.2.2 Community structure and dissimilarity

Species diversity (Observed ASVs, Shannon, and Simpson indices) were determined for all samples at each location. When subsampled to 1000 sequences per sample, rarefaction curves reached a plateau indicating that the sequencing was sufficient to capture the diversity of these communities (**Figure A11**). First Flush samples tended to have higher ASV diversity than samples taken after supply had stabilized in the exact location and at the entrance, but these differences were not significant (Kruskal-Wallis, $p > 0.05$); however, the differences in sample numbers further complicate the comparison.

Community analysis using weighted UniFrac did not reveal a strong pattern of clustering of community structure by sample type, due to the high dispersion of the samples within each group (PERMDISP, F-value = 0.522, $p > 0.05$). A number of samples from First Flush, Stable supply, and Entrance clustered together, while others seem to have different bacterial community structure. It is likely that seasonal and other environmental factors influenced the bacterial community composition, and these influences are not evenly distributed among the sample types given our inability to collect paired samples on each date (**Figure 4.4C**). Nonetheless, while a strong clustering patterned is not observed, PERMANOVA suggests significant differences between First Flush samples, Entrance and Stable supply (PERMANOVA: for FF/entrance, pseudo-F = 3.28, $p < 0.05$; for FF/stable, pseudo-F = 2.5, $p < 0.05$. ANOSIM: for FF/entrance, R = 0.60, $p < 0.01$; for FF/stable, R = 0.36, $p < 0.01$). The bacterial communities in the six paired samples (First Flush and Stable supply) were also analyzed using weighted UniFrac. Using PERMANOVA, significant differences were observed between the sample pairs (PERMANOVA, pseudo-F = 1.68 $p < 0.05$).

4.4.3 Potential Pathogens, the abundance of Pseudomonas, and other bacteria of interest in the DWDS

A closer analysis of taxa was undertaken to explore the potential for IWS to be a source of pathogens (including opportunistic pathogens) (**Figure 4.6**). Although their relative abundances were low, *Aeromonas* spp. were detected in seven (26.9%) samples (one from the Entrance and six from the IWS zone) with the highest abundance in first-flush samples. *Legionella* spp. were observed in four samples (< 0.15% relative abundance) from the IWS zone (three First Flush samples and one sample taken 24 hours later). *Mycobacterium* spp. were also observed with the highest relative abundance in First Flush samples, while *Nitrospira* was only found in First Flush samples (**Figure 4.6**). Members of the genus *Pseudomonas* spp. were the most common of all ASVs in samples taken 24 hours after supply restart (26% relative abundance). If HPC measurements are indicative of total bacterial abundance, bacterial abundance in samples taken 24 hours after the restart of the supply was significantly lower than in first flush samples.

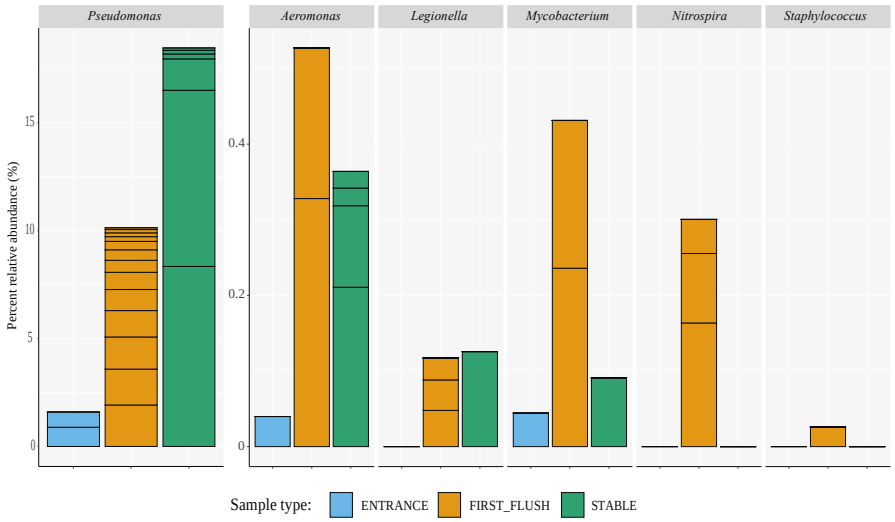

Figure 4.6: Relative abundance of genera containing opportunistic pathogens and nitrifying bacteria in IWS based on 16S rRNA gene sequencing. Lines represent the number of ASVs. A higher number of ASVs classified as Pseudomonas were found in samples from first flush (12) as compared to stable IWS (6) and at the entrance where supply is continuous (2).

4.4.4 Relationship between water quality parameters and bacterial community composition

Significant correlations were 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. Canonical correspondence analysis (CCA) also revealed relationships between these water quality metrics and bacterial community structure (**Figure 4.6)**.

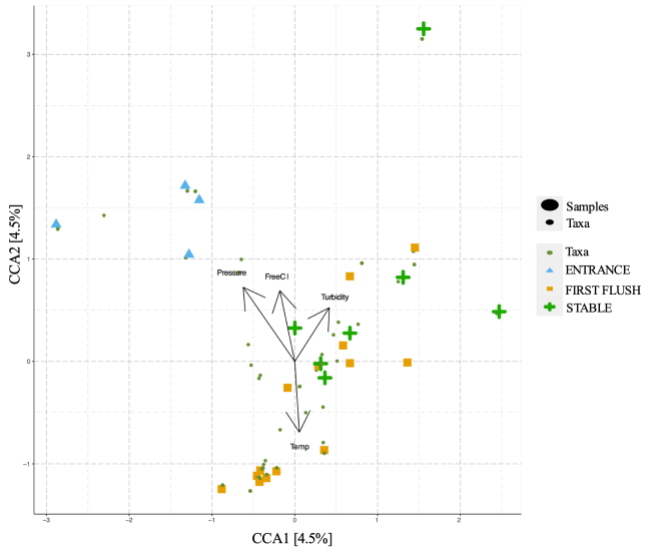

Figure 4.7. Canonical correspondence analysis of bacterial communities in IWS and drinking water quality metrics.

4.5 Discussion

Bacterial communities in DWDSs are important as they can have impacts not only on public health but also on the distribution system infrastructure. Analysis of culture-based indicator bacteria is insufficient to understand the impact of IWS on the microbial communities found in water as they represent a very small fraction of the whole microbial community. The characterization of microbiomes in DWDSs with IWS, however, has been lacking, perhaps in part due to the difficulty of obtaining IWS samples from full-scale distribution systems because water delivery can be infrequent and unpredictable. The inherent complexity of IWS makes it frustrating for experimental design, and the occurrence of IWS in different locations from where the majority of researchers studying the drinking water microbiome are based (Bertelli et al., 2018; Bivins et al., 2017; Dai et al., 2020; Jia et al., 2015; Ley et al., 2020; Perrin et al., 2019; Stanish et al., 2016; Wang et al., 2012). In this study, bulk water from two locations, the Entrance to the IWS zone and a downstream location (the First Flush and 24-h later with Stable supply) was sampled to better understand how IWS influences bacterial communities. The results provide novel information on the possible ecological effects of IWS on the microbiome of DWDSs.

Overall, the analysis revealed a highly variable bacterial community that is likely at least partially a result of the dynamic conditions created by IWS. While none of the sample types were strongly clustered by PCoA, significant differences between bacterial communities were found in the First Flush, Stable supply, and Entrance samples. Paired First Flush and Stable supply samples also showed high variability in community structure. In addition to the unique features of intermittent supply, other factors likely influenced the bacterial communities, including seasonal effects on the water source and conditions in the distribution system as well as operational changes at the DWTP and in the DWDS. The high degree of variability within each sample type was not evident from analysis of traditional water quality metrics (e.g., free chlorine residual in Stable supply was consistent).

Several water quality parameters were associated with the observed differences between microbial communities sampled in this study. Pearson's correlation and CCA analysis revealed chlorine concentrations, pressure, and temperature as measured factors that significantly influenced community structure. These parameters have been previously demonstrated to influence drinking water microbiomes (Dai et al., 2020; Ley et al., 2020; Liu et al., 2014; Zhang et al., 2020). However, this is the first time, to our knowledge, these parameters are correlated with microbial community structures found in IWS. Further research is needed to understand the effects of these water quality metrics on microbial communities that are part of drinking water systems in tropical regions, where distribution systems can be exposed to higher temperatures, lower pressures, and lower chlorine residuals. In addition, further investigation is needed into water quality measures that were not measured, such as the quantity and diversity of organic carbon compounds, sulfate, and dissolved oxygen that have been shown to impact community structure and composition (Emmanuelle I. Prest et al., 2016, results from Chapter 2).

Based on grab samples, significantly lower chlorine concentrations (both free chlorine residual and total chlorine) were observed in First Flush samples than in the Entrance and Stable supply samples. During the time of this study, Panama's residual chlorine standard for piped drinking

water was set in the range of 0.8 - 1.5 mg/L; new water quality standards were released in 2019, and the free chlorine residual standard was revised to be in the range of 0.3 - 0.8 mg/L (COPANIT, 2019, 1999) as previously mentioned in Chapter 3. Further research is necessary to understand how new water quality standards affect water quality and water microbiomes in IWS in this zone. HPC concentrations in IWS during the initial First Flush were significantly higher than after the supply was Stable. There is no clear evidence that HPC bacteria pose a public health risk (Robertson and Brooks, 2003); however, HPC can be used as a tool to assess conditions such as bacterial regrowth and intrusion in the drinking water distribution system, especially in DWDSs with low risk of fecal contamination.

Previous studies have shown that the observed taxa found in this study are often associated with freshwater lakes, which are the primary water source in this area. Genera such as *Rheinheimera, Silvanigrella, Undibacterium, DSSF69* and *Pseudomonas* have been found in drinking water microbiomes (Chao et al., 2015; Kämpfer et al., 2007; Perrin et al., 2019; Stanish et al., 2016) and were genera also found in water streams in the Panama Canal Watershed (Chapter 2). Interestingly, *Rheinheimera* has been found primarily in biofilm drinking water samples and stagnant water in pipes and thus could be an indicator of biofilm detachment during first flush and/or microbial growth inside pipes between IWS cycles (Chao et al., 2015; Perrin et al., 2019). Although we found *Rheinheimera* in all water sample types, its relative abundance was higher in the First Flush samples. Other genera primarily found in IWS samples, *Hyphomicrobium* and *Phreatobacter*, have only been documented in drinking water microbial communities with low chlorine concentrations (Perrin et al., 2019; Stanish et al., 2016). We speculate that low chlorine concentrations found in IWS could allow the growth of *Hyphomicrobium* and *Phreatobacter* in the Arraiján DWDS. A "core microbiome" has been defined by previous studies (Henne et al., 2012; Pinto et al., 2012; Caitlin R. Proctor and Hammes, 2015). Here we found a small number of taxa as being part of a "core microbiome." Compared to other studies looking at microbial communities in drinking water where "core microbiomes" have been found to contain a much higher number of members (El-Chakhtoura et al., 2015, 2018; Pinto et al., 2012), it is clear that due to the variability in microbial communities found in an IWS system, a "core microbiome" was found to be minimal.

While we found genera that contain species that are opportunistic pathogens, the taxonomic resolution of 16S amplicon sequencing was not sufficient to distinguish pathogenic and non-pathogenic species. Furthermore, the detection of DNA does not provide information about whether the organisms are viable. Nonetheless, the observed trend that the relative abundance of these genera was higher in downstream samples (**Figure 4.6**) is consistent with research that opportunistic pathogens can proliferate in stagnant water (Falkinham et al., 2015; Ley et al., 2020; Razzolini et al., 2010; Richards et al., 2018), and motivates for additional research to understand whether IWS conditions foster the growth of opportunistic pathogens.

We found *Pseudomonas* to be prevalent in samples from IWS, both in our First Flush and Stable sampling. *Pseudomonas* can survive in many different environments due in part to its minimal nutritional requirements (Klockgether et al., 2011). In addition, *Pseudomonas* spp. have a tremendous ability to form biofilms which makes them a model for biofilm-forming organisms (Klockgether et al., 2011). Prior research has shown that their ability to grow on a wide range of organic compounds and their resistance to chlorine allows them to colonize distribution networks,

including premise plumbing (Grobe et al., 2001; Mena and Gerba, 2009). These results suggest that IWS provides conditions for the proliferation of *Pseudomonas* in the distribution system. Fish and Boxall (2018) reported *Pseudomonas* to be the most abundant genus present in the biofilm of continuous-flow drinking water pipes (12.4% of sequences) with slightly higher abundance in low-chlorine regimes. Previous research also suggests that the abundance of *Pseudomonas* is higher in biofilms and suspended solids when compared to bulk water (Bertelli et al., 2018; Walters et al., 2016; Wang et al., 2007). This suggests that the high relative abundance of *Pseudomonas* in downstream samples could be due to detachment of biofilm during supply cycles in IWS. In addition to detachment as pipes fill, the high relative abundance in Stable supply samples suggests that biofilm detachment could be occurring during the entire supply cycle, possibly due to fluctuations in pressure (**Figure 4.2**). The higher number of ASVs classified as *Pseudomonas* in First Flush compared to Stable supply samples suggest the presence of *Pseudomonas* due to intrusion and biofilm detachment in First Flush samples, while Stable supply might only have the presence of *Pseudomonas* due to biofilm detachment. Key molecular pathways that drive its survival and persistence could allow *Pseudomonas* to withstand chlorine concentration changes, pressure changes, and other selective pressures generated by IWS. High temperatures, of 30 °C, in drinking water and roof-harvested rainwater systems, have also been associated with increased growth of *Pseudomonas* (Zhang et al., 2020).

The lower relative abundance of *Pseudomonas* in First Flush samples compared to Stable samples may be due to the higher abundance of other bacteria due to intrusion or growth in the distribution system. 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 were due to growth or intrusion of cells. We attempted to determine total bacterial cells by flow cytometry in this study, but unfortunately, the instrument did not provide reliable data.

Given the presence of unique ASVs in first-flush samples, an intriguing idea for future research is to investigate whether any taxa could be used to determine the source of the organisms, in particular, to distinguish between intrusion versus in-situ growth between supply cycles (e.g., in stagnant water, biofilms, or loose deposits). Based on the higher number of observed ASVs in some First Flush samples, ASV number may also hold promise as a signal for intrusion/growth between supplies; however, more studies are needed to determine if higher ASVs are consistently observed during First Flush compared to Stable supply.

4.6 Conclusions

- Based on conventional water quality parameters, water quality at the downstream sampling location was similar to the upstream location during stable supply, whereas water quality during the first flush had lower chlorine and higher HPC and temperature.
- Chlorine concentrations, temperature, and pressure were correlated with the observed differences in beta diversity, suggesting that the unique conditions in First Flush samples, which are indicative of conditions in the pipes between supply cycles, can at least partially explain the observed differences in bacterial composition.

- The presence of taxa in First Flush samples that were not present upstream or after 24 hours of stable supply and not part of the core microbiome across sample types could indicate intrusion and/or growth of microorganisms between supply cycles.
- *Pseudomonas* was among the most abundant taxa in IWS. 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 critical for this proliferation to occur.

Chapter 5

Bacterial community dynamics in bench-scale annular reactors simulating stagnation and drainage in drinking water distribution systems supplying water intermittently

5.1 Introduction

Compared to continuous water supply, several characteristics of IWS have the potential to increase the deterioration of drinking water. As described in Chapter 4, low and negative pressures between supply cycles can lead to intrusion and/or backflow; growth of microorganisms in biofilms, loose deposits, and bulk water in IWS may differ from that of continuous water supply due to the unique conditions that exist between supply cycles and during the first flush of water when supply is restarted. IWS forces consumers to store water, leading to recontamination of stored water (Bautista-de los Santos et al., 2019; Gonzalez et al., 2020; Kumpel and Nelson, 2016; Liu et al., 2014). Although the deterioration of water quality in IWS has been well documented through the use of culture-based techniques and traditional water quality measurements (Kumpel and Nelson, 2016, 2014, 2013), a limited number of studies have shown how IWS impacts microbial communities in DWDSs. Understanding how unique features of IWS can impact water microbial communities can help better characterize the risks of IWS and provide insight into strategies to protect water quality in DWDSs operating intermittently.

Within the unique factors of IWS, stagnation and drainage (between supply cycles) are important mechanisms that can influence microbial communities (**Figure 5.1**); however, to our knowledge, no studies have been conducted to understand the influence of these conditions on microbial communities in IWS. While drained pipes are unique to IWS, stagnation also occurs during continuous supply. However, the conditions that lead to stagnation in continuous water supply may vary from those in IWS. In continuous supply, stagnation usually occurs overnight due to diurnal demand patterns. Consumption tends to be high during the morning and early evenings, decreasing during working hours and at night when people are sleeping (Ling et al., 2018). Stagnation in continuous supply also tends to occur mainly in premise plumbing rather than the

distribution pipes, where even at night, there is some flow due to aggregated water demand and leakage. Thus, water in distribution pipes is seldom stagnant for long periods of time. In IWS, stagnation depends on factors such as the severity of the intermittency, consumer demand, leaks, structural deficiencies, and topography (Henne et al., 2012; Klingel, 2012; Tokajian and Hashwa, 2003). Stagnation occurs between supply cycles in low-lying pipes and other sections of the network that do not drain entirely. Unique to IWS, some pipes may drain completely between supply cycles, depending on the length of the stoppage, topography, consumer demand, and leakage rates.

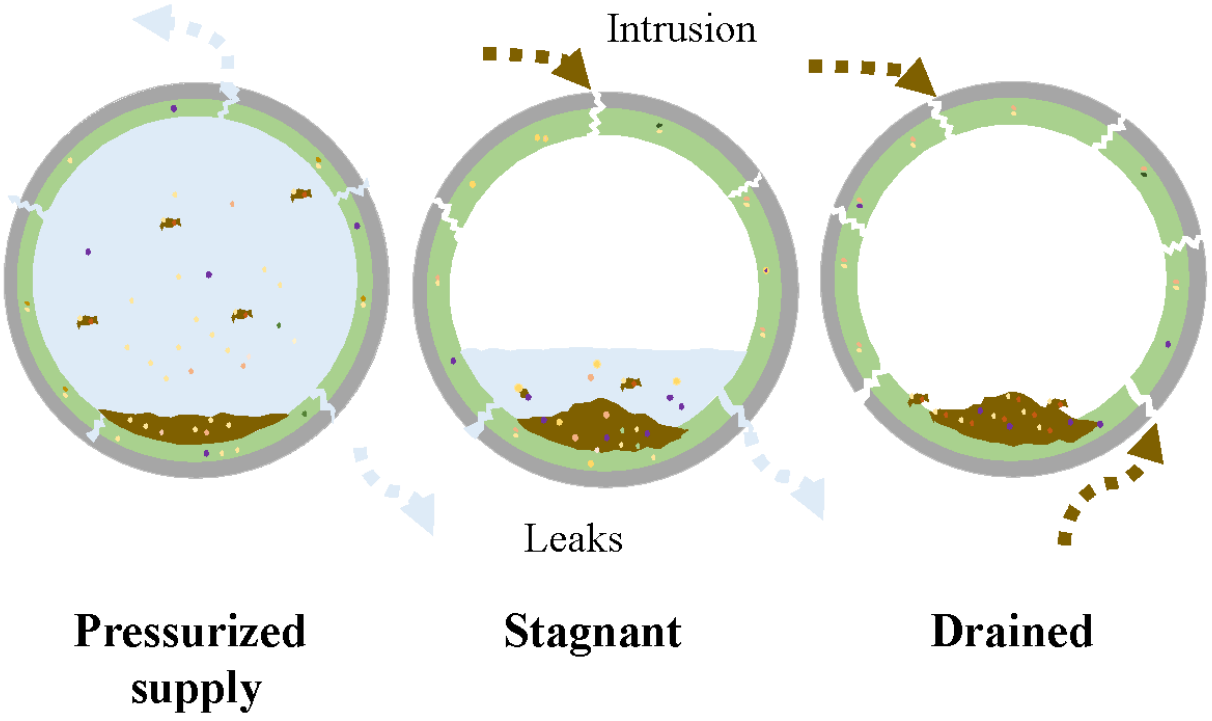

Figure 5.1. Sketch of a cross-section of drinking water pipe showing different stages of IWS

The influence of stagnation (**Figure 5.1**) on microbial communities in drinking water (i.e., premise plumbing) has been well documented in the last few years. Microbial growth due to overnight stagnation has been reported in continuous water supply with (Lautenschlager et al., 2010) and without disinfectant residual (Ling et al., 2018; Lipphaus et al., 2014). Differences in community structure between premise plumbing samples affected by stagnation and water from mains were reported by Ling et al., 2018. Furthermore, Ling et al., 2018 also found that microbial communities influenced by stagnation (i.e., stagnant water samples closer to the taps) were more similar to biofilm communities collected from water meters in the network compared to water from the mains. In IWS, microbial community structure changes due to stagnation are likely to occur;

however, the influence of stagnation in distribution system pipes (where the size and the material of pipes differ from premise plumbing) is still an unexplored subject, perhaps due to the inherit complexities of studying IWS systems. Regardless, the influence of stagnation in IWS, the magnitude of public health implications will depend on the duration of the stagnation period, the composition of the microbial community (both autochthonous and introduced), and the persistence of disinfectant residual.

Contrary to stagnation, there is no information from continuous supply research regarding the effects of drained periods (**Figure 5.1**) that can be used to conclude the potential influence of this mechanism on microbial communities in drinking water (Bautista-de los Santos et al., 2019). Pipes in IWS often experience drained periods followed by re-wetting periods. Thus, biofilms and loose deposits in pipes could experience dry periods that could last from hours to days. In freshwater streams, for example, flow intermittency has been shown to decrease and change microbial diversity (Sabater et al., 2016), increase the abundance of phyla that produce endospores (genera such as Firmicutes and Actinobacteria), and show the sensitivity of phyla that do not produce endospores (such as Proteobacteria and Bacteroidetes) (Potter et al., 2017; Timoner et al., 2014). In addition, desiccation in temperate streambed sediments has been reported to shift bacterial community towards the composition of typical soils and decrease extracellular enzymatic activity; upon re-wetting, there was bacterial community recovery but not return to its original composition, with a higher abundance of Bacteroidetes and Alphaproteobacteria (Pohlon et al., 2013). Saltmarsh sediments have been reported to demonstrate a similar behavior (McKew et al., 2011). Although the information from intermittent freshwater streams illustrates an influence of dry periods on microbial communities, microbiomes in drinking water are different in composition and are exposed to different environmental conditions that could result in unique changes to the microbial community structure.

Understanding stagnation and drainage effects on bacterial communities in IWS can help elucidate how each mechanism affects drinking water and help guide the daily operations of DWSs with IWS. Here, we present a laboratory bench-scale experiment of bacterial community assembly in simulated DWDSs with continuous and IWS to explore the influence of stagnation and drainage. Bench-scale rotating annular reactors, high-throughput sequencing, and flow cytometry were used to evaluate stagnation and drainage independently. The main objectives of this study were to: 1) understand how each mechanism influences bacterial communities in bulk water; 2) assess bacterial community structure in biofilms exposed to stagnation and drainage; and 3) draw conclusions that can aid in the management of IWS systems and identify research priorities for understanding the IWS microbiome. The use of a laboratory bench-scale experiment allowed us to evaluate the effects of stagnation and drainage independently and eliminate confounding factors commonly found in full scale DWDSs; however, it is important to note that there were limitations with this simplified system, such as mimicking realistic conditions for the filling and draining of pipes.

5.2 Material and methods

5.2.1 Annular reactor operations

Rotating annular reactors ("ARs"; Biosurface Technologies Corp., Bozeman, MT) were used to investigate the effects of stagnation and drainage on the bacterial composition of biofilm and bulk water (**Figure 5.2 A-C**). These ARs present various advantages, including a simple sampling protocol and the ability to exert different shear stress conditions by the rotational speed of its inner rotating cylinder (Gomez-Alvarez et al., 2014). In addition, the liquid phase is well mixed, ensuring a uniform bacterial distribution in the liquid phase. Three ARs were used to simulate hydraulic conditions typically found in DWDSs (1 L volume, RD 128-PVC, BioSurface Technologies Corporation, Bozeman, MT) (**Figure 5.2 A-C)**. Twenty PVC slides were installed in each AR to allow biofilm formation and simulate PVC piping. PVC was used in this experiment as it was the piping material encountered in all sampling locations in the DWDS in Arraiján, Panama. These ARs were run in parallel (**Figure 5.2 A&B**) and fed from a reservoir (used for flow control) that was continuously fed by an adjacent drinking water tap supplied water by the East Bay Municipal Utility District's (EBMUD) chloraminated municipal supply.

ARs: Cleaning and Preparation

Before starting this experiment, the ARs were treated overnight with 40% bleach solution and sterilized by autoclaving at 121ºC and 15 PSI for 30 minutes. The PVC biofilm coupons were sterilized with 95% ethanol and air-dried before inserting into the reactors. Assembled ARs were filled with sterile, distilled water and operated for eight hours prior to feeding with tap water. ARs were acclimated by feeding with tap water for four weeks before starting the stagnation/drainage simulations (Characklis and Marshall, 1990; Gomez-Alvarez et al., 2014).

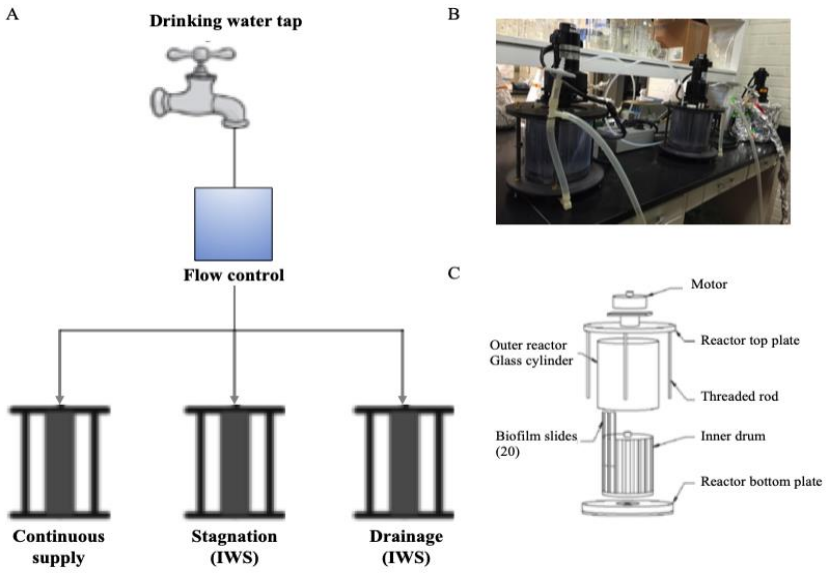

Figure 5.2. Sketch of ARs operations during this study (A); picture of actual ARs used (B) and schematic of an AR and its components (C).

ARs: Operating Conditions During Continuous and IWS Supply

The operation of the ARs during continuous supply was similar to those that simulate conditions of mains found in DWDS and used by other studies simulating DWDS using ARs (Gomez-Alvarez et al., 2014; Peyton, 1996) (**Table 5.1**). At the beginning of the experiment, the three ARs were operated continuously and identically for four weeks to establish a baseline. Once the baseline was established, each AR was operated to simulate one of three conditions: i) Continuous supply (labeled Continuous); ii) IWS where pipes do not drain causing water stagnation (labeled Stagnant); and iii) IWS where pipes drain completely (labeled Drained). Stagnant and Drained events were set to last three days (three days with stagnation or drainage and four days with continuous supply). Three-day events were chosen to simulate IWS conditions similar to those found in the full-scale DWDS in Arraiján, Panama (previously described in Chapter 4). ARs were operated at this stagnant/drained schedule for seven months.

Table 5.1. Operation conditions of 1-L ARs during continuous supply

Hydraulic Residence Time (HRT)	6 hours (Under continuous condition)
Flow Rate	~ 2.7 mL/min
Shear Stress	0.154 N/m^2
Rotational Speed	50 rpm
Temperature	Ambient (~20°C)
Intermittency	4/3 cycle (4 days on, 3 days off)

5.2.2 Water quality, cell quantification, and sample collection

During the seven months of operating the ARs on a weekly stagnant/drained schedule, water quality parameters and flow cytometry measurements were carried out twice weekly: before and after each stagnant/drained event (**Figure 5.3**). Sampling for 16S rDNA sequencing was conducted after stagnant/drained events during the last 10 weeks of the experiment. Details are provided in the following sections.

Water Quality Measurements

Basic water quality parameters were measured for each AR twice weekly, at the end of continuous supply (beginning of each stagnant/drained event) and at the end of each stagnant/drained event (**Figure 5.3**). The following parameters were analyzed: free and total chlorine (Hach Portable Colorimeter II), pH, and temperature (Cole-palmer laboratory multiparameter meter). To carry out such measurements without major disturbances to the supply, all reactors were turned off and sampled for less than ten minutes at a time.

Cell counts by flow cytometry

Bacterial cell concentrations (total = TCC and intact = ICC) were measured by flow cytometry also twice weekly (**Figure 5.3**), before and after each stagnant/drained event, following established protocols by our laboratory (Kantor et al., 2019; Kennedy et al., 2020; Miller et al., 2020). Briefly, to distinguish between TCC and ICC, a combination of the dyes (SYBR® Green I (Sigma-Aldrich;

S9430) and propidium iodide (Life Technologies; P1304MP)) were used. TCC samples were stained with SYBR® Green I working solution (10,000x in DMSO) that was 100x diluted in buffer (10mM TRIS in 0.1 µm filtered nanopore water); ICC samples were additionally stained with propidium iodide (30mM; P1304MP) at a working propidium iodide concentration of 0.6 mM. Measurements were performed on an Accuri C6 flow cytometer (BD Biosciences), equipped with a 50 mW laser (and can emit a fixed wavelength of 488 nm). Samples were run under the "fast" flow rate (66 µL/minute on 50 µL sample volumes). Detection and enumeration of the bacterial cell were performed on density plots of green (FL1; 533 ± 30 nm) and red (FL3; >670 nm) fluorescence using an electronic gating system provided by the Accuri C6 software. Gate positions were used from a template that is publicly available for the BD Accuri C6 (Gatza et al., 2013) with some modifications.

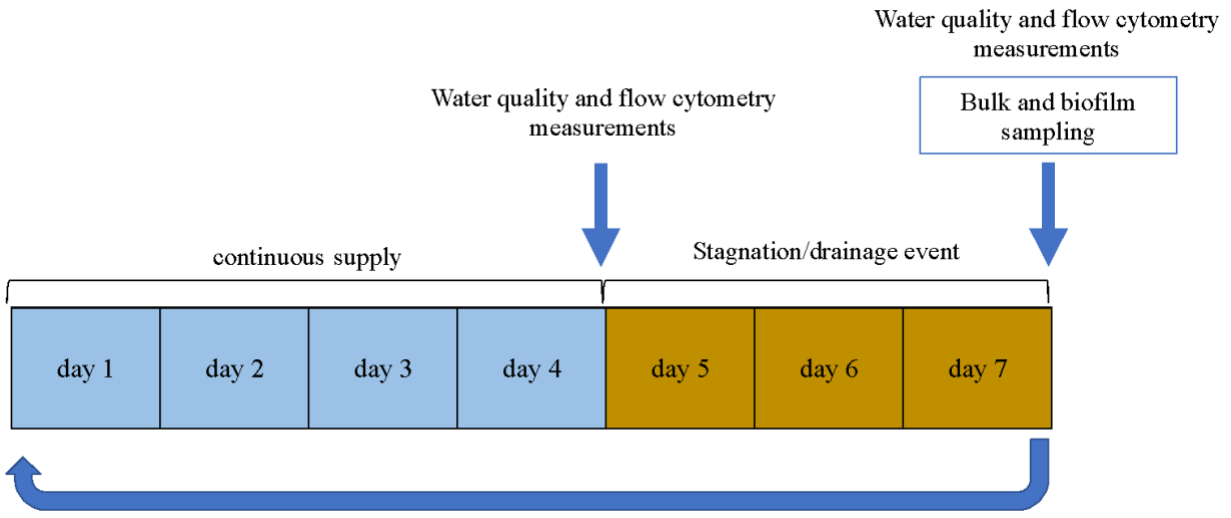

Figure 5.3. Schematic of sampling schedule for water quality and for cell quantification by flow cytometry as well as sampling of bulk water and biofilm for 16S rDNA sequencing.

Bulk Water and Biofilm Sample Collection for Sequencing Analysis

ARs were operated on a weekly stagnant/drained schedule for approximately 18 weeks prior to sampling for sequencing. Bulk water (1L) and biofilm samples for 16S rDNA sequencing were collected at the end of stagnant/drained events for all three ARs (**Figure 5.3**) during the last ten weeks of the experiment. Initially, the experiment was planned to produce ten samples for bulk water and biofilm each (ten (1L) bulk water samples and ten biofilm -two PVC slides from a total of 20- samples). However, during preliminary analysis, it was concluded that this volume (for water samples) and the number of slides (for biofilm samples) were insufficient to produce sufficient DNA for sequencing. For this reason, two bulk water samples (two liters of bulk water)

were processed together as one bulk water sample and three PVC slides were combined as one biofilm sample for DNA extractions and subsequent 16S rDNA sequencing. These changes resulted in five (2L) bulk water samples and six (three PVC slides each) biofilm samples for each AR.

For bulk water samples, reactors were drained by gravity and water was collected in sterile borosilicate glass PYREX® bottles with sodium thiosulfate to quenched residual chlorine. Water samples were stored at 4°C and processed within eight hours of collection. Bulk water samples were concentrated by membrane filtration (0.22 um sterile filters by Millipore) in a biological safety cabinet previously sterilized by UV and ethanol. Once the sample from each reactor was collected, sterile borosilicate glass PYREX® bottles (1L) were filled with tap water to refill the ARs, and after, the continuous supply cycle was restarted for all three reactors. The refilling process was performed very slowly to avoid disturbances to the biofilm in the ARs. To sample the Drained AR, the AR was filled with tap water and allowed to operate (rotate) for three minutes. Then, the AR was turned off, and the bulk water was sampled. This process was done to simulate the first flush of water after the restart of the supply. For reference, the dynamics of the first flush in a real-world distribution system are described in detail in Chapter 4.

For biofilm samples, Three PVC slides were removed from each AR (sacrificial sampling), and the recovered biomass was pooled for each reactor. Following Gomez-Alvarez et al., 2014, sterile Teflon scrapers were used to scrape each slide into sterile 50 mL conical tubes containing filter-sterilized 10mM phosphate-buffered saline (PBS) solution. The solution was centrifuged and resuspended in 10mM PBS. Each sample was subsequently processed for DNA extraction.

5.2.3 DNA isolation and 16S rDNA sequencing

DNA for sequencing was extracted from bulk and biofilm samples using DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions with the following modifications: 0.22-µm mixed cellulose filters were cut into pieces in sterile conditions and combined in 5-mL low-bind tubes before extractions. Samples were incubated with lysis buffer at 65°C for 15 minutes prior to the extraction protocol. Bead-beating was performed at maximum speed for 8 minutes using a vortex adapter, and the isolated DNA was eluted in a final volume of 60 uL. After DNA extraction, all samples were stored at -20°C prior to the preparation of the sequencing librariy. DNA quantification was determined via the Qubit dsDNA HS assay kit (Thermo Fisher Scientific).

Library preparation for 16S rDNA amplicon sequencing was performed following the Schloss Lab Miseq wet-lab protocol to amplify the V4 region of the 16S rRNA gene with modifications (Schloss, Patrick D., Baxter, N., Jenior, M., Koumpouras, C., Bishop, 2018). Briefly, the V4 region was amplified using a one PCR protocol with barcoded primers 515F and 806R with Phusion Hot Start II polymerase, HF buffer, dimethyl sulfoxide (3.75% final concentrations), and 2.5 µL of DNA. Reactions (Triplicate 25 µL) were pooled and concentrated via SpeedVac. The final dual-barcoded library was normalized using SequalPrep Normalization Plate kit (Invitrogen), pooled, and sequenced using a MiSeq with V3 chemistry for 600 cycles, 250 paired-end reads.

5.2.4 Data analysis

Analysis of water quality parameters was performed as described in previous chapters and Kumpel and Nelson, 2014, performing tests for significance based on non-parametric pairwise Kruskal-Wallis test with Benjamini-Hochberg corrections. For 16S rDNA data, raw FASTQ sequencing reads were demultiplexed and mapped to (23 %) PhiX. The QIIME2 platform v2020.6 and R v.3.6.3 were used for the majority of the analysis of single-end sequence reads. Single-end sequence reads were used due to the low quality of the corresponding single-end sequence reads; however, this does not affect the analysis outcomes (Tremblay et al., 2015). Preprocessing was performed using QIIME2's Cutadapt and DADA2 plugins to produce ASVs (Bolyen et al., 2019)(Callahan et al., 2016), and taxonomy was assigned using a naïve Bayesian classifier trained on the SILVA 99% sequence similarity database (Pruesse et al., 2007; Wang et al., 2007). Sequences were filtered as previously described: using BLAST (97% confidence), ASVs with low abundance (0.005%), and ASVs classified as mitochondria or chloroplast.

AS described in previous chapters, diversity (Alpha and Beta) were calculated using the diversity core-metrics function in QIIME2 from the resulting ASV table standardized to a sequencing depth of 7000 sequences per sample. Differences in alpha diversity (Observed ASVs and Shannon diversity indices) were tested by Kruskal-Wallis with Benjamini-Hochberg corrections, with pairwise differences between sample types. Permutation-based ANOSIM tests (with 999 permutations on weighted UniFrac distances) determined statistical significance between beta diversity of the different types of supply (Anderson, 2017; Lozupone et al., 2011). To explore the structure of microbial communities and visualize beta-diversity dissimilarity, an ordination approach was adopted using PCoA using both Bray-Curtis and weighted UniFrac distances generated in R with the package Phyloseq. The QIIME2 plugin ANCOM and linear discriminant analysis Effect Size (LEfSe) were used to detect differentially abundant taxa accounting for compositional constraints (Mandal et al., 2015; Segata et al., 2011). Differences were considered to be significant if p-value < 0.05.

5.3 Results and discussion

5.3.1 Disinfectant residual is an important changing factor

Water temperature and pH showed little change across the different supply types simulated in this study (**Figure 5.4**). Temperatures ranged from 18.3 °C to 25 °C (Mean ± SD, Continuous = 20.8 ± 1.6 °C; Stagnant = 21 ± 1.7 °C; Drained = 20.9 ± 1.6 °C) and showed no significant differences across ARs. Similarly, pH measurements ranged from 7.1 to 9.4 (Mean ± SD, Continuous = 8.4 ± 0.5; Stagnant = 8.2 ± 0.6; Drained = 8.4 ± 0.6). The lowest pH values were measured after water being stagnant (pH values around 7). As the supply started back again in the stagnant AR, the pH increased to an average of 8.4. These variations in pH are within previously reported ranges in water samples taken from building premise plumbing (Bédard et al., 2018; Boppe et al., 2016), and other studies looking into stagnation in premise plumbing have also found no significant changes in temperature and pH (Lautenschlager et al., 2010). For the drained AR, it was expected to find no significant changes in these parameters as the key differential feature is the lack of water

during drained periods. Pre- and post-drained events exhibited similar temperature and pH measurements.

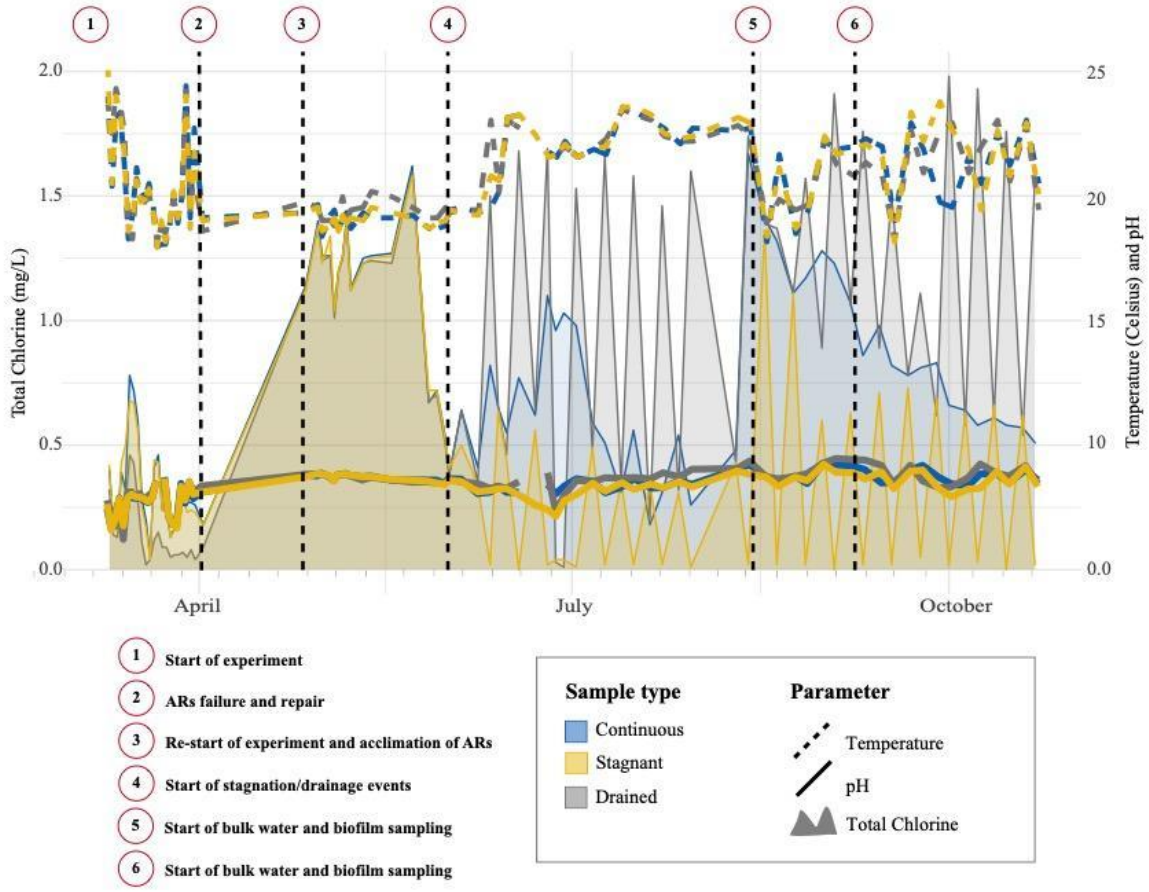

Figure 5.4. Total chlorine, pH and temperature measurements during experimental study. Numbers in circles represent important stages along the study.

Chlorine (total and free) concentrations, on the other hand, varied significantly across the different types of simulated supply (continuous vs. stagnation in IWS vs. drainage in IWS) and pre- and post- stagnant/drained events (Kruskal-Wallis, $p < 0.01$, respectively) (Mean ± SD, Continuous = 0.72 ± 0.4 mg/L; Stagnant = 0.48 ± 0.4 mg/L; Drained = 0.81 ± 0.6 mg/L) (**Figure 5.4**). Concentrations of disinfectant residual during stagnation depend on many factors, including the duration that the water stays stagnant and reactions with pipe material and other constituents in the water (Lautenschlager et al., 2010; Lipphaus et al., 2014; Rhoads et al., 2016). In this experiment, water stagnation was simulated with a stagnation period of three days for each stagnant event. After 24 hours (tested for nine cycles only), chlorine measurements were below the detection limit and remained below 0.2 mg/L within the first two hours post-stagnant events. Mean chlorine concentration in the AR simulating stagnation was the lowest compared to other simulated supplies (**Figure 5.4**).

During simulated drained events (Drained AR), a different chlorine concentration pattern was observed (**Figure 5.4**). The highest chlorine concentrations were found immediately after the drained event (first-flush simulations), which was similar to the chlorine concentration directly from the water tap used, and decreased over time until the next drained event. This result is inconsistent with our previous study of chlorine concentrations immediately after water restarted in an IWS system and other IWS studies (results in Chapter 4, Erickson et al., 2017, Kumpel and Nelson, 2013, 2014). In these previous studies, we found that chlorine concentrations were lowest immediately after supply restarts. As for our simulated drained event, it is very likely that we were unable to simulate properly all the dynamics that occur during a first flush. Most importantly, by using this type of AR, we were unable to simulate how water travels across a section of pipe, in many instances traveling long distances, exerting shear forces on pipe walls (biofilm slough off) and also carrying contamination that could have entered the system during the supply stoppage. However, it is important to note that in studies of full-scale DWDSs on chlorine residual during the first flush, it is not possible to differentiate between the impacts of stagnation, drainage, and intrusion of contaminated water in between supply cycles. In any case, the influence of these different factors is likely to vary dramatically depending on the local operating conditions.

During simulated continuous supply (continuous AR), there was high variability in total chlorine concentrations. The lowest concentrations were measured during the summer months (**Figure 5.4**). This effect may be because the water source for this experiment comes from a building premise plumbing and is influenced by water usage patterns commonly found in buildings (Lautenschlager et al., 2014; Ling et al., 2018; Lipphaus et al., 2014). The variability in chlorine concentrations in the continuous supply could also be influenced by the pattern of students in the building during the school year and absence during school breaks, such as during summer. It is important to note that measurements were taken during Friday and Monday mornings, which could also have a different water usage pattern than the rest of the week. Notably, the water source for this experiment was continuously running during the entire experiment, which minimized the effects of stagnation and diurnal patterns commonly found in this building; we observed that chlorine concentrations in the drained reactor just after filling with tap water (to simulate the first flush) were higher than those found in the continuous supply reactor. Thus, numerous factors influenced the chlorine concentrations in the continuous supply reactor, including decay while in the reactor, including disinfectant demand exerted by the PVC slides and other AR materials, and variable chlorine concentrations in feed tap water that may not have been captured due to the sampling schedule.

5.3.2 Cell quantification changes driven by stagnation and drainage

Here we present total (TCC) and intact (ICC) cell concentrations that were measured after the ARs were acclimated and during stagnation/drainage events (**Figure 5.5**). The geometric mean of TCC and ICC concentrations in the source (tap) water were 1.3×10^4 cells/mL (SD = 1.1) and 2.1×10^3 cells/mL (SD = 1.5), respectively. For the AR simulating continuous supply, TCC and ICC geometric mean concentrations were 1.6×10^5 cells/mL (SD = 0.5) and 7.7×10^4 cells/mL (SD = 0.7), respectively. We observed an increase in TCC and ICC of about one order of magnitude between the source (tap) water and simulated continuous supply. The highest concentrations of

TCC and ICC were measured during the summer months and at the end of the experiment, consistent with the lowest chlorine concentrations observed in continuous supply.

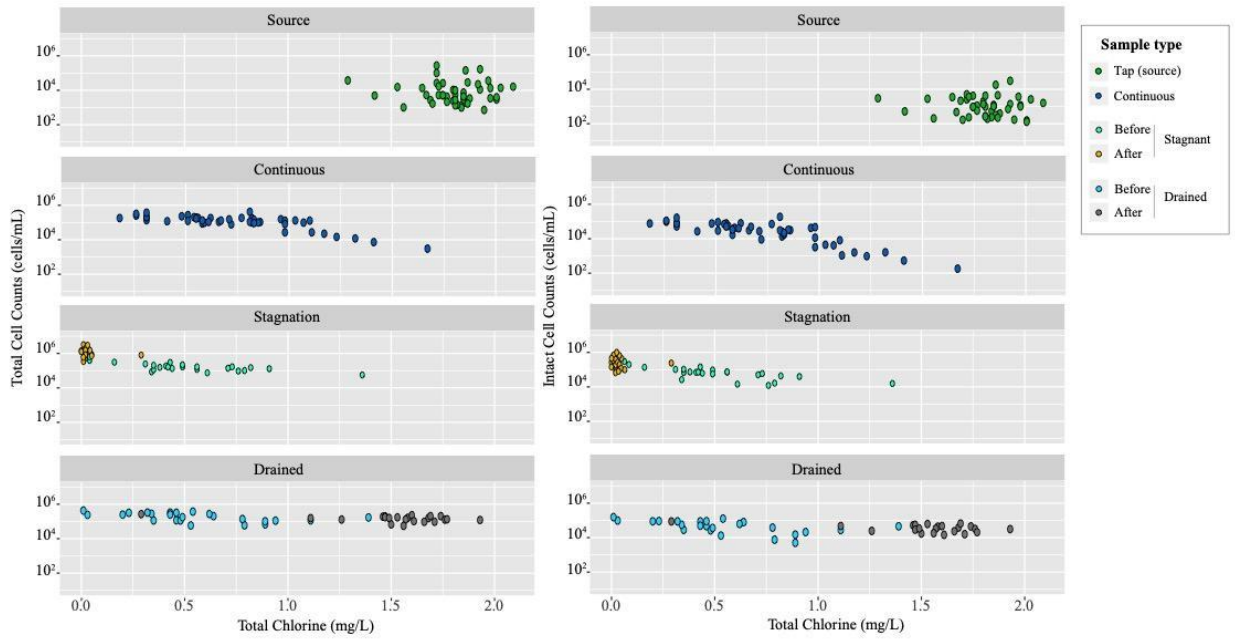

Figure 5.5. Effect of total chlorine concentrations and total (A) and intact (B) cell counts in the different simulated supply types and stagnation/drainage event. Data shown only include samples taken after period of acclimation. All samples were analyzed in technical triplicates.

During stagnation simulations (stagnant AR), samples were taken during continuous supply (water had been continuously running for four days) and after stagnant events (where water had been stagnant for three days before sampling) (**Figure 5.5**). Before stagnation, samples had a TCC and ICC geometric mean concentrations of 1.7 x 10^5 cells/mL (SD = 0.52) and 7.6 x 10^4 cells/mL (SD = 0.76), respectively. After 3-day stagnant events, TCC and ICC geometric mean concentrations were 4.2 x 10^5 cells/mL (SD = 0.51) and 2.0 x 10^5 cells/mL (SD = 0.72), respectively. There were no significant differences in cell counts (TCC and ICC) between simulated continuous supply (continuous AR) and concentrations in the stagnant AR when the AR was operated continuously during the acclimation phase. Thus, the significant increase in TCC and ICC of about 0.42$\log_{10}$ after stagnation events was due to stagnation (Kruskal-Wallis, $p < 0.01$).

Samples were taken in a similar fashion as previously described to measure cell concentrations for drained events (drained AR) after four days of continuous supply and after refilling the AR simulating a first flush event. Cell concentrations (both TCC and ICC) exhibited a different pattern than those found in the continuous AR and stagnant AR. The highest TCC and ICC concentrations were found during continuous supply and the lowest during first flush simulated events. We found the TCC and ICC geometric mean concentrations before drained events were 1.2 x 10^5 cells/mL

(SD = 0.62) and 6.7 x 10^4 cells/mL (SD = 0.99), respectively. After drained events, geometric means of TCC and ICC concentrations were 1.2 x 10^5 cells/mL (SD = 0.81) and 3.3 x 10^4 cells/mL (SD = 1.02), respectively. Although there were some differences observed between TCC and ICC concentrations before and after drained events, only ICC concentrations before and after drained events were significantly different (ICC measurements were higher before drained events, Kruskal-Wallis, $p < 0.05$, respectively). TCC measurements before or after a drained event did not show significant differences between TCC measurements from the continuous supply (continuous AR); however, ICC concentrations were significantly higher in the drained AR than ICC concentrations measured in the continuous AR (Kruskal-Wallis, $p < 0.05$).

Overall, TCC and ICC concentrations significantly increased from the source water to concentrations found in all three ARs (Kruskal-Wallis, $p < 0.001$, respectively). Within each supply type, stagnation was the only supply that exhibited a significant difference between TCC measurements taken before and after stagnant/drained events (Kruskal-Wallis, $p < 0.0001$). However, both ICC measurements from the stagnant and drained ARs differ before and after stagnant/drained events (Kruskal-Wallis, $p < 0.01$, respectively) and were also significantly different from measurements found in the continuous AR.

Flow cytometry has been shown to be a powerful tool for quantifying planktonic cells in drinking water (Kantor et al., 2019; Kennedy et al., 2020; Lautenschlager et al., 2014; Lipphaus et al., 2014). Flow cytometry has the advantage of being a sensitive, rapid, and reproducible approach that can give water operators and researchers information, in a short period of time, on total and intact planktonic cell counts up to nearly seven orders of magnitude. We found that stagnation and drainage influenced bacterial concentrations differently. Stagnation increased both TCC and ICC concentrations. These results agree with previous research on the effects of water stagnation in premise plumbing (Lautenschlager et al., 2010; Zlatanović et al., 2017). On the other hand, the influence of drainage on cell counts after the reactors were re-filled was minimal (slight increase in ICC). These results are not surprising given that the bulk water was sampled only 3 min after the reactor was filled with tap water following a drained period. It is important to note as well that we did our best to simulate a first flush (when a supply cycle begins in IWS); however, there are several limitations to our efforts, they include the small working volumes, the direction of flow when AR was filled after each drainage event and the rotational speed used. The hydraulic conditions in actual pipes that are re-filling after a stoppage may be more aggressive. Field studies have documented higher bacterial counts in water from the first flush compared to samples collected during continuous supply periods.

5.3.3 Stagnation and drainage drive changes in bacterial community diversity

Illumina 16S rDNA amplicon sequencing was used to profile the bacteria within bulk and biofilm in simulated DWDs with different supply dynamics commonly found in IWS. Thirty-three total samples were sequenced, but 31 were sequenced successfully (10-11 samples per AR, 5-6 samples per each sample type (bulk or biofilm)). After processing, the data set included a total of 1,447,964 high-quality sequence reads. There was a significant variation in the number of sequences obtained for each sample, despite normalization procedures during library preparation, from a low of 7,414

to a high of 36,854. Rarefaction curves for all samples reached a plateau (**Appendix A.10**), which suggested that the sequencing depth was sufficient in this study to capture the diversity within these communities. Hellinger transformation to normalize the sequencing data was also explored to analyzed the data set, and the results were similar to those shown here.

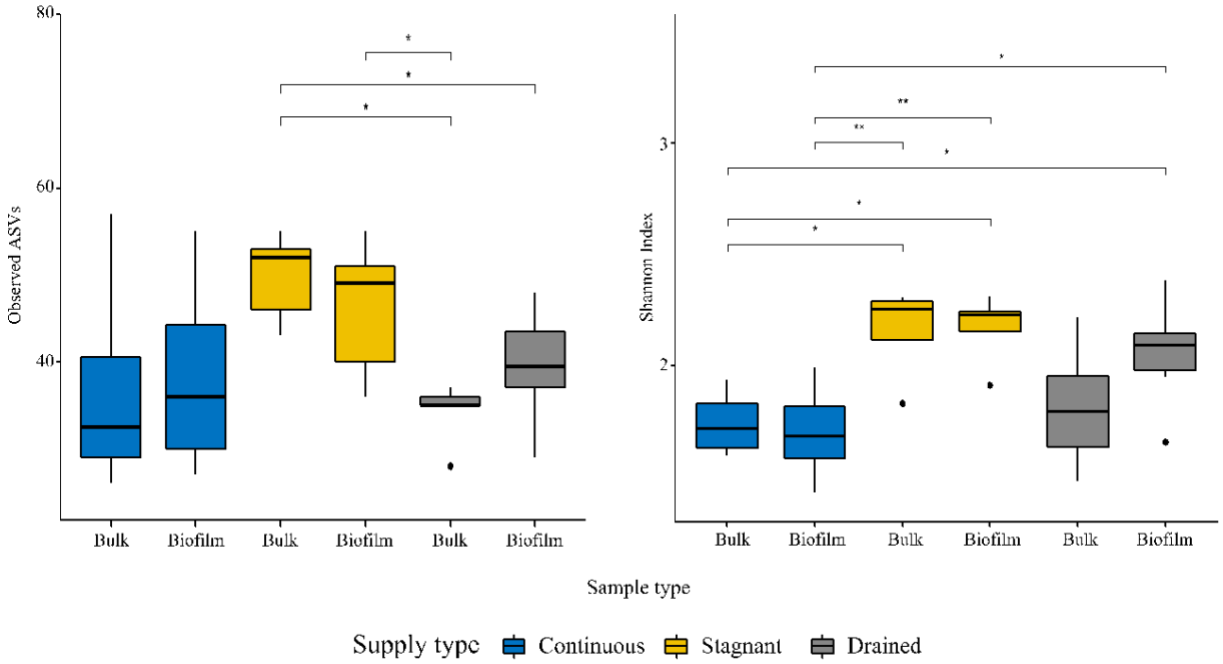

Figure 5.6. Alpha diversity measures. Faith's Observed ASVs (A) and Shannon Diversity Index (B). Statistical significance by Kruskal-Wallis and pairwise Wilcox tests are shown in asterixis: * = *p* < *0.05*; ** = *p* < *0.01*; * = *p* < *0.001*; **** = *p* < *0.0001*; not shown = *no statistical significance.***

Alpha diversity indices are provided in **Figure 5.6**. Bulk and biofilm samples in continuous supply had the lowest alpha diversity, followed by bulk water samples exposed to drainage. Bulk and biofilm samples exposed to stagnation had the highest alpha diversity, followed by biofilm samples exposed to drained events. Thus, the biofilm community was affected by drainage, even though this was not evident in the bulk water sample because it was collected immediately after refilling with tap water. Pair-wise statistical analysis between the different supply types and sample types showed differences across alpha diversity indices **Figure 5.6**. Observed ASVs were only statistically different between samples exposed to stagnant and drained events (Kruskal-Wallis, $p < 0.05$). Shannon diversity was statistically different across supply type and sample type; however, these differences indicate that the diversity of bulk and biofilm samples exposed to stagnant and drained events differed from bulk and biofilm samples in continuous supply. Higher alpha diversity after stagnation, like the results presented here, is contrary to the results published by Ling et al. 2018, where the influence of a week-long stagnation event on microbiome diversity was studied in a university building at the University of Illinois at Urbana Champaign. Although Ling

et al. 2018 found significantly higher cell concentrations after stagnation, alpha diversity was significantly lower than the diversity in tap water. However, Chen et al., 2020 found that alpha diversity in drinking water increased during the first 72 hours of a stagnation event. Thus, stagnation duration is likely an important factor in the assembly of microbial communities in drinking water exposed to stagnation. In addition, the type of disinfectant residual used, pipe material, and size of pipes can also be important factors that affect the microbial diversity in drinking water exposed to stagnation (Bédard et al., 2018; Lautenschlager et al., 2010; Lipphaus et al., 2014; Zlatanović et al., 2017). The lack of differences between alpha diversity of bulk drained samples and continuous supply could be due to our sampling and experimental scheme.

Principal Coordinates Analysis (PCoA) using weighted UniFrac distances revealed differences in bacterial composition between continuous supply and other supply types (**Figure 5.7A**). The first two PCoA axes accounted for over 85 % of the variation, with bulk and biofilm samples exposed to stagnation and biofilm samples exposed to drainage separating from other samples along both axes but primarily the first (PCoA 1 = 61.8 %). This difference was statistically significant (ANOSIM and PERMANOVA, $p < 0.05$). Interestingly, in the continuous reactor, the bulk and biofilm samples clustered together, along with some of the bulk water samples from the drained reactor, presumably because they were collected right after re-filling with fresh feed water. Distances based on compositional relationship of community members (i.e., Bray-Curtis) yielded similar results (**Figure 5.7B**). Two distinct clusters were observed. The first cluster was composed of bulk and biofilm samples from the simulated continuous supply and bulk water samples exposed to drained events. Beta and alpha diversity analysis demonstrated high similarities between these three types of samples. These results can be in part due to factors such as the time required for a different biofilm profile to develop in continuous supply (Lehtola et al., 2004; Pedersen, 1990; Percival et al., 1999), which might not have been reached in this experiment, and our sampling and experimental scheme as previously mentioned. Biofilms exposed to desiccation and rewetting (in other environments) have demonstrated an overall resistance and resilience of their microbial community (McKew et al., 2011; Timoner et al., 2012). The second cluster was composed of bulk and biofilm samples exposed to stagnation and biofilm samples exposed to drainage. Stagnant and drained events influenced biofilm microbial diversity and composition. Like previously mentioned, other studies have demonstrated the potential for stagnation (in DWDSs) and desiccation/rewetting events (in other water environments) to change the composition of microbial communities in biofilms (Lautenschlager et al., 2010; McKew et al., 2011; Prest et al., 2013; Timoner et al., 2012; Zlatanović et al., 2017). Between bulk and biofilm microbial communities from stagnant AR samples, we found a high degree of similarity that has also been observed in other studies comparing bulk and biofilms exposed to stagnation (Lautenschlager et al., 2010; Ling et al., 2018; Lipphaus et al., 2014). The high similarity in diversity and composition in stagnant bulk water and biofilms might be explained by the likelihood that microbiomes in surface-associated biofilms are in equilibrium with planktonic microbiomes in stagnant water (Lipphaus et al., 2014). This is also consistent with higher cell concentrations found after stagnant events. Interestingly, biofilms exposed to drained events are more similar to those exposed to stagnant events than continuous supply. Further research into the dynamics of biofilm development in drinking water under desiccation and rewetting is needed to further understand this key feature in IWS.

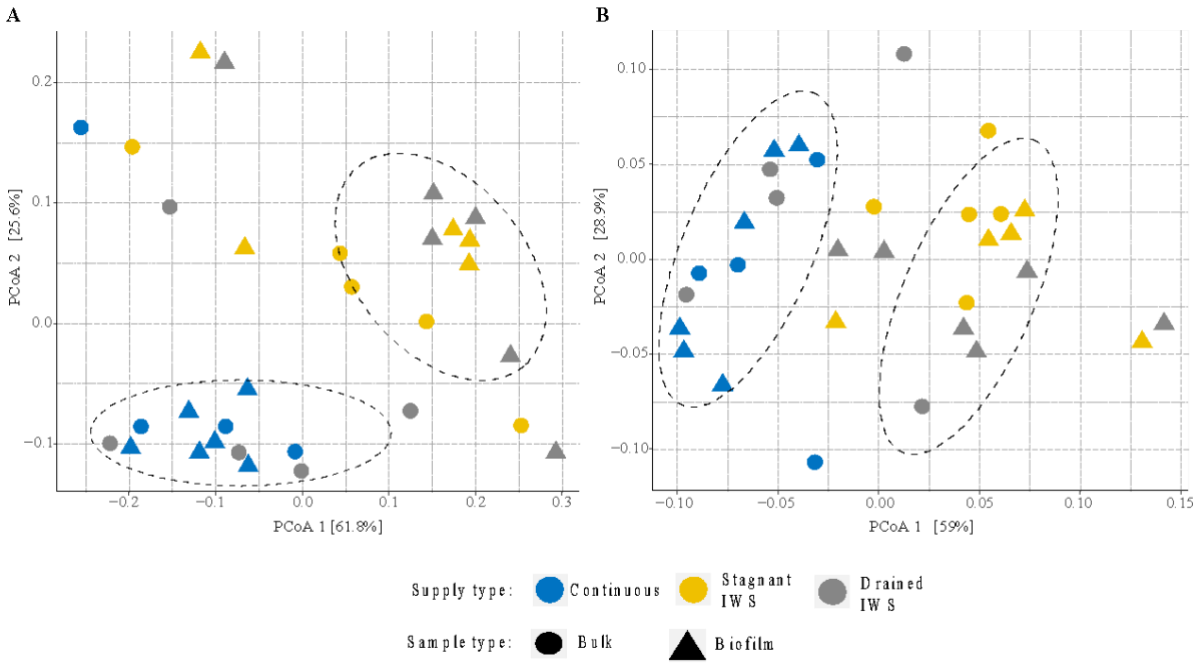

Figure 5.7. Principal coordinates analysis (PCoA) based on weighted UniFrac distances (A) and Bray-Curtis dissimilarity distances (B) for the different supply types investigated in this study. Distinct similarity clusters are indicated with dotted circles.

5.3.4 Taxonomic profiles in bulk and biofilms in simulated IWS

16S rDNA sequencing analysis identified a total of 107 ASVs (agglomerated at the genus level), and the number of ASVs ranged from 30 to 58. A total of 56 ASVs (52% of ASVs) were shared by all three ARs, representing over 90% of the total reads. There were ten phyla represented in the dataset, but over 99 % of the relative abundance could be attributed to six phyla (**Table 5.2**). Actinobacteria and Proteobacteria represented over 90 % of relative abundance. At the Class level, Actinobacteria, Alphaproteobacteria, and Gammaproteobacteria were the most abundant in samples from the continuous supply (72.4 %, 23.4 %, and 1.5 %, respectively). Actinobacteria, Alphaproteobacteria, and Melainabacteria (previously classified as Cyanobacteria) were the most abundant among samples exposed to stagnant and drained events (Stagnant: 61.5%, 24.4%, 4.4%; Drained: 66.4%, 21.87%, 3.3%, respectively). Among top families, Mycobacteriaceae, Sphingomonadaceae, Beijerinckiaceae, Gemmataceae, Hyphomicrobiaceae, Obscuribacteraceae, and Nitrospiraceae were most abundant. Mycobacteriaceae, Sphingomonadaceae were abundant across all samples (Continuous: Biofilms, 42.4%, 7.4%, Bulk, 28.6%, 4.9%; Stagnation: Biofilms, 32.7%, 7.7%, Bulk, 27.5%, 7.3%; Drainage: Biofilms, 38.0%, 7.3%, Bulk, 27.3%, 5.1%). Beijerinckiaceae and Hyphomicrobiaceae were abundant in biofilm samples, and Beijerinckiaceae and Gemmataceae were abundant in bulk samples in continuous supply. For samples exposed to stagnant events, Obscuribacteraceae and Nitrospiraceae were abundant in biofilms, and Obscuribacteraceae and Beijerinckiaceae were abundant in bulk water. For samples exposed to

drained events, Obscuribacteraceae and Nitrospiraceae were abundant in biofilms and Beijerinckiaceae, Gemmataceae were abundant in bulk water. All samples were unevenly distributed – skewed by three genera: *Mycobacterium*, *Sphingomonas*, and an unspecified Beijerinckiaceae (**Figure 5.8**). Three genera were found in samples (both bulk and biofilm) exposed to stagnant and drained events but were not found in continuous supply (continuous AR): *Methylomonas*, *Bosea*, and *Rhodovarius* (**Figure 5.8**). *Methylomonas* was only observed in biofilm samples from the continuous supply (continuous AR), but *Bosea* and *Rhodovarius* were not found in continuous supply (neither in bulk or biofilm samples from continuous AR) (**Figure 5.8**).

Table 5.2. Mean relative abundance (within site percentage) and standard deviation of phyla found in bulk and biofilm samples from three ARs simulating different supply types.

Supply	Continuous		Stagnant		Drained	
Sample type	Bulk	Biolfilm	Bulk	Biolfilm	Bulk	Biolfilm
Phylum	Mean ± SD		Mean ± SD		Mean ± SD	
Actinobacteria	29.3 ± 17.0	43.1 ± 15.9	28.0 ± 15.3	33.5 ± 14.9	27.7 ± 16.5	38.7 ± 16.9
Proteobacteria	9.2 ± 2.3	15.6 ± 2.8	13.0 ± 1.8	14.6 ± 1.9	10.5 ± 2.9	13.6 ± 1.6
Cyanobacteria	< 0.1	0.7 ± 0.4	1.9 ± 1.2	2.6 ± 1.4	0.3 ± 0.2	3.0 ± 1.7
Planctomycetes	0.6 ± 0.4	0.3 ± 0.1	1.2 ± 0.5	1.9 ± 0.6	0.8 ± 0.6	2.3 ± 0.8
Nitrospirae	0.5 ± 2.0	0.1 ± 0.1	1.3 ± 1.7	2.0 ± 2.7	0.6 ± 2.9	2.2 ± 2.0
Firmicutes	< 0.1	0.1 ± 0.1	< 0.1	< 0.1	< 0.1	< 0.1

Analysis of composition of microbes **(**ANCOM) and linear discriminant analysis (LefSE) were also used to determine the taxa that described the most variance between supply and sample types (**Figure 5.9**). Comparisons of ASVs showed several taxa whose proportions were significantly different among water samples (bulk and biofilm) from continuous supply and the rest of the supply types. *Mycobacterium* (Actinomycetales) and several unclassified genera are among the taxa that were highly enriched in biofilm samples from the continuous supply. *Methylobacteium* (Rhizobiales) was the only genera enriched in bulk water samples from the continuous supply. Within samples exposed to stagnation, *Nitrospira* (Nitrospirales), *Cenothrix* (Sphingobacterales), *Methyloglobus* (Rhizobiales), *Sphingopixis* (Sphingomonadales) were enriched in biofilms, and *Hyphomicrobium* (Rhizobiales), *Novosphingobium* (Sphingomonadales), *Methylomonas* (Methylococcales) were significantly different among bulk water samples. In samples exposed to drainage, *Obscuribacter* (Obscuribacterales), *Phreatobacter* (Rhizobiales), and three unclassified genera were significantly enriched in biofilms while no genera were notably different in bulk water samples.

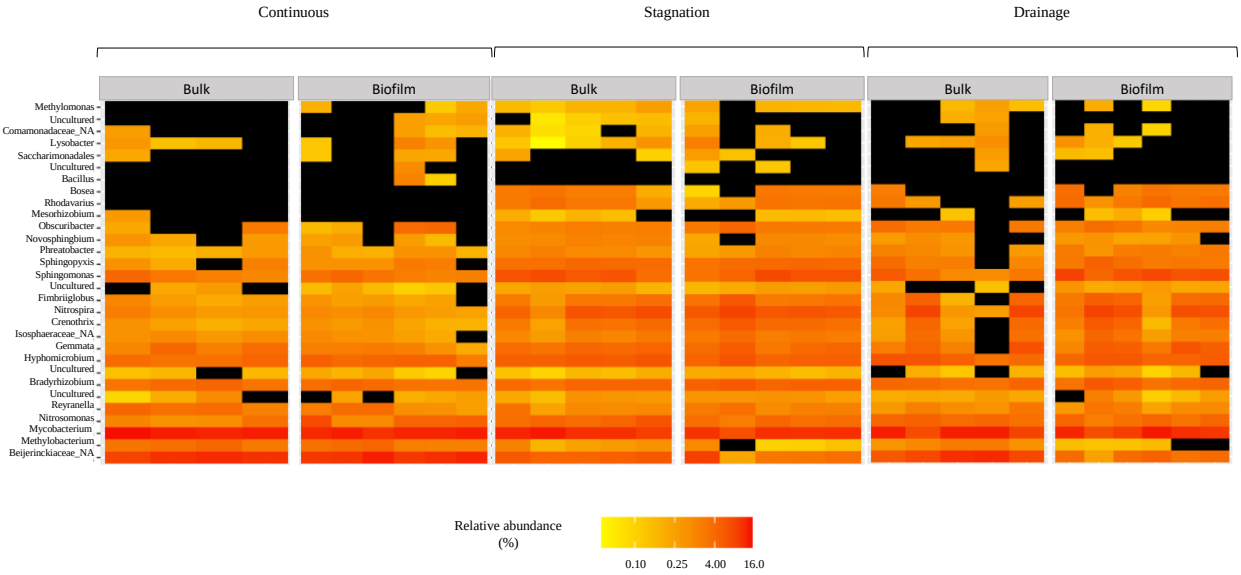

Figure 5.8. Heatmap of relative abundance of top genera in bulk and biofilm samples in different supply types: continuous, stagnation, and drainage.

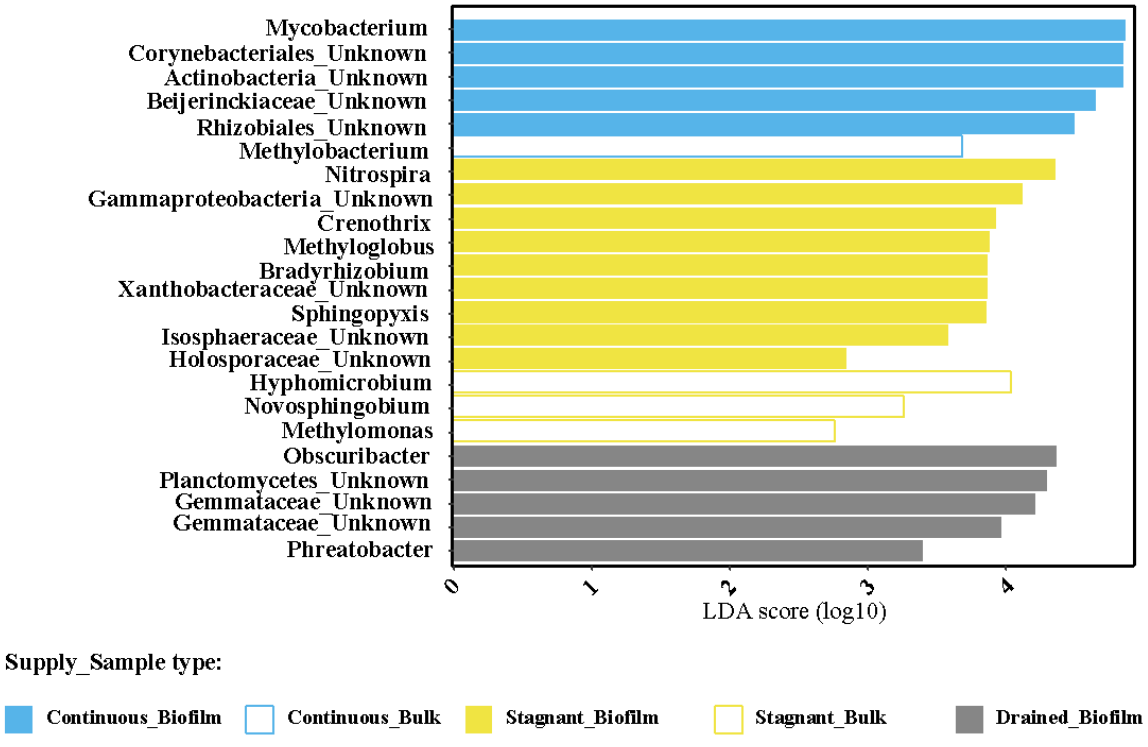

Figure 5.9. Bacterial taxa identified as differentially abundant between samples from four different land-type uses as analyzed by LefSe with LDS values above 2.0. No differentially abundant taxa were found in bulk water exposed to drained events.

Detailed characterizations of microbial communities in full-scale DWDS and DWDS with IWS are lacking, in part, due to the difficulty of obtaining bulk and biofilm samples from water pipes. In this study, bulk water and biofilms were sampled from three ARs simulating different supply types to characterize and compare their bacterial communities. The results provide novel information on the relationships between microbial communities (in bulk water and biofilms) and disinfection, stagnation, and drainage within simulated DWDS and suggest significant differences, mainly driven by stagnation. Based on the dominant taxa, bulk and biofilm samples appeared to be adapted for nutrient scarcity. Across supply types, taxa that are considered facultative methylotrophs were common in both sample types. Methylotrophs have the ability to use single-carbon compounds as a sole carbon and energy source. *Mycobacterium*, *Methylobacterium*, and *Hyphomicrobium* spp., (which are facultative methylotrophs) have been associated with biofilm in DWDSs, water meters, and premise plumbing (Stanish et al., 2016). In addition, *Mycobacterium* and *Methylobacterium* have been primarily associated with chloraminated DWDS (Gomez-Smith et al., 2015; Kelly et al., 2014), while *Hyphomicrobium* has been associated with DWDS with no residual disinfectant (Stanish et al., 2016). The results presented here were consistent with two surveys of DWDSs that found *Mycobacterium* and *Methylobacterium* spp. enriched in drinking water with measurable chloramine concentrations. *Hyphomicrobium* and other genera, on the other hand, were found to be abundant in drinking water with low or no chlorine residual (Stanish et al., 2016; Waak et al., 2019).

The presence of *Mycobacterium* and *Methylobacterium* together, which compete for a similar ecological niche, is interesting; however, *Mycobacterium* was found to be enriched in biofilm samples while *Methylobacterium* was enriched in bulk water (both in continuous supply). The enrichment of *Mycobacterium spp.* in continuous supply has also been found in other chloraminated systems (Waak et al., 2019). If chloramination-induced enrichment of *Mycobacterium* is common in chloraminated systems, this may indicate a potential public health concern. However, 16S rDNA sequencing cannot be used to distinguish between pathogenic and non-pathogenic mycobacteria. Many species of Mycobacterium have been associated with drinking water systems (Beye et al., 2018; Waak et al., 2019), and it has been suggested that this may be due to its cell membrane composition, a waxy cell membrane that likely allows them to tolerate disinfection residual (Luh et al., 2008).

Stagnant and drained events appeared to differentiate bulk and biofilm drinking water further. Several genera were enriched in biofilms exposed to stagnation, including several unclassified genera, *Nitrospira, Cenothrix, Methyloglobus*, and *Sphingopixis.* These genera have all been found during drinking water treatment (Jia et al., 2015; C. Li et al., 2017; Ling et al., 2018; Oswald et al., 2017; Vaz-Moreira et al., 2011). *Nitrospira* has been identified in many environments, including drinking water treatment plants (Fujitani et al., 2020)(Gruber-Dorninger et al., 2015). In addition, the presence of comammox *Nitrospira* in DWTPs and DWDSs has been recently discovered (Pinto et al., 2016; Wang et al., 2017). In drinking water systems, nitrification plays an critical role in decreasing chloramine residual and the production of nitrates (Wang et al., 2014b). Genera enriched in bulk water samples exposed to stagnant events, *Hyphomicrobium, Novosphingobium, Methylomas,* have been associated with drinking water systems with no chlorine residual (Stanish et al., 2016). This study demonstrated that stagnation leads to the enrichment of certain bacteria (both qualitative and quantitative) that significantly changes the diversity and structure of bulk drinking water and biofilms. The influence of drained events is

more complex and not straightforward, leading to the enrichment of only biofilm bacteria and higher ICC concentrations.

No particular genera were found to be significantly different for bulk water samples exposed to drained events; but, *Obscuribacter* and *Phreatobacter* were enriched in biofilms along with unclassified bacteria. The enrichment of these two genera is of particular interest as they were both also found in samples from the distribution system in Arraiján, Panama. Although not much is known about these genera in DWSs, recent studies have also reported a high abundance of these genera in drinking water (Bruno et al., 2018; Liu et al., 2018; Perrin et al., 2019).

The results from this study on the effects of stagnation and drainage only captured the impact of one specific set of conditions, and more research is needed to understand the impacts on microbial communities of the wide range of IWS operating conditions in real-world systems. In particular, it was difficult to simulate the dynamics of pipes draining and re-filling. The shear forces and other factors that influence biofilm detachment during the first flush may be much more significant in some real systems than what was simulated in the AR in this study. The first flush of water in full-scale IWS systems could contain a mixture of microorganisms from stagnant water, detached biofilms, re-suspended loose deposits, and the bulk water supply. Further research under controlled experimental conditions is necessary to continue elucidating these unique features of IWS in microbial water quality. These efforts should take into consideration: i) different shear forces during flushing; (ii) environmental factors that could also strongly influence microbial communities in drinking water, such as temperature changes, humidity levels, and disinfectant residual; (iii) the influence of the duration and scheme of stagnant/drained events that are comparable to IWS cycles found in full-scale systems; and (iv) the development and morphology of biofilms under stagnant and drained conditions with alternate continuous supply. Another important consideration is the use of other experimental approaches, such as the use of pipe loops and other operating ARs, that can better simulate features of IWS and can elucidate the influence of IWS on microbiomes to a greater detail.

5.4 Conclusions

- Chloramine concentrations varied significantly between supply types and can potentially lead to chloraminated-induced enrichment of *Mycobacterium.*

- Stagnation increased bacterial cell concentrations and strongly influenced the diversity and composition to enrich bacterial genera such as *Nitrospira*, *Cenothrix*, *Methyloglobus*, *Sphingopixis* in biofilms and *Hyphomicrobium, Novosphingobium, Methylomas* in bulk water.

- The effect of drained periods was more evident in the biofilms than the bulk water because the bulk water was fresh feed water (added and mixed for three min), and the filling conditions likely did not cause significant detachment of biofilm. *Obscuribacter, Phreatobacter* were significantly abundant in biofilms and were also found in the DWDS of Arraiján, Panama.

Chapter 6

Conclusions

There is no doubt that increasing water scarcity, climate change, population growth, and urbanization will continue to pose significant challenges to the provision of safe water around the world. It is estimated that global water demand will significantly grow over the next 20 years in three main areas: industry, domestic, and agriculture (United Nations, 2018). In Central and South America alone, water demand is estimated to increase by more than 200% by 2050 (Flachsbarth et al., 2015; Wada et al., 2016). With increasing water demand and declining water resources and water quality, it is imperative to understand further how vulnerable water resources are affected by changing landscapes and how water quality is affected by drinking water systems with deficiencies and unreliable supply. The research objective presented in this dissertation was to evaluate water quality and bacterial communities in water at various stages of a drinking water system in a resource-constrained region, from source to tap.

Starting with water sources, I carried out a two-year field survey to evaluate water quality and water microbial communities in streams that are part of the Panama Canal watershed and are impacted by different land uses commonly found in Latin America and the Caribbean (Chapter two). After the water source, the next stage of supply is water treatment and distribution; however, there is limited information on how microbial communities are impacted by centralized drinking water treatment and distribution in resource-constrained tropical regions. Thus, I carried out a one-year field survey to assess the effects of treatment and distribution in the drinking water system serving the peri-urban region of Arraiján, Panama, part of the Panama Canal watershed (Chapter three).

This dissertation research also focused on a common substandard water delivery practice, intermittent water supply (IWS). Temporal (e.g., droughts, pipe failures, pollution accidents, electrical outages, maintenance) and full-time (e.g., planned water rationing) supply intermittency is common in many low- and middle-income countries, and thus it is essential to further understand how IWS impacts microbial communities in drinking water. To bring new insights into the effects of IWS on drinking water microbial communities, I conducted a field survey in a section of Arraiján's drinking water distribution network with IWS (Chapter four). As I was conducting this field research in Arraiján, I discovered that it was very challenging to answer key questions regarding IWS in the field due to the inherent complexities of actual full-scale distributions networks and the difficulty of sampling. Thus, I conducted a controlled laboratory experiment focusing specifically on how two key features of IWS (stagnation and drainage) affect microbial communities in water and biofilms (Chapter five).

This research was an effort to collect as much microbial data as possible using 16S rDNA sequencing and begin answering initial questions about the microbial composition in water sources

and drinking water in a tropical environment. I hope this work expands our knowledge regarding water microbiomes in resource-constrained tropical regions and can inform strategies that improve current water management strategies at various levels of water provision.

6.1 Key Findings

In Chapter Two, the influence of land use on water quality and microbial communities in stream water was explored, namely, the impacts of tropical forest restoration efforts and improved agricultural practices on stream microbiomes. I studied bacterial communities in the water column of four streams, each influenced by a different land use type - primary mature forest, secondary forest, silvopasture and traditional cattle pasture - over two years. These streams are part of the drinking water sources in the Panama Canal watershed that serve Panama City and surrounding regions. Nearby land uses primarily influenced community diversity and composition of stream water bacteria, and seasonal effects were also observed. Streams influenced by forested areas exhibited higher diversity and similar community structure to each other, demonstrating that they have a robust community that is more resistant to seasonal changes. On the other hand, the stream influenced by traditional cattle pasture had the lowest diversity, with a high abundance of only a few members, making it less resilient. The stream influenced by silvopasture showed the strongest seasonal shifts, with similar communities to forested catchments during the wet seasons and cattle pasture during dry seasons. During the dry season, cattle tended to congregate near the stream where there is more shade to avoid heat and drink. The increased congregation of cattle near the stream and reduced dry-season flow could have led to seasonal changes and bacterial composition. These results highlight the importance of maintaining a forest buffer with fencing and also tree buffers that are away from stream channels to reduce the influence of cattle in nearby streams. These results can be helpful in the design and management of silvopastures systems and encourage the restoration and protection of riparian zones, expansions of gallery forests that serve as corridors connecting forest patches, and other considerations that increase the forest content in agroforestry systems. The overall findings highlight the benefits that forest regrowth and riparian corridors can provide to freshwater streams in the tropics.

Indicator bacteria (total coliforms and *E. coli*) by traditional culturing methods were found across all sites, showing seasonal shifts identified in both forested streams and those influenced by cattle. Although culture-based indicator bacteria remain the standard for water quality assessment, we found (through the sequencing results) several genera that could interfere with the interpretation of indicator bacteria results because previous research has shown they can produce false-positive results using the IDEXX Colilert (total coliform/*E.coli*) culture-based test. When evaluating the water quality of water sources, where we are likely to encounter a higher diversity in microbial communities, the question arises of how effective are standard culture-based tests at measuring fecal contamination. Based on the results presented here, combining more general culture-based tests with more specific microbial targets might be more useful at monitoring water quality and public health risks.

In Chapter Three, the impact of source water and treatment processes on bacterial diversity, taxonomy, and abundance of DW microbial communities serving the peri-urban region of Arraiján, Panama, was assessed by surveying three DWTPs and the interconnected distribution network that had operational and infrastructure deficiencies and IWS, which are commonly

encountered in developing countries. A diverse bacterial community was found at each of the three DWTPs, dominated by Proteobacteria but with significant differences along the treatment train. Treatment processes were found to have varied effects on bacterial diversity and structure at the different DWTPs, perhaps driven by DWTP operations. The correlations of water quality parameters with bacterial community composition indicate the possibility to control bacterial community structure by operating DWTPs differently. The abundance of the relatively new 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 environmental conditions present in the distribution network on bacterial communities in piped drinking water.

In Chapter Four, an investigation of how IWS influenced bacterial communities in a portion of the full-scale DWDS in Arraiján, Panama, revealed significant changes in composition. Over the course of one year, a field survey was carried out to assess the water quality and microbial communities in the first flush (immediately after supply resumed) and stable supply (after ~24 h of continuous supply at the same location). Based on conventional water quality metrics, water quality was similar in continuous and IWS when supply was stable; however, bacterial community composition was different. Water quality during the first flush had lower chlorine, higher HPC concentrations, and higher temperatures. First Flush samples also revealed the presence of unique taxa that could be the result of intrusion or in-situ growth between supply cycles (e.g., in stagnant water, biofilms, or loose deposits). Interestingly, *Pseudomonas* was among the most abundant taxa in IWS; however, further research on other IWS systems is needed for validation and 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. 16S rDNA sequencing data may hold promise as a tool to reveal signals for intrusion/growth between supplies; however, more studies are needed to determine if these results are consistently observed during first flush compared to stable supply. Key water quality parameters such as chlorine concentrations, temperature, and pressure were correlated with the observed differences in beta diversity, which suggests that the unique conditions in first flush samples can at least partially explain observed differences in bacterial composition.

In Chapter Five, to further understand the influence of key IWS features on microbial communities in bulk water and biofilms, I carried out a laboratory bench-scale experiment to specifically explore the influence of stagnation and drainage. Bench-scale rotating annular reactors (simulating three types of water supply: continuous, IWS where water is left stagnant in pipes between supply cycles, and IWS where pipes drain completely), high-throughput sequencing, and flow cytometry were used to evaluate stagnation and drainage independently. The results from this study on the effects of stagnation and drainage only captured the effects of one specific set of conditions, and more research is needed to understand the impacts on microbial communities of the wide range of IWS operating conditions in real-world systems. However, from this research, we determined that stagnation significantly influenced bacterial concentrations, diversity, and structure. Stagnation enriched bacterial genera such as *Nitrospira*, and *Sphingopixis* in biofilms and *Hyphomicrobium*, *Novosphingobium, Methylomas* in bulk water. The effects of drained periods were more ambiguous but more evident in the biofilms than the bulk water. *Obscuribacter* and *Phreatobacter* were significantly abundant in biofilms exposed to drained periods; interestingly, these taxa were also highly abundant in the bulk water samples from the DWDS of Arraiján, Panama.

6.2 Limitations

Although this dissertation research has provided new insights into microbial communities in water sources and drinking water in a resource-constrained setting, several challenges limited the outcomes of this research, from sample collection to the methods chosen to assess microbial communities. Sampling IWS systems effectively is a difficult practice that requires resources, time, and collaboration. In many DWDS, IWS is unplanned and may only affect some sections of the DWDS. From my experience, even in sections of the DWDS with planned IWS, water supply delivery schedules were unreliable. This unreliability creates distrust of the water utility by consumers, and by proxy, can manifest as distrust of researchers that require the utility's partnership to undertake their research. The unreliability also makes it very difficult to collect samples because it is unpredictable when the water supply will start and end. These are significant challenges for any water quality monitoring program and research effort. To overcome the unpredictability, real-time monitoring equipment can be installed for measuring some water quality parameters and has been proven effective in water quality microbial surveys (Erickson et al., 2020, 2017). Due to the challenge of determining the timing of IWS supply cycles and the fact that sampling locations were located far from the laboratory, this research could only focus on one type of IWS in the DWS of Arraiján, Panama.

It is also important to note that for the research work in the DWS in Arraiján, Panama, we worked with a limited number of samples which decreased the power of the statistical analysis, although each sample from the DWS represented a large volume of water collected. More robust assessments of microbiomes in DWSs in developing countries are needed in order to understand how water microbiomes are influenced by operations, environmental factors, and common deficiencies found.

Simulating IWS features was also challenging. In particular, it was difficult to simulate the dynamics of pipes draining and re-filling accurately. The shear forces and other factors that influence microbial communities in IWS during the first flush may be much more significant in some full-scale systems than what was simulated in the ARs in this study. The first flush of water in real IWS systems most likely contains a mixture of microorganisms from stagnant water, detached biofilms, re-suspended loose deposits, and the bulk water supply. In addition, only one set of conditions was able to be investigated for each supply type (continuous, stagnant, drained). Furthermore, the first flush of water in the pipes of a full-scale system might spend much longer in the pipes than the time used in these simulations (three minutes). Depending on where the first flush of water is sampled in a real system, water might travel from minutes to hours before this first flush of water reaches the tap.

Next-generation sequencing has been widely used in recent years to assess microbial communities in drinking water, from freshwater sources to taps (Bertelli et al., 2018; Douterelo et al., 2016; Q. Li et al., 2017; Ma et al., 2017; Montoya-Pachongo et al., 2018; Pinto et al., 2012; Van Assche et al., 2019; Vignola et al., 2018; Xu et al., 2017). Although tremendous progress has been made in 16S rDNA sequencing (DNA metabarcoding), there are still limitations to this nucleic-acid based method. As with any analysis involving PCR amplification, biases can exist due to secondary structure or GC content, resulting in sequencing error or chimeras. The choice of primers and the

presence of multiple copies of the small subunit rRNA gene targeted can also result in biases and errors (Kozich et al., 2013; Quail et al., 2012; Schirmer et al., 2016; van Dijk et al., 2014). Detection and quantification of DNA in water that may be free-floating is very likely, especially in chlorinated drinking water systems where the issue of DNA damage and the presence of free-floating DNA is critical. The inability to incorporate accurate quantification into 16S rDNA sequencing resulted in a qualitative assessment of microbial communities in this research work. To combat the issue of free-floating DNA in water samples and target viable cells, the use of propidium monoazide in conjunction with PCR and Illumina sequencing has been used to identify and quantify microbial communities (Guo et al., 2021; Q. Li et al., 2017; Pang et al., 2016; Tantikachornkiat et al., 2016). In addition, several studies on drinking water have used amplification-independent metagenomic shotgun sequencing because of PCR amplification concerns, which offers higher resolution and functional power (Brumfield et al., 2020; Quince et al., 2017; Tan et al., 2015).

16S rDNA analysis captures shifts in community diversity but with limited resolution and lower sensitivity compared to metagenomics. 16S rDNA sequencing lacks the resolution for pathogen detection and hinders direct microbial risk assessment (Rizal et al., 2020), and provides limited functional analysis (Sun et al., 2020). Different library preparation protocols can generate sequencing errors on the Illumina platform (Schirmer et al., 2016), and downstream bioinformatic data processing can also produce biases in the relative abundance and diversity of microbial taxa (Straub et al., 2020). Despite these concerns, several studies have demonstrated that 16S rDNA sequencing provides a reasonable estimation of microbial composition and structure (Ong et al., 2013; R. Wang et al., 2013). Nonetheless, the sequencing efforts performed in this research are a first step in more specific assessments regarding microbial communities in water in tropical regions in the future.

6.3 Future work

Deterioration of the surviving rainforest and climate change impacts are rising in humid tropical regions. The distribution of tropical rainfall in this area is highly variable, and in many regions, the supply of safe potable water is still inadequate. The need for source protection programs in these regions is imperative. Source water protection programs are essential for the protection of watershed characteristics, water quality, and public health. The protection of forest areas must be at the forefront of protecting water resources. In addition, opportunities exist to improve water quality conditions in the studied area after water has been sourced and treated. The reality is that IWS will continue to be a method of water delivery in many parts of the world and a water delivery shortfall for many water utilities. This work provides direction for future research to continue investigating microbial communities in water in tropical regions.

Understanding the relationship between land use and water quality is necessary for effective water management. Further research into the influence of land use changes, reforestation, and different agricultural practices commonly found in the Neotropics on water quality and microbial communities in water could aid in the management of water resources frequently used for drinking water. Future research in this region should consider stream biofilms as they are hot spots for microbial activity. In addition, functional data by metagenomic analysis could be beneficial to

further understand nutrient cycling and functions in stream ecosystems. Another important research question is how microbial communities in streams near the different land use types that were part of this research differ when compared to other similar catchments in the area that are not experimental catchments (like those studied here). A robust assessment of several catchments with similar characteristics would provide significant insights into the impact of land use on stream ecosystems in the area.

Although the proposed guidelines to ensure safe drinking water have evolved consistently, drinking water treatment processes and the approaches commonly used to monitor water quality have remained almost unchanged. Monitoring of water quality relies on culture-based tests and indicator organisms to determine potability and process efficiency. However, in addition to challenges such as operational and infrastructure inefficiencies, supply unreliability, and recontamination, the provision of drinking water faces new challenges such as emerging pathogens, antibiotic resistance, and climate change. The application of molecular approaches, which have expanded and improved in terms of the amount and quality of data they can provide, could further enhance our understanding of DW microbial ecology in developing countries and tropical settings.

A better understanding of how microbial communities in drinking water are influenced by the different mechanisms of IWS is imperative to protect water quality in intermittent systems. Further research is needed to understand disinfectant residual in IWS, the importance of intrusion and regrowth, antibiotic resistance, and the abundance of disinfection byproducts. In parallel, improved data collection on IWS systems and a better understanding of the hydraulics of IWS are needed to realize the true scope of IWS and develop strategies to maintain water quality standards in IWS systems. In addition, further research under controlled experimental conditions is necessary to continue elucidating the influence of key features of IWS on microbial water quality.

Although it is not the intent of this research to recommend IWS as a way of operation, the reality is that IWS will continue to be the way many people receive drinking water now and in the near future. Thus, developing effective strategies for minimizing the problematic effects and microbial risks of IWS is essential. Effective monitoring and operation strategies have the potential to safeguard water quality and perhaps develop a consistent IWS microbiome, as well as protect pipe infrastructure and ensure the reliability of supply. Technical and practical research should also be integrated with policy changes to promote effective management of IWS systems.

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Appendix A

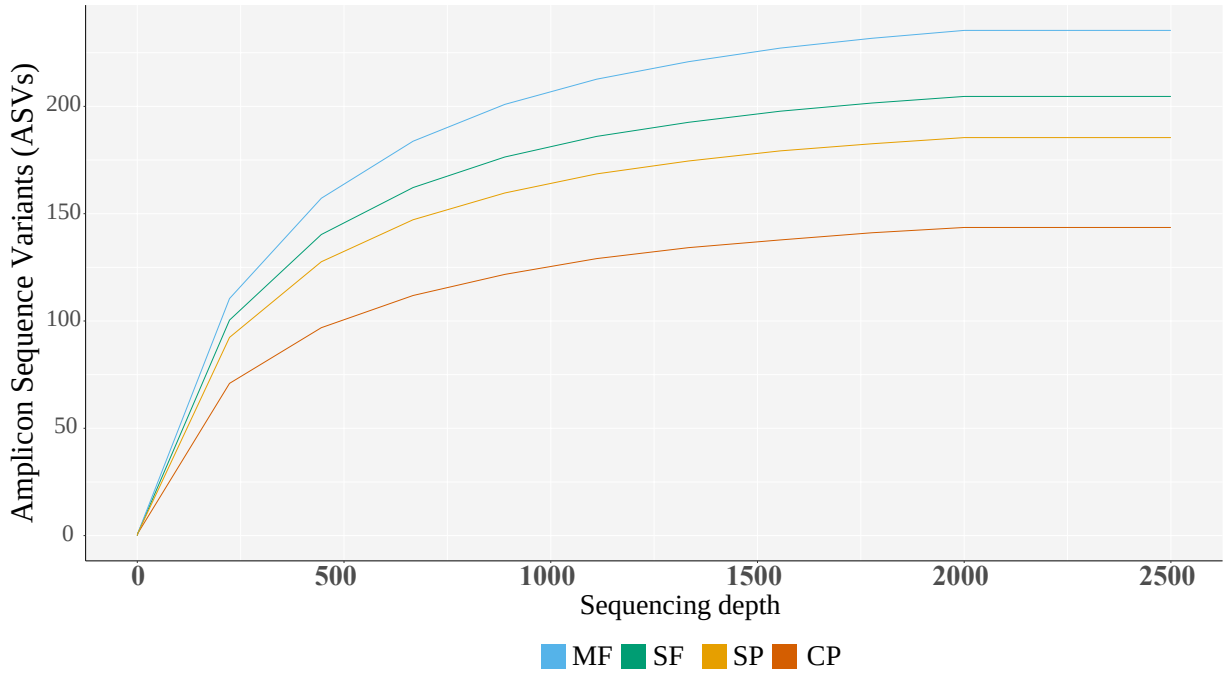

Figure A1. Rarefaction curves of average amplicon sequence variants at each site. Mature Forest, SF = Secondary Forest, SP = Silvopasture, CP = Cattle Pasture.

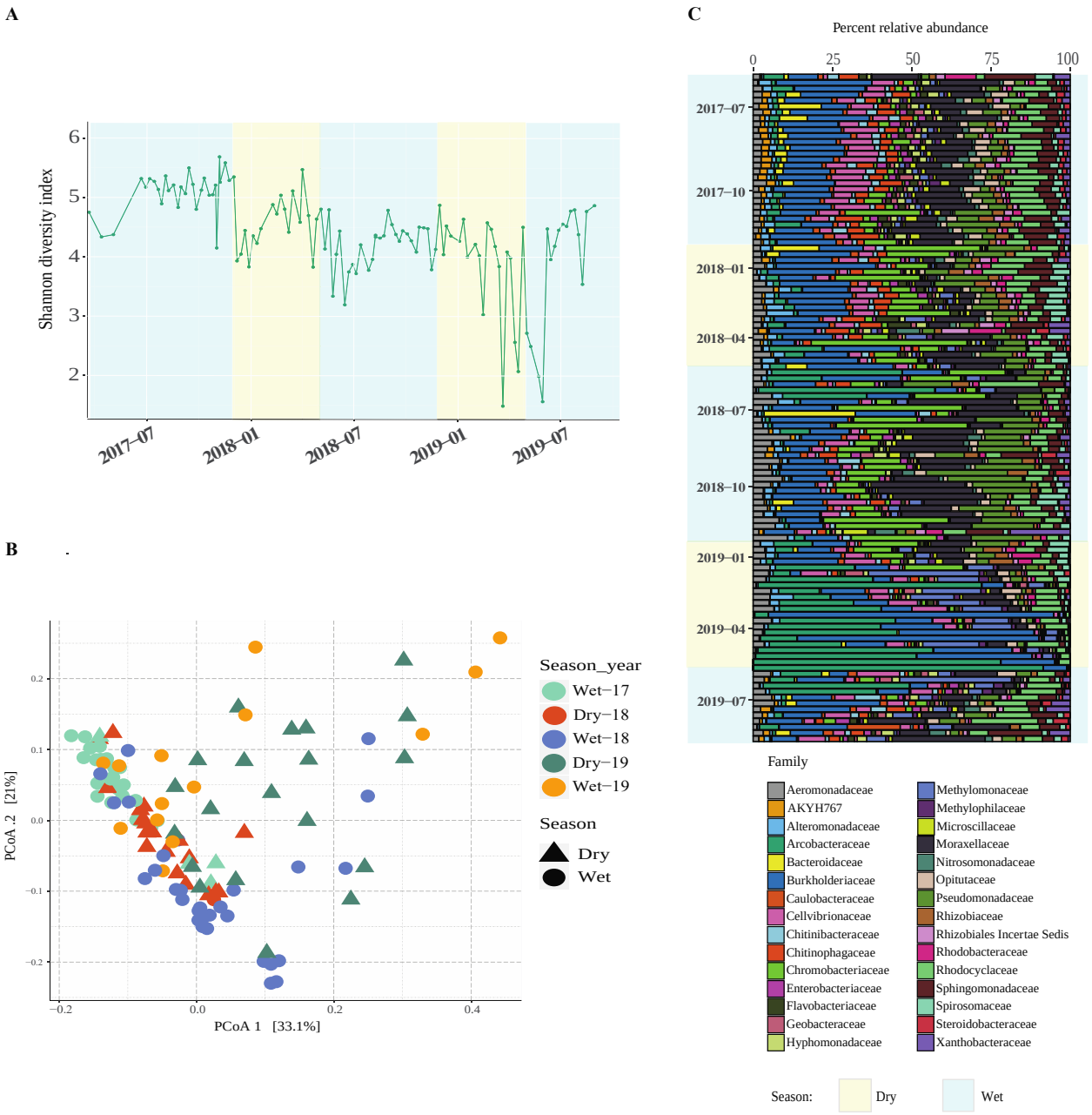

Figure A2. Mature Forest (MF). A) Shannon diversity across seasons throughout the study time; B) PCoA based on weighted UniFrac distances of samples across seasons; C) Relative abundance of top 20 families over a two year period based on weekly sampling. Diversity calculations were determined from samples rarefied to 2100 sequences/sample.

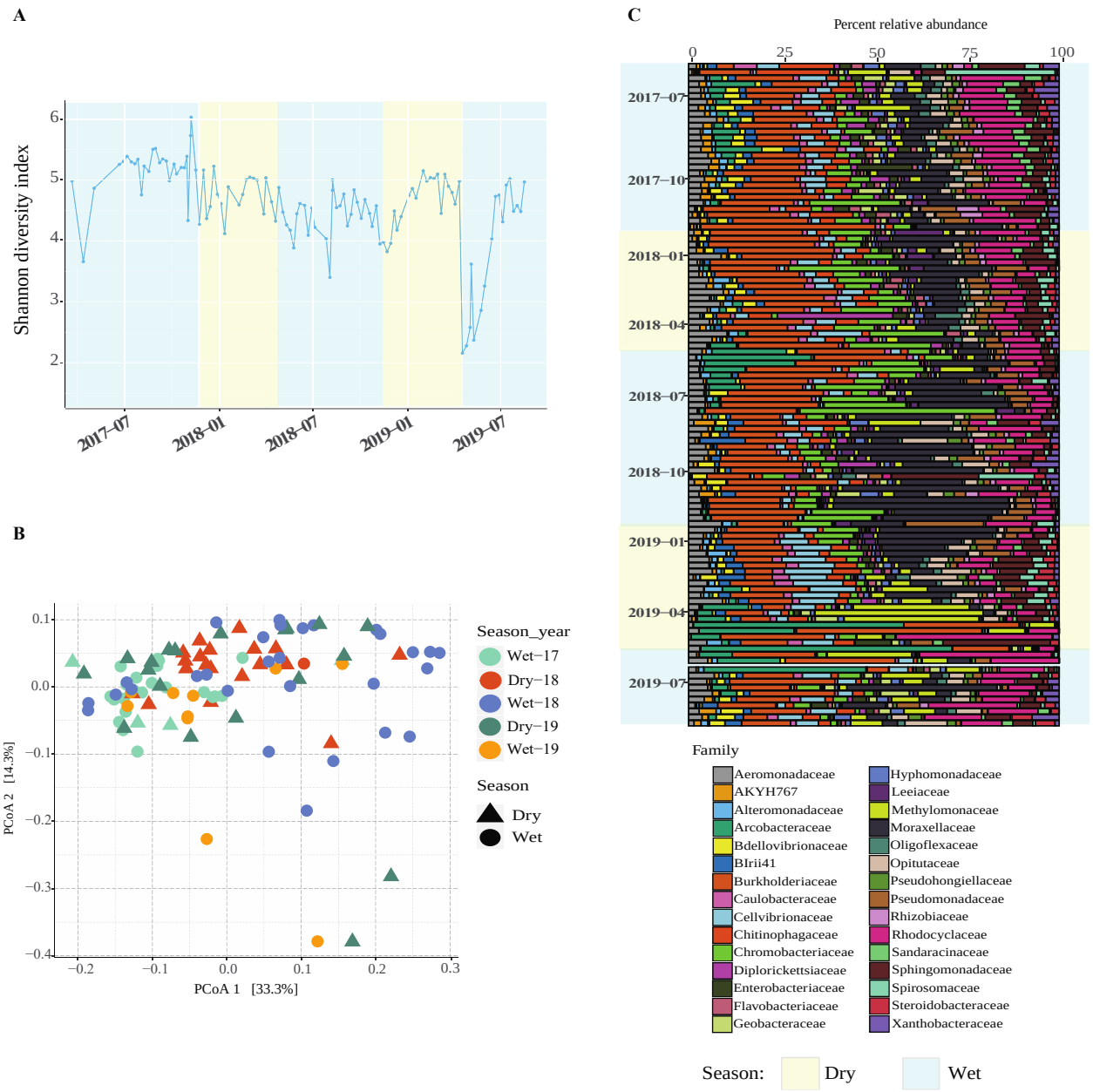

Figure A3. Secondary Forest (SF). A) Shannon diversity across seasons throughout the study time; B) PCoA based on weighted UniFrac distances of samples across seasons; C) Relative abundance of top 20 families over a two year period based on weekly sampling. Diversity calculations were determined from samples rarefied to 2100 sequences/sample.

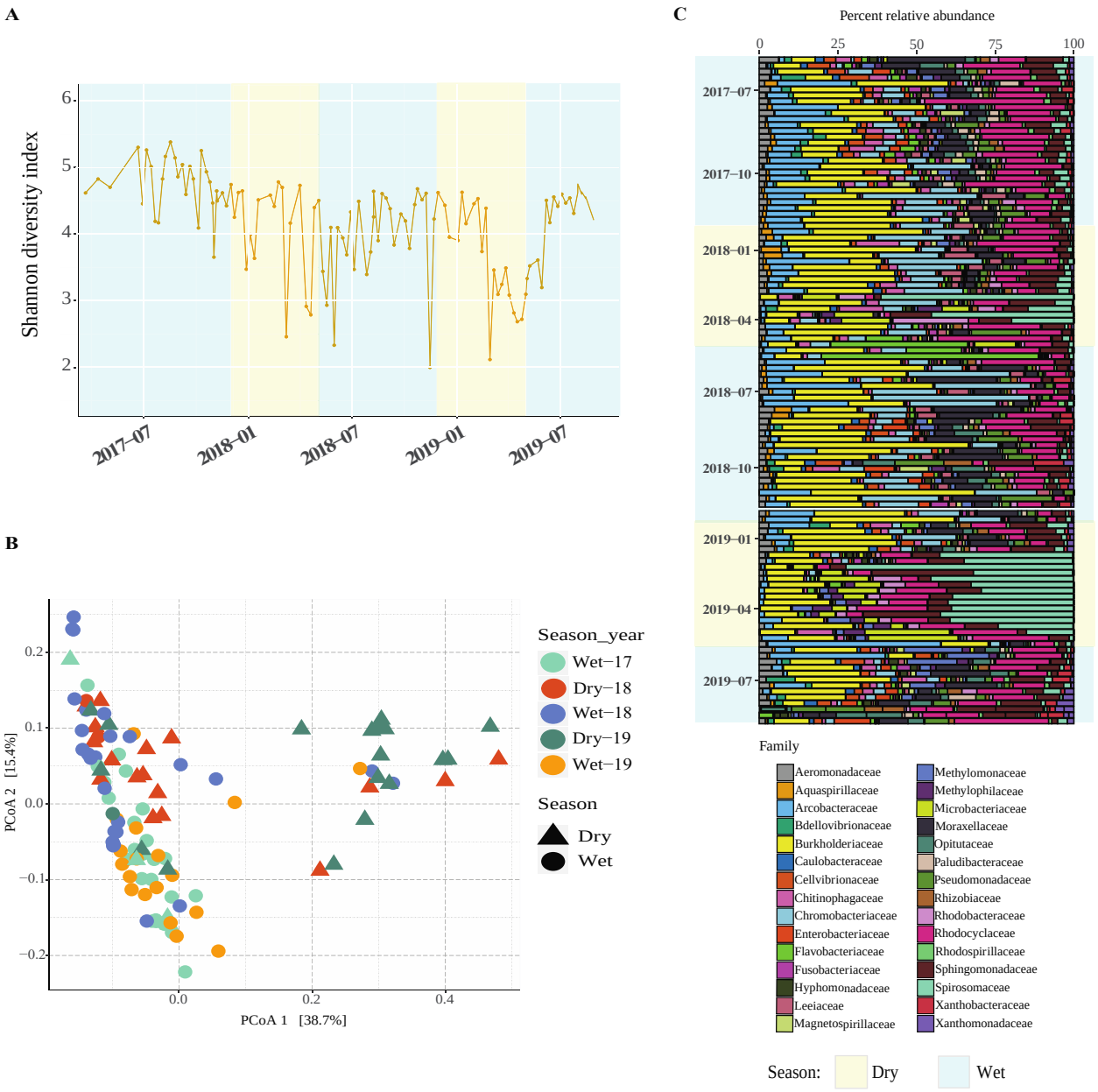

Figure A4. Silvopasture (SP). A) Shannon diversity across seasons throughout the study time; B) PCoA based on weighted UniFrac distances of samples across seasons; C) Relative abundance of top 20 families over a two year period based on weekly sampling. Diversity calculations were determined from samples rarefied to 2100 sequences/sample.

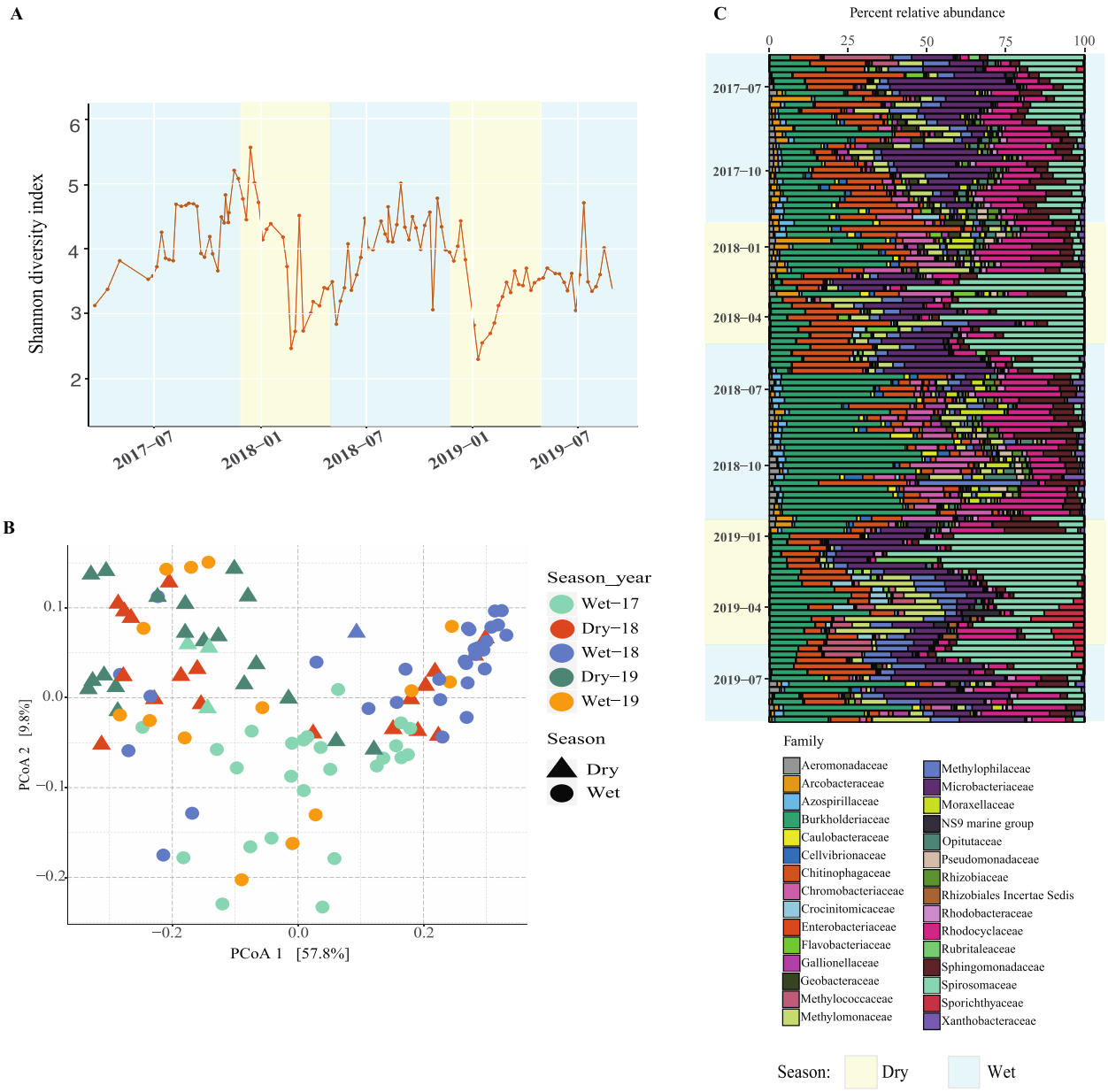

Figure A5. Traditional cattle pasture (CP). A) Shannon diversity across seasons throughout the study time; B) PCoA based on weighted UniFrac distances of samples across seasons; C) Relative abundance of top 20 families over a two year period based on weekly sampling. Diversity calculations were determined from samples rarefied to 2100 sequences/sample.

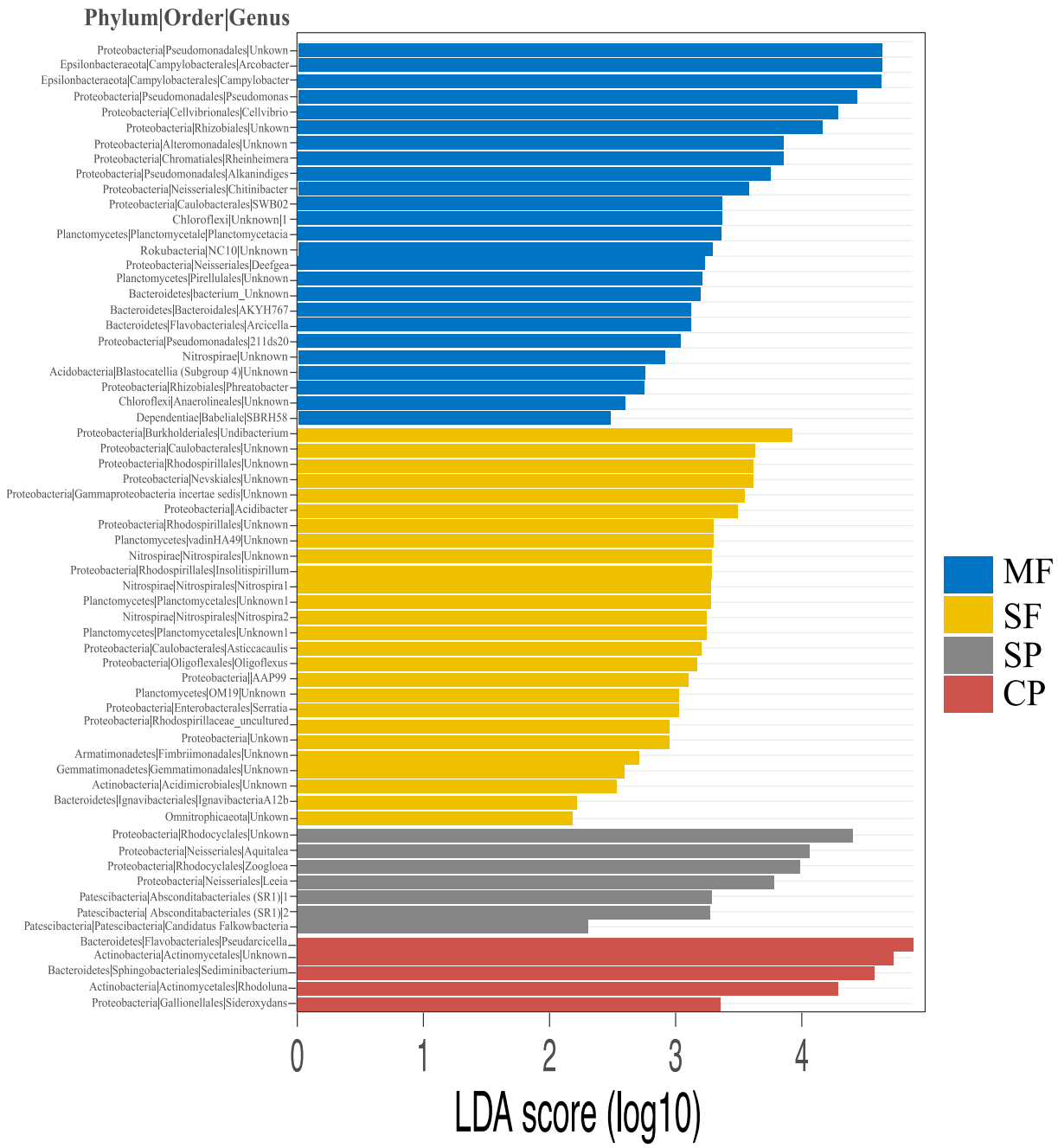

Figure A6. Bacterial taxa identified as differentially abundant between samples from four different land-type uses as analyzed by LefSe with LDA values above 2.0 and $p < 0.05$. MF = Mature Forest, SF = Secondary Forest, SP = Silvopasture, CP = Cattle Pasture.

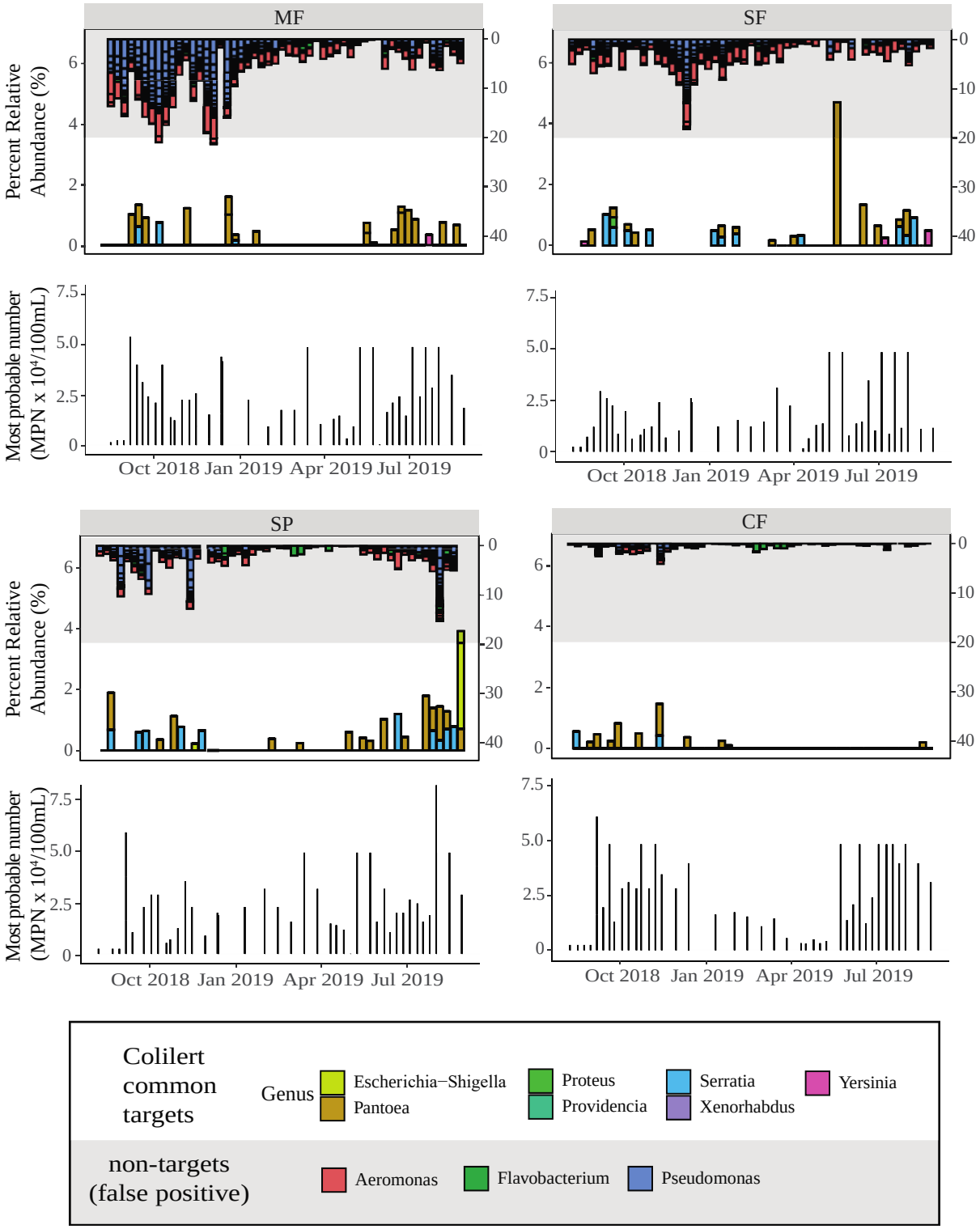

Figure A7. Bacterial taxa targeted by cultured-based Colilert (coliforms) (IDEXX, USA). Relative abundance of both targeted genera and non-targets for the detection of coliforms and *E.coli* as well as Colilert 2000 most probable number results.

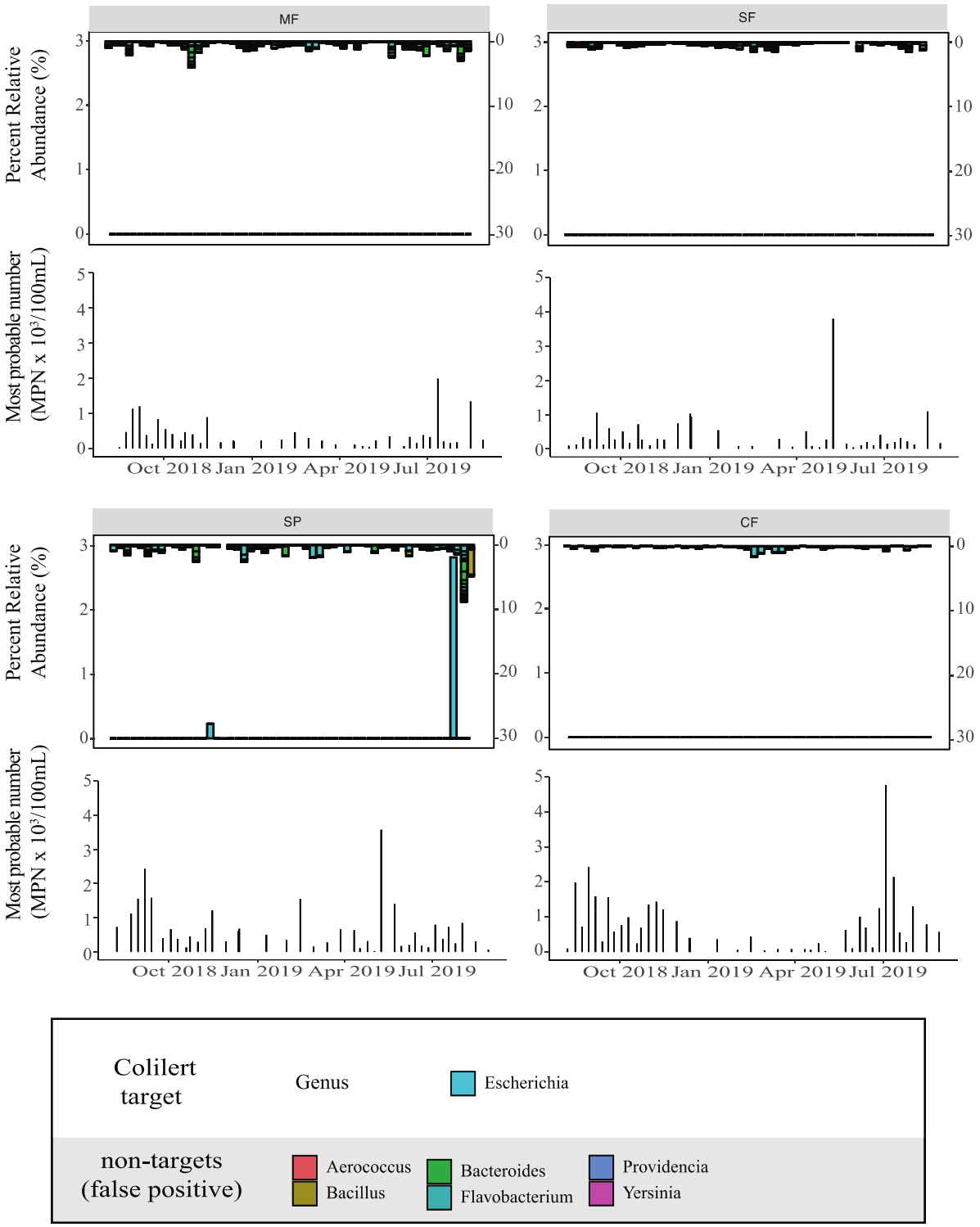

Figure A8. Bacterial taxa targeted by cultured-based Colilert (*Escherichia coli*) (IDEXX, USA) for each land use. Relative abundance of both target and non-target genera based on 16S sequencing as well as Colilert 2000 most probable number results.

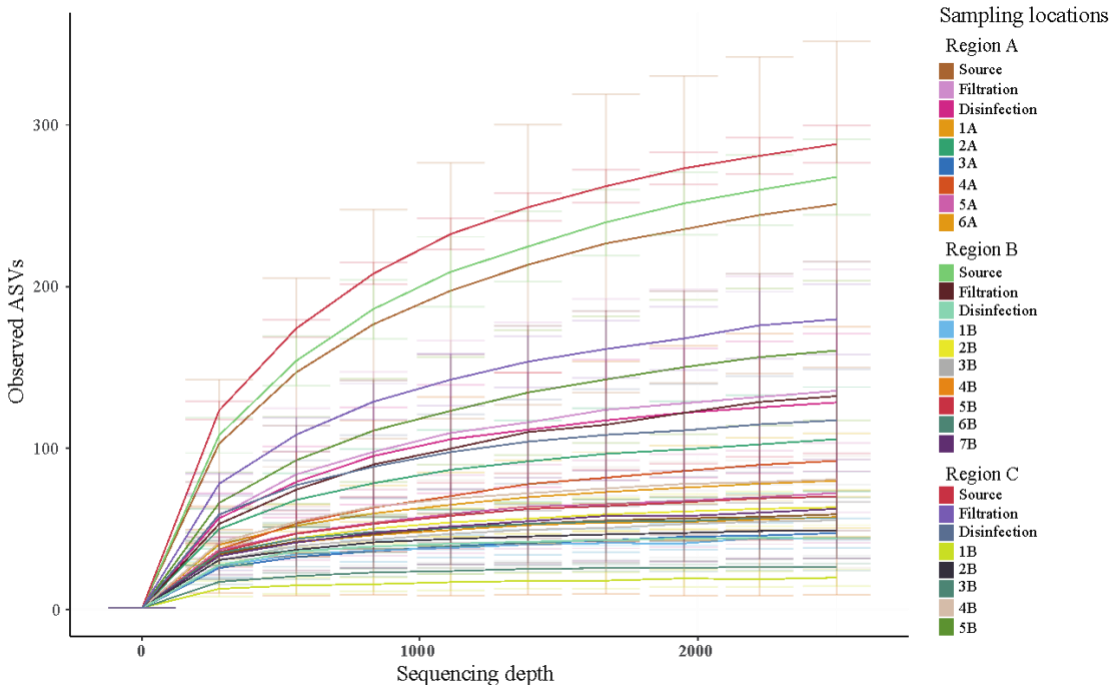

Figure A9. Rarefaction curves for all sample locations. The plateau of curves demonstrates that sequencing depth was sufficient to capture the sample's diversity.

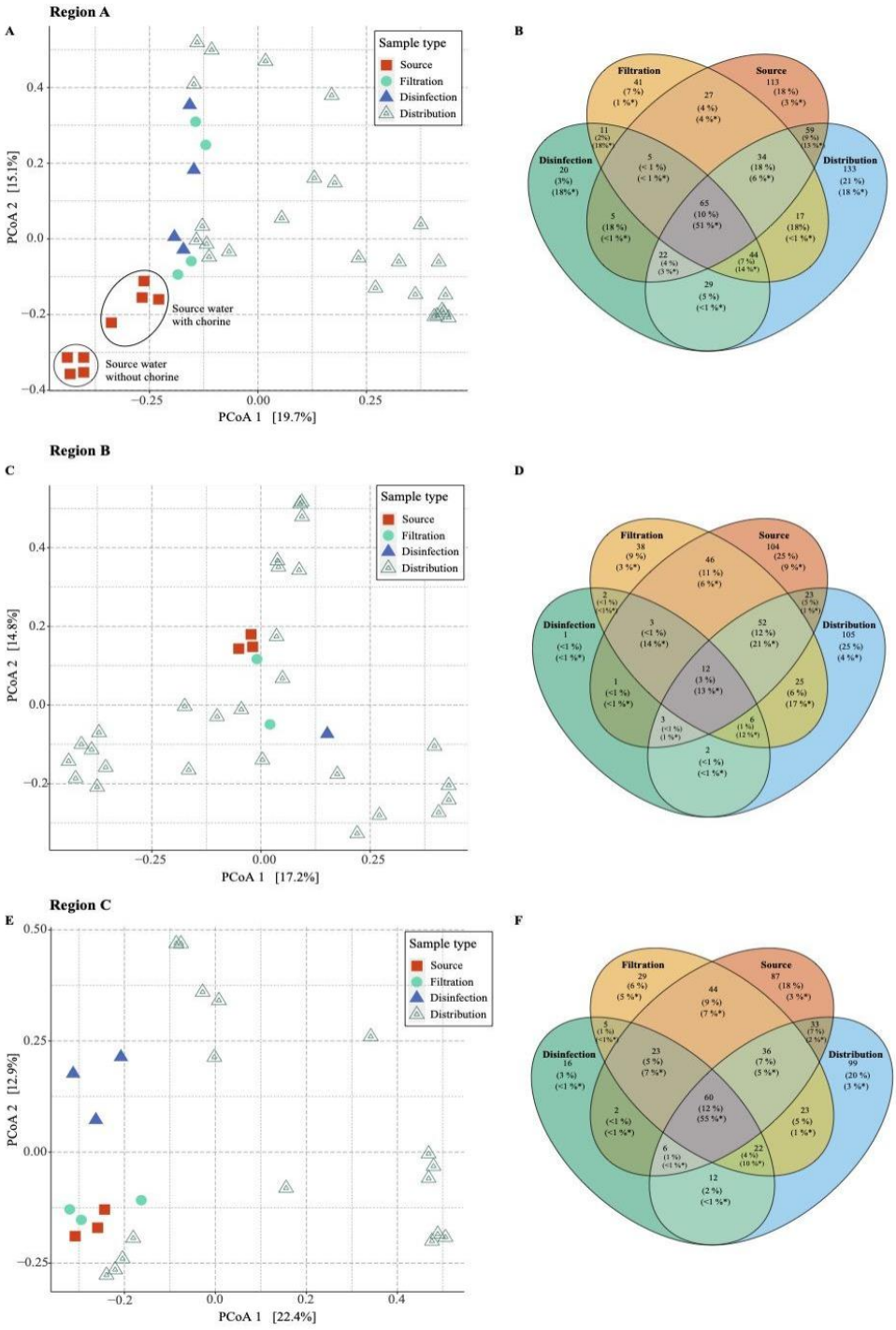

Figure A10. Weighted UniFrac-based principal coordinates analysis (PCoA) for each region (A, C, and E), and Venn diagrams demonstration shared and unique ASVs in each region (B, D, and F).

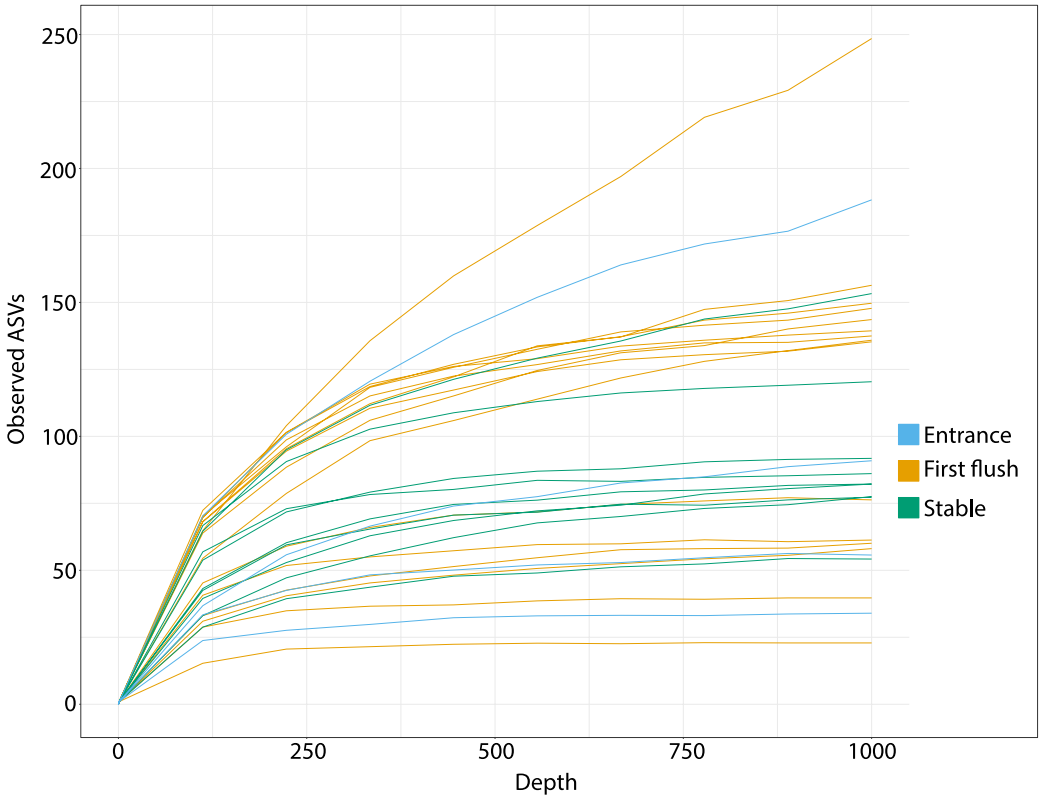

Figure A11. Rarefaction curves of average amplicon sequence variants for each sample type in IWS in the drinking water supply system of Arraiján, Panama.

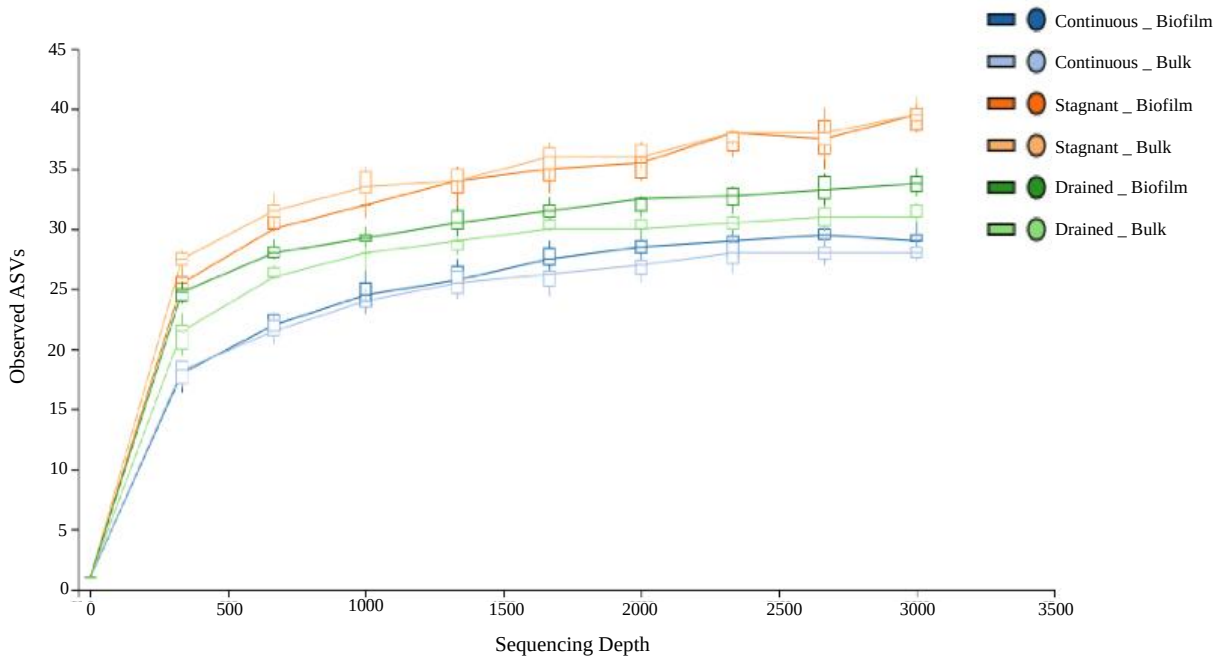

Figure A12. Rarefaction curves of average amplicon sequence variants for each sample type in simulated drinking water distribution.

Table A1. Comparison of overall community composition between locations in the DWDS of Arraiján, Panama. Analysis of similarity (ANOSIM) results with Bonferroni corrections.

Location 1	Location 2	Permutations	R	p-value
1A	2B	999	0.48	0.034
1A	3B		0.71	0.004
1A	4B		0.44	0.030
1A	5C		0.70	0.006
2A	5A		0.34	0.037
2A	2B		0.42	0.012
2A	3B		0.46	0.001
2A	4B		0.38	0.033
2A	5B		0.43	0.032
2A	6B		0.46	0.015
2A	7B		0.46	0.017
2A	2C		0.47	0.020
2A	3C		0.51	0.015
2A	4C		0.37	0.028
2A	5C		0.71	0.006
3A	3B		0.65	0.007
3A	5C		0.53	0.027
4A	4B		0.72	0.036
4A	4C		0.83	0.032
4A	5C		0.64	0.024
1B	2A		0.45	0.030
2B	3A		0.42	0.022
2B	4A		0.72	0.029
2B	6B		0.69	0.040
2B	3C		0.69	0.030
2B	5C		0.61	0.024
3B	4A		0.67	0.003
3B	5A		0.43	0.045
3B	6A		0.66	0.040
3B	5B		0.43	0.036
3B	6B		0.49	0.017
3B	5C		0.87	0.003
4B	5A		0.50	0.030
4B	5B		0.44	0.016
4B	6B		0.87	0.026
4B	5C		0.60	0.033
1C	2A		0.52	0.015
1C	4A		0.81	0.026
1C	2B		0.87	0.029
1C	5C		0.50	0.022
2C	3B		0.36	0.019
2C	5C		0.63	0.039
3C	4A		0.65	0.030
3C	5C		0.50	0.026
5C	6B		0.44	0.032
5C	7B		0.50	0.031

Table A2. Mean relative abundance and standard deviation of phyla found in Arraiján's DWDS by sample type and region.

Region A				
Phylum	**Source**	**Filtration**	**Disinfection**	**Distribution**
Proteobacteria	55.9 ± 2.7	64.9 ± 4.4	63.9 ± 8.1	54.3 ± 6.0
Actinobacteria	12.9 ± 0.7	8.1 ± 2.2	2.8 ± 0.3	1.4 ± 2.3
Bacteroidetes	12.5 ± 0.8	1.5 ± 0.2	2.3 ± 0.3	1.3 ± 1.1
Cyanobacteria	0.6 ± 0.1	3.9 ± 1.2	0.6 ± 0.2	38.3 ± 12.1
Firmicutes	0.2 ± 0.1	9.7 ± 0.4	19.15 ± 0.7	2.2 ± 0.4
Verrucomicrobia	3.6 ± 0.3	1.2 ± 0.1	2.1 ± 1.5	0.6 ± 0.4
Planctomycetes	5.1 ± 0.4	5.8 ± 3.1	2.5 ± 1.3	0.1 ± 0.1
Acidobacteria	2.3 ± 0.2	1.9 ± 0.7	3.0 ± 0.9	0.4 ± 0.2
Chloroflexi	1.3 ± 0.1	1.6 ± 1.2	0.7 ± 0.2	0.1 ± 0.1
Patescibacteria	0.2 ± 0.1	0.1 ± 0.0	< 0.1	0.2 ± 0.1
Myxococcota	0.3 ± 0.0	0.7 ± 0.4	1.6 ± 0.3	0.5 ± 0.3
Nitrospira	1.2 ± 0.5	-	0.5 ± 0.5	0.1 ± 0.3
SAR324_clade	1.2 ± 0.9	-	-	< 0.1
Gemmatimonadetes	0.9 ± 0.2	0.1 ± 0.1	0.2 ± 0.0	< 0.1
Latescibacteria	0.6 ± 0.5	0.1 ± 0.0	< 0.1	< 0.1
Region B				
Phylum	**Source**	**Filtration**	**Disinfection**	**Distribution**
Proteobacteria	29.4 ± 0.5	74.2 ± 3.9	88.3 ± 0.4	87.2 ± 6.6
Actinobacteria	34.4 ± 1.4	6.1 ± 4.0	-	0.1 ± 0.2
Bacteroidetes	12.5 ± 0.3	1.6 ± 0.4	8.0 ± 2.7	5.0 ± 4.7
Cyanobacteria	4.5 ± 1.0	14.6 ± 4.0	1.8 ± 0.2	5.3 ± 1.8
Firmicutes	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	1.2 ± 0.8
Verrucomicrobia	3.4 ± 0.1	1.3 ± 0.1	-	0.4 ± 0.3
Planctomycetes	0.6 ± 0.1	1.0 ± 0.2	1.7 ± 0.3	0.4 ± 0.2
Acidobacteria	0.6 ± 0.1	0.4 ± 0.1	-	0.1 ± 0.2
Chloroflexi	12.1 ± 2.5	0.3 ± 0.1	-	< 0.1
Patescibacteria	0.3 ± 0.1	0.1 ± 0.0	-	< 0.1
Myxococcota	0.4 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.2
Nitrospira	< 0.1	0.1 ± 0.0	-	< 0.1
SAR324_clade	1.0 ± 0.1	-	-	-
Gemmatimonadetes	0.2 ± 0.1	0.1 ± 0.1	-	< 0.1
Latescibacteria	0.1 ± 0.1	-	-	-
Region C				
Phylum	**Source**	**Filtration**	**Disinfection**	**Distribution**
Proteobacteria	39.6 ± 0.8	50.0 ± 1.7	72.7 ± 1.7	84.8 ± 7.6
Actinobacteria	12.9 ± 0.6	6.5 ± 0.5	0.7 ± 0.1	0.5 ± 0.3
Bacteroidetes	16.3 ± 0.4	30.3 ± 3.4	11.1 ± 2.5	4.3 ± 4.0
Cyanobacteria	7.8 ± 1.4	2.0 ± 0.3	7.0 ± 1.0	1.6 ± 1.4
Firmicutes	0.1 ± 0.1	0.1 ± 0.0	1.1 ± 0.1	0.3 ± 0.2
Verrucomicrobia	6.4 ± 0.3	5.2 ± 0.6	1.0 ± 0.1	3.0 ± 0.7
Planctomycetes	6.0 ± 0.5	1.5 ± 0.3	5.5 ± 0.5	1.6 ± 1.4
Acidobacteria	1.5 ± 0.4	2.0 ± 0.4	0.1 ± 0.0	0.3 ± 0.2
Chloroflexi	3.4 ± 0.7	0.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.1
Patescibacteria	0.4 ± 0.3	0.3 ± 0.2	0.4 ± 0.2	2.2 ± 0.2
Myxococcota	0.6 ± 0.1	0.4 ± 0.4	0.4 ± 0.3	0.3 ± 0.3
Nitrospira	0.4 ± 0.3	< 0.1	0.1 ± 0.1	0.1 ± 0.1
SAR324_clade	0.7 ± 0.1	< 0.1	-	0.1 ± 0.1
Gemmatimonadetes	0.2 ± 0.2	0.1 ± 0.0	-	0.1 ± 0.3
Latescibacteria	0.1 ± 0.0	-	-	< 0.1

Table A3. Bacterial taxa identified as differentially abundant between samples from four different sample-type and three regions as analyzed ANCOM (shaded blue) and by LefSe with LDA values above 2.0 and p < 0.05 (*); p < 0.01 (**)

					Region A				Region B				Region C			
Phylum	Class	Order	Family	Genus	Source	Filtration	Disisnfection	Distribution	Source	Filtration	Disisnfection	Distribution	Source	Filtration	Disisnfection	Distribution
Acidobacteria	Acidobacteriae	Acidobacteriae	Acidobacteriae	Paludibaculum												
	Vicinamibacteria	Vicinamibacterales	Vicinamibacteraceae	Uncultured												
	Holophagae	Holophagales	Holophagaceae	marine_group												
				Geothrix										*		
Actinobacteria	Acidimicrobiia	IMCC26256	IMCC26256_Unknown	IMCC26256_Unknown												
		Microtrichales	Ilumatobacteraceae	CL500-29_marine_group	*											
		Corynebacteriales	Mycobacteriaceae	Mycobacterium		*										
		Frankiales	Sporichthyaceae	hgcI_clade	*				*				*			
		Micrococcales	Microbacteriaceae	Candidatus_Planktoluna					*							
			Microbacteriaceae	Rhodoluna					*							
			Microbacteriaceae	MWH-Ta3					*							
		PeM15	PeM15	Uncultured					*							
		Streptomycetales	Streptomycetaceae	Streptomyces												
Armatimonadetes	Fimbriimonadia	Fimbriimonadales	Fimbriimonadaceae	Uncultured												
Bacteroidetes	Bacteroidia	Chitinophagales	Chitinophagaceae	Dinghuibacter												
				Sediminibacterium										*		*
				Ferruginibacter												
				Terrimonas						*						
				Edaphobaculum										*		
				Rurimicrobium												
				Parasediminibacterium										*		
			Saprospiraceae	Uncultured												
			uncultured	Uncultured												
		Cytophagales	Microscillaceae	Uncultured	*											
			Spirosomaceae	Pseudarcicella										*		
				Emticicia										*		
		Flavobacteriales	Crocinitomicaceae	Fluviicola												
			Cryomorphaceae	Uncultured												
			NS9_marine_group	NS9_marine_group												
		Sphingobacteriales	AKYH767	Uncultured												
			env.OPS_17	Uncultured										*		
			NS11-12_marine_group	Uncultured										*		
Chloroflexi	Anaerolineae	SBR1031	SBR1031	Uncultured						*						
	Chloroflexia	Chloroflexales	Roseiflexaceae	Uncultured					*							
	SL56_marine_group	SL56_marine_group	SL56_marine_group	Uncultured					*				*			
Cyanobacteria	Cyanobacteriia	Cyanobacteriales	Phormidiaceae	Cephalothrix_SAG_75.79					*				*			
		Synechococcales	Cyanobiaceae	Cyanobium_PCC-6307						*				*		
	Vampirivibrionia	Obscuribacterales	Obscuribacteraceae	Candidatus_Obscuribacter				*								
Deinococcota	Deinococci	Thermales	Thermaceae	Meiothermus									*			
Firmicutes	Bacilli	Alicyclobacillales	Alicyclobacillaceae	Alicyclobacillus												
		Paenibacillales	Paenibacillaceae	Paenibacillus			*									
	Clostridia	Clostridia	Hungateiclostridiaceae	Ruminiclostridium			*									
Planctomycetes	Planctomycetes	Gemmatales	Gemmataceae	Uncultured			*									
				Gemmata												
		Isosphaerales	Isosphaeraceae	Uncultured								*				
		Pirellulales	Pirellulaceae	Uncultured	*											
				Pirellula												
		Planctomycetales	Unknown	Uncultured		*										
Proteobacteria	Alphaproteobacteria	Acetobacterales	Acetobacteraceae	Uncultured												
		Caulobacterales	Hyphomonadaceae	Uncultured				*				*				
		Elsterales	Unknown	Uncultured												
		Paracaedibacterales	Paracaedibacteraceae	Candidatus_Paracaedibacter						*						
		Parvibaculales	PS1_clade	Uncultured												
		Reyranellales	Reyranellaceae	Reyranella												
		Rhizobiales	Beijerinckiaceae	FukuN57												
				Methylocystis												
				Methylobacterium-Methylorubrum		*	*	*								
				alphaI_cluster												
			KF-JG30-B3	Uncultured												
			Rhizobiales_Incertae_Sedis	Uncultured												
				Phreatobacter				**				**				**
			Xanthobacteraceae	Uncultured												
		Rickettsiales	Fokiniaceae	Uncultured				*								
			Rickettsiaceae	Uncultured								*				
		SAR11_clade	Clade_III	Clade_III						**						
		Sphingomonadales	Sphingomonadaceae	Uncultured					*							
				Porphyrobacter								*				
				Parablastomonas										*		
				DSSF69						**						
	Gammaproteobacteria	Burkholderiales	Alcaligenaceae	Ampullimonas										*		
			Burkholderiaceae	Limnobacter												
				Polynucleobacter					*	*			*	*		
			Comamonadaceae	Curvibacter										*		
				Comamonas						*						
			Methylophilaceae	Candidatus_Methylopumilus	*											
				Uncultured					*							
			Nitrosomonadaceae	GOUTA6												
			TRA3-20	Uncultured												*
		CCD24	CCD24	Uncultured												
		Cellvibrionales	Spongiibacteraceae	BD1-7_clade												
		Diplorickettsiales	Diplorickettsiaceae	Aquicella					*							
		Pseudomonadales	Moraxellaceae	Acinetobacter												
			Pseudomonadaceae	Pseudomonas								**				
			Salinisphaerales	Solimonadaceae	Nevskia							*				
		Steroidobacterales	Steroidobacteraceae	Uncultured												
	Oligoflexia	Bdellovibrionales	Bdellovibrionaceae	Bdellovibrio										*		
		Silvanigrellales	Silvanigrellaceae	Silvanigrella							*	*			*	
SAR324	clade (Marine_group_B)	Unknown	Unknown	Unknown	*											
Verrucomicrobia	Verrucomicrobiae	Chthoniobacterales	Chthoniobacteraceae	Chthoniobacter					*							
		Methylacidiphilales	Methylacidiphilaceae	Uncultured										*		
		Pedosphaerales	Pedosphaeraceae	Uncultured	*											
				SH3-11										*		