Critical Factors Controlling Regrowth of Opportunistic Pathogens in Premise Plumbing

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ABSTRACT

Opportunistic pathogens (e.g., *Legionella pneumophila, Mycobacterium avium complex, Acanthamoeba polyphaga, Pseudomonas aeruginosa*) residing in human-made water systems, particularly premise plumbing, are now the primary source of water-borne disease in developed countries. The prevention and control of opportunistic pathogens is a new challenge in premise plumbing due to the limited knowledge concerning the factors driving their occurrence and regrowth mechanisms, and also the complexity of premise plumbing conditions. The goal of this study is to identify key factors governing occurrence of opportunistic pathogens in drinking water distribution systems, particularly premise plumbing, via field investigations and lab-scale experiments.

A molecular survey of three opportunistic pathogens (*L. pneumophila*, *M. avium*, *P. aeruginosa*), related groups (*Legionella* and mycobacteria) and two amoeba hosts (*Acanthamoeba* spp. and *Hartmanella vermiformis*) was performed in two real-word chloraminated drinking water distribution systems using quantitative polymerase chain reaction (q-PCR). A high occurrence of *Legionella* ($\geq$ 69.0%) and mycobacteria (100%), lower occurrence of *L. pneumophila* ($\leq$ 20%) and *M. avium* ($\leq$ 33.3%), and rare detection of *Pseudomonas aeruginosa* ($\leq$ 13.3%) was observed in both systems. *Hartmanella vermiformis* was more prevalent than *Acanthamoeba*. Three-minute flushing resulted in reduced gene copies of *Legionella*, mycobacteria, *H. vermiformis* and 16S rRNA genes ($P<0.05$) and distinct microbial community structure in postflushing water, implying strong regrowth potential of opportunistic pathogens in premise pluming.

In order to examine the influence of pipe material, disinfectant type, and water age on occurrence and persistence of the target microorganisms, triplicate simulated distribution systems (SDSs) comparing iron, cement and PVC pipe materials were fed either chlorinated or chloraminated tap water, and were sampled at water ages ranging from 1d to 5.7d. q-PCR quantification of target microorganisms in both biofilm and bulk water revealed that *Legionella*, mycobacteria, *P. aeruginosa* and both amoebas naturally colonized the six SDSs, but *L. pneumophila* and *M. avium* were not detected. Disinfectant type and dose have the strongest influence on the microbiota. Disinfectant decay was noted with water age, particularly in chloraminated SDSs (due to nitrification), generally resulting in increased microbial detection frequencies and densities with water age. Influence of pipe material became apparent at water ages corresponding to low disinfectant residual.

Natural colonization of *Legionella* spp., *Mycobacterium* spp., *Acanthamoeba* spp., *H. vermiformis* and *M. avium* was also observed in biofilms from five annular reactors, which were used to investigate effects of prior granular activated carbon (GAC) biofiltration and disinfectant type (chlorine, chloramine) on opportunistic pathogens under premise plumbing conditions. GAC pre-treatment effectively reduced total organic carbon (TOC). In most cases, total bacteria and opportunistic pathogens were higher in undisinfected annular reactors, but the levels were not proportional to the level of GAC pre-treatment/TOC. Chlorine was more effective for controlling mycobacteria and *Acanthamoeba*, whereas chloramine was more effective for controlling *Legionella*. Both chlorine and chloramine effectively reduced *M. avium* and *H. vermiformis* numbers. Pyrosequencing of 16S rRNA genes in biofilms revealed a significant effect of GAC pre-treatment and disinfectant type on the microbial community structure.

Overall, the study provides insights to critical factors triggering proliferation of opportunistic pathogens in drinking water systems. Knowledge gained from this study can assist in formulating practical guidance for drinking water utilities and water consumers in terms of opportunistic pathogen prevention and control.

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CHAPTER 1

Introduction

1.1 Background

Opportunistic pathogens are microorganisms that can cause diseases when the host's immune system is weakened by underlying diseases and/or medication. In recent years, waterborne opportunistic pathogens such as *Legionella pneumophila*, nontuberculous mycobacteria (NTM), *Pseudomonas aeruginosa*, *Acanthamoeba* have attracted growing public attention, especially in developed countries.

Legionella is the cause agent of legionellosis, which refers to a potentially fatal form of pneumonia called Legionnaires' disease or a milder respiratory illness called pontiac fever. According to the U.S. Centers for Disease Control (CDC, http://www.cdc.gov/legionella/top10.htm), *Legionella* infection hospitalized 8000-18000 people each year in the U.S. *Legionella* was added into EPA drinking water candidate contaminant list (CLL) in 2009 as a result of increased numbers of Legionnaire's diseases and association with public drinking water systems (Brunkard et al., 2011; CDC, 2011).

NTM refers to mycobacterial species other than *Mycobacterium tuberculosis* complex. Most of NTM are confirmed opportunistic pathogens (Falkinham, 2009), which can cause a spectrum of diseases including pulmonary infection, lymphadenitis, skin infection and disseminated disease (Griffith et al., 2007). NTM commonly infect people with AIDS; however, increase incidence of NTM has been noticed in U.S. (Billinger et al.,

2009) and other countries (Al Houqani et al., 2011; Lai et al., 2010) in both AIDS and non-AIDS population in recent years (Billinger et al., 2009; Henry et al., 2004). Several epidemiological studies have linked the NTM infection to tap water (Conger et al., 2004; Falkinham, 2011; Falkinham et al., 2008), implying that the drinking water system is a potential route of NTM exposure.

P. aeruginosa is a leading cause of nosocomial (i.e., hospital-acquired) infection in individuals with cancer, cystic fibrosis and burns (Engleberg et al., 2007). *P. aeruginosa* infection can lead to severe diseases such as pneumonia and bacteremia, which are associated with high mortality rates (Rossolini and Mantengoli, 2005). Two independent studies reviewing recorded nosocomial *P. aeruginosa* infection from 1966-2001 and 1998-2005 demonstrated the strong link between hospital tap water and *P. aeruginosa* infection outbreaks. It has been suggested that 14.2-50% of *P. aeruginosa* infection/colonization in patients were due to strains found in hospital water (Anaissie et al., 2002; Trautmann et al., 2005).

Free-living amoebae (FLA) are a group of eukaryotic microorganisms that are commonly found in aquatic environment and soil. Recent increasing attention cast on FLA are associated with outbreaks of *Acanthamoeba* keratitis (AK) (Fraser et al., 2012; Yoder et al., 2012b) and occasional occurrences of fatal amebic meningoencephalitis (Yoder et al., 2012a). Tap water is also suggested as a transmission route for pathogenic FLA, as evidenced by identical mtDNA profiles of clinical and water isolates (Kilvington et al., 2004) and revisits of patients' daily activities (Yoder et al., 2012a).

Drinking water opportunistic pathogens have very unique ecological niches and transmission routes compared to traditional fecal source pathogens. Instead of escaping from the drinking water treatment processes, opportunistic pathogens are natural inhabitants of fresh water and able to survive and proliferate under oligotrophic drinking water environments. Their features of heat-tolerant (Allegra et al., 2011; Falkinham, 2009), disinfectant-resistant (Coulon et al., 2010; Taylor et al., 2000), and biofilm-loving (Falkinham, 2009; Taylor et al., 2009) increase their survivability under drinking water and household plumbing conditions. They are also able to interact and communicate with each other and/or other indigenous microorganisms, which may enhance their growth potential. Figure 1.1 serves as an illustrative model for understanding opportunistic pathogen ecological niche in drinking water system, using *Legionella* spp. as an example. Biofilm-loving free-living amoebae graze on indigenous bacteria for food by phagocytosis. However, some amoeba-resisting bacteria, such as *Legionella* spp. and *Mycobacterium* spp., are able to evade phagosolysosomal fusion and amplify within phagosome. After depletion of nutrients in amoebae, these amplified cells are released into the drinking water by lysing amoeba cells (Taylor et al., 2009). Cell-to-cell interactions also widely exist between *Legionella* and other indigenous bacteria in the forms of competition (Declerck et al., 2005), antagonism (Guerrieri et al., 2008), and symbiosis (Wadowsky and Yee, 1983). It was also reported that *Legionella* is able to undergo extracellular growth by taking up nutrients from other dead cells (i.e., necrotrophic growth) (Temmerman et al., 2006). Further, it is of importance to note that their exposure pathways are also unique: inhalation of aerosols and direct contact (e.g.,

during the shower) are the primary transmission routes for these opportunistic pathogens instead of ingestion, which is the typical route for fecal source pathogens.

Premise plumbing includes the portions of the potable water distribution system beyond the service pipe (i.e., pipeline connecting a building to a main pipe) and inside of buildings (Figure 1.1), where low disinfectant residual is more likely to occur as a result of high surface area to volume ratio, long retention times, and warmer temperature (NRC, 2006). The colonization of opportunistic pathogens in premise plumbing is well documented, especially in hospital buildings (Arvand et al., 2011; Brown-Elliott et al., 2011; Rivera et al., 2007; Thomas et al., 2006) and hotels (Bonetta et al., 2010; Borella et al., 2005; Mouchtouri et al., 2007). They were widely found in cold and hot water (Falkinham et al., 2008), biofilms from taps or shower heads (Feazel et al., 2009), shower aerosol (Perkins et al., 2009), eye wash stations (Paszko-Kolva et al., 1998), and occasionally water filters (Falkinham, 2010). It is worth pointing out that ubiquitous biofilms in premise plumbing provide an ideal ecological niche for opportunistic pathogens to attach, proliferate, and/or graze (e.g., protozoa), as well as a shelter to protect them from disinfection. NTM and other opportunistic pathogens were able to enrich to as much as 100-fold above background water level in drinking water biofilms (Feazel et al., 2009).

In pursuit of effective pathogen prevention and control strategies, the relationships between opportunistic pathogens and several risk factors have been previously explored. Water heater temperature is considered to be one critical factor controlling colonization

of *Legionella* and NTM in household plumbing (Falkinham, 2011; Flannery et al., 2006; Lasheras et al., 2006; Mathys et al., 2008; Mouchtouri et al., 2007). Setting water heater ≥ 60℃ and maintaining cold water ≤25℃ has been recommended for prevention of Legionnaires' disease by Word Health Organization (WHO, 2007). Water heater capacity and type also appears to play a role in favoring *Legionella* growth in premise plumbing. Instantaneous water heater is less likely to be colonized by *Legionella* compared to water heaters with storage tanks or circulating systems (Martinelli et al., 2000; Mathys et al., 2008). Independent water heater is a better option compared to centralized heating system in terms of *Legionella* colonization (Leoni et al., 2005).

Occurrences of opportunistic pathogens were often associated with low level or absence of disinfectant residual (Brousseau et al., 2013; Edagawa et al., 2008; Sebakova et al., 2008), which frequently occurs at the dead ends of distribution systems and premise plumbing. A number of current studies investigated various disinfection methods including hyperchlorination, chlorination, chloramination, chlorine dioxide disinfection, and copper-silver ionization in individual buildings where occurrences of *L. pneumophila* were found. Chlorine dioxide disinfection and copper-silver ionization were highly recommended for building-level disinfection in some cases (Lin et al., 2011; Marchesi et al., 2011; Tesauro et al., 2010; Zhang et al., 2009; Zhang et al., 2007).

Trace metals are essential nutrients for microbes, but they can become toxic when the concentrations are higher than the threshold values. With regard to opportunistic pathogens, negative association of Cu >50 mg/L and *Legionella* colonization was

observed in hot water systems (Bargellini et al., 2011; Borella et al., 2004). Similarly, a Cu level of 0.066 mg/L was found to impair the cultivability of *P. aeruginosa* (Dwidjosiswojo et al., 2011). However, reverse results were observed in one *Legionella* survey of German residences, where plumbing systems with copper pipes were more frequently contaminated with *Legionella*, and positive correlation was observed between Cu concentration and *Legionella* growth in hot water (Mathys et al., 2008). The contradictory Cu effect might be ascribed to different levels of bioavailable Cu, and/or other uninvestigated factors in different premise plumbing. Colonization of *Legionella* was also found to be positively associated with other trace metal concentrations such as Mn, Zn, Fe (Bargellini et al., 2011; Borella et al., 2004; Edagawa et al., 2008; Rakic et al., 2012), which are essential nutrients for *L. pneumophila* growth (Reeves et al., 1981).

Assimilable organic matter (AOC) is used an indicator of bacterial regrowth potential in drinking water as it provides carbon resources for heterotrophic bacteria. Positive relationships were found between mycobacterial abundance and AOC concentration in U.S. and Finland real-world drinking water distribution systems (Falkinham et al., 2001; Torvinen et al., 2004). However, no clear effect of AOC was found on NTM numbers in a recent investigation of eight Dutch drinking water plants and distribution systems. In contrast, *L. pneumophila* was found to be more prevalent in water with AOC concentrations above 10 μg/L than in water with AOC levels below 5 μg/L (van der Wielen and van der Kooij, 2013). In a pilot study, though *M. avium* was found to be recovered at an AOC level as low as 50 μg/L, no significant quantitative relationship

could be established between *M. avium* and AOC levels of 50-228 μg/L (Norton et al., 2004).

Premise plumbing condition and components can also affect opportunistic pathogen colonization and regrowth. It was found that newly constructed buildings were less likely to be colonized by *Legionella* (Mathys et al., 2008). A recent study in a 1000-bed university hospital showed that electronic faucets were more commonly contaminated with *Legionella* species and other bacteria and were less likely to be disinfected after chlorine dioxide remediation (Sydnor et al., 2012). Electronic faucets were also considered as a health risk factor for *P. aeruginosa* infection in hospitals (Yapicioglu et al., 2012).

Although a significant body of intriguing research pertaining to opportunistic pathogen occurrence and control exist, there is still a lack of sound, scientific understanding of key factors governing opportunistic pathogen growth in premise plumbing. For example, effects of several important factors such as disinfectant, water age, and pipe materials on multiple drinking water opportunistic pathogens have not been fully explored. These are especially of interest because there is some potential for control by utilities or building owners. Unfortunately, the existing literature does not provide clear guidance for practical means of opportunistic pathogen control. Therefore, a key research need is the identification and establishment of critical factors driving opportunistic pathogen proliferation in the premise plumbing in support of development of practical opportunistic pathogen control and prevention.

1.2 Research Questions

This dissertation addresses three key research questions.

Question 1**:** *How common are opportunistic pathogens and associated microorganisms in premise plumbing, and at what level? Are there any factors (e.g., total organic matter (TOC), water age) associated with occurrence of opportunistic pathogen?* Though there is a body of literature monitoring opportunistic pathogens in premise plumbing as stated before, there is still a lack of a simultaneous, comprehensive molecular examination of multiple opportunistic pathogens. Such a study is of value considering complex interactions between different opportunistic pathogens (e.g., host-parasite interaction (Thomas et al., 2006)) and that the factors that inhibit one pathogen may actually favor the growth of others (Moore et al., 2006). Application of molecular method also has advantages of low detection limit, high specificity and high reproducibility, which can overcome the limitations of culture-based methods that were commonly used in the past studies. For a better understanding of the contributions of abiotic (e.g., TOC, disinfectant residual) and biotic factors (e.g., interactions among opportunistic pathogens) to opportunistic pathogen regrowth, exploring potential relationships between these factors and opportunistic pathogens abundance is of interest by using the first-hand quantitative investigation data.

Question 2**:** *How will the disinfectant, water age and pipe material affect the occurrence and persistence of opportunistic pathogens in drinking water distribution systems, and to what extent?* Though disinfectant and pipe materials are known to be important factors

affecting drinking water microbiota (mainly heterotrophic bacteria) by previous studies (Lechevallier et al., 1993; Camper et al., 2003), their precise effects on the drinking water opportunistic pathogen group are unknown. It has been advocated that *Legionella* can be better controlled by chloramine (Moore et al., 2006). However, the mechanism driving this phenomena has neither been fully understood nor been confirmed by lab studies. Only a few recent studies investigated pipe effects on opportunistic pathogens (Dwidjosiswojo et al., 2011; Norton et al., 2004; van der Kooij et al., 2005). Elucidating the responses of opportunistic pathogens and related microorganisms to these factors in engineering water system will be critical for better understanding of opportunistic pathogen ecology and ultimate identification of engineering solutions for pathogen prevention and control.

Question 3**:** *What is the effect of GAC- biofiltration on opportunistic pathogen occurrence? How effective will the residual disinfectant for limiting opportunistic pathogen regrowth in premise plumbing?* GAC filtration is commonly used in drinking water plant to remove dissolved organics by active biofilm attached on the filters (Chien et al., 2008), or in point-of-use buildings to remove taste, odors, and sediments. GAC filtration was also reported to be capable of removing pathogens such as *Escherichia coli* and protozoan (oo)cysts (Hijnen et al., 2010). However, it is not clear that GAC filtration will actively inhibit opportunistic pathogens regrowth by providing low organic carbon water, or instead serve as a pathogen reservoir. Meanwhile, although previous lab studies have indicated distinct effects of chlorine and chloramine towards *L. pneumophila*, *M. avium*, and *Acanthamoeba* (e.g., effective dosages, mechanisms, and disinfection models)

by using lab-cultivated cells or under nutrient rich environments (Dupuy et al., 2011; Luh and Marinas, 2007; Luh et al., 2008; Mogoa et al., 2011), there is still a lack of direct comparison of disinfectant residual effects on limiting opportunistic pathogen growth under representative drinking water conditions. Understanding of these information is crucial for practical opportunistic pathogen control and risk management in premise plumbing.

1.3 Annotated Dissertation Outline

Chapter 1: *Introduction*

Chapter 2: *Molecular Survey of Occurrence of Legionella spp., Mycobacterium spp., Pseudomonas aeruginosa and Amoeba Hosts in Two Chloraminated Drinking Water Distribution Systems* This manuscript described a molecular survey of multiple opportunistic pathogens and related microorganisms in two real-world chloraminated distribution systems. Question 1 was addressed in this manuscript. Portions of this work were presented at AEESP Education & Research Conference, July 10, 2011. The manuscript has been accepted for publication:

Wang H, Edwards M, Falkinham JO, III, Pruden A. 2012. Molecular survey of occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa* and amoeba hosts in two chloraminated drinking water distribution systems. Appl. Environ. Microbiol. 78:6285-6294

Chapter 3: *Effect of Disinfectant, Water Age, and Pipe Material on Occurrence and Persistence of Legionella, mycobacteria, Pseudomonas aeruginosa, and Two Amoebas* In this study, six parallel simulated distribution systems containing different pipe materials (cement, iron, and PVC) were constructed and fed with either chlorinated or chloraminated tap water. This provides a controlled, head-to-head matrix for determining how these factors affect drinking water opportunistic pathogens. Varying drinking water conditions from best-case (e.g., high disinfectant level) to the worst-case (e.g., depletion of disinfectant) scenarios were achieved by sampling at different water ages (1 d, 2.3 d, 3.6 d, and 5.7 d). Question 2 was addressed in this chapter. Portions of this work were presented at American Water Works Association ACE12 Conference in Dallas, Texas, June 14, 2012. This manuscript has been accepted for publication:

Wang H, Masters S, Hong Y, Stallings J, Falkinham JO, Edwards MA, Pruden A. 2012b. Effect of disinfectant, water age, and pipe material on occurrence and persistence of *Legionella*, mycobacteria, *Pseudomonas aeruginosa*, and two amoebas. Environ. Sci. Technol. 46:11566-11574.

Chapter 4: *Effect of GAC pre-treatment and disinfectant on microbial community structure and opportunistic pathogen regrowth* This manuscript examined effects of GAC biofiltration and disinfectant type on the occurrence and persistence of opportunistic pathogens using annular reactors, and addressed Question 3. Portions of this work were presented the American Water Works Association's Water Quality

Technology Conference in Toronto, Ontario, November 6, 2012. The manuscript of this work is currently under peer-review in Water Research.

Chapter 5: *Conclusions* This chapter summarized key conclusions drawn from this study, and highlighted the scientific contributions of this work to the understanding of factors trigging opportunistic pathogen growth and drinking water microbial ecology in general.

1.4 Figures

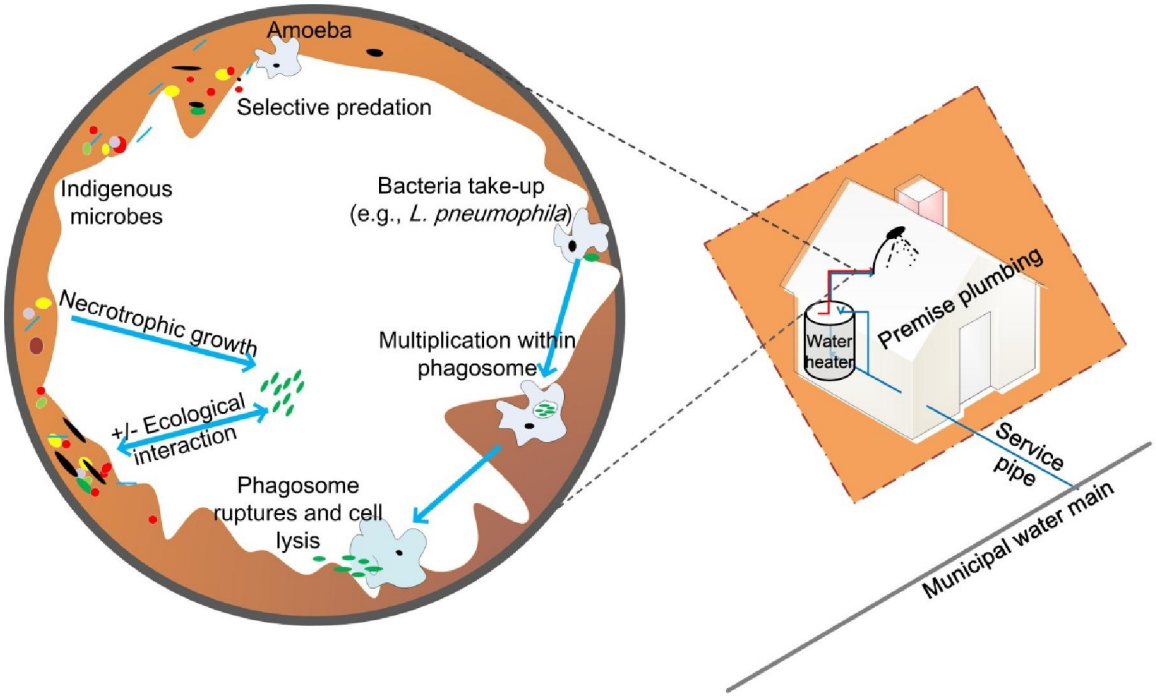

Figure 1.1 Opportunistic pathogens ecological niche in building plumbing

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CHAPTER 2

Molecular Survey of Occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa* and Amoeba Hosts in Two Chloraminated Drinking Water Distribution Systems

2.1 Authors

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2.2 Abstract

The spread of opportunistic pathogens via public water systems is of growing concern. The purpose of this study was to identify patterns of occurrence among three opportunistic pathogens (*Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa*) relative to biotic and abiotic factors in two representative chloraminated drinking water distribution systems using culture-independent methods. Generally, a high occurrence of *Legionella* (≥ 69.0%) and mycobacteria (100%), lower occurrence of *L. pneumophila* (≤ 20%) and *M. avium* (≤ 33.3%), and rare detection of *Pseudomonas aeruginosa* (≤ 13.3%) was observed in both systems according to quantitative polymerase chain reaction. Also, *Hartmanella vermiformis* was more prevalent than *Acanthamoeba*, both of which are known hosts for opportunistic pathogen amplification, the latter itself containing pathogenic members. Three-minute flushing served to distinguish distribution system water from premise (i.e., building) plumbing water and resulted in reduced copies of *Legionella*, mycobacteria, *H. vermiformis* and

16S rRNA genes ($P<0.05$), while yielding distinct terminal restriction fragment polymorphism (T-RFLP) profiles of 16S rRNA genes. Within certain subgroups of samples, some positive correlations were noted, including: mycobacteria and total bacteria (16S rRNA genes); *H. vermiformis* and total bacteria; mycobacteria and *H. vermiformis*; and *Legionella* and *H. vermiformis*, emphasizing potential microbial ecological relationships. Overall the results provide insight into factors that may aid in controlling opportunistic pathogen proliferation in real-world water systems.

2.3 Keywords

Legionella pneumophila, *Mycobacterium avium*, *Pseudomonas aeruginosa*, *Hartmanella vermiformis*, *Acanthamoeba*, drinking water, chloramine

2.4 Introduction

In recent years, opportunistic pathogens including *Legionella pneumophila*, non-tuberculosis mycobacteria (NTM), *Pseudomonas aeruginosa,* and *Acanthamoeba* spp. have become a leading source of waterborne disease in developed countries. A growing incidence of Legionnaires diseases was reported in the U.S.A. from 2000-2009 (CDC, 2011) and France from 1998-2005 (Campese et al., 2011). In the U.S.A., *Legionella* has been the single most commonly reported pathogen identified in drinking water-associated outbreaks since its addition to Waterborne Disease and Outbreak Surveillance System in 2001 (Brunkard et al., 2011). Multiple studies also linked NTM infection to drinking water systems by employing genetic and epidemiological methods to compare clones isolated from patients and drinking water (e.g.,(Brown-Elliott et al.,

2011; Conger et al., 2004; Falkinham, 2011; Falkinham et al., 2008)), highlighting drinking water as a potential exposure route for NTM infection. A study reviewing waterborne nosocomial infection from 1966-2001 suggested that outbreaks of nosocomial *P. aeruginosa* infection are commonly related to hospital tap water (Anaissie et al., 2002). Recent outbreaks of *Acanthamoeba* keratitis (AK) in the U.S.A. were also suspected to be associated with drinking water (CDC, 2007; Qian et al., 2010). Further evidence linking AK to tap water demonstrated identical *Acanthamoeba mt*DNA profiles for the clinical and home tap water isolates in 75% (6 out of 8) of *Acanthamoeba* positive (8 out of 27) patients' homes in the U.K. (Kilvington et al., 2004). The incidences of waterborne NTM, *P. aeruginosa*, and *Acanthamoeba* spp. infection and their association with drinking water are likely to be underestimated since they are non-reportable diseases.

Premise (i.e., building) plumbing is an important reservoir for opportunistic pathogens and represents a direct route for transmission and exposure to humans, typically via inhalation of aerosols or skin contact. The unique characteristics of premise plumbing include high surface area to volume ratio, long retention times, presence of reactive pipe materials (e.g., corrosion), and warm temperatures, all of which contribute to low disinfectant residual in household water and therefore promote bacterial colonization and multiplication (NRC, 2006). On the other hand, some water-borne opportunistic pathogens, such as *M. avium* complex, are slow-growing oligotrophs capable of resisting heat and disinfectants, which makes them strong competitors in the drinking water environment (Falkinham, 2009). The occurrence of *Legionella* and mycobacteria in premise plumbing and potential relationships of occurrence with environmental factors,

such as water chemistry (Bargellini et al., 2011; Borella et al., 2004; Falkinham et al., 2001; Torvinen et al., 2004), temperature (Falkinham, 2011; Flannery et al., 2006; Lasheras et al., 2006; Mathys et al., 2008; Mouchtouri et al., 2007), water heater capacity and type (Leoni et al., 2005; Mouchtouri et al., 2007), and premise plumbing characteristics (Mathys et al., 2008) has been previously reported. Water heater temperature is considered to be the most critical determining factor for *Legionella* and NTM colonization in household plumbing (Falkinham, 2011; Flannery et al., 2006; Lasheras et al., 2006; Mathys et al., 2008; Mouchtouri et al., 2007). A positive relationship between mycobacterial abundance and assimilable organic carbon (AOC) concentration was observed by Falkinham et al. (Falkinham et al., 2001) and Torvinen et al. (Torvinen et al., 2004) in U.S. and Finnish drinking water distribution systems. The colonization of *Legionella* was also recently found to be associated with trace metals. Negative association of copper levels >50 mg/L and *Legionella* colonization and positive association of Zn and Mn and *Legionlla* colonization in hot water systems have been proposed in some studies (Bargellini et al., 2011; Borella et al., 2004). In contrast, a *Legionella* survey of German residences suggested a positive effect of copper pipes towards *Legionella* colonization (Mathys et al., 2008).

In general, the driving factors of opportunistic pathogen occurrence and regrowth in premise plumbing remain elusive due to the complexity of premise plumbing and limited knowledge of pathogen transmission and life cycles in engineered water systems. Few studies have considered the influence of microbial ecology, which is likely to be particularly critical in governing occurrence and regrowth of opportunistic pathogens in

drinking water systems. For example, the ecological niche of *Legionella* and mycobacteria overlaps with those of amoebae and protozoa (Adekambi et al., 2006; Declerck et al., 2009) and infection of protozoan hosts can enhance reproduction and virulence in these and other pathogens (Cirillo et al., 1999; Cirillo et al., 1997). Certain aquatic bacteria have also been reported to exert a negative influence towards *L. pneumophila* (Guerrieri et al., 2008). Therefore, advancing understanding of the microbial ecology of multiple representative opportunistic pathogens is critical to developing appropriate guidance and controls to broadly limit their proliferation. In particular, there is need for a simultaneous, comprehensive molecular examination of multiple opportunistic pathogens. Such a study is of value considering that the factors that inhibit one pathogen may actually favor the growth of others. For example, in the Pinellas County, Florida (PCF) drinking water distribution system, Moore and colleagues (Moore et al., 2006) previously reported that switching from chlorine to chloramines mitigated *Legionella* colonization, but favored mycobacteria. However, the full extent and implications of such phenomena are unknown.

This study provides a comprehensive molecular survey of the occurrence of *L. pneumophila* and other *Legionella*, *M. avium* and non-tuberculosis mycobacteria, and *P. aeruginosa*, as well as two known amoeba hosts (*Acanthamoeba* spp. and *Hartmanella vermiformis*) in two chloraminated drinking water systems in the U.S.A. The first, PCF, has been subject to prior characterization of *L. pneumophila* and *M. avium,* as noted above, and is representative of a warm climate. The second, Blacksburg-Christiansburg-VPI Water Authority (BCV), located in Virginia, has not been

previously characterized and is representative of a temperate climate. Chloramination is of particular interest given that there is a general movement, particularly in the U.S.A., to switch away from chlorine to reduce the risk of disinfection by-product formation. The present study primarily employed quantitative polymerase chain reaction (q-PCR) as a culture-independent approach for pathogen enumeration, the advantages of which include low detection limit, high specificity, and high throughput. Differing sampling techniques were implemented to estimate the relative influence of premise plumbing (first draw samples) versus the main water distribution system (post three minute flushing) environments. To explore the potential relationship between factors such as water age, opportunistic pathogen numbers, and microbial ecology, the broader microbial community structure was profiled in the bulk water and biofilm using terminal restriction fragment length polymorphism (T-RFLP) targeting 16S rRNA genes.

2.5 Materials and Methods

2.5.1 Site Location and Sampling Procedures

Blacksburg-Christiansburg-VPI Water Authority (BCV): BCV is located in southwest Virginia and serves a population of about 65,000, treating surface water by chlorination, flocculation, sedimentation and dual media filtration. Chloramines have served as the disinfectant since June, 2005, prior to which chlorine was used. Samples were collected from September, 2010 to November, 2010. The sampling plan was designed based on a water age model provided by the utility. Three to eight houses were selected for each of five water age ranges (3 to 6 d (n=4), 6 to 8 d (n=8), 8 to 10d (n=5), 10 to 12d (n=6), and ≥ 17d (n=3); 3 had unknown water age). One liter samples were collected before flushing

the sampled tap (first draw), after flushing for 3 min (post flushing), and from the safety valve and bottom drain valve of corresponding water heaters, if available. The sampling procedure was performed in accordance with the U.S. Environmental Protection Agency (U.S. EPA) total coliform sampling guide, except a first draw sample was included (EPA, 2001). Collected water samples were transported to the lab on ice within 2 h.

Pinellas County (PCF): *Legionella* spp. and *Mycobacterium* spp. were historically reported to be prevalent in the PCF drinking water distribution system (Moore et al., 2006; Pryor et al., 2004), which serves over 640,000 people. Details of PCF were reported in a previous U.S. Center for Disease Control (CDC) study (Moore et al., 2006) except that the source water has been adjusted to a blend of surface, ground, and desalinated water (at the time of the previous CDC study the source water was 100% groundwater). Sampling took place in May, 2011 targeting eight sites that were positive and seven sites that were negative for *Legionella* in the previous CDC study (Moore et al., 2006). Among the eight positive sites, three were reported positive only when chlorine was used as the disinfectant, but negative following the switch to chloramination. Four sites that were *Legionella* positive when chloramine was utilized were also previously positive when chlorine was used. The water sampling procedure was identical to BCV, except shower water samples were collected when available and biofilm samples were collected from taps and shower heads by swabbing the inner surfaces with sterile cotton. These additional samples facilitated comparison with a previous CDC study (Moore et al., 2006).

2.5.2 Water-Quality Analysis

Temperature, pH, total ammonia, and total chlorine residual were measured at the time of collection. pH was monitored using a portable pH 110 meter (Oakton Research, Vernon Hills, IL). Total ammonia was measured using a DR2700 spectrophotometer (HACH, Loveland, CO) according to standard method 4500-NH_3 (APHA, 1998). Total chlorine residual was measured using a HACH chlorine pocket colorimeter according to HACH DPD colorimetric method 8167. NO_3^- was measured using a Dionex (Sunnyvale, CA) DX-120 ion chromatography according to standard method 4110 (APHA, 1998). Total organic carbon (TOC) was measured on a SIEVERS 800 portable TOC analyzer (GE, Boulder, CO) using standard method 5310A(APHA, 1998).

2.5.3 Water Sample Processing and DNA Extraction

One liter of water was filtered through 0.22 μm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA), which were fragmented prior to extraction. DNA was extracted directly from cotton swabs for biofilm samples. DNA extraction was carried out using a FastDNA® SPIN Kit (MP Biomedicals, Solon, OH) according to manufacturer protocol. For PCF samples, only half of the membrane was used for DNA extraction, and the other half was reserved for culturing.

2.5.4 Quantitative Polymerase Chain Reaction (q-PCR)

Legionella spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *Acanthamoeba* spp., *H. vermiformis*, *P. aeruginosa*, and total bacteria were enumerated by q-PCR using previously published methods (Anuj et al., 2009; Kuiper et al., 2006; Nazarian et al.,

2008; Radomski et al., 2010; Riviere et al., 2006; Suzuki et al., 2000; Wilton and Cousins, 1992). All reactions were performed using a Bio-Rad (Hercules, CA) CFX96 real time system in a final volume of 10 µl. Detailed information about primers, probes, and Q-PCR programs is provided in Table S2.1. The specificity of all q-PCR assays except total bacteria was confirmed by cloning and sequencing of q-PCR products from select positive samples collected in this study (Table S2.2-S2.8). For Taqman™ assays, each 10 µl reaction mixture contained 5 µl of 2× SsoFast™ Probes Supermix (Bio-Rad), 250 nM of each primer, 93.75 nM of probe, and 1 µl DNA template. For EvaGreen assays, each 10 µl reaction mixture contained 5µl of 2×SsoFast ™ EvaGreen® Supermix (Bio-Rad), 400 nM of each primer, and 1 µl DNA template. DNA extracts, negative DNA controls (template DNA replaced by sterile Nanopure™ water), and 10-fold serial dilutions of standard DNA were included in triplicate in each q-PCR run. Based on a serial dilution analysis, a sample dilution of 1:5 was determined to be effective for elimination of q-PCR inhibition. Melt curve analysis was implemented on EvaGreen q-PCR assays in order to verify specificity by ramping the temperature from 65 to 95 °C at a rate of 0.5 °C / 5 s. The limit of quantification (LOQ) for all q-PCR assays ranged from 1-10 gene copy/ reaction and was implemented as appropriate for each specific run. For samples having gene copy concentration near the LOQ (0-5 gene copies/ µ l), only samples all three triplicates yielded a detectable threshold cycle (Ct) was considered positive. To determine the effective LOQ and recovery efficiency considerate of upstream sample processing (i.e., membrane filtration and DNA extraction), *L. pneumophila*, *Legionella* spp., *M. avium*, *Mycobacterium* spp., *P. aeruginosa*, *H. vermiformis*, and *Acanthamoeba* spp. were spiked at defined concentrations in 500 ml water and analyzed. Using this

approach, LOQs were determined to be 32, 32, 170, 170, 114, 2.4, and 0.85 CFU or cells/ml, respectively. Linear models were established, which provide conversion between CFUs and q-PCR for targeted organisms as assayed in this study (Figure S2.1, Table S2.9).

2.5.5 *Legionella* Cultivation

Legionella in PCF water samples were enumerated by colony count on Buffer Charcoal Yeast Extract (BCYE) agar (Edelstein, 1981) following a 30 minute pretreatment at 50 °C (Leoni and Legnani, 2001). Heat pretreatment was selected instead of acid based on preliminary experiments demonstrating impairment of *Legionella* culturability by acid pre-treatment. After 10 d incubation at 37 °C, the identity of *Legionella*-like colonies was verified by both *Legionella* spp. and *L. pneumophila* q-PCR (Table S2.1).

2.5.6 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

16S rRNA genes were amplified on a Bio-Rad C1000 thermal cycler via nested-PCR using fluorescently labeled primer 27f (5'-FAM-AGAGTTTGATCMTGGCTCAG-3') (Sipos et al., 2007) and 907r (CCGTCAATTCCTTTRAGTTT) (Kim et al., 2008) with an annealing temperature of 50 °C. The first round and second round amplification cycles were optimized to 15 and 30, respectively, in order to reach a balance between increasing T-RFLP profile resolution and minimizing PCR bias (Osborn et al., 2000; Sipos et al., 2007). PCR products were purified using a GeneClean® spin kit (MP Biomedicals, Solon, OH). Purified PCR products (10 µl) were digested with 20 U *Hha* I (Promega, Madison, WI). Digested PCR products (1 µl) were mixed with 8.75 µl formamide and 0.25 µl

GeneScan^{TM} 500 LIZ^{TM} size standard [Applied Biosystems (ABI), Foster, CA] and denatured at 95 °C for 5 min followed by snap cooling in an ice bath prior to electrophoresis on an ABI 3130 genetic analyzer. T-RFs between 50 to 500 bp with peak heights of ≥ 50 fluorescence units were identified using GeneMapper V 4.0 (ABI).

2.5.7 Statistical Analysis

The Shapiro–Wilk test was used to test the normality of datasets. The Student's t test was used to compare means of physiochemical parameters. Since log transformed gene copies numbers were not normally distributed, non-parametric Wilcoxon Rank Sum test was used to compare gene copies of targeted organisms. An equal or given proportion (prop. Test) was used to compare the detection rates. Correlation analyses were conducted between different targeted organisms and physiochemical parameters using Spearman Rank Correlation analysis. The differences of Shannon diversity index between samples with different water age were compared using One Way Analysis of Variance (ANOVA) followed by pairwise comparison (pairwise t test). The differences in chloramine concentration for samples with different water age were compared using non-parametric Kruskal-Wallis rank sum test followed by multiple comparison test (kruskalmc). All above statistical testes were implemented by R (http://www.r-project.org/). Primer-E (Plymouth, United Kingdom) was employed to retrieve univariate indices (i.e., evenness, richness, Shannon diversity index) and perform multivariate statistical analysis of T-RFLP profiles. The similarity of T-RF profiles was examined using Bray-Curtis (Clarke et al., 2006) as a measure of similarity based on T-RF peak heights, which generated bacterial community resemblance matrices for cluster analysis,

multi-dimensional scaling (MDS) and analysis of similarity (ANOSIM). Global R values, generated by ANOSIM, fall between 0 and 1, indicating the degree of discrimination of sample groups. R=1 indicates that all samples within the group are more similar to each other than any sample from other groups. An R value of 0 indicates that the similarity between and within the groups is the same on average (Clarke and Warwick, 2001). Analysis of > 999 random permutations tested the null hypothesis that the bacterial community structures were similar. Biota and/or environmental matching (BEST) analysis was used to conduct correlation analyses of environmental parameters and microbial community data (Clarke et al., 2008). Significance was set at $P \leq 0.05$.

2.6 Results

2.6.1 Water Quality Characteristics

The water quality characteristics of the two distribution systems are presented in Table 2.1. The average temperature of the BCV water samples collected after flushing was approximately 7 ºC lower than the PCF samples ($P<0.001$). Except for pH ($P= 0.002$), no other significant differences in water quality constituents were found between the two systems in post flushing water. However, a higher variance of the total chlorine concentration was noted in the PCF distribution system.

2.6.2 Occurrence of *Legionella*, mycobacteria, *P. aeruginosa* and two amoebas

The frequencies of detection (FOD) and densities of *Legionella*, mycobacteria, *P. aeruginosa*, and two amoebas for the two water systems are presented in Tables 2.2 and 2.3. The highest FOD in BCV was *Mycobacterium* spp. (94% of samples), followed by

Legionella spp. (30% of samples). *L. pneumophila* was detected in 4 out of 27 (15 %) *Legionella* positive samples. *M. avium* was detected in 8 of 85 (9 %) mycobacteria positive samples. The average proportions of *L. pneumophila* and *M. avium* were approximately 15% and < 0.1% of the total *Legionella* spp. and *Mycobacterium* spp., respectively by assuming all *Legionella* species carry 3 genome copies of 23S rRNA gene, as does *L. pneumophila,* and all mycobacteria carry 1 genome copy of 16S rRNA gene, as does *M. avium* (http://rrndb.mmg.msu.edu/search.php). The FOD of *H. vermiformis* was noted to be twice that of *Acanthamoeba* spp., with a significantly higher average density ($P < 0.001$). Only one sample was positive for *P. aeruginosa*, a water heater sample with a very low number of gene copies of 1.8 ±0.3 gene copies/ml.

In the PCF distribution system (Table 2.3), all 15 sites yielded positive detections for *Legionella* spp. and *Mycobacterium* spp. The FOD of *L. pneumophila* and *M. avium* were 20% and 33% of sites sampled, respectively. *Acanthamoeba* spp. were not detected in any water sample and only in one biofilm sample. As observed in BCV, *H. vermiformis* was more prevalent than *Acanthamoeba* ($p<0.001$). *P. aeruginosa* was detected at 2 of 15 sites: 1 biofilm and 3 water samples. Generally, no preference of biofilm versus bulk water was observed for any of the bacterial groups monitored ($p = 0.11$).

2.6.3 Detection of *Legionella* by Cultivation

Legionella spp. colonies, as confirmed by q-PCR, were recovered from only 1 of 56 water samples (2%), at a density of 2 CFU/ml in PCF.

2.6.4 Effect of 3-min Flushing

Figure 2.1 compares the average gene copies of *Legionella* spp., *Mycobacterium* spp., *H. vermiformis,* and total bacteria measured in first draw and post-flushing samples in BCV. The average densities of the targeted genes post-flushing were 6 to 45-fold lower than corresponding first draw samples. These differences were significant for *Legionella* spp. ($P=0.002$), *Mycobacterium* spp. ($P<0.001$), *H. vermiformis* ($P=0.018$), and total bacteria ($P<0.001$). However, a significant effect of flushing was only observed for total bacterial 16S rRNA genes in PCF ($P=0.032$), where it was not possible to impose an 8 hour stagnation period prior to sampling. It was also noted that none of the post-flushing samples were positive for *L. pneumophila* or *M. avium* in BCV. In PCF, the only site positive for *M. avium* in the first draw sample was no longer positive after flushing; about 10-fold reduction was observed for the only positive sample for *P. aeruginosa.*

2.6.5 Associations with Biotic Factors

Moderate to strong correlation between *Mycobacterium* spp. and total bacterial 16S rRNA genes were observed in BCV water samples. (ρ =0.6216-0.7729, $P<0.001$) (Table 2.4); however, at PCF this same correlation was only observed in biofilm samples (ρ =0.7308, $P<0.001$). Low to moderate correlations between *H. vermiformis* and total bacterial 16S rRNA genes were displayed in water heater samples and biofilm samples in both distribution systems (ρ =0.3863-0.6911, $P<0.05$). Low to moderate positive correlations were found between *Legionella* spp. and *H. vermiformis* (ρ =0.3550-0.5907, $P<0.05$) in BCV but not PCF. *Mycobacterium* spp. also displayed weak to moderate correlations (ρ =0.3697-0.5560, $P<0.05$) with *H. vermiformis* in some sample types at

both BCV and PCF. The only observed correlations for *Legionella* spp. were weak to moderate correlations with total bacterial 16S rRNA genes (ρ =0.4593, P=0.008) and *Mycobacterium* spp. (ρ =0.3786, P=0.032) in BCV water heater samples (Table 2.4).

2.6.6 Associations with Abiotic Factors

Moderate negative correlations were noted between *Mycobacterium* spp. gene copies and total chloramines (ρ =-0.52, P=0.004) and between total bacterial 16S rRNA genes and total chloramines (ρ =-0.49, P=0.007) for the first draw BCV samples. For PCF samples, no correlations of either of these groups with total chloramines were found. In BCV first draw samples, *Mycobacterium* spp., *H. vermiformis*, and total bacterial 16S rRNA genes displayed low to moderate correlations with TOC (ρ = 0.4, P<0.05). However, in PCF, only *Legionella* spp. was found to correlate with TOC, and only in water heater samples (ρ =0.73, P=0.01). No correlations with temperature were found for either system.

2.6.7 Characteristics of the Broader Bacterial Community

The broader bacterial communities of all samples were profiled by T-RFLP. Post-flushing samples from BCV and PCF were pooled together for ANOSIM analysis, which demonstrated that the bacterial community compositions were significantly different between the BCV and PCF systems (Global R=0.298, P=0.001) (Table 2.5). 3-D multi-dimensional scaling (MDS) plots illustrated the separation of samples by location (Figure 2.2). No significant clustering was observed among samples pooled into categories of first draw, post flushing, or water heater (Global R=0.009, P=0.297). Of the

twenty-six biofilm samples collected from PCF taps and showerheads, no significant difference in the T-RFLP patterns were observed with corresponding water samples ($P=0.636$).

The average values of the Shannon diversity index increased from 1.0±0.8 at water age of 3~6d to 2.1±0.3 at water age of >17d. Significantly lower values of the Shannon diversity index were observed in 3~6d and 6~8d samples compared to >17d ($P<0.05$). ANOSIM indicated a weak water age effect on bacterial community structure (Global $R=0.097$, $P=0.003$) (Table 2.5). Further, pair wise tests revealed weak separation between samples with water ages of 3~6 d and 6~8d, 6~8 d and 8~10d, 8~10 d and 10~12 d, 3~6 d and 10~12 d ($R=0.091$-0.222, $P<0.05$), which was also confirmed by MDS (Figure S2.2). BEST analysis was applied to determine the correspondence of bacterial community profiles to the environmental parameters reported in Table 2.1; however, no relationships were identified.

The wide variability observed in MDS plots (Figure 2.3) indicate that the microbial community compositions of the main distribution systems varied dramatically from location to location. For all sampled BCV and PCF sites, the difference in bacterial community composition between first draw and post-flushing samples varied from 16 to 100%. More than half (55%) of the sampled sites demonstrated greater than a 50% change after flushing (Figure 2.3).

2.7 Discussion

2.7.1 Water Quality Characteristics

The relatively lower average temperature in BCV post-flushing samples was as expected, considering the difference in season and latitude (10°), and may account for some of the differences observed between the two systems. Water heater temperatures in both systems were also lower on average than the recommended range for pathogen control (49-60 °C) (Lévesque et al., 2004). Temperature stratification has recently been noted to be a widespread phenomenon in water heaters, typically resulting in colder temperatures at the bottom (Brazeau and Edwards, 2011), highlighting a key issue of concern for better protecting public health.

2.7.2 Occurrence of *Legionella*, mycobacteria, *P. aeruginosa* and two amoebas

It is interesting that both distribution systems shared similar patterns of targeted organism occurrence, despite their geographic separation. Both systems were characterized by high FODs of *Legionella* spp. and *Mycobacterium* spp., low FODs of *L. pneumophila* and *M. avium,* and rare detection of *P. aeruginosa.* It is not clear that if the season contributes to the pattern similarity since both sampling occurred during warm seasons (Spring or Fall). Seasonal effect on *Legionella* diversity or distribution has been found in one location of a French pristine river (Parthuisot et al., 2010) and cooling towers(Turetgen et al., 2005). It is possible that seasonal factors could have been at play in the present study by influencing the bacterial growth environment (e.g., temperature, humidity, and water chemistry) and operation of premise plumbing (e.g., water heater usage).

In addition to *L. pneumophila*, several members of *Legionella* such as *L. longbeachae*, *L. micdadei*, *L. bozemanii*, and *L. dumooffii* are documented human pathogens (Muder and Yu, 2002). High diversities of *Legionella,* including pathogenic species, have been observed in drinking water treatment plants and treated drinking water in the Netherlands (Wullings et al., 2011; Wullings and van der Kooij, 2006). In these studies, *L. pneumophila* only accounted for 0.1-1% of the total *Legionella* spp. and instead *L. bozemannii* predominated in the clone library, which is in agreement with the finding of the present work. However, *L. pneumophila* has been observed to be the most frequently isolated species in several other premise plumbing field surveys (Bargellini et al., 2011; Borella et al., 2005; Legnani et al., 2002; Mathys et al., 2008). For example, *L. pneumophila* accounted for 93 % of *Legionella* spp. positive samples in a culture-based survey of hot water systems in Italy (Bargellini et al., 2011). The physiologic and genetic basis for the survival and persistence of *Legionella* species in drinking water systems remains elusive. Many factors such as water source, water treatment processes and conveyance, and the characteristics of the premise plumbing likely play a role in permitting certain *Legionella* species to persist.

Similarly, a high diversity of mycobacteria has been demonstrated to be characteristic of the drinking water environment, some of which are established opportunistic pathogens, such as *M. avium, M. intracellulare, M. kansasii, M. abscessus, and M. chelonae* (Falkinham et al., 2001; Lehtola et al., 2006; Shin et al., 2008; Tsintzou et al., 2000; von Baum et al., 2010), of which *M. avium* is currently on the U.S. EPA candidate contaminant list. In the present study, a considerable number of mycobacteria were

detected, accounting for 0.1% to 68% of total bacterial estimate, assuming all bacteria have a single copy of 16S rRNA gene. *Mycobacterium* spp. in post-flushing samples ranged from 15-3.0$\times 10^3$ gene copies/ml (average = 495 gene copies/ml), which is comparable to q-PCR estimation of *Mycobacterium* spp. in hospital tap water (1.0$\times$ 10^3-2.0$\times 10^7$ CFU/500ml) (Hussein et al., 2009), but relatively higher than reported *Mycobacterium* spp. by culturing methods in drinking water systems (e.g., 10-7$\times 10^5$ CFU/L (Falkinham et al., 2001); 15-140 CFU/L (Torvinen et al., 2004); 1-1000CFU/500ml (von Baum et al., 2010)) by assuming all mycobacteria have one genome copy of 16S rRNA gene. Important to note is that all q-PCR assays applied in this study yielded slightly higher estimates than CFU counts (slope of 1.2-1.6 fold), except the *Mycobacterium* spp. and *M. avium* q-PCR assays, which yielded lower estimates (slope of 0.8-0.9 fold) (Figure S2.1, Table S2.9). This is likely due to greater losses of mycobacteria during upstream processing, such as filtration and DNA extraction, accounted for in the q-PCR estimate. Mycobacteria are notorious for such analytical challenges imposed by the unique hydrophobic and impermeable nature of their cell envelope (Amita et al., 2002). Nonetheless, the results reported here support the conclusion of the previous CDC study that switching to chloramines favors the growth of *Mycobacterium* spp. (Moore et al., 2006). The prevalence of *Mycobacterium* spp. is likely to further increase the incidence of disease, particularly through aerosol inhalation by susceptible individuals. Identical DNA fingerprints found for patients with mycobacterial pulmonary disease and household plumbing isolates has implicated that *Mycobacterium* spp. inhabiting premise plumbing is a probable source of pulmonary infection (Falkinham, 2011; Falkinham et al., 2008).

A recent literature review examining drinking water systems across 14 different countries provides evidence of ubiquitous free-living amoeba in treated drinking water, especially in reservoirs and in premise-plumbing tanks. In particular, *Acanthamoeba* and *Hartmanella* were the most frequently identified genera in tap water (Thomas and Ashbolt, 2011). In the present study, the FOD and abundance of *Acanthamoeba* spp. were significantly lower than those of *H. vermiformis*. It has been suggested that *Hartmannella* spp. are more ecologically relevant than *Acanthamoeba* spp. in premise plumbing. Thomas and colleagues (Thomas et al., 2008) reported that the dominance of *Acanthamoeba* spp. over *Hartmanella* spp. was reversed in a drinking water treatment plant after sand filtration. In a biofilm batch test examining protozoan hosts for *L. pneumophila* under a range of controlled water conditions, indigenous *H. vermiformis* grew whereas *Acanthamoeba* spp. did not (Valster et al., 2010).

2.7.3 Detection of *Legionella* by Cultivation

Legionella is of particular interest due to the severe pneumonia that it can cause and the relatively high number of reported outbreaks associated with drinking water systems (CDC, 2011). To provide some comparison to the previous CDC study (Moore et al., 2006), PCF water samples were cultured for recovery of *Legionella* spp. It is of interest to note that the only culture-positive sample was collected from a water heater where *L. pneumophila* water had been detected during both the chlorine and chloramine disinfection phases of the previous CDC study (Moore et al., 2006). Consistent with these observations, the culture-positive sample also yielded the highest number of *Legionella*

spp. and *L. pneumophila* gene copies according to q-PCR. However, the q-PCR estimate (220 cells/ml based on one gene copy per cell (Koide et al., 1993)) was about 100 fold higher than the culture-based estimate. A similar difference in magnitude between q-PCR and culturing of *L. pneumophila* was reported for cooling towers (Lee et al., 2011).

One possible explanation for the lower estimates of frequency and density of culture-positive *Legionella* samples is a higher detection limit of the cultivation method used here compared to the CDC method (Moore et al., 2006). A recent pilot study for the Environmental *Legionella* Isolation Techniques Evaluation (ELITE) program demonstrated that inter-laboratory differences in *Legionella* culture enumeration were as high as 1,660-fold (Lucas et al., 2011). Importantly, the optimal method for isolation from environmental samples appears to be sample-specific and *Legionella* concentration-dependent (Bartie et al., 2003; De Luca et al., 1999; Leoni and Legnani, 2001), and can vary greatly as a result of sample shipping and processing (McCoy et al., 2012). q-PCR is also generally subject to a higher estimate of *Legionella* due to detection of DNA from dead cells (Slimani et al., 2012; Yanez et al., 2011), but also because it can detect cells in a viable but non-culturable (VBNC) status, which has been widely reported in other drinking water studies (Diederen et al., 2007; Wullings et al., 2011; Wullings and van der Kooij, 2006). Another possible explanation for the low recovery of *Legionella* isolates could have been due to the reported fall in *Legionella* spp. numbers following the switch to chloramine disinfection (Moore et al., 2006). Finally, additional sample pre-treatment steps required for culturing could diminish recovery relative to q-PCR.

2.7.4 Effect of 3-min Flushing

Post-flushing samples are representative of the water distribution system, whereas first draw samples are indicative of the premise plumbing. The reduction of *Legionella* spp., *Mycobacterium* spp., *H. vermiformis*, and 16S rRNA gene densities in post-flushing samples suggests that even when the distribution system itself is highly chloraminated, conditions in premise plumbing may still permit the persistence and growth of bacteria and protozoa harbored in the biofilm. Greater differences were observed between first draw samples and post-flushing samples in BCV relative to PCF, which is likely due to longer water stagnation time for BCV. Residents in BCV were advised to abstain from water use for 8 h prior to the sampling. However, it was impractical to impose the same restriction in PCF due to the limits of sampling time and building complexity.

Impacts of flushing on bacteria and amoeba are consistent with findings of a study investigating the influence of overnight stagnation on bacterial densities assayed by flow cytometry, adenosine tri-phosphate (ATP) concentration, and heterotrophic plate counts (HPC) (Lautenschlager et al., 2010). Samples taken after overnight stagnation (first draw) yielded 2-3 fold, 2-18 fold, and 4-580 fold higher cell numbers, ATP, and HPC, respectively. The difference of water quality in the premise plumbing and main distribution system reinforces prior research indicating that routine monitoring as employed by water utilities does not reflect conditions (e.g., stagnant periods) encountered in premise plumbing (NRC, 2006). This difference also demonstrated

regrowth of bacteria and protozoa in premise plumbing, indicating that q-PCR is capable of capturing information about live cells in the system.

2.7.5 Associations with Biotic and Abiotic Factors

The positive correlation between *H. vermiformis* and total bacteria is likely a direct reflection of *H. vermiformis* grazing on bacteria for food (Weekers et al., 1993). However, this relationship was only observed in water heaters and was not strong ($r<0.7$), indicating that other environmental factors may account for *H. vermiformis* growth. Furthermore, *H. vermiformis* and other amoeba can serve as hosts for amoeba-resisting bacteria like *Legionella* spp. and *Mycobacterium* spp. (Berry et al., 2010; Buse and Ashbolt, 2011). Observed correlations between *Legionella* spp. and *H. verimformis* in BCV was possibly a reflection of pathogen-host effect. However, the absence of strong correlations between pathogens and hosts in either BCV or PCF suggests the importance of the broader microbial ecology of drinking water systems, involving complex interactions involving both abiotic and biotic factors. For example, cell-to-cell interaction between microbes can either stimulate or inhibit the growth of opportunistic pathogens in drinking water. Specifically, one isolate of *B. subtilis* was able to reduce *L. pneumophila* numbers in either the absence or presence of amoebae by lysing *Legionella* cells or inhibiting internal replication in amoebae, respectively (Temmerman et al., 2007). Among one collection of aquatic bacteria, 66% inhibited *L. pneumophila* growth (Guerrieri et al., 2008).

Limiting TOC has been proposed as a potential control measure for limiting pathogen re-growth in drinking water distribution systems. Previous studies linked the heterotrophic plate counts (HPC) and mycobacterial abundance to AOC (Escobar et al., 2001; Falkinham et al., 2001; Torvinen et al., 2004; Vanderkooij, 1992). This study also identified a correlation between 16S rRNA genes, *Mycobacterium* spp., and *H. vermiformis* with TOC in BCV first draw samples. However, low ρ values indicated that TOC is not the only factor contributing to their growth. Furthermore, the fact that the correlation only existed in first draw samples and that 16S rRNA genes also correlated suggests that the true correlation may have been with biofilm constituents, which are detectable by TOC, but do not represent actual TOC from the distribution system. The strong correlation between *Legionella* spp. and TOC in PCF water heater samples, but absence of such relationship in other samples implies that TOC may be a factor favoring *Legionella* spp. growth under certain conditions. Further lab-control experiments will be needed in order to better delineate potential relationships with TOC.

While temperature is known to be a critical factor for controlling opportunistic pathogen occurrence (Falkinham, 2011; Flannery et al., 2006; Lasheras et al., 2006; Mathys et al., 2008; Mouchtouri et al., 2007), no correlation was observed in this study between *Legionella* or mycobacteria and temperature. This is not unexpected because temperature typically elicits a threshold response at extremes, and the temperatures captured in this study were not necessarily representative of the narrow range corresponding to a linear growth rate response.

2.7.6 Characteristics of the Broader Bacterial Community

ANOSIM analysis demonstrated that the bacterial community compositions were significantly different between the BCV and PCF systems, which could be explained by the geographic separation, water source difference as well as the different physical characteristics of these two systems. In BCV, all of the transmission lines are cement lined ductile iron pipe. In contrast, PCF pipes are mainly made of copper (70-75%) and PVC (25-30%). Several studies have previously demonstrated significant influence of pipe materials on microbial community structure in simulated drinking water systems (Jang et al., 2011; Lehtola et al., 2004; Yu et al., 2010).

The low Shannon diversity index in samples with water age of 3-6 d might be attributable to relatively higher concentrations of chloramine (average=2.6±0.2mg/L, $P<0.05$). ANOSIM analysis demonstrated that water age has a weak effect on the bacterial community structure (Table 2.5). Even within the same water age group, the similarity of the bacterial community profile among the samples was as low as 0, indicating that water age is not the sole driving factor. Further, BEST analysis failed to identify any relationship between microbial communities and environmental parameters measured in Table 2.1. It is likely that other characteristics of the main distribution systems and premise plumbing not investigated in the present study, such as pipe age and materials, may also be significant drivers of the broader microbial community composition.

The 3-min flush caused visible microbial community change in post-flushing water samples in this study. Likewise, a considerable change was noted in bacterial community

composition by denaturing gradient gel electrophoresis after a 5-min flush in household water (Lautenschlager et al., 2010). Slight increases in evenness and richness indices were also noted in the present study after flushing. The visible microbial community changes between first draw and post-flushing samples further question the validity of routine monitoring methods, which may overlook important microbes residing in premise plumbing (NRC, 2006) and that are the source of direct human exposure.

2.8 Conclusions

This study provides a comprehensive and quantitative snapshot of the prevalence of *L. pneumophila* and other *Legionella*, *M. avium* and other mycobacteria, *P. aeruginosa*, and two amoeba hosts in two representative chloraminated drinking water distribution systems. The results confirmed that drinking water systems, especially premise plumbing, are a reservoir for microorganisms and pathogens even in the presence of high chloramine residuals. Overall, this study demonstrated the value of q-PCR for simultaneously monitoring genetic markers of multiple opportunistic pathogens and their protozoan hosts. These microorganisms are unique from traditional pathogens of concern (i.e., those leaving the drinking water treatment plant, typically of fecal origin) because they readily inhabit drinking water distribution systems and in-home plumbing. Thus, although q-PCR does not provide a direct measure of viable organisms, it does provide a measure of organisms that were presumably viable at one point in time in the distribution system. Regrowth of organisms in first draw samples representative of premise plumbing illustrates this principle and also highlights the fact that general water distribution system monitoring approaches likely overlook regrowth of opportunistic pathogens in premise

plumbing, which is the frontline of exposure to consumers. Nonetheless, future effort is merited to translate gene copy numbers into meaningful risk criteria, which currently are based on culture-based measurements. The relationships identified among targeted bacteria and amoebas, as well associations between their numbers and abiotic environmental factors identified in the study shed light on the importance of understanding drinking water pathogen ecology in order to better discern the mechanisms of opportunistic pathogen persistence in drinking water. Further, investigations of the influence of disinfectant type, pipe materials, water age, in conjunction with microbial ecology, on opportunistic pathogen occurrence and persistence under controlled laboratory conditions would be beneficial in order to identify the most critical factors that could be modified to limit opportunistic pathogen proliferation in drinking water systems.

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2.11 Tables

Table 2.1 Physicochemical properties of water in distribution systems BCV and PCF ±SD

	T (^{0}C)	pH	NH_4^+ (mg/L)	NO_3^- (mg/L)	Total Cl_2 (mg/L)	TOC (mg/L)
BCV						
First draw	20.8 ±2.8	7.85 ±0.24	0.58 ±0.15	0.35 ±0.05	2.02 ±0.63	2.56 ±1.18
Post flushing	19.7 ±2.4*	7.85 ±0.23*	0.63 ±0.14	0.34 ±0.05	2.21 ±0.63	2.36 ±2.96
Water heater	37.3 ±8.4	8.02 ±0.32	0.64 ±0.10	0.37 ±0.05	1.81 ±0.62	2.87 ±3.00
PCF						
Post flushing	26.8 ±0.9*	7.63 ±0.21*	0.45 ±0.36	0.25 ±0.21	2.15 ±1.13	3.51 ±2.68
Water heater	43.2 ±6.0	7.66 ±0.24	0.47 ±0.33	0.24 ±0.22	1.27 ±0.93	5.84 ±2.51

Note: The first draw data from PCF is not available. * indicates significant difference with 95% confidence ($P<0.05$) in post-flushing samples between BCV
and PCF.

Table 2.2 Opportunistic pathogen survey of drinking water distribution system BCV

	Occurrence rate (%)		Highest concentration	Average Concentration of positive samples
	Sites (N=29)	Samples (N=90)	(Gene copies/ml, ±SD)	(Gene copies/ml, ±SD)
Legionella	69.0	30.0	$2.3\times10^3\pm9.7\times10^2$	186.6±458.2
L. pneumophila	13.7	4.4	13.7±5.1	9.8±4.4
Mycobacterium	100	94.4	$1.8\times10^5\pm9.6\times10^4$	$1.4\times10^4\pm3.7\times10^4$
M. avium	24.1	8.9	1.9±0.3	1.1±0.6
H. vermiformis	27.6	14.4	$7.1\times10^4\pm4.4\times10^3$	$1.2\times10^4\pm2.0\times10^4$
Acanthamoeba	13.7	6.7	6.8±2.9	2.2±2.4
P. aeruginosa	3.4	1.1	1.8±0.3	1.8

Table 2.3 Opportunistic pathogen survey of drinking water distribution system PCF

	Occurrence rate (%)				Highest concentration, ±SD		Average concentration of positive samples, ±SD	
	Sites (N=15)	Samples (N=80)	Water (N=54)	Biofilm (N=26)	Water (Gene copies/ml)	Biofilm (Gene copies/swab)	Water (Gene copies/ml)	Biofilm (Gene copies/swab)
Legionella	100	67.5	83.3	34.6	759.6±285.7	$1.5\times10^{6}\pm1.8\times10^{5}$	100.8±184.2	$2.2\times10^{5}\pm4.7\times10^{5}$
L. pneumophila	20	5.0	5.6	3.8	219.4±23.8	$1.9\times10^{4}\pm1.1\times10^{4}$	90.4±111.9	1.9×10^{4}
Mycobacterium	100	93.7	98.1	84.6	$2.1\times10^{4}\pm4.2\times10^{3}$	$2.9\times10^{7}\pm8.1\times10^{5}$	$1.4\times10^{3}\pm3.5\times10^{3}$	$3.8\times10^{6}\pm8.1\times10^{6}$
M. avium	33.3	10	11.1	7.7	850.1±458.7	$4.3\times10^{5}\pm3.9\times10^{4}$	38.4±166.1	$9.1\times10^{4}\pm1.9\times10^{5}$
H. vermiformis	73.3	28.7	29.6	26.9	$5.1\times10^{3}\pm2.2\times10^{2}$	$4.6\times10^{6}\pm2.3\times10^{5}$	781.7±1408.0	$1.8\times10^{6}\pm1.9\times10^{6}$
Acanthamoeba	6.7	1.25	0	3.8	N/A	$3.0\times10^{4}\pm5.2\times10^{4}$	N/A	3.0×10^{4}
P. aeruginosa	13.3	5.0	5.6	3.8	700.3±158.7	$5.3\times10^{4}\pm5.5\times10^{3}$	340.6±363.0	5.3×10^{4}

Table 2.4 Correlation analysis of relationship between different potential opportunistic pathogens in BCV and PCF*

	***Legionella* spp.** **vs** **16S rRNA gene**	***Mycobacterium* spp.** **vs** **16S rRNA gene**	***H. vermiformis*** **vs** **16S rRNA gene**	***Legionella* spp.** **vs** ***Mycobacterium* spp.**	***Legionella* spp.** **vs** ***H. vermiformis***	***Mycobacterium* spp.** **vs** ***H. vermiformis***
BCV						
First draw	0.3243 (0.086)	**0.7729 (<0.001)**	0.2650 (0.165)	0.3619 (0.054)	**0.5907 (<0.001)**	**0.4020 (0.031)**
After flushing	0.0756 (0.699)	**0.6216 (<0.001)**	0.3089 (0.103)	0.2932 (0.123)	**0.4356 (0.018)**	**0.3697 (0.048)**
Water heater	**0.4593 (0.008)**	**0.7401 (<0.001)**	**0.3863 (0.029)**	**0.3786 (0.033)**	**0.3550 (0.046)**	0.2283 (0.209)
PCF						
First draw	0.4372 (0.103)	0.5107 (0.054)	0.4633 (0.082)	0.0860 (0.760)	0.4124 (0.127)	**0.5560 (0.031)**
After flushing	0.2901 (0.294)	-0.0036 (0.995)	-0.2017 (0.471)	0.0090 (0.975)	0.3839 (0.158)	0.0733 (0.795)
Water heater	0.4231 (0.152)	0.5494 (0.055)	**0.6911 (0.009)**	0.5549 (0.052)	0.3195 (0.287)	0.4644 (0.110)
Biofilm	0.2516 (0.215)	**0.7308 (<0.001)**	**0.5107 (<0.001)**	0.1972 (0.334)	0.3541 (0.076)	**0.4853 (0.012)**

* Correlation results were presented in the form of Spearman's Rank Correlation (ρ). P values were indicated in parenthesis. Bold values indicate significant difference with 95% confidence (P<0.05).

Table 2.5 ANOSIM analysis of microbial community structures in different sample groups*

Factor	Group	Global R value	P value
Site	BCV, PCF (post-flushing samples)	0.298	**0.001**
Water age	3 to 6 d, 6 to 8d, 8 to 10d, 10 to 12d, ≥17d	0.097	**0.003**
	Pairwise tests		
	3 to 6d, 6 to 8d	0.147	**0.041**
	6 to 8d, 8 to 10d	0.095	**0.028**
	8 to 10d, 10 to 12d	0.118	**0.01**
	10 to 12d, ≥17d	0.078	0.148
	3 to 6d, 8 to10d	0.103	0.054
	3 to 6d, 10 to 12d	0.222	**0.002**
	6 to 8d, 10 to 12d	0.06	0.09
	3 to 6d, ≥17d	0.12	0.057
	6 to 8d, ≥17d	0.069	0.205
	8 to 10d, ≥ 17d	-0.031	0.603
Sample type	Water sample, Biofilm sample	-0.016	0.636
	First draw sample, Flushing sample, Water heater sample	0.009	0.297

*Bold values indicate significant difference with 95% confidence (P<0.05).

2.12 Figures

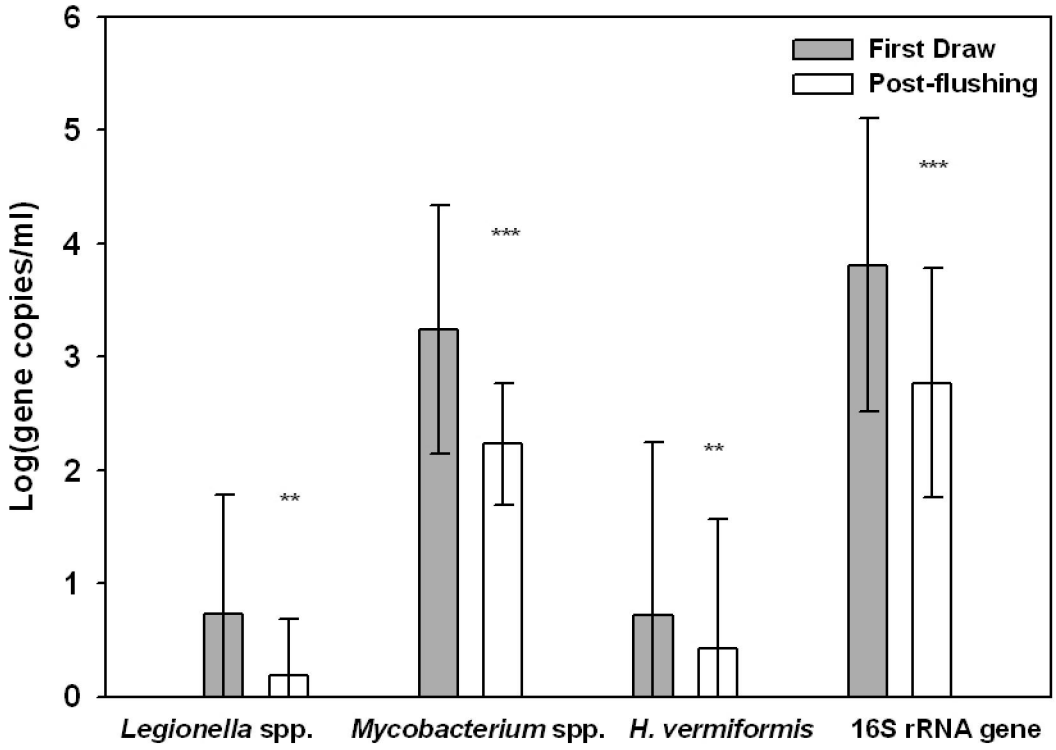


Figure 2.1 Average copy numbers of *Legionella* spp., *Mycobacterium* spp., *H. vermiformis*, and the 16S rRNA gene in first-draw and postflushing samples. Error bars represent the standard deviations of 29 log-transformed q-PCR measurements [log(x+1)] of target organisms in all first-draw and postflushing samples collected from BCV. ** and *, significant differences according to paired Wilcoxon rank sum testing at the P<0.01 and P<0.001 levels, respectively.**

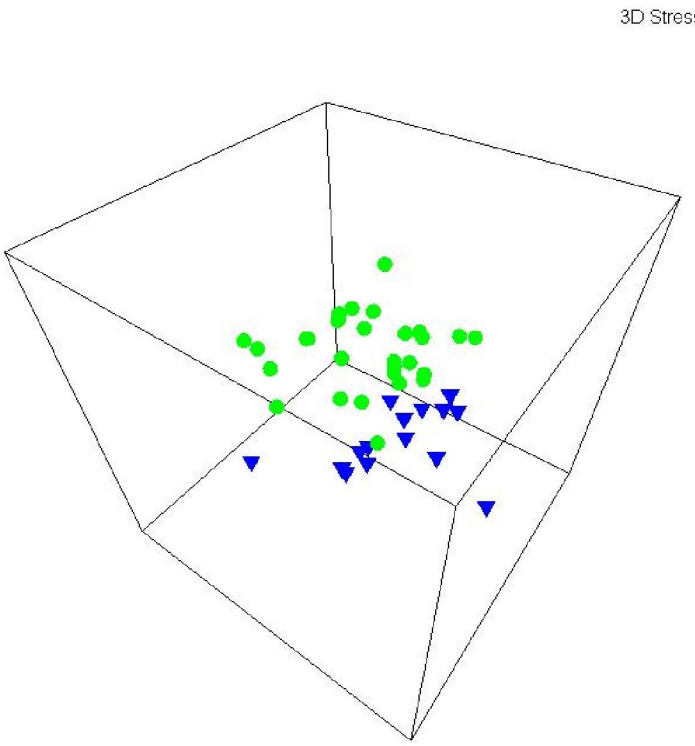


Figure 2.2 Multidimensional scaling analysis of bacterial community composition (T-RFLP profiles) for postflushing samples from BCV and PCF. Green round symbols represent samples from BCV. Blue inverted triangle symbols represent samples from PCF. Note: one sample from BCV was excluded from analysis due to an absence of T-RFLP peaks.

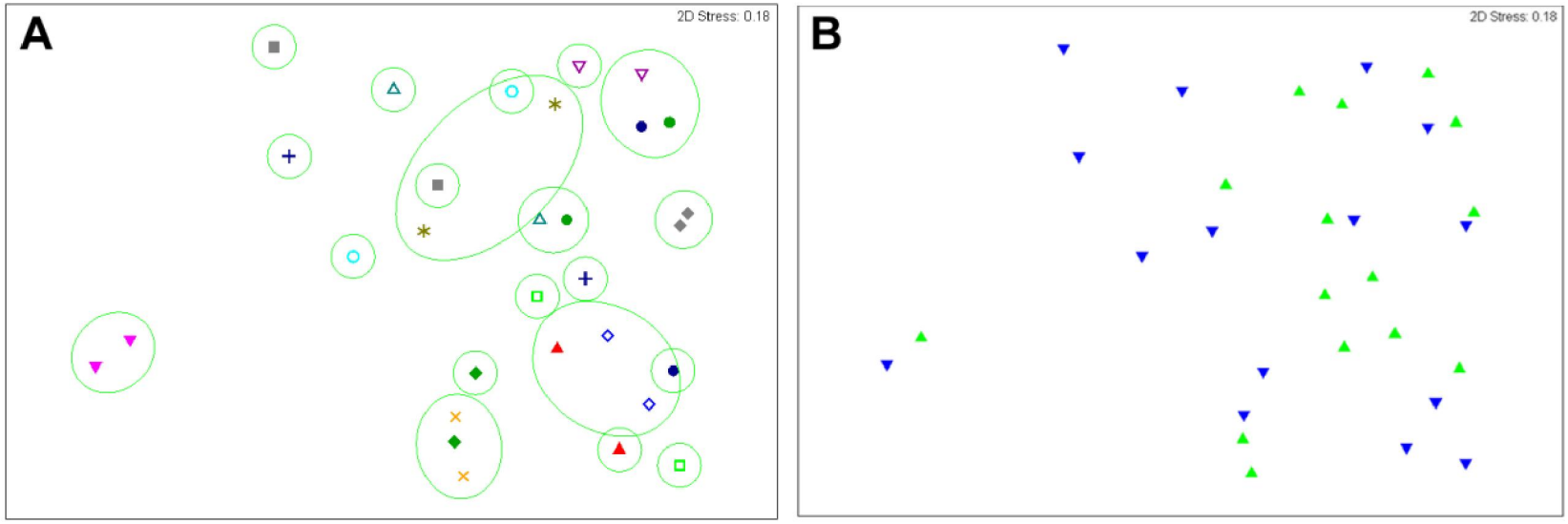


Figure 2.3 Multidimensional scaling analysis of bacterial community composition (T-RFLP profiles) for first-draw and postflushing samples from PCF. (A) Each symbol pair represents paired first-draw and postflushing samples from the same location (n=15). The green circles represent a similarity level of 50% as determined by cluster analysis. (B) Green symbols represent first-draw samples and blue symbols postflushing samples.

2.13 Supplemental Materials

Table S2.1 q-PCR primers, probes, and assay conditions used in this study

Targeted organisms	Targeted genes	Sequences (5'-3')	Program		Amplicon (bp)	Reference
			Initial denaturation and enzyme activation	Denaturing / Annealing / Extension		
***Legionella* spp.**	23S rRNA	Leg23SF: CCCATGAAGCCCGTTGAA Leg23SR:ACAATCAGCCAATTAGTACGAGTTAGC Probe: HEX-TCCACACCTCGCCTATCAACGTCGTAGT	95 ^{0}C for 2 min	40 cycles of 95 ^{0}C for 5 s and 58.5 ^{0}C for 10 s	92	Nazarian et al., 2008
L. pneumophila	*mip*	LmipF:AAAGGCATGCAAGACGCTATG LmipR: GAAACTTGTTAAGAACGTCTTTCATTTG Probe: FAM-TGGCGCTCAATTGGCTTTAACCGA	95 ^{0}C for 2 min	40 cycles of 95 ^{0}C for 5 s and 60 ^{0}C for 10 s	78	Nazarian et al., 2008
***Mycobacterium* spp.**	16S rRNA	110F: CCTGGGAAACTGGGTCTAAT I571R: CGCACGCTCACAGTTA H19R: FAM-TTTCACGAACAACGCGACAAACT	95 ^{0}C for 2 min	45 cycles of 95 ^{0}C for 5 s, 55 ^{0}C for 15 s and 72 ^{0}C for 10 s	462	Radomski et al, 2010
M. avium	16S rRNA	MycavF: AGAGTTTGATCCTGGCTCAG MycavR: ACCAGAAGACATGCGTCTTG	98 ^{0}C for 2 min	40 cycles of 98 ^{0}C for 5 s and 68 ^{0}C for 18 s	180	Wilton and Cousins, 1992
H. vermiformis	18S rRNA	Hv1227F: TTACGAGGTCAGGACACTGT Hv1728R: GACCATCCGGAGTTCTCG	98 ^{0}C for 2 min	40 cycles of 98 ^{0}C for 5 s and 72 ^{0}C for 18 s	502	Kuiper et al., 2006
Acanthamoeba	18S rRNA	TaqAcF1: CGACCAGCGATTAGGAGACG	95 ^{0}C for 2	40 cycles of 95	66	Riviere et

spp.		TaqAcR1: CCGACGCCAAGGACGAC Probe: FAM-TGAATACAAAACACCACCATCGGCGC	min	^{0}C for 5 s and 60 ^{0}C for 10 s		al., 2006
P. aeruginosa	*ecf*X & *gyr*B	*ecf*X-F: CGCATGCCTATCAGGCGTT *ecf*X-R: GAACTGCCCAGGTGCTTGC Probe: HEX-ATGGCGAGTTGCTGCGCTTCCT *gyr*B-F: CCTGACCATCCGTCGCCACAAC *gyr*B-R: CGCAGCAGGATGCCGACGCC Probe: FAM-CCGTGGTGGTAGACCTGTTCCCAGACC	95 ^{0}C for 2 min	50 cycles of 95 ^{0}C for 5 s and 60 ^{0}C for 10 s	63 (*ecf*X) 220 (*gyr*B)	Anuj et al, 2009
Total Bacteria	16S rRNA	BACT1369F: CGGTGAATACGTTCYCGG PROK: GGWTACCTTGTTACGACTT	98 ^{0}C for 2 min	40 cycles of 98 ^{0}C for 5 s and 55 ^{0}C for 5 s	124	Suzuki et al., 2000

q-PCR specificity validation using drinking water samples

DNA amplicons obtained from each q-PCR amplification were cloned to TOPO® TA cloning® kit for sequencing (Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Inserts were amplified from each clone using vector-specific primer M13F and M13R. Only M13 PCR products with insert length >100 bp were visualized on a 2% agarose gel using a Molecular Image® ChemiDocTM XRS system (Bio-Rad, CA) to eliminate clones without inserts prior to sequencing. M13 PCR products with insert length ≤100 bp were sequenced without evaluating insert presence. Sequences of M13 amplicons were analyzed using the NCBI Basic Local Alignment Search Tool (BLAST).

Table S2.2 Blast results for *Legionella* spp. q-PCR assay

Sample	bp	%	gap	Acc #	Highest similarity
C1	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C2	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C3	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C4	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C5	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C6	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)

C7	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C8	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C9	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C10	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C11	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C12	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C13	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C15	83/92	90	4	Z30458.1	*L.rubrilucens* WA-270A-C2 genes for 23S ribosomal RNA and 5S ribosomal RNA
C16	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C17	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C18	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C19	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C20	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C21	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S

					ribosomal RNA)
C23	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C24	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)
C25	90/91	99	0	CP003192.1	*Legionella pneumophila* subsp. *pneumophila* ATCC 43290 (rRNA-23S ribosomal RNA)

Note: Sequencing of C14 and C22 failed.

Table S2.3 Blast results for *L. pneumophila* q-PCR assay

Sample	bp	%	gap	Acc #	Highest similarity
C26	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C27	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C28	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C29	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C30	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C31	76/78	97	1	JN697588.1	*Legionella pneumophila* culture-collection DMST:17221 macrophage infectivity potentiator (*mip*) gene, complete cds
C32	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C33	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C34	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C35	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600

					macrophage infectivity potentiator (*mip*) gene, complete cds
C36	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C37	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C38	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C39	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C40	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C41	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C42	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C43	76/78	97	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C44	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C45	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600

					macrophage infectivity potentiator (*mip*) gene, complete cds
C46	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C47	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C48	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C49	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds
C50	77/78	99	1	JN697590.1	*Legionella pneumophila* culture-collection DMST:24600 macrophage infectivity potentiator (*mip*) gene, complete cds

Table S2.4 Blast results for *Acanthamoeba* spp. q-PCR assay

Sample	bp	%	gap	Acc #	Highest similarity
C51	63/63	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C52	64/64	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C53	229/239	96	0	BA000040.2	*Bradyrhizobium japonicum* USDA 110 DNA, complete genome
C54					No match
C55	63/63	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C56	63/63	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C57	64/64	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C58	63/63	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C59	63/63	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C60	63/63	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C61	63/63	100	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C62	62/63	98	0	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C63	65/65	100	0	JQ408990.1	*Acanthamoeba* sp. A-T4-3-146 18S ribosomal RNA gene, partial sequence
C64	64/65	98	1	JQ408990.1	*Acanthamoeba* sp. A-T4-3-146 18S ribosomal RNA gene, partial sequence

C65	60/64	94	1	FJ042636.1	*Acanthamoeba* sp. Had_008 18S ribosomal RNA gene, partial sequence Length=425
C68	62/65	95	2	JQ408985.1	*Acanthamoeba* sp. A-T4-1-89 18S ribosomal RNA gene, partial sequence
C69	63/64	98	1	JQ031557.1	*Acanthamoeba* sp. AcaKM01 18S ribosomal RNA gene, partial sequence
C70	121/125	97	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, two-component sensor
C71	63/64	98	1	JQ031557.1	*Acanthamoeba* sp. AcaKM01 18S ribosomal RNA gene, partial sequence
C72	64/65	98	1	JQ408990.1	*Acanthamoeba* sp. A-T4-3-146 18S ribosomal RNA gene, partial sequence
C73					No match
C74	63/64	98	1	JQ268236.1	Acanthamoeba sp. P4 18S ribosomal RNA gene, partial sequence
C75	63/64	98	1	JQ031557.1	*Acanthamoeba* sp. AcaKM01 18S ribosomal RNA gene, partial sequence
C76	63/64	98	1	JQ031557.1	*Acanthamoeba* sp. AcaKM01 18S ribosomal RNA gene, partial sequence
C77	64/65	98	1	JQ031557.1	*Acanthamoeba* sp. AcaKM01 18S ribosomal RNA gene, partial sequence
C78	62/64	97	1	JQ268236.1	*Acanthamoeba* sp. P4 18S ribosomal RNA gene, partial sequence

Note: Sequencing of C66 and C67 failed. M13 PCR products of C53, C54, C70, C73 can't be amplified using *Acanthamoeba* spp. q-PCR (with a probe).

Table S2.5 Blast results for *Mycobacterium* spp. q-PCR

Sample	bp	%	gap	Acc #	Highest similarity
C91	460/464	99	2	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C92	452/462	98	2	NR_043002.1	*Mycobacterium massiliense* strain CCUG 48898 16S ribosomal RNA
C93	460/464	99	2	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C94	467/477	98	0	NR_043588.1	*Mycobacterium arupense* strain AR30097 16S ribosomal RNA
C95	460/464	99	2	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C96	455/462	98	1	NR_042280.1	*Mycobacterium llatzerense* strain : MG13 16S ribosomal RNA
C97	460/463	99	1	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C98	458/462	99	1	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C99	456/462	99	1	NR_042280.1	*Mycobacterium llatzerense* strain : MG13 16S ribosomal RNA
C100	470/477	99	1	NR_043588.1	*Mycobacterium arupense* strain AR30097 16S ribosomal RNA
C101	461/463	99	0	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C102	457/463	99	0	NR_042923.1	*Mycobacterium farcinogenes* strain NCTC 10955 16S ribosomal RNA
C103	459/463	99	0	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C104	463/470	99	2	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C105	465/470	99	2	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C106	459/463	99	0	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C107	457/462	99	1	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C108	470/476	99	1	NR_043588.1	*Mycobacterium arupense* strain AR30097 16S ribosomal RNA
C109	460/463	99	1	NR_042919.1	*Mycobacterium mucogenicum* strain

					ATCC 49650 16S ribosomal RNA
C111	454/462	98	1	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C112	460/462	99	1	NR_042919.1	*Mycobacterium mucogenicum* strain ATCC 49650 16S ribosomal RNA
C113	456/462	99	1	NR_042280.1	*Mycobacterium llatzerense* strain : MG13 16S ribosomal RNA
C114	459/463	99	1	NR_042923.1	*Mycobacterium farcinogenes* strain NCTC 10955 16S ribosomal RNA
C115	468/476	98	1	NR_043588.1	*Mycobacterium arupense* strain AR30097 16S ribosomal RNA
C116	466/477	98	1	NR_043588.1	*Mycobacterium arupense* strain AR30097 16S ribosomal RNA
C117	466/477	98	1	NR_043588.1	*Mycobacterium arupense* strain AR30097 16S ribosomal RNA

Note: Sequencing of C110 and C118 failed

Table S2.6 Blast results for *H. vermiformis* q-PCR

Sample	bp	%	gap	Acc #	Highest similarity
C119	497/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C124	500/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C125	501/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C128	500/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C129	500/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C130	500/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C131	498/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C132	498/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C133	501/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C134	501/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C135	498/499	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C136	497/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C137	496/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial

					sequence
C138	496/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C139	498/499	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C140	501/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C141	497/499	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C142	501/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C143	500/502	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence
C144	492/499	99	0	DQ407609.1	*Hartmannella vermiformis* isolate CT8.4 18S ribosomal RNA gene gene, partial sequence

Note: Sequencing of C120, C121, C122, C123, C126, C127 failed.

Table S2.7 Blast results for *P. aeruginosa* q-PCR

Sample	bp	%	gap	Acc #	Highest similarity
ecf1	62/63	98	1	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf2	63/63	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf3	56/56	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf4	65/65	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf5	63/63	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf6	61/64	95	1	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf7	62/63	98	1	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf9	62/63	98	1	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf10	65/65	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf11	64/65	98	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf12	63/63	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf13	65/65	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf14	65/65	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily

					sigma-70 factor
ecf15	69/70	99	1	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf16	63/63	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf17	63/63	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf18	62/63	99	1	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf19	63/63	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
ecf20	65/65	100	0	AP012280.1	*Pseudomonas aeruginosa* NCGM2.S1 DNA, RNA polymerase ECF-subfamily sigma-70 factor
gyr1	70/79	89	0	AP012224.1	*Pseudogulbenkiania* sp. NH8B DNA, DNA protecting protein DprA
gyr2					No match
gyr3					No match
gyr4					No match
gyr5	70/79	89	0	AP012224.1	*Pseudogulbenkiania* sp. NH8B DNA, DNA protecting protein DprA
gyr6	222/222	100	0	CP002496.1	*Pseudomonas aeruginosa* M18, complete genome, DNA gyrase subunit B
gyr7	291/328	89	0	CP000356.1	*Sphingopyxis alaskensis* RB2256, phosphoribosylformylglycinamidine synthase subunit II
gyr8	70/79	89	0	AP012224.1	*Pseudogulbenkiania* sp. NH8B DNA, DNA protecting protein DprA
gyr9					No match
gyr10	133/156	85	0	CP002417.1	*Variovorax paradoxus* EPS, major facilitator superfamily MFS_1

gyr11	141/166	85	1	CP002417.1	*Variovorax paradoxus* EPS, major facilitator superfamily MFS_1
gyr13					No match
gyr14	139/163	85	1	CP002417.1	*Variovorax paradoxus* EPS, major facilitator superfamily MFS_1
gyr15	139/163	85	1	CP002417.1	*Variovorax paradoxus* EPS, major facilitator superfamily MFS_1
gyr17	187/218	86	16	AP012206.1	*Bradyrhizobium j*, aponicum USDA 6 DNA, glucarate dehydratase 5-dehydro-4-deoxyglucarate dehydratase
gyr18	70/79	89	0	AP012224.1	*Pseudogulbenkiania* sp. NH8B DNA, DNA protecting protein DprA
gyr20	70/79	89	0	AP012224.1	*Pseudogulbenkiania* sp. NH8B DNA, DNA protecting protein DprA

Note: Sequencing of ecf8, gyr16 and gyr19 failed. gyr12 is an empty vector without insert. q-PCR results of the M13 PCR products of gyr clones demonstrated that only gyr6 has amplification, indicating that the specificity of gyrB q-PCR assay strongly relied on the probe.

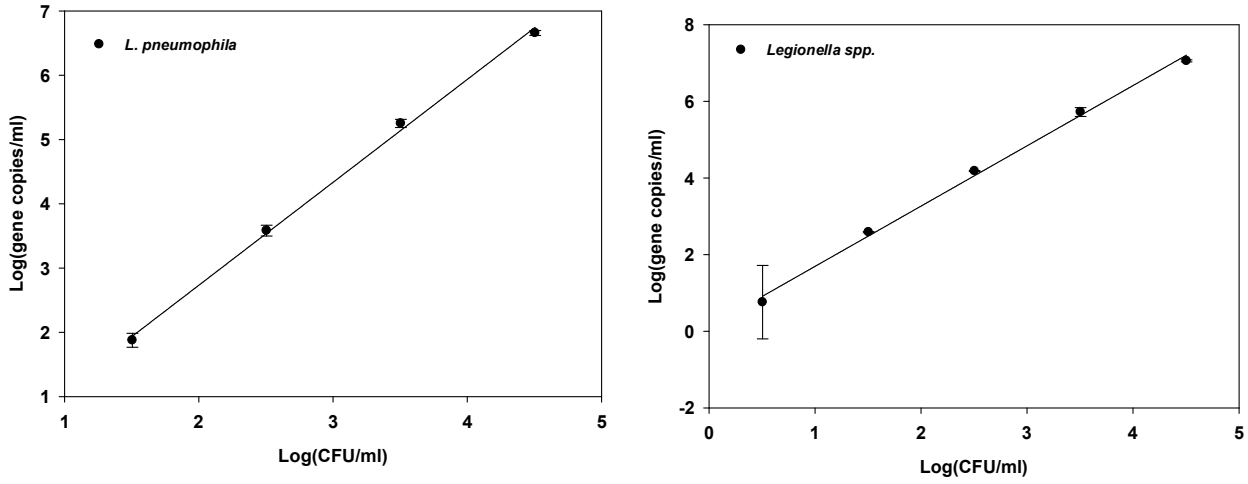
Table S2.8 Blast results for *M. avium* q-PCR

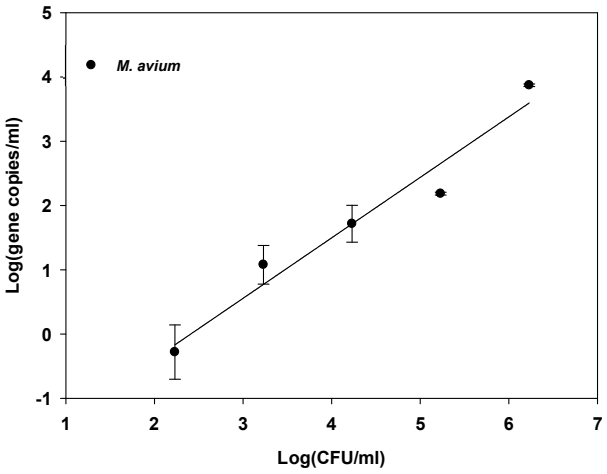
Sample	bp	%	gap	Acc #	Highest similarity
MAC1	189/191	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC2	189/191	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC3	191/191	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC4	186/187	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC5	190/193	98	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC6	187/187	100	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC7	190/193	98	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC8	186/187	98	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC9	192/193	99	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC10	189/191	99	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC11	187/187	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC12	191/191	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC13	191/191	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC14	190/193	98	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC15	193/193	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC16	192/194	99	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC17	185/187	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC18	190/191	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC19	191/191	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S

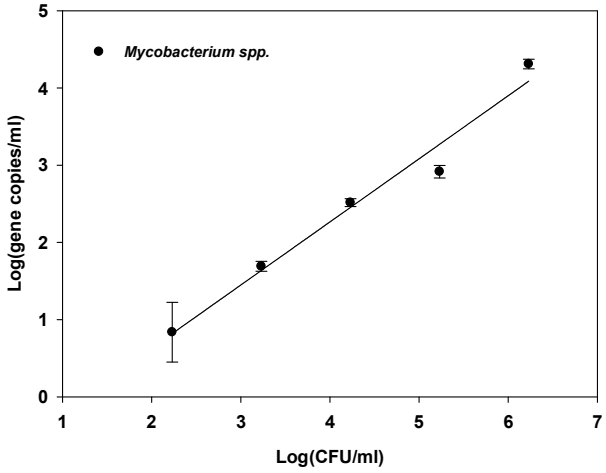
					ribosomal RNA gene, partial sequence
MAC20	189/191	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC21	190/191	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC22	189/191	99	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC23	193/193	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC24	192/193	99	1	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence
MAC25	193/193	100	0	JN899580.1	*Mycobacterium avium* strain E6 16S ribosomal RNA gene, partial sequence

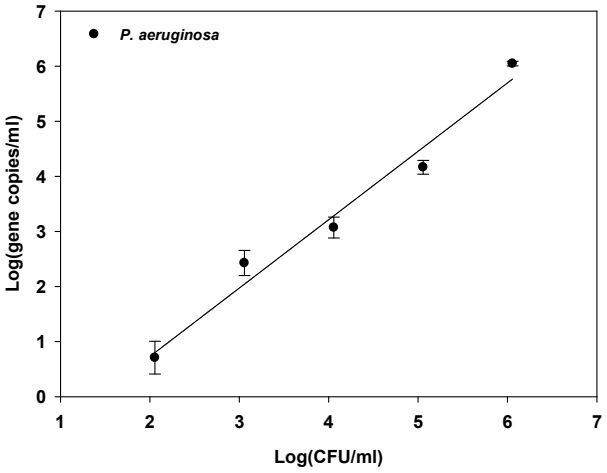
Comparison of Recovery Efficiency and Limit of Quantification Using Culture and q-PCR

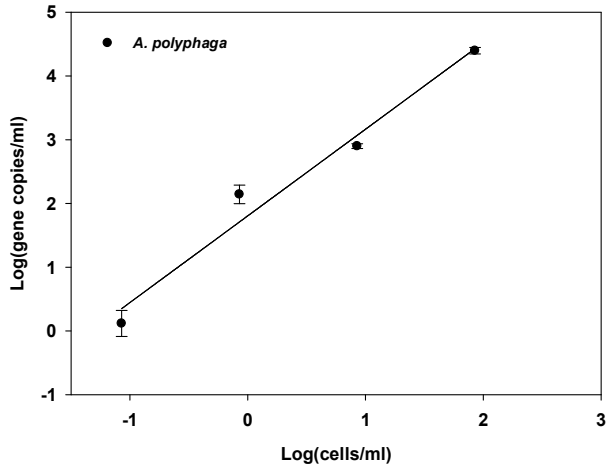
Cultures of *L. pneumophila*, *M. avium*, *A. polyphaga*, and *H. vermiformis* of known concentrations were serially diluted into 500 ml autoclaved drinking water, which were subjected to membrane filtering concentration using 0.22 μm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA). The membranes were subsequently fragmented and subjected to DNA extraction using a FastDNA® SPIN Kit (MP Biomedicals, Solon, OH) according to manufacturer protocol. The relationship between q-PCR and culturing were provided in Figure S2.1. The limit of quantitation was defined as the lowest detectable concentration with three positive threshold cycle (Ct) reading. The detection limit in 500 ml water samples for *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *P. aeruginosa*, *Acanthamoeba* spp. and *H. vermiformis* were reported to be 32, 32, 170, 170, 114, 0.85, 2.4 CFU or cells/ml. Regression analysis were performed between log (gene copies/ml) and log (CFU or cells/ml). The model equations were summarized in Table S2.2.











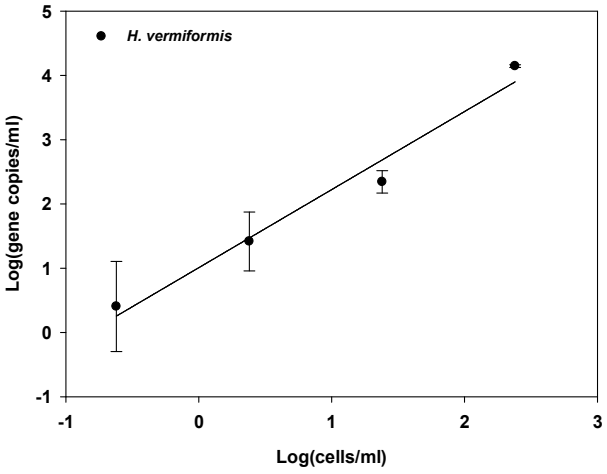


Table S2.9 Regression analysis of q-PCR and culturing data

Targeted organisms	Model Equation	R^2
Legionella spp.	Y=1.4X+0.6	0.9968
L. pneumophila	Y=1.6X+0.1	0.9981
Mycobacterium spp.	Y=0.8X-1.0	0.9734
M. avium	Y=0.9X-2.3	0.9565
P. aeruginosa	Y=1.2X-1.8	0.9740
Acanthamoeba spp.	Y=1.4X+1.8	0.9715
H. vermiformis	Y=1.2X+1.0	0.9734

Note: Y=log (gene copies/ml)
X=log (CFU or cells/ml)

Figure S2.1 Enumeration of microbial targets in serial dilutions via culturing versus q-PCR.

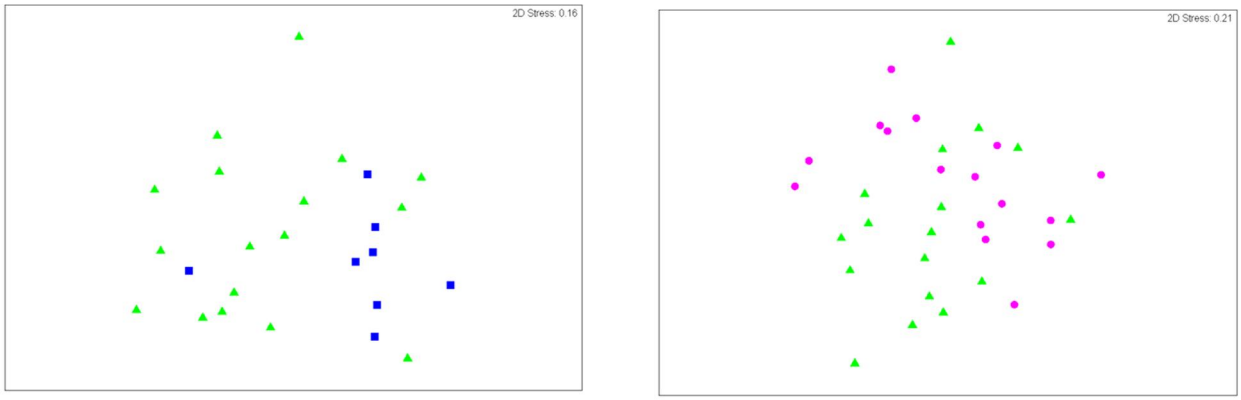


Figure S2.2 Multi-dimensional scaling analysis of bacterial community composition (T-RFLP profiles) for samples from BCV with different water age. Green triangle symbols represent samples with water age of 10-12 d. Blue square symbols represent samples with water age of 3-6d. Pink round symbols represent sample with water age of 8-10d. Note: samples without T-RFLP peaks were excluded from analysis.

CHAPTER 3

Effect of Disinfectant, Water Age, and Pipe Material on Occurrence and Persistence of *Legionella*, mycobacteria, *Pseudomonas aeruginosa*, and Two Amoebas

3.1 Authors

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3.2 Abstract

Opportunistic pathogens represent a unique challenge because they establish and grow within drinking water systems, yet the factors stimulating their proliferation are largely unknown. The purpose of this study was to examine the influence of pipe materials, disinfectant type and water age on occurrence and persistence of three opportunistic pathogens (*Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa*), broader genera (*Legionella* and mycobacteria) and two amoeba hosts (*Acanthamoeba* spp. and *Hartmanella vermiformis*). Triplicate simulated distribution systems (SDSs) compared iron, cement and PVC pipe materials fed either chlorinated or chloraminated tap water and were sampled at water ages ranging from 1d to 5.7d. Quantitative polymerase chain reaction quantified gene copies of target microorganisms in both biofilm and bulk water. *Legionella*, mycobacteria, *P. aeruginosa* and both amoebas naturally colonized the six SDSs, but *L. pneumophila* and *M. avium* were not

detected. Disinfectant type and dose was observed to have the strongest influence on the microbiota. Disinfectant decay was noted with water age, particularly in chloraminated SDSs (due to nitrification), generally resulting in increased microbial detection frequencies and densities with water age. Influence of pipe material became apparent at water ages corresponding to low disinfectant residual. Each target microbe appeared to display a distinct response to disinfectant type, pipe materials, water age and their interactions. Differences between the first and second samplings (e.g., appearance of *Legionella*, reduction in *P. aeruginosa* and *Acanthamoeba*) suggest a temporally dynamic drinking water microbial community.

3.3 Keywords

Legionella, mycobacteria, *Pseudomonas aeruginosa*, drinking water distribution systems, chlorine, chloramine, water age

3.4 Introduction

Waterborne opportunistic pathogens, such as *Legionella pneumophila*, non-tuberculosis mycobacteria (NTM), *Pseudomonas aeruginosa,* and *Acanthamoeba* spp., are natural inhabitants of drinking water distribution systems (DWDSs) (Brunkard et al., 2011; Falkinham, 2009; Thomas and Ashbolt, 2011; von Baum et al., 2010) and pose potential health threats to humans, especially immunocompromised populations. Recent epidemiological investigations highlight DWDSs as an important exposure route for disease transmission (Anaissie et al., 2002; Brown-Elliott et al., 2011; Brunkard et al.,

2011; Kilvington et al., 2004). For example, *Legionella* is the most commonly reported pathogen identified in drinking water-associated outbreaks in the U.S. since 2001 (Brunkard et al., 2011).

In the U.S., drinking water treatment plants typically use chlorine or chloramine as secondary disinfectants, which are capable of inhibiting the regrowth of microorganisms in DWDSs by maintaining a disinfectant residual. However, loss of disinfectant can be an ongoing challenge. Disinfectant decay and efficacy are affected by many factors, including disinfectant types (Lee et al., 2011; Neden et al., 1992), water constituents (e.g., dissolved organic matter, ammonia, iron, manganese) (Fisher et al., 2011), pipe materials (e.g., plastic, metal) (Lehtola et al., 2005), conditions (e.g., pipe age, corrosion) (Al-Jasser, 2007; Zhang and Andrews, 2012), biofilm characteristics (Lu et al., 1999), and water age (Maul et al., 1985). Commonly cited advantages of chloramines are less production of disinfectant by-products and longer stability compared to chlorine (Seidel et al., 2005). However, chloramine can trigger severe nitrification in DWDSs, which accelerates chloramine decomposition and subsequently promotes regrowth of microorganisms (Zhang and Edwards, 2009).

The ability of pipe materials to support drinking water biofilm varies dramatically from plastic to metal pipes as they exhibit different degrees of surface roughness and chemical

activity (Niquette et al., 2000). Moreover, the interaction between different disinfectant and pipe materials may govern microbial proliferation. For example, application of free chlorine resulted in greater biofilm heterotrophic plate count (HPC) inactivation than chloramine in copper and PVC pipes, while chloramine resulted in greater inactivation in galvanized pipe (Lechevallier et al., 1990) . Though previous studies have investigated the influence of various environmental factors on drinking water HPCs, only a few studies have focused on opportunistic pathogens (Dwidjosiswojo et al., 2011; Norton et al., 2004; van der Kooij et al., 2005).

The aim of this study was to examine the influence of disinfectant type (chlorine, chloramine), pipe materials (cement, iron, PVC), water age (1d, 2.3d, 3.6d, 5.7d), and their interactions on the occurrence and persistence of opportunistic pathogens (*Legionella pneumophila*, *Mycobacterium avium*, and *Pseudomonas aeruginosa*), two amoeba hosts (*Acanthamoeba* spp. and *Hartmanella vermiformis*), and genus level *Legionella* and mycobacteria. Genus level *Legionella* and *Mycobacterium* were targeted because many of their members are established human pathogens (e.g., *L. longbeachae*, *L. micdadei*, *L. bozemanii*, *L. dumooffii*, *M. intracellulare, M. kansasii, M. abscessus, and M. chelonae* (Falkinham, 2009; Muder and Yu, 2002)). Experiments were carried out for six disinfectant/pipe conditions in triplicate simulated DWDSs (SDSs), which provided a controlled matrix of drinking water conditions from best-case (e.g., high disinfectant

level) to the worst-case (e.g., depletion of disinfectant) scenarios as a function of water age. Culture-independent quantitative polymerase chain reaction (q-PCR) was used for opportunistic pathogen enumeration due to its advantages of low detection limit, high specificity, high reproducibility, and high throughput (Wang et al., 2012). The ultimate goal was to gain insight into factors that may trigger proliferation of opportunistic pathogens in the DWDS and potential practical engineering controls.

3.5 Materials and Methods

3.5.1 Simulated Distribution System (SDS) Set-up and Operation

Six triplicate SDSs comparing iron, cement and PVC pipe materials fed either chlorinated or chloraminated Blacksburg, VA tap water were constructed in October, 2010. Iron, PVC, and cement were epoxied into a cumulative length of 62–inch long PVC sleeves, which were inserted into each 1.5 inch-diameter PVC pipe (ASTM D1785 Schedule 40), respectively. This achieved a surface:volume ratio of 0.30-inch^{-1} of active pipe material (i.e., iron, cement), equaling to that in a 13.5-feet diameter main. The total surface:volume ratio was about 3.2 inch^{-1} for each pipe. Each PVC pipe consisted of 4 chambers connected by check valves, with an internal volume measured at 550, 700, 700, and 1175ml in each chamber in the direction of flow. Water in a 20L Hedwin cubitaner (Hedwin, Baltimore, MD) was pumped into pipes using a 12-channel low-speed digital peristaltic pump (Ismatec, Vancouver, WA) with a flowrate of 0.40±0.005 ml/min. The

water ages from the reservoir to the sampling ports were approximately 1d, 2.3d, 3.6d, and 5.7d. All six SDSs were rinsed with 500mg/L sodium hypochlorite prior to operation to reduce background microorganisms, satisfy chlorine demand, and simulate new main disinfection practices. Tap water after breakpoint chlorination was subsequently adjusted to 4.0mg/l chlorine or 4.8mg/l chloramine and pH of 8.0. Disinfectant concentration in the water tank was maintained by replacing the water daily or every other day. All six SDSs were operated in parallel under 20ºC for about 6 months prior to the first biofilm and water sampling event (03/31/2011). In order to investigate persistence of opportunistic pathogens in SDSs, a second sampling was performed 8 months after the first (11/27/2011).

3.5.2 Water-Quality Analysis

Disinfectant, pH, dissolved oxygen (DO), and total organic carbon (TOC) were measured every two weeks. Free chlorine and chloramine were measured according to Standard Method 4500-Cl (APHA, 1998) using a DR2700 spectrophotometer (HACH, Loveland, CO). pH was measured using a pH 110 meter (Oakton Research, Vernon Hills, IL) and DO was monitored using an Orion Star DO probe (Thermo Scientific, Beverly, MA). TOC was measured on a SIEVERS 800 portable analyzer (GE, Boulder, CO) using Standard Method 5310 (APHA, 1998).

3.5.3 Sample Processing and DNA Extraction

Fifty-milliliter water samples were transferred aseptically from each sampling port to a sterilized glass bottle, immediately frozen to -80^{0}C, and then freeze dried using a FreeZone 2.5L Benchtop system (Labconco, Kansas, MO). Biofilm samples were collected by swabbing the inside area of sampling port (surface area= 60cm^2) completely with sterile cotton swabs. This was intended to provide consistent technique across a smooth surface exposed to the unique water chemistry at that age. Freeze dried pellets (after evaporation of water) and cotton tips were subject to DNA extraction using a FastDNA®SPIN Kit (MP Biomedicals, Solon, OH) according to manufacturer's protocol. To evaluate recovery efficiency, serial dilutions of *L. pneumophila* were spiked into 50ml water and subject to freeze drying, DNA extraction, and q-PCR. Recovery efficiency ranged from 13%-31% relative to equivalent cells spiked directly into DNA extraction tubes and subject to q-PCR (Figure S3.1). The limit of quantification (LOQ) of the freeze drying method was comparable to that obtained with membrane filtration of 1000ml water (e.g., ~30 CFU/ml) (Wang et al., 2012).

3.5.4 q-PCR

Legionella spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *Acanthamoeba* spp., *H. vermiformis*, *P. aeruginosa* and bacterial 16S rRNA genes were enumerated by q-PCR assays using previously established methods (Wang et al., 2012). In brief, all q-PCRs

were performed in 10µl reaction mixtures containing 1×SsoFast™ Probes or Evagreen® supermix (Bio-Rad, Hercules, CA), 250nM or 400nM primer, 93.75nM probe (Taqman assay only), and 1µl of DNA template. DNA extracts (1:5 diluted), negative control, and 10-fold serial dilutions of standard were included in triplicate in each q-PCR run. Melt curve analyses were performed for each Evagreen® q-PCR. The LOQ for all q-PCR assays ranged from 1-10 gene copies/reaction and was implemented as appropriate for each specific run. Samples yielding a detectable threshold cycle in at least two experimental triplicates were scored as positive.

3.5.5 Statistical Analysis

Student's t-test or non-parametric Wilcoxon test was used to compare two groups of physiochemical parameters or log transformed biological data [$\log_{10}(x+1)$] based on the normality of the dataset. One-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis rank sum test was used for multiple comparisons (groups≥3) based on the normality of the dataset. Two-way ANOVA evaluated the effect of disinfectant type, pipe materials, and their interaction on target microorganisms categorized by water age. Linear regression characterized the relationship of 16S rRNA genes between sampling events. Non-parametric Spearman rank correlation analysis compared targeted organisms and disinfectant concentrations. All above statistical analyses were performed in R (http://www.r-project.org/). Principal component analysis (PCA) was applied to

physiochemical data using Primer-E 6.0 (Plymouth, United Kingdom). Data were reported as average±standard error. Significance was set at $P < 0.05$.

3.6 Results

3.6.1 Patterns in Water Chemistry

PCA explained 84.7% of the total physiochemical data variance and illustrated general trends in water quality as a function of SDS conditions (Figure 3.1). For example, the samples clearly separated along PC1 according to water age, with disinfectant and DO driving the overall trend (Eigenvectors = -0.661 for disinfectant, -0.649 for DO) (Figure 3.1a). PC2 was primarily driven by pH and clustered according to pipe material (Figure 3.1b). TOC contributed to both PC1 (Eigenvectors = 0.377) and PC2 (Eigenvectors= -0.590).

Chloramine concentrations decreased faster than chlorine in all SDSs ($P<0.05$) (Figure S3.2). At 1d, the chloramine residual was 35-43% higher in cement than the other SDSs ($P=0.017$), but chloramine decreased ≧90% by 2.3d for all pipe materials. For chlorinated SDSs, the chlorine residual was significantly higher in the PVC SDS on days 1, 2.3, and 3.6 ($P<0.05$), followed by cement and iron SDSs, respectively.

In all SDSs, disinfectant and DO decreased, while TOC increased with increasing water age (Table S3.1). Variation in pH was consistent with expectations dependent on pipe materials, their interaction with disinfectants, and occurrence of nitrification in the chloraminated system. For example, water samples collected from the chlorinated cement SDS at 5.7d had the highest average pH of 9.0±0.1 due to lime leaching, in contrast to the typical pH range of 7.1-7.8. The lowest average pH, 7.2±0.03, was measured in the chloraminated iron SDS at 3.6d.

3.6.2 Total Bacteria (16S rRNA genes) in SDSs

Bacterial density, as indicated by q-PCR employing universal 16S rRNA gene primers, increased in both biofilm and bulk water across all SDSs with increasing water age (Figure 3.2). The lowest 16S rRNA gene copies in biofilm and water were 4.7×10^2 gene copies/cm^2 and 3.7×10^2 gene copies/ml, respectively, both observed in chlorinated SDS 1d samples. The arithmetic mean 16S rRNA gene copies across all SDSs increased to $1.9\times10^6\pm0.38\times10^6$ gene copies/cm^2 and $3.3\times10^5\pm0.68\times10^5$ gene copies/ml for 5.7d biofilm and water samples, respectively. Similar bulk water 16S rRNA gene levels were found in Blacksburg and Pinellas county DWDS samples in a previous survey (i.e., ~10^2 to 10^5 gene copies/ml)(Wang et al., 2012).

Negative correlations were observed between disinfectant concentrations and 16S rRNA gene copy numbers in the biofilm and water across all six SDSs (r_s=-0.9454~-0.6421, P<0.05), except for chlorinated cement SDS water (Table 3.1). Further, 16S rRNA gene copies in biofilm and water samples were positively correlated when all samples were pooled together (r_s=0.7196, P<0.001).

Disinfectant type imposed a significant effect on 16S rRNA gene levels in 1d, 2.3d, and 3.6d biofilm samples, when disinfectants were not completely depleted (ANOVA; P<0.05). The degree of disinfectant effect depended on pipe material and water age. Effect of pipe materials and their interaction with disinfectant type on 16S rRNA gene levels were significant for 1d and 5.7d biofilm samples (P<0.01). In contrast, in the bulk water, disinfectant and pipe material each individually only had a significant effect on 16S rRNA genes at 1d, whereas their interactive effect remained significant throughout the SDSs (P<0.05).

Linear regression revealed consistent numbers of 16S rRNA genes in the bulk water between the first and second samplings (slope=0.89-0.95, P<0.05) of chloraminated SDSs. However, chlorinated SDSs yielded lower 16S rRNA gene copy numbers during the second sampling (Figure S3.3). Negative associations between 16S rRNA gene copy numbers and disinfectant concentration (r_s=-0.8392~-0.6667, P<0.05) were also

identified in SDSs during the second sampling, except for chlorinated PVC SDSs (P=0.21).

3.6.3 Effect of SDS Conditions on Opportunistic Pathogens and Other Microorganisms

q-PCR confirmed the presence of all targeted organisms during the first sampling, except *Legionella* and *M. avium* (Figure 3.2). Mycobacteria and *Acanthamoeba* were detected in all biofilm samples in chloraminated SDSs, with numbers ranging from 63 to 1.7×10^6 and 172 to 3.8×10^6 gene copies/cm^2, respectively. Detection frequencies of targeted organisms were lower in chlorinated SDSs. Mycobacteria were not detected in 1d biofilm regardless of pipe material, nor in 2.3d PVC biofilm. *Acanthamoeba* were not detected in chlorinated iron 1d biofilm. *P. aeruginosa* was detected in all SDS biofilms, except for 1d chlorinated SDSs, with numbers varying from <8 to 3.5×10^4 gene copies/cm^2. *H. vermiformis* was least prevalent across all SDSs, predominantly detected in biofilm samples with ≧3.6d water age and ranging from 13 to 4.7×10^5 gene copies/cm^2. Increased gene copy number of targeted organisms was observed with increased water age in most cases. Notably, all microorganisms naturally colonized the SDSs, none were intentionally inoculated. Examination of chloraminated and chlorinated feed reservoirs demonstrated all opportunistic pathogens and related organisms were below detection limit except that mycobacteria varied between <10-70 gene copies/ml.

The detection pattern of mycobacteria, *P. aeruginosa*, *Acanthamoeba* and *H. vermiformis* in bulk water was similar to the biofilm, but generally with lower detection frequencies, especially in chlorinated SDSs (Figure 3.2b). Similar to 16S rRNA genes, their copy numbers in bulk water and biofilm were positively correlated (r_s = 0.6387~0.7683, $P<0.001$).

Two-way ANOVA revealed significant disinfectant type effect on mycobacteria, *Acanthamoeba*, and *P. aeruginosa* at each water age ($P<0.05$) in both biofilm and water, with few exceptions (i.e., *P. aeruginosa* in 3.6d biofilm and 1d and 5.7d water samples; mycobacteria in 5.7d biofilm samples). The degree of disinfectant effect depended on pipe material and water age. Pipe material effect on these three microorganisms was only noted for 5.7d biofilm and water samples ($P<0.05$).

3.6.4 Effect of Disinfectant Concentration

In the biofilm, mycobacteria and *Acanthamoeba* were negatively correlated with chloramine in cement SDSs (r_s=-0.6154-~0.6083, $P<0.05$, Table S3.2-S3.3) and with chlorine in all three chlorinated SDSs (r_s=-0.8336~-0.6786, $P<0.05$). *Acanthamoeba* demonstrated a similar relationship with disinfectants in the bulk water as observed in the biofilm. For mycobacteria in bulk water, negative correlations were found with

disinfectant in both chloraminated and chlorinated SDSs (r_s =-0.8951~-0.7499, P<0.01), except for chloraminated PVC SDSs (P=0.10) and chlorinated cement SDSs (P=0.18).

P. aeruginosa and *H. vermiformis* appeared to be particularly susceptible to disinfectant. *P. aeruginosa* in the bulk water was predominantly detected in samples with disinfectant concentration <0.3mg/L (31 out of 33). Similarly, about 81% (17/21) and 93% (14/15) of *H. vermiformis* positive biofilm and water samples, respectively, corresponded to a disinfectant residual ≤ 0.1mg/L. In the biofilm, *P. aeruginosa* were negatively correlated with chloramine in PVC SDSs (r_s=-0.7894, P=0.002, Table S3.4) and chlorine in cement and PVC SDSs (r_s=-0.6523~-0.6043, P<0.05). In bulk water, negative correlations between *P. aeruginosa* and disinfectant were found in all except for iron SDSs (P=0.24).

3.6.5 Effect of Pipe Materials

Because both chlorine and chloramine residuals were depleted by 5.7d, this water age was selected for more detailed comparison of pipe effects (Figure 3.3). In chloraminated SDS biofilm, the gene copies of mycobacteria, *P. aeruginosa*, and *Acanthamoeba* were significantly lower (at least 10-fold) in iron SDSs compared to cement and/or PVC SDSs (P<0.05). This is in contrast to the highest 16S rRNA gene copies detected in iron SDSs (P<0.05). A similar pattern was observed in chlorinated

SDS biofilm, but significant differences were only noted for *Acanthamoeba* and 16S rRNA gene copies ($P<0.05$). In bulk water, the pipe effects on targeted microorganisms were distinct from those observed in the biofilm (Figure 3.3).

3.6.6 Effects of Interactions among Conditions

A significant interaction between pipe material and disinfectant type was observed for mycobacteria in 2.3d water and biofilm samples ($P<0.05$) and *Acanthamoeba* in 1d and 2.3d biofilm and 1d and 5.7d water samples ($P<0.05$) (Figure S3.4). Pipe material and disinfectant interactions were also found for *P. aeruginosa* in 3.6d water samples ($P<0.05$) (Figure S3.4).

3.6.7 Relationships Among Targeted Microorganisms

Moderate to strong correlations ($r=0.5518$-0.8603, $P<0.05$) existed among gene copy numbers of mycobacteria, *P. aeruginosa*, *Acanthamoeba*, and 16S rRNA in both biofilm and bulk water samples (Table S3.6). The correlations between *H. vermiformis* and other targeted organism were relatively weak ($r<0.5$, $P<0.05$) or insignificant ($P>0.05$), which may be an artifact of the low overall detection rate of *H. vermiformis*.

3.6.8 Persistence in Second Sampling Event

The second sampling (Figure 3.4) focused on the bulk water, based on the significant correlations of 16S rRNA gene and other targeted organisms in biofilm and bulk water. *Legionella* was newly detected in the second sampling in both chloraminated and chlorinated SDSs. *Legionella* was detected throughout all three chloraminated SDSs, except for 1d cement and iron samples, with gene copy numbers ranging from 214 to 1.1×10^5 copies/ml. In chlorinated SDSs, *Legionella* was detected in 2 (out of 3) 5.7d cement and iron water samples and 1 (out of 3) 3.6d PVC water samples. *Legionella* numbers were negatively associated with chloramines (r_s=-0.9078~-0.6927, P<0.01).

Both 16S rRNA and mycobacteria genes in the second sampling were comparable to those in first (Figure S3.5) in chloraminated SDSs, whereas reduced prevalences of *P. aeruginosa* and *Acanthamoeba* were noted. Only 3 samples (out of 36) were positive for *P. aeruginosa*. The detection frequency of *Acanthamoeba* in chloraminated SDSs decreased from 100%, 100%, and 83% to 58%, 16.7%, and 25% in PVC, iron, and cement SDSs, respectively. Conversely, *H. vermiformis* detection frequency increased by 16.7%, 50% and 25% in PVC, iron and cement SDSs, respectively. In chlorinated SDSs, mycobacteria were eradicated from cement water samples with water ages ≤ 3.6d and all PVC water samples in the second sampling, which is consistent with the 16S rRNA gene detection pattern between samplings. Only one sample was positive for *P. aeruginosa*, *Acanthamoeba*, and *H. vermiformis* in chlorinated SDSs. Similar to the first event,

moderate to strong correlations between targeted organisms were also found in the second event, except for *Acanthamoeba* versus *H. vermiformis* (r_s<0.4) (Table S3.7).

3.7 Discussion

3.7.1 SDS Water Chemistry

PCA (Figure 3.1) explained most of the variance of drinking water physiochemical properties throughout the six triplicate SDSs and revealed key patterns. The SDSs covered a range of drinking water chemistries, enabling a head-to-head investigation of the effect of pipe material, disinfectant, and water age on opportunistic pathogens.

Chloramine decayed faster than chlorine across all SDSs, which was unexpected as chloramine is considered to be more stable (Seidel et al., 2005). Further investigation ascribed rapid chloramine decay to ongoing nitrification, which was confirmed by presence of nitrifiers by q-PCR targeting ammonia monooxygenase (*amoA*) genes (Figure S3.6). Faster decay of chloramine versus free chlorine when nitrification is occurring has been observed by others in a few cases (Zhang et al., 2009). For example, Zhang & Edwards (Zhang and Edwards, 2009) reported that in the presence of nitrification, much more chloramine decayed than chlorine, even in relatively inert pipe materials (e.g., PVC, stainless-steel). Significantly higher free chlorine demand was observed in iron SDSs

compared to PVC due to high reactivity of iron, in agreement with previous studies (Hallam et al., 2002; Munavalli et al., 2009).

It was noted that disinfectants were nearly depleted when water age reached 5.7days for all SDSs. Faster depletion rates compared to real-world DWDSs may be related to the higher (room) temperature and lower flow rate (0.40 ml/min) used in this study, which was necessary to achieve 5.7day water age in lab-scale pipe systems. Although this likely had some effect on microbial growth, the SDSs in this study still generated a range of scenarios occurring in real-world DWDSs (e.g., drinking water nitrification).

3.7.2 Variance among Triplicates

For biofilm 16S rRNA gene copy numbers, reproducible results were observed in all three chloraminated SDSs. Higher variance was observed among chlorinated cement and PVC SDSs, which was likely a result of high variance in chlorine residuals among the triplicate SDS for each condition. Relative to total bacteria, similar or higher variances were observed for other targeted microorganisms. For example, *P. aeruginosa* was not consistently recovered from 3.6d iron SDS biofilm in either chloraminated or chlorinated SDSs, although the corresponding 16S rRNA gene copy numbers were all greater than 10^6 gene copies/cm^2. Higher variances of certain target microorganisms are possibly associated with their own unique characteristics, including susceptibility to

environmental factors, growth rates, and interactions with other microorganisms. Slight variance in physiochemical properties among triplicate pipes might turn a hostile environment into a favorable one for certain microorganisms. Moreover, heterogeneous distributions of microorganisms in biofilm (Wimpenny et al., 2000) and water samples also contribute to variance.

3.7.3 Efficacy of Drinking Water Disinfection for Total Bacteria and Opportunistic Pathogens

Negative correlations between total bacteria in biofilm and bulk water with disinfectant concentration confirmed expected dose-dependent limitation of bacterial growth. The importance of secondary disinfectant residual in controlling opportunistic pathogens was also apparent. The precise effects on opportunistic pathogens and/or their broader genera were dose-dependent and species-specific. For example, the presence of mycobacteria and *Acanthamoeba* in SDS sampling ports where relatively high disinfectants were measured (1d samples) confirmed their high resistance to standard drinking water disinfection (Coulon et al., 2010; Falkinham, 2003; Le Dantec et al., 2002; Taylor et al., 2000). The present study provides a benchmark with respect to other key target organisms.

Significant correlations between 16S rRNA gene copies and other targeted organisms in biofilm versus bulk water implies similar trends of bacterial/protozoa growth in water and biofilm or their release from biofilm. There was no apparent trend in the ratio of 16S rRNA gene copies in biofilm versus bulk as a function of disinfectant concentration (Figure S3.7, $P>0.05$), which is in contrast with one study reporting increased bulk HPC:total HPC ratio as chlorine residual decreased in DWDSs (Srinivasan et al., 2008). The difference may be associated with different experimental design (e.g., relatively higher surface to volume ratio in the present study) and that the present study employed q-PCR, which estimates the total number of bacteria, including non-cultivable and dead cells, whereas HPC only takes into account a certain sub-set of cultivable cells.

Both chlorine and chloramine are considered effective at reducing bacterial growth in DWDSs (Neden et al., 1992; Zhang and DiGiano, 2002). However, different efficiencies against opportunistic pathogens have been observed. For example, chloramine was reported to favor mycobacteria colonization, but inhibit *Legionella* (Moore et al., 2006). Different disinfection mechanisms of chlorine and chloramine against *M. avium* and *Acanthamoeba castellanii* were also reported (Dupuy et al., 2011; Luh and Marinas, 2007; Luh et al., 2008; Mogoa et al., 2011). To achieve 99.9% inactivation of *A. castellanii*, the Ct value of monochloramine is approximately 18 times of that of chlorine (Mogoa et al., 2011). In the present study, nitrification caused faster decay of chloramine,

which in turn resulted in relatively higher gene copy numbers of target organisms (*Legionella*, mycobacteria, *P. aeruginosa*, *Acanthamoeba*) compared to the chlorinated SDSs, especially in samples with water age ≤3.6d. Previous studies reported that the occurrence of nitrifying bacteria was correlated positively with the numbers of HPCs in drinking water (Lipponen et al., 2002; Skadsen, 1993). To the authors' knowledge, this is the first study demonstrating a link between nitrification and increased prevalence of opportunistic pathogens in drinking water systems.

Our results demonstrated that *H. vermiformis* were more susceptible to disinfectant compared to *Acanthamoeba*. However, the greatly decreased prevalence of *Acanthamoeba* as well as the persistence of *H. vermiformis* in the second sampling indicate that relative tolerance to disinfectant is not the only factor contributing to amoeba survival in drinking water. In spite of high susceptibility of *H. vermiformis*, some research still indicates relatively higher detection frequency and abundance of *H. vermiformis* compared to *Acanthamoeba* spp. in real-world DWDSs (Menard-Szczebara et al., 2008; Wang et al., 2012).

3.7.4 Pipe Material Influence on Drinking Water Biofilm and Interaction with Disinfectants

The effect of varying pipe materials and different reactors on total biofilm bacteria has been extensively studied (Camper et al., 2003; Jang et al., 2011; Manuel et al., 2007; Niquette et al., 2000). However, only a few studies have examined material influences on opportunistic pathogens (Dwidjosiswojo et al., 2011; Norton et al., 2004; van der Kooij et al., 2005). In the present study, significant material effects towards opportunistic pathogens and related organisms were mainly found for 5.7d samples, where the disinfectant residuals were depleted. This is in agreement with a previous study that pipe influence on biofilm density was far less than disinfectant type and concentration (Hallam et al., 2002). In both chlorinated and chloraminated biofilms, highest bacterial gene copy numbers were observed in iron SDSs, which is consistent with the general ranking of bacterial regrowth potential: iron>cement>PVC (Camper et al., 2003; Niquette et al., 2000). Iron rust not only increases pipe surface porosity and roughness for microbial attachment, but also can serve as a nutrient (Morton et al., 2005). Also, iron corrosion was found to interfere with both chlorine and chloramine disinfection (Lechevallier et al., 1990; Lechevallier et al., 1993), causing faster deterioration of water quality in iron SDSs. Thus, we did not observe improved "penetrating power" of chloramine versus chlorine for biofilm control in iron SDSs, as observed by LeChevallier et al. (Lechevallier et al., 1990; Lechevallier et al., 1993). When nitrification is absent, chloramine is expected to better penetrate iron biofilms (Zhang and Edwards, 2009).

In contrast to total bacteria, mycobacteria, *P. aeruginosa*, and *Acanthamoeba* were found to be the lowest in chloraminated iron SDSs (Figure 3.3), suggesting potential persistent benefits of chloramine for some opportunistic pathogens even after it is gone. The reasons for this are not clear. It is possible that unique conditions created in iron SDSs (e.g., relatively lower DO (Table S3.1), release of phosphorus (Morton et al., 2005), bioavailable Fe) facilitated the proliferation of some other dominant microbes that better compete for available ecological niches. Less pipe material influence was observed in chlorinated SDSs, which could relate to the relatively high variance of chlorine residual in 5.7d water samples. Pipe influence in bulk water and biofilm differed for total bacteria and other microorganisms, which is in agreement with previous work indicating that pipe effects were significant for total bacteria in biofilm, but not bulk water, in non-disinfected pipes (Norton et al., 2004).

Significant interactions between disinfectant type and pipe material on opportunistic pathogens and related organisms found in the present study demonstrate the complexity of chemical and biological processes in DWDSs. A similar interaction was also found for *M. avium* in a previous study, with lower *M. avium* numbers on copper pipes in chlorinated SDSs and on iron pipes in chloraminated SDSs (Norton et al., 2004).

3.7.5 Persistence and Temporal Variation of SDS Microorganisms

The emergence of *Legionella* and decreased prevalence of *P. aeruginosa* and *Acanthamoeba* during the second sampling demonstrated dynamic microbial communities in the SDSs. Similar 16S rRNA gene levels (mainly in chloraminated SDSs), but distinct patterns of other targeted organisms, indicates that the shift was in terms of the community composition and structure, not total biomass quantity. The difference could be a result of water source dynamics. The first sampling and the second sampling occurred in spring and winter, respectively. Season is linked to fluctuations in water chemistry (Mazari-Hiriart et al., 2005), metabolism (e.g., ATP) (van der Wielen and van der Kooij, 2010), and pathogen numbers (Mazari-Hiriart et al., 2005) in DWDSs, although in this work temperature was held constant. Another possibility is that drinking water biofilm continued to undergo succession with time. A three-year investigation of a model DWDS suggested that >500d may be required for the biofilm community structure to stabilize (Martiny et al., 2003). Moreover, aging of pipes affects succession of microorganisms. For example, Van der Kooij and co-workers reported that the inhibitory effect of copper pipes towards *Legionella* disappeared along with the accumulation of corrosion products in a >2-year study (van der Kooij et al., 2005). Therefore, long-term sampling (e.g., >2 year operation) is of interest in future studies in order to elucidate the complex temporal variation of opportunistic pathogens as the biofilm matures with the time.

3.7.6 Insight into SDS Management and Research Needs

This study provides a head-to-head comparison of disinfectant, pipe material, and water age on occurrence and persistence of opportunistic pathogens and related organisms. Natural occurrences of *Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*, *H. vermiformis* and *Acanthamoeba* spp. in the disinfected, lab-controlled SDSs indicate that even well-maintained and operated DWDSs can be a reservoir of opportunistic pathogens. Chloraminated SDSs, due to severe nitrification, harbored relatively higher bacteria and protozoa numbers at shorter water ages compared to chlorinated SDSs, especially in the second sampling, illustrating the importance of nitrification control when the chloramine is used as the secondary disinfectant. Disinfectant type and dose was demonstrated to exert the strongest influence on the microbial community, with species specific tolerances and responses. Pipe material, and its interaction with disinfectants, was also observed to be an important factor in governing microbial proliferation. Variation of microorganisms among triplicate SDSs and temporal variation between two sampling events demonstrated complexity and dynamics of microbial community structure and composition. Thus, microbial ecology is in great need of further study as a potential governing factor in stimulating or inhibiting opportunistic pathogen survival and persistence.

3.8 Acknowledgements

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3.10 Tables

Table 3.1 Spearman correlation rank analysis between 16S rRNA gene and disinfectant concentration

SDS	Sample type	Chloramine		Chlorine	
		r_s	p	r_s	p
Iron	Biofilm	-0.7478	0.005	-0.7368	0.01
	Water	-0.9454	<0.001	-0.9018	<0.001
Cement	Biofilm	-0.7272	0.01	-0.7460	0.005
	Water	-0.9371	<0.001	-0.4620	0.18
PVC	Biofilm	-0.6771	0.02	-0.8021	0.002
	Water	-0.6421	0.02	-0.6690	0.02

3.11 Figures

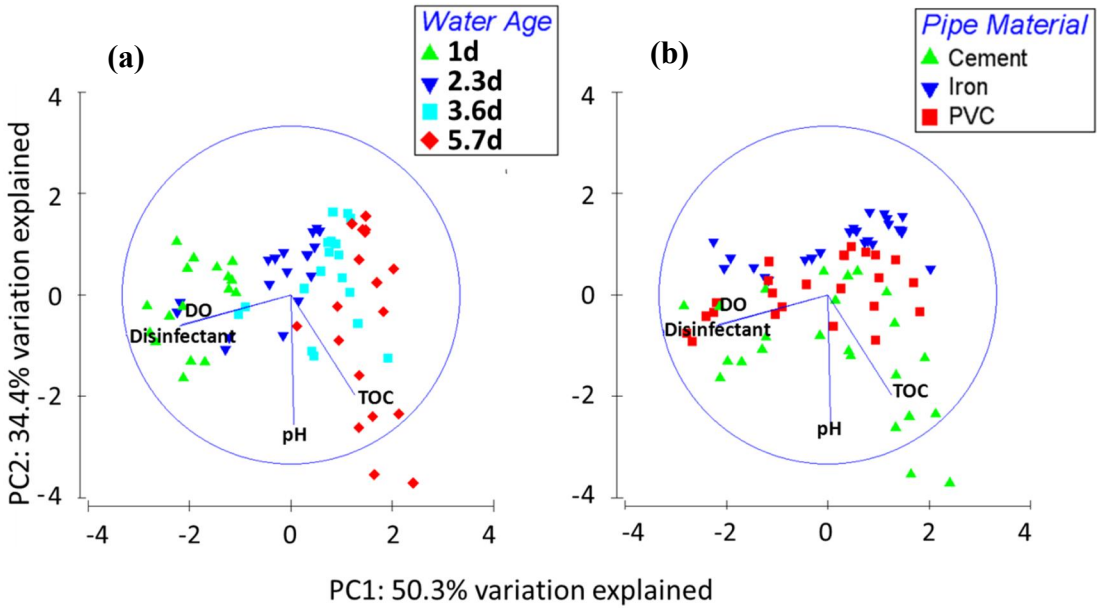
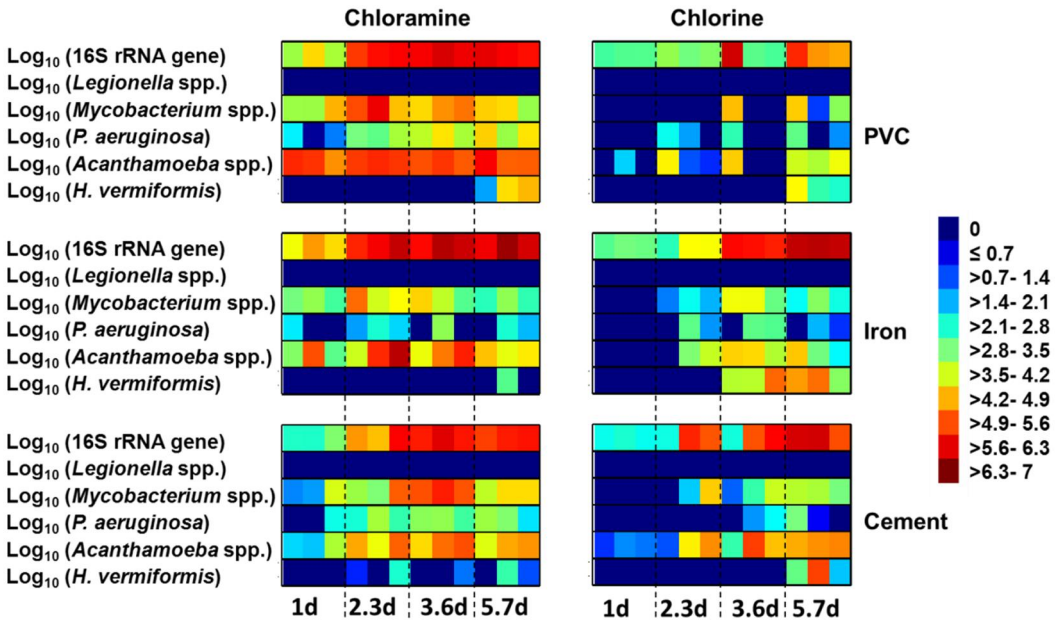
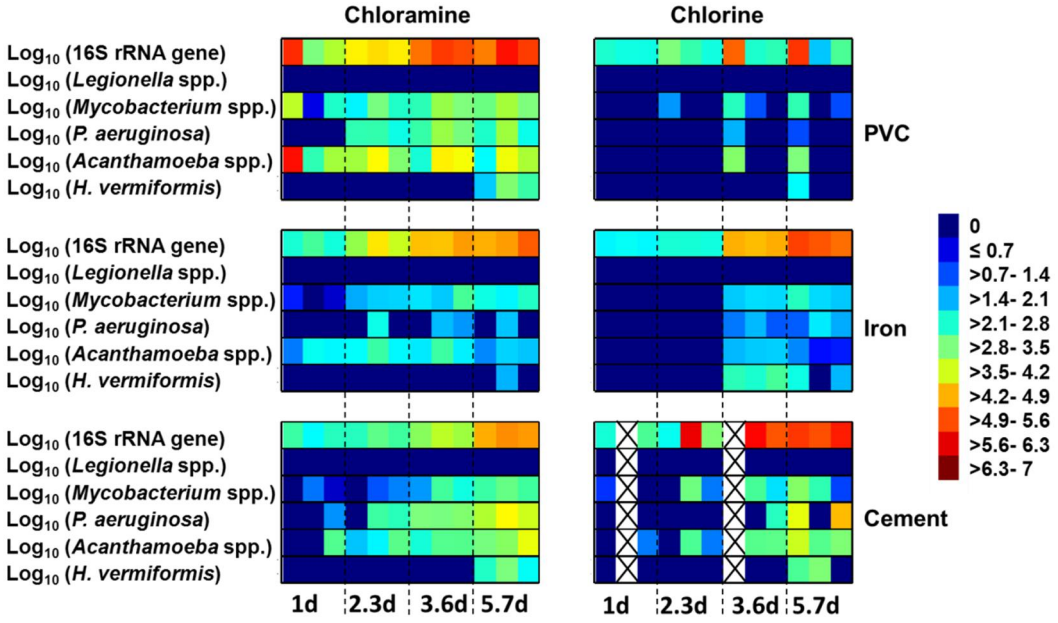


Figure 3.1 Principal component analysis (PCA) of pH, disinfectant concentration, DO, and TOC of water samples from simulated water distribution systems collected during the first sampling. (a) color-coded according to water age and (b) pipe material effect. PC1 and PC2 together explained 84.7% variance.



(a) Biofilm (gene copies/cm^2)



(b) Bulk water (gene copies/ml)

Figure 3.2 Detection of 16S rRNA, *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba* spp., and *H. vermiformis* genes in SDS (a) biofilm (gene copies/cm^2) and (b) bulk water (gene copies/ml) in the first sampling (6 months after SDS start-up). Data were $\log_{10}(x+1)$ transformed. ☒ indicates lost sample during processing.

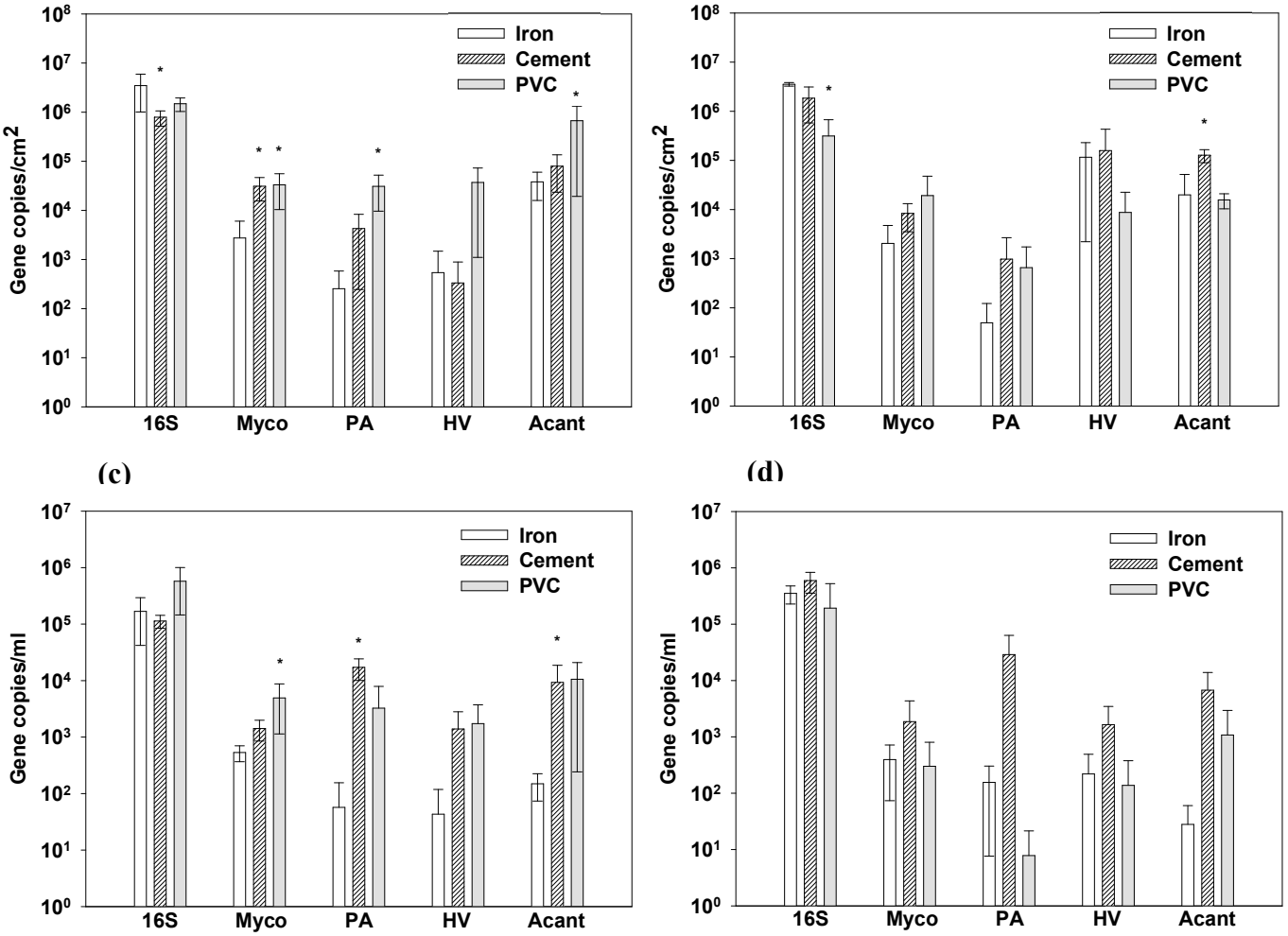


Figure 3.3 Gene copy numbers of 16S rRNA and other targeted microorganisms in 5.7d biofilm and bulk water samples. (a) chloraminated SDS biofilm; (b) chlorinated SDS biofilm; (c) chloraminated SDS water; (d) chlorinated SDS water. 16S: 16S rRNA gene, Myco: *Mycobacterium* spp., PA: *P. aeruginosa*, HV: *H. vermiformis*, Acant: *Acanthamoeba* spp. Error bars represent standard error of gene copy number of targeted microorganisms from triplicate pipes. * indicates significant difference compared to iron pipes (P<0.05).

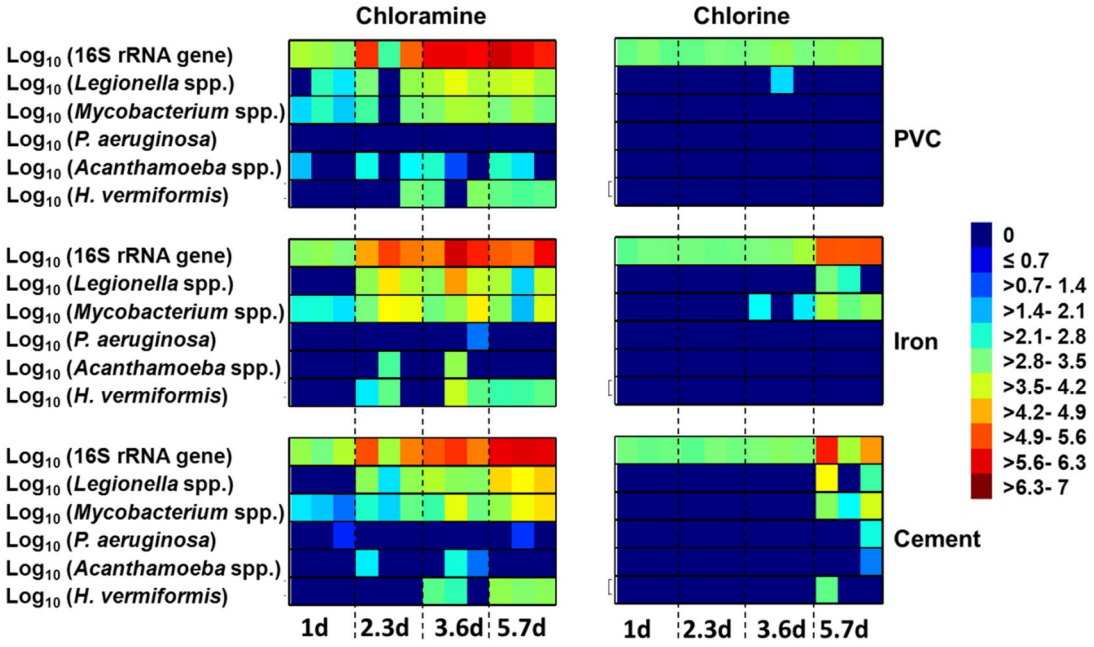


Figure 3.4 Detection of 16S rRNA, *Legionella* spp., *Mycobacterium* spp., *P. aeruginosa*, *Acanthamoeba* spp., and *H. vermiformis* genes in SDS bulk water (gene copies/ml) in the second sampling (14 months after SDS start-up). Data were $log_{10}(x+1)$ transformed.

3.12 Supplemental Materials

Table S3.1 Physiochemical parameters in SDSs (± standard error)

	Disinfectant concentration (mg/L)	pH	TOC (mg/L)	DO (mg/L)
		Chloraminated SDSs		
Iron 1d	1.73±0.17	7.56±0.09	1.49±0.10	6.85±0.13
Iron 2.3d	0.15±0.03	7.24±0.06	1.72±0.14	4.08±0.27
Iron 3.5d	0.07±0.01	7.17±0.03	1.70±0.15	2.32±0.23
Iron 5.7d	0.02±0.01	7.19±0.04	1.93±0.13	1.68±0.32
Cement 1d	2.65±0.32	7.63±0.10	1.61±0.41	8.12±0.20
Cement 2.3d	0.36±0.11	7.45±0.06	2.28±0.13	5.89±0.25
Cement 3.5d	0.06±0.01	7.37±0.04	3.31±0.82	4.53±0.32
Cement 5.7d	0.03±0.01	8.37±0.13	3.30±0.24	3.21±0.23
PVC 1d	1.52±0.16	7.50±0.06	1.72±0.17	7.16±0.20
PVC 2.3d	0.17±0.05	7.24±0.05	2.10±0.14	5.24±0.36
PVC 3.5d	0.04±0.004	7.20±0.03	2.51±0.23	4.41±0.47
PVC 5.7d	0.02±0.01	7.28±0.09	2.98±0.23	2.94±0.30
		Chlorinated SDSs		
Iron 1d	2.52±0.21	7.48±0.06	1.09±0.23	7.30±0.04
Iron 2.3d	0.75±0.13	7.44±0.05	1.64±0.14	5.58±0.16
Iron 3.5d	0.08±0.01	7.40±0.07	1.73±0.12	3.23±0.05
Iron 5.7d	0.08±0.01	7.38±0.11	1.99±0.23	0.92±0.10
Cement 1d	3.42±0.17	7.79±0.14	2.68±0.26	8.30±0.04
Cement 2.3d	1.99±0.37	7.66±0.03	2.69±0.44	7.21±0.11
Cement 3.5d	0.58±0.15	7.78±0.08	2.91±0.53	4.78±0.36
Cement 5.7d	0.06±0.01	8.96±0.13	3.36±0.54	2.97±0.32
PVC 1d	3.60±0.18	7.82±0.07	1.70±0.14	8.40±0.12
PVC 2.3d	2.57±0.43	7.55±0.04	1.91±0.16	7.25±0.50
PVC 3.5d	1.32±0.38	7.47±0.02	2.40±0.31	6.84±0.24
PVC 5.7d	0.40±0.13	7.68±0.14	2.73±0.36	5.07±0.32

Table S3.2 Spearman correlation rank analysis between mycobacteria and disinfectant concentration

SDS	Sample type	Chloramine		Chlorine	
		r_s	p	r_s	p
Iron	Biofilm	0.2610	0.40	-0.7208	0.01
	Water	-0.8677	<0.001	-0.8951	<0.001
Cement	Biofilm	-0.6083	0.04	-0.7772	0.003
	Water	-0.8441	<0.001	-0.4573	0.18
PVC	Biofilm	0.2105	0.51	-0.8336	<0.001
	Water	-0.4912	0.10	-0.7499	0.005

Table S3.3 Spearman correlation rank analysis between *Acanthamoeba* and disinfectant concentration

SDS	Sample type	Chloramine		Chlorine	
		r_s	p	r_s	p
Iron	Biofilm	-0.1904	0.55	-0.6786	0.02
	Water	0.3563	0.26	-0.8465	<0.001
Cement	Biofilm	-0.6154	0.04	-0.7671	0.004
	Water	-0.9001	<0.001	-0.8354	0.003
PVC	Biofilm	0.1649	0.61	-0.6899	0.01
	Water	0.0596	0.85	-0.6408	0.03

Table S3.4 Spearman correlation rank analysis between *P. aeruginosa* and disinfectant concentration

SDS	Sample type	Chloramine		Chlorine	
		r_s	p	r_s	p
Iron	Biofilm	-0.2487	0.44	-0.5348	0.07
	Water	-0.3672	0.24	-0.7528	0.005
Cement	Biofilm	-0.4098	0.18	-0.6043	0.04
	Water	-0.9296	<0.001	-0.7180	0.02
PVC	Biofilm	-0.7894	0.002	-0.6523	0.02
	Water	-0.6254	0.03	-0.6408	0.02

Table S3.5 Spearman correlation rank analysis between *H. vermiformis* and disinfectant concentration

SDS	Sample type	Chloramine		Chlorine	
		r_s	p	r_s	p
Iron	Biofilm	-0.4406	0.15	-0.9101	<0.001
	Water	-0.7343	0.01	-0.7238	0.01
Cement	Biofilm	-0.5380	0.07	-0.7310	0.01
	Water	-0.7343	0.01	-0.5207	0.12
PVC	Biofilm	-0.7599	0.004	-0.6528	0.02
	Water	-0.7599	0.004	-0.4812	0.11

Table S3.6 Correlation analysis between different targeted microorganisms in SDS in the 1st sampling

		mycobacteria	*P. aeruginosa*	*Acanthamoeba*	*H. vermiformis*
P. aeruginosa	B	0.6845 (<0.001)			
	W	0.6031 (<0.001)			
Acanthamoebae	B	0.7472 (<0.001)	0.5682 (<0.001)		
	W	0.7938 (<0.001)	0.6802 (<0.001)		
H. vermiformis	B	0.1966 (0.1)	0.2463 (0.04)	0.1531 (0.2)	
	W	0.4630 (<0.001)	0.4129 (<0.001)	0.2970 (0.012)	
16S rRNA gene	B	0.6291 (<0.001)	0.5518 (<0.001)	0.6515 (<0.001)	0.4398 (<0.001)
	W	0.8603 (<0.001)	0.5552 (<0.001)	0.7250 (<0.001)	0.4897 (<0.001)

Note: Correlation results were presented in the form of Spearman's Rank Correlation (r_s).
B= biofilm, W=bulk water.

Table S3.7 Spearman's Rank Correlation (r_s) between targeted microorganisms in SDS water in the 2nd sampling

	Legionella	mycobacteria	*Acanthamoeba*	*H. vermiformis*
mycobacteria	0.8564			
	P<0.001			
Acanthamoeba	0.4619	0.4436		
	P<0.001	P<0.001		
H. vermiformis	0.7836	0.6597	0.3294	
	P<0.001	P<0.001	P=0.005	
16S rRNA gene	0.8570	0.8768	0.4932	0.7099
	P<0.001	P<0.001	P<0.001	P<0.001

Cultures of *L. pneumophila* of known concentrations (3370, 337, 33.7, 3.4 cfu/ml) were serially diluted into 50 ml autoclaved drinking water, which were subjected to freeze drying and/or DNA extraction. The detection limit in 50 ml water samples by freeze drying for *L. pneumophila* is 34 CFU/ml, which is comparable to the membrane filtration method by filtering 1000 ml water (e.g., 32 CFU/ml, [1]). The recovery efficiency of freeze drying method varied from 13-31% depending on the concentration of the sample (compared to the direct DNA extraction).

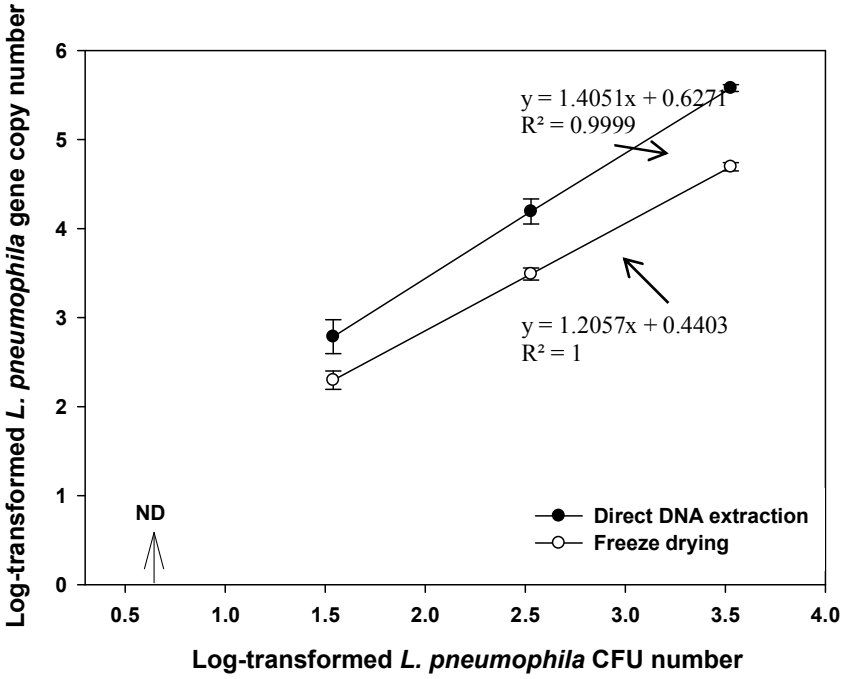


Figure S3.1 DNA recovery efficiency and detection limit of freeze drying concentration method prior to DNA extraction. Cells were spiked into 50 mL and subject to freeze drying versus spiking equivalent cells directly into DNA extraction tube. *L. pneumophila* was used as an example. ND=not detected. Linear regression was performed between gene copies numbers and CFU numbers.

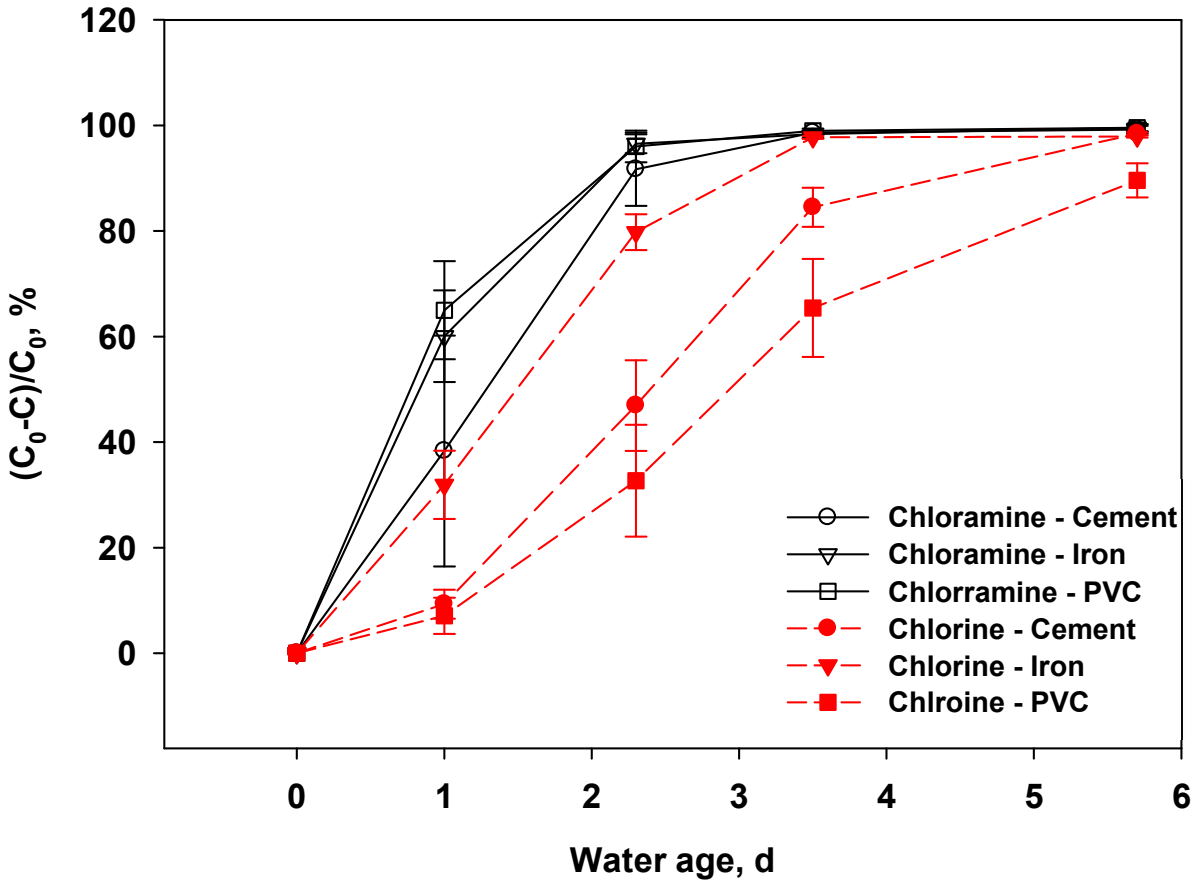


Figure S3.2 Chloramine and chlorine decay in six simulated distribution systems. Error bars represents standard error of nine measurements from triplicate pipes (3 ×3).

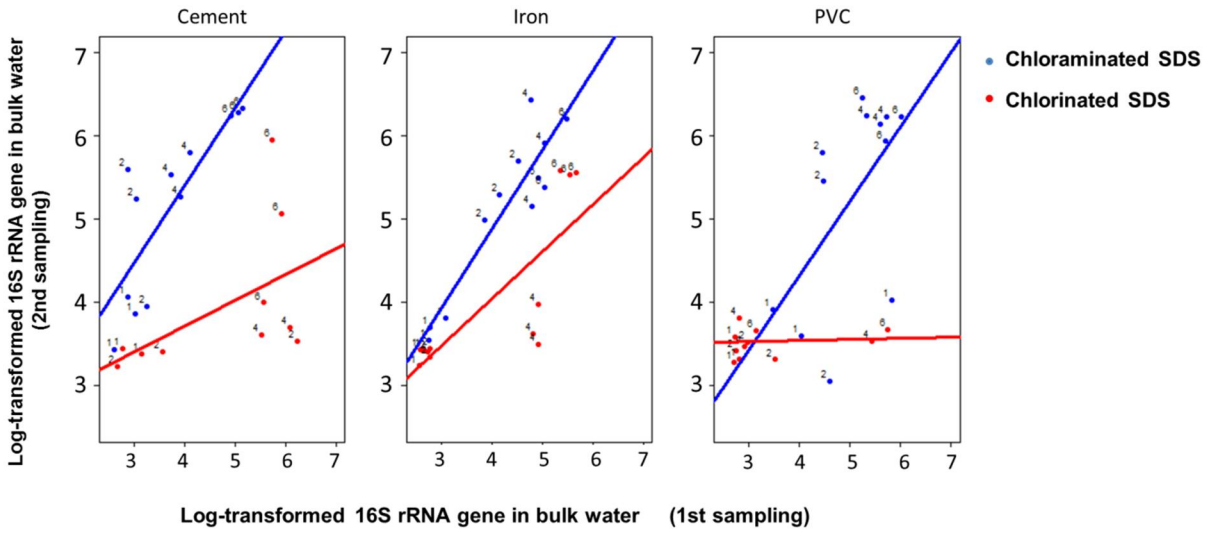
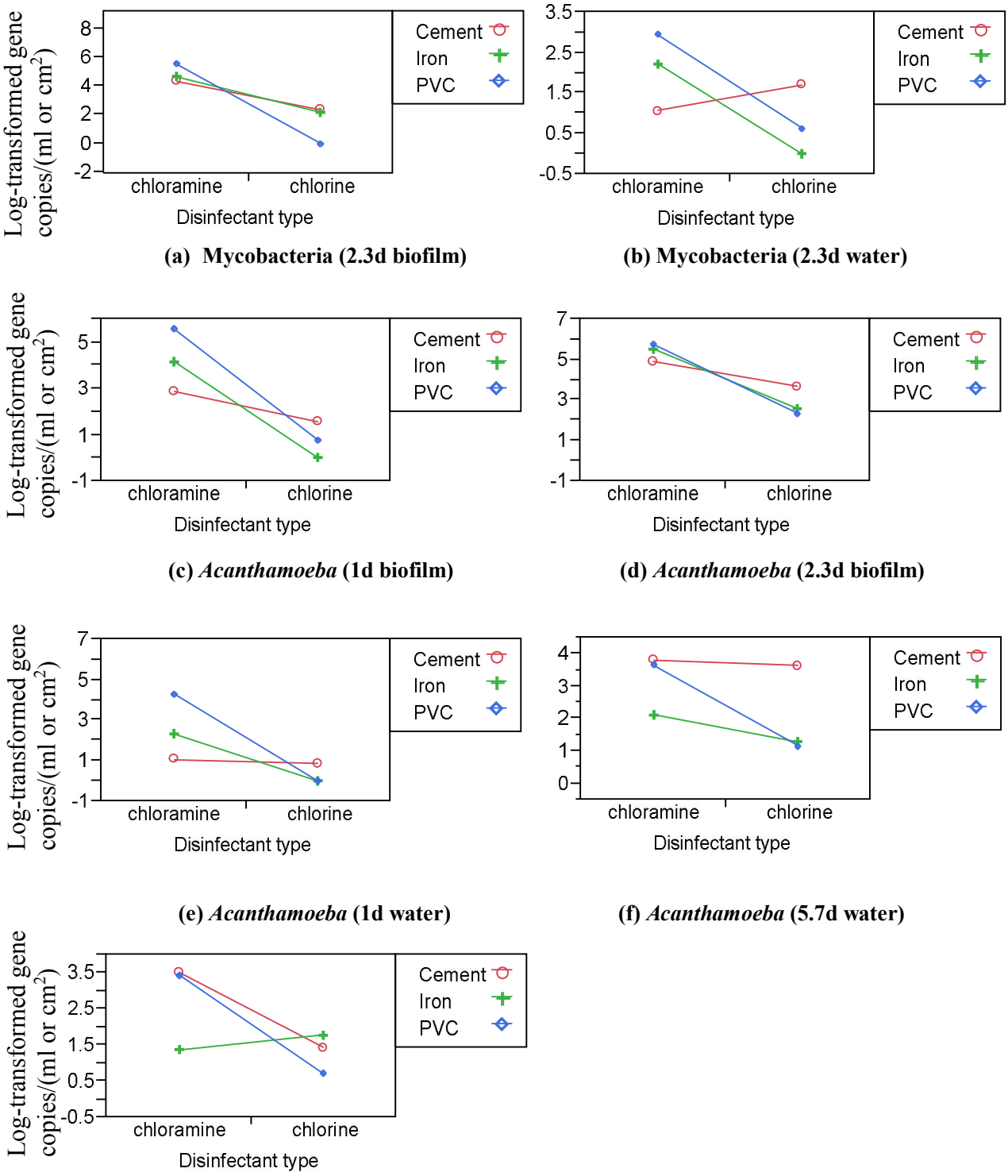


Figure S3.3 Log-transformed [$\log_{10}(x+1)$] 16S rRNA gene (gene copies/ml) in the bulk water during the 1st and 2nd sampling. The numbers beside the points indicate corresponding water age: 1=1d, 2=2.3d, 4=3.5d, 6=5.7d. Lines were generated by performing linear regression between 1st and 2nd sampling using after log-transformed data.



(a) Mycobacteria (2.3d biofilm)

(b) Mycobacteria (2.3d water)

(c) *Acanthamoeba* (1d biofilm)

(d) *Acanthamoeba* (2.3d biofilm)

(e) *Acanthamoeba* (1d water)

(f) *Acanthamoeba* (5.7d water)

(g) *P. aeruginosa* (3.6d water)

Figure S3.4 Interactions between disinfectant type and pipe material on targeted organisms (least square mean plots)

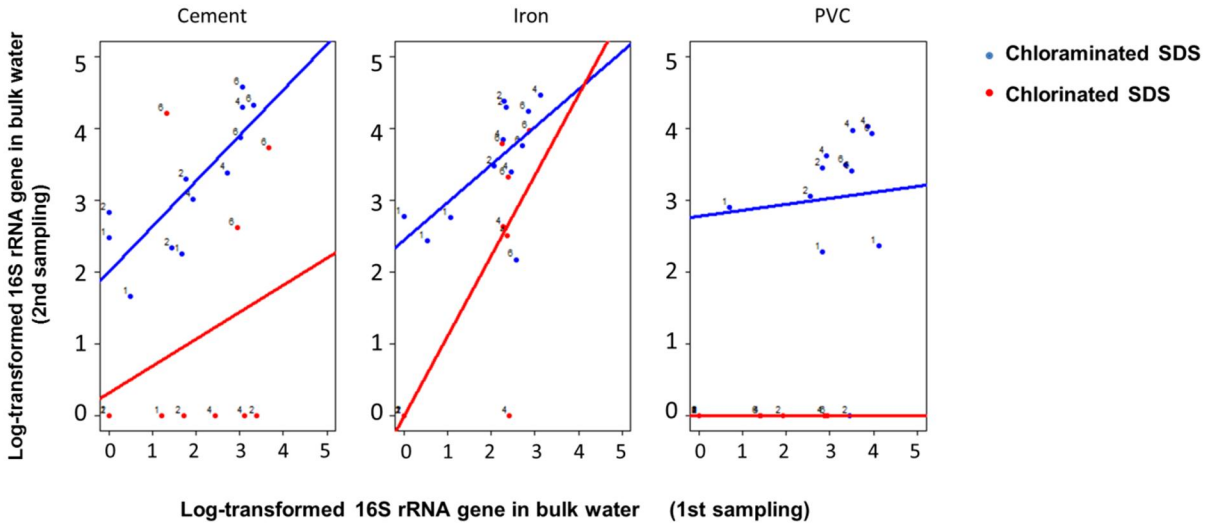


Figure S3.5 Log-transformed [$\log_{10}(x+1)$] *Mycobacterium* spp. (gene copies/ml) in the bulk water during the 1st and 2nd sampling. The numbers beside the points indicate corresponding water age: 1=1d, 2=2.3d, 4=3.5d, 6=5.7d. Straight lines were generated by performing linear regression between 1st and 2nd sampling using after log-transformed data.

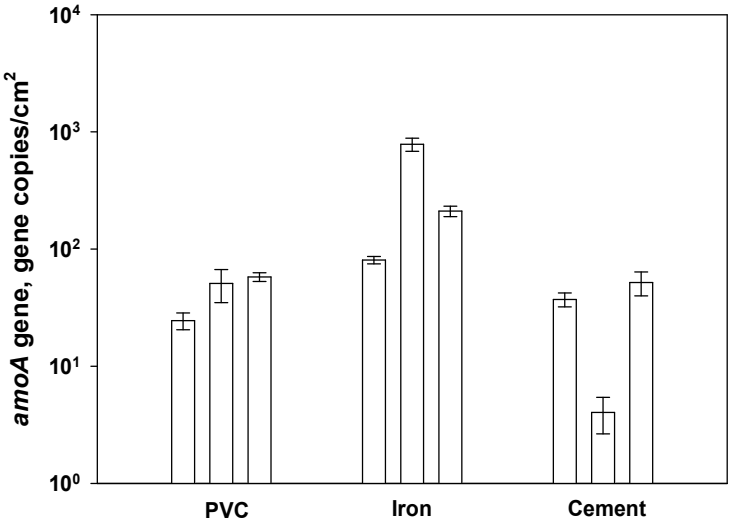


Figure S3.6 Ammonia monooxygenase (*amoA*) gene copy numbers in chloraminated SDSs 2.3d biofilm samples. Non-detection of *amoA* in chlorinated SDS 2.3d biofilm samples was also confirmed by q-PCR[1]. Three bars for each material represent three measurements from triplicate pipes. Error bars indicate standard error of triplicate q-PCR measurements for each sample.

[1] a*moA* q-PCR used a*mo*A gene specific primer set amoA1-F and amoA2-R (Rotthauwe et al., 1997) with annealing temperature of 60^0C. Primer specificity has been examined by cloning/sequencing of PCR products using drinking water samples.

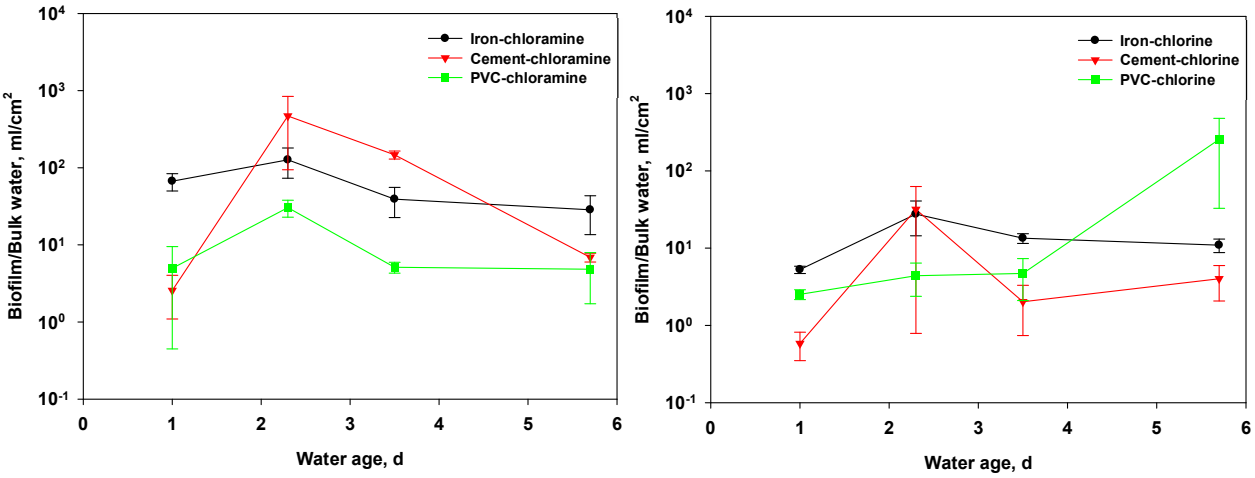


Figure S3.7 Biofilm to bulk water ratio as a function of water age in six SDSs. Left: chloraminated SDSs. Right: chlorinated SDSs

CHAPTER 4

Effect of GAC Pre-treatment and Disinfectant on Microbial Community Structure and Opportunistic Pathogen Regrowth

4.1 Authors

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4.2 Abstract

Opportunistic pathogens in potable water systems are an emerging health concern; however, the factors influencing their proliferation are poorly understood. Here we investigated the effects of prior granular activated carbon (GAC) biofiltration [GAC-filtered water, unfiltered water, and a blend (30% GAC filtered and 70% unfiltered water)] and disinfectant type (chlorine, chloramine) on opportunistic pathogen occurrence using five annular reactors (ARs) to simulate water distribution systems, particularly premise plumbing. GAC pre-treatment effectively reduced total organic carbon (TOC), resulting in three levels of influent TOC investigated. Quantitative polymerase chain reaction demonstrated natural colonization of *Legionella* spp., *Mycobacterium* spp., *Acanthamoeba* spp., *H. vermiformis* and *M. avium* on AR coupons. Cultivable mycobacteria and amoeba, including pathogenic species, were also found in bulk water and biofilm samples. In most cases, total bacteria and opportunistic pathogens were higher in the three undisinfected ARs, but the levels were not proportional to the level of GAC pre-treatment/TOC. Chlorine was more effective for controlling mycobacteria and

Acanthamoeba, whereas chloramine was more effective for controlling *Legionella*. Both chlorine and chloramine effectively reduced *M. avium* and *H. vermiformis* numbers. Pyrosequencing of 16S rRNA genes in coupon biofilms revealed a significant effect of GAC pre-treatment and disinfectant type on the microbial community structure. Overall, this study suggests that GAC treatment alone will not control pathogens, but that water treatment strategies do impact the microbial community, with associated trade-offs in terms of relative effectiveness against co-occurring opportunistic pathogens.

4.3 Keywords

Legionella, mycobacteria, amoeba, annular reactor, disinfectant, GAC, pyrosequencing

4.4 Introduction

Waterborne opportunistic pathogens, including *Legionella pneumophila,* non-tuberculosis mycobacteria (NTM), *Pseudomonas aeruginosa*, and *Acanthamoeba* spp., have become an emerging public health concern, as evidenced by elevated clinical and outbreak cases (Campese et al., 2011, CDC, 2011, Griffith et al., 2007). Opportunistic pathogens represent a unique challenge, particularly because they establish and grow in drinking water systems (Anaissie et al., 2002; Brunkard et al., 2011; Falkinham, 2011; Qian et al., 2010). For example, in the U.S., *Legionella* was the most frequently reported etiologic agent among drinking water outbreaks since 2001, most of which were associated premise (i.e., building) plumbing (Brunkard et al., 2011). Genetic and epidemiological evidence has also linked instances of NTM, *P. aeruginosa*, and *Acanthamoeba* infection to household or clinical tap water (Anaissie et al., 2002; Falkinham, 2011; Qian et al.,

2010). Thus, drinking water distribution systems (DWDSs), especially premise plumbing, demand attention as a significant transmission route for opportunistic pathogens. However, universally effective mitigation strategies have not yet been identified, in large part because of limited knowledge concerning the factors governing their proliferation in drinking water systems.

Granular activated carbon (GAC) filtration can be used either at the water treatment plant to remove dissolved organics by adsorption and biodegradation, or by consumers, using whole-building or point-of-use filters, to remove taste, odors, and sediments. Microbes readily colonize the high surface area of GAC, which may actually decrease downstream microbial regrowth by degrading assimilable organic carbon (Chien et al., 2008; Bouwer and Crowe, 1988). Notably, GAC filtration can sometimes remove pathogens, in one case resulting in <0.1-1.1 log and 1.3~2.7 log reduction for *Escherichia coli* and protozoan (oo)cysts, respectively (Hijnen et al., 2010). At the same time, GAC colonized by microbes could also influence microbial ecology by releasing attached biofilm cells into the water. GAC can also remove residual disinfectant (Sorlini and Corrivignarelli, 2005), which may further stimulate microbial growth. Considering this range of influence of GAC on water quality, it is not clear whether high level GAC/biological treatment will act as a barrier or a reservoir for opportunistic pathogens, or will otherwise alter the water chemistry in a manner that enhances or inhibits their regrowth in premise plumbing.

Secondary disinfectant residuals are the primary strategy for limiting microbial regrowth in DWDSs. In the U.S., many water utilities recently switched from chlorine to chloramines, since chloramines are generally more stable and thought to produce less

disinfection by-products (Seidel et al., 2005). DWDS field studies suggest that switching from chlorine to chloramines may aid in decreasing *Legionella* colonization; however, preliminary field data indicate that mycobacteria prevalence can correspondingly increase (Moore et al., 2006; Pryor et al., 2004). Lab studies have also indicated a range of effective dosages, mechanisms, and disinfection models among *L. pneumophila*, *M. avium*, *Acanthamoeba* (Dupuy et al., 2011; Luh and Marinas, 2007; Luh et al., 2008; Mogoa et al., 2011), suggesting that the disinfectant type is an important factor affecting opportunistic pathogen control. However, such studies were mainly performed using batch reactors with high concentrations of lab-cultivated cells and high nutrient levels, which may not be representative of typical drinking water environments. Environmental strains can behave entirely distinct compared to medium-grown strains in terms of their susceptibility to disinfectant (Kuchta et al., 1985), growth environment (Wadowsky et al., 1991), and other properties [e.g., resistance to infection (Berry et al., 2010)]. Thus, there is a need to understand the effect of disinfectants under drinking-water relevant conditions, especially with naturally-occurring environmental strains and under the flow and chronically low disinfectant residual conditions encountered in premise plumbing systems (Zhang et al., 2009; Zhang et al., 2008).

The aim of this study was to investigate the effects of GAC biofiltration and disinfectant type on the occurrence and persistence of opportunistic pathogens (*L. pneumophila*, *Mycobacterium avium*, and *P. aeruginosa*) and two amoeba hosts (*Acanthamoeba* spp. and *H. verimiformis*) using annular reactors (ARs) under no or low disinfectant conditions representative of near ends of DWDSs or in premise plumbing. Genus-level *Legionella* and mycobacteria were also investigated because they contain many

pathogenic species other than *L. pneumophila* and *M. avium* (Falkinham, 2009; Muder and Yu, 2002). ARs were maintained in Pinellas County, FL, as a special emphasis of the study was to conduct the first lab confirmation of trends from an earlier field sampling there, which suggested that a switch to chloramines might have decreased prevalence of *L. pneumophila* while increasing *Mycobacterium* in premise plumbing (Moore et al., 2006). Quantitative polymerase chain reaction (q-PCR) was used to quantify target microbes because of its advantages of low detection limit, high specificity, and high throughput (Wang et al., 2012b). To explore the effects of GAC filtration and disinfectant type on the broader bacterial community, bacterial 16S rRNA genes were profiled by pyrosequencing.

4.5 Materials and Methods

4.5.1 Simulated Distribution System Setup and Operation

Five ARs (Biosurface Technologies, Bozeman, MT) were operated at the Pinellas County Utility Lab (Figure 4.1). Each AR housed twenty PVC coupons, which allowed biofilm to establish under conditions representative of the end of the DWDS/premise plumbing. Prior to the experiment, each reactor was sterilized in an autoclave at 121ºC and 15 pounds per square inch (psi) for 30 min. PVC slides were sterilized with 70% ethanol and air-dried before placing into the reactors. The assembled ARs were finally rinsed by pumping sterile DI water through the system for 3h. All reactors were operated at a hydraulic residence time of 1.9 h (flow rate = 8 ml/min) at 24°C during study. The ARs were acclimated about 6 weeks prior to commencing weekly sampling of biofilm on PVC coupons, over an additional 6 week period.

4.5.2 Source Water and Preparation of Annular Reactor (AR) Feed

The AR feed water was collected in 55-gallon plastic drums (designated for drinking water) from two sampling locations at the Pinellas drinking water treatment plant (Figure 4.1). Chlorinated water (0.5-1 mg/L chlorine residual) collected from the Above Ground Storage Tank effluent line was stagnated for 24-48 h to remove chlorine and then passed through two GAC filters in a recirculating mode (the 2nd filter) to create a highly treated GAC-biofiltered water. The GAC-biofiltered water was mixed with unfiltered dechlorinated water at ratios of 100:0, 30:70, and 0:100 to prepare feed water of AR1-AR3, respectively. The feed water for the remaining two ARs was collected after the Forced Draft Aerators (which remove sulfur from the water) and prior to the addition of chlorine. Ten-liter batches of water were prepared by adjusting the total chloramine concentration to a typical target of 1.0 mg/L (1.0 mg/L residual with 1 mg/L NH_3-N) or the free chlorine to a target of 0.4 mg/L. The levels of disinfectant in the feed water were adjusted to the target values daily, ensuring that coupons and water in each AR water was regularly exposed to disinfectant. However, consistent with field experiences monitoring premise plumbing in this water system and known persistence of each disinfectant, free chlorine was often absent from the reactor effluent whereas chloramine residual was always (> 0.4 mg/L) present. Thus, the disinfectant regime of premise plumbing or ends of distribution systems in which opportunistic pathogens amplify was successfully replicated. Daily monitoring and adjustment of chlorine and chloramine residuals was performed in order to maintain a relatively constant disinfectant level in the feed water for AR4 and AR5.

4.5.3 Water Quality Analysis

Influent and effluent temperature, pH, conductivity, turbidity, total organic carbon (TOC), free chlorine and total chlorine residual were measured weekly. pH and temperature were monitored using an Oakton pH 10 series meter (Oakton Instruments, Vernon Hills, IL). Conductivity was measured using an Oakton CON6 meter (Oakton Instruments). Turbidity was measured using a 2100P portable turbidimeter (HACH, Loveland, CO). Free chlorine and total chlorine residual were measured using a HACH DR 890 colorimeter according to HACH DPD colorimetric method based on manufacturer's instruction. TOC was measured on an Aurora 1030W Analyzer (OI Corporation, College Station, TX) according to standard method 5310C (APHA, 1998).

4.5.4 Sample Processing and DNA Extraction

AR influents and effluents were sampled on a weekly basis for biological analysis. One-liter water samples were filtered through sterile 0.22 μm-pore-size mixed cellulose ester filters (Millipore), which were fragmented using sterilized tweezers and placed in 2 ml Lysing Matrix A tubes (MP Biomedicals, Solon, OH). For biofilm samples, three PVC coupons were aseptically extracted from each AR on each sampling event, after which three new sterilized PVC coupons were replaced in order to maintain the same surface area. Sterile cotton swabs were used to scrape off the front part of the PCV coupons and the tips placed into a 2 ml Lysing Matrix A tube for DNA extraction. Lysing Matrix A tubes containing filters and swab tips were shipped overnight to Virginia Tech on dry ice. DNA was extracted using a FastDNA® SPIN Kit (MP Biomedicals) according to

manufacturer instruction.

4.5.5 q-PCR

q-PCR was used to quantify *Legionella* spp., *L. pneumophila*, *Mycobacterium* spp., *M. avium*, *Acanthamoeba* spp., *H. vermiformis*, *P. aeruginosa* and 16S rRNA genes using a CFX96 real-time system (Bio-Rad, Hercules, CA) in a final volume of 10 µl. All Q-PCR assays were subject to extensive validation with respect to specificity, limit of quantification, and relationship between colony forming units and genome units, as described recently (Wang et al., 2012a).

4.5.6 Pyrosequencing and Phylogenetic Analysis

Biofilm samples from the 4th sampling was selected for microbial community profiling by pyrosequencing targeting 16S rRNA genes, which was performed at the Research and Testing Laboratory, Lubbock, TX, USA (http://www.researchandtesting.com/). Briefly, diluted biofilm DNA extracts (20 µ g/ µ l) were amplified with barcode-labeled forward primer 341f and 907r as follows: initial denaturation at 95℃ for 5 min, 30 cycles of 94℃ for 30 s, 54℃ for 45s, 72℃ for 60s, followed by a final extension 72℃ for 10min. PCR product from each sample was quantified and mixed proportionally with each other prior to pyrosequencing on a Roche 454 FLX Titanium platform (Roche, Nutley, NJ). The average number of pyrotags for these samples (n=15) was 5,105±3,435 (1,187-14,413).

Sequences were processed and analyzed according to company protocol (http://www.researchandtesting.com/docs/Data_Analysis_Methodology.pdf) (Ishak et al., 2011). Briefly, sequences were subject to de-noising, chimera check, and quality check

prior to taxonomic identification. Sequences were classified at the appropriate taxonomic levels based on the following criteria: 97% for species, 95-97% for genus, 90-95% for family, 85-90% for order, 80-85% for class, and 77-80% for phyla. Sequences with all blast hits falling below 77% identity or with a high score pair coverage below 75% were excluded from further study (n=0-14 for each sample, <0.25%). Taxonomic output for each sample (i.e., relative abundance) was used to calculate the Shannon diversity index and Bray-Curtis similarities using Primer-E 6.0 (Plymouth, United Kingdom), based on the latter, multidimensional scaling (MDS) and analysis of similarity (ANOSIM) were performed as described previously (Wang et al., 2012a). 2-stage MDS was used to examine the effects of taxonomic aggregation on data analysis using Primer-E 6.0 (Somerfield and Clarke, 1995).

4.5.7 Enumeration of *Legionella* spp., Mycobacteria and Amoeba Species by Culturing and Microscopy

Two batches of water and biofilm samples were cultivated for heterotrophic plate count (HPC), *Legionella*, mycobacteria, *P. aeruginosa* or examined for amoeba species by microscopy. Water samples were directly plated without further concentration. Biofilm samples were prepared by suspending cotton swabs of PVC coupons in 2 ml phosphate-buffered saline solution. One hundred microliters of the suspension was used for plating water and biofilm samples. Bulk water HPC were monitored according to Standard Method 9215 using the spread plate method with R2A medium (APHA, 1998). Cultivation of *Legionella* was performed on Buffer Charcoal Yeast Extract agar following a 30 minute pretreatment at 50℃ (Leoni and Legnani, 2001). Mycobacteria were

recovered and identified as described by Falkinham et al. (Falkinham et al., 2008). Amoebae were enumerated using a Petroff-Hauser counter.

4.5.8 Statistical Analysis

All statistical analyses were performed using R (http://www.r-project.org/). Gene copy numbers were log transformed (log(x+1)) prior to analysis. Parametric one-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis one-way ANOVA was performed to compare target microbes in biofilm and bulk water sample groups depending on the normality and variance of the data, which was followed by multiple comparison analysis (pairwise t test or kruskalmc in R) for further paired comparison of sample groups. The nonparametric Wilcoxon rank sum test was used to compare relative abundance of bacteria in AR biofilm. Correlation analysis was used to identify the associations between pyrosequencing results and q-PCR results using non-parametric Spearman Rank Correlation analysis (r_s). Statistical significance was set at $p<0.05$.

4.6 Results

4.6.1 Feed Water Quality

The average values of pH, temperature, turbidity, and conductivity varied in the range of 8.05-8.34, 22.8-24.0℃, 0.24-0.45 NTU and 385-415 μS/cm among the five ARs, respectively. The pH of AR3 was significantly lower than AR1 and AR2 in influent and effluent ($p<0.05$). Except slightly higher turbidity observed in AR5 effluent relative to AR2 effluent ($p<0.05$), no significant differences were observed in temperature, turbidity, or conductivity or influents and effluents among the five ARs ($p>0.05$).

Figure 4.2 presents the influent TOC concentrations. As expected, the GAC/biological pre-treatment successfully reduced the TOC by 37-71% over the 6 week study. Overall TOC removal was highest when the filter was newest (week 1). The first GAC filter clogged frequently, presumably due to rapid bio-growth, as evidenced by formation of thick slimes, which required the filter to be replaced almost monthly. The extent of TOC reduction in the blended 30% GAC filtered and non-filtered water was consistent with expectations based on the dilution factor.

4.6.2 Total Bacterial 16S rRNA Genes

There was little effect of GAC biofiltration on 16S rRNA gene copy numbers in the biofilm or bulk water (Figure 4.3). The only significant difference was that AR2 (30% GAC-filtered water) yielded a slightly higher biofilm density of 16S rRNA genes ($p < 0.05$, compared to all other annular reactors).

Both chlorine and chloramine tended to slightly decrease the levels of 16S rRNA genes in the biofilm, influent, and effluent (Figure 4.3). The chlorinated water resulted in the lowest levels of 16S rRNA genes in the biofilm ($10^{5.4}$ gene copies/cm^2) ($p < 0.05$, compared to the reactors without disinfection, but not for chlorine versus chloramine). The lack of statistical difference for the disinfected water influents and effluents (AR4 and AR5) is likely due to high variation in 16S rRNA genes from week to week (5×10^2-2×10^5 gene copies/ml for chloraminated water and 6×10^3-3×10^5 gene copies/ml for chlorinated water).

Similarly, HPCs in corresponding influents and effluents of AR1-3 were not significantly

different from each other (P>0.05) (Figure S4.1). The average effluent HPC was about 1.2 log higher and 0.8 log higher than the influent, for AR4 and AR5, respectively, but the differences were not statistically significant (P>0.05).

4.6.3 Natural Occurrence of *Legionella* spp., *Mycobacterium* spp., *Acanthamoeba* spp. and *H. vermiformis* in Annular Reactors by q-PCR

Legionella spp., *Mycobacterium* spp., *Acanthamoeba* spp., *H. vermiformis*, *L. pneumophila*, *P. aeruginosa, M. avium* were enumerated in biofilm and water samples using q-PCR (Figure 4.4). However, *L. pneumophila* and *P. aeruginosa* were not detected in any of the annular reactors throughout this investigation.

4.6.3.1 *Legionella* spp.

GAC biofiltration did not influence *Legionella* spp. biofilm densities, with average densities from $10^{4.1}$ to $10^{4.5}$ gene copies/cm^2 in AR1-AR3 (Figure 4.4a). Chlorination of unfiltered water also did not significantly affect *Legionella* spp. in biofilm. (AR3 vs AR4, P>0.05). However, AR5 (fed with chloraminated water) had significantly lower *Legionella* spp. density in the biofilm than the other annular reactors (P<0.05), with an average density of $10^{3.2}$ gene copies/cm^2. The weak disinfection effect of chlorine towards *Legionella* spp. in AR4 biofilm was in agreement with the significant increase of *Legionella* spp. in AR4 effluent (P<0.05).

4.6.3.2 *Mycobacterium* spp.

The densities of *Mycobacterium* spp. in the biofilm of all five ARs were relatively higher compared to the other organisms, averaging $10^{5.1}$, $10^{5.1}$, $10^{4.6}$, $10^{4.5}$, and $10^{4.9}$ gene

copies/cm^2 in AR1 to AR5, respectively (Figure 4.4c). AR3 and AR4 harbored lower *Mycobacterium* spp. densities than AR1, AR2 and AR5 ($P<0.05$). Growth of *Mycobacterium* spp. was evidenced in AR4 and AR5 by comparing the influent and effluent, with an average increase of 1.4 log and a 1.0 log in AR4 and AR5, respectively, though only the increase in AR4 was significant ($P=0.005$, Figure 4.4d).

4.6.3.3 *M. avium*

The abundances of *M. avium* were relatively low in the biofilms of the five ARs, with the highest density of 404 gene copies/cm^2 detected in AR2 (30% GAC- filtered water) (Figure 4.4i). Both chlorine and chloramine disinfection dramatically decreased *M. avium* densities in the biofilms of AR4 and AR5 ($P<0.05$). There was no significant difference between chlorine and chloramine disinfection towards *M. avium* in the biofilm ($P>0.05$). *M. avium* was not detected in the bulk water.

4.6.3.4 *Acanthamoeba* spp.

The average densities of *Acanthamoeba* spp. in the biofilm were approximately $10^{4.8}$, $10^{5.1}$, and $10^{4.5}$ gene copies/cm^2 in AR1, AR2, and AR3, respectively (Figure 4.4e). *Acanthamoeba* biofilm density in AR2 was significantly higher than AR3 ($P<0.05$). Chlorination of water (AR4) dramatically reduced the average *Acanthamoeba* density to $10^{2.1}$ gene copies/ cm^2 ($P<0.05$, compared to AR1-AR3). However, chloramine did not have a significant effect on *Acanthamoeba* spp., relative to AR3 (0% GAC-filtered water) ($P>0.05$).

In the bulk water, both chlorine and chloramine decreased *Acanthamoeba* to <18 gene copies/ml in the influent (AR4 and AR5). However, about 3 log increase was observed in chloraminated water effluent (Figure 4.4f). This was in agreement with high levels of *Acanthamoeba* in AR5 biofilm.

4.6.3.5 *H. vermiformis*

The average densities of *H. vermiformis* varied from $10^{4.0}$-$10^{5.7}$ gene copies/ml in AR1-3, with the lowest detection in AR2 (Figure 4.4g). Both chlorine and chloramine significantly decreased the *H. vermiformis* densities in the biofilm of AR4 and AR5, relative to ARs1-3 ($P<0.05$). Lower *H. vermiformis* densities (approximately 1 log less) were also observed in influents and effluents in AR4 and AR5, respectively, compared to ARs1-3 (Figure 4.4h).

4.6.4 Detection of *Legionella* spp., *Mycobacterium* spp. *P. aeruginosa* and Amoeba by Culturing and Microscopy

No cultivable *Legionella* were detected after 10-day incubation of biofilm or water samples. Mycobacteria were frequently detected in AR biofilm and water. However, poor reproducibility of plate counting for mycobacteria prevented accurately reporting numbers. Plating variance was associated with overgrowth of non-target microorganisms, which is a major challenge in recovering mycobacteria from environmental samples (Radomski et al., 2010). Identification of mycobacteria isolates revealed that there were viable *M. chelonae*, *M. mucogencum*, *M. gordonae*, *M. abscessus*, and *M. kansasii* in AR biofilm. Amoeba species were sporadically detected in the influents, effluents, and

biofilms of the five ARs. Positive readings occurred only in one or two of five repeated measurements for one sample, with the maximal density in water (influent/effluent) about 2×10^4 cells/ml and 2.3×10^3 cells/cm^2 in biofilm.

4.6.5 Effect of GAC Filtration and Disinfectant on Microbial Communities

A total of 76,571 sequences distributed among 15 bacterial phyla were retrieved from the biofilm samples, with Proteobacteria as the most abundant in all of the samples (relative abundance > 77%) (Figure S4.2). Other commonly detected phyla (relative abundance >1%) included Bacteroidetes, Actinobacteria, Firmicutes, Acidobacteria, Planctomycetes, and Verrucomicrobia, the latter three were reduced below detection in disinfected ARs, except that Acidobacteria were detected in one out of three samples from disinfected ARs (<1%). AR biofilm phyla distributions were similar to other pyrosequencing studies of drinking water treatment plants and DWDS biofilms (Hong et al., 2010; Pinto et al., 2012).

A total of 30 classes were detected among the ARs (Figure S4.3). The common dominant classes were α-Proteobacteria (26-72%), β-Proteobacteria (10-34%), γ-Proteobacteria (3-12%), and Actinobacteria (2-6%). GAC biofiltration decreased the relative abundance of α-Proteobacteria, but increased that of δ-Proteobacteria, Sphingobacteria, and Cytophaga (AR1 vs AR3, $P<0.05$). Reduced β-Proteobacteria, δ-Proteobacteria, Sphingobacteria, Cytophaga, Clostridia and Acidobacteria, as well as elevated α-Proteobacteria, γ-Proteobacteria, Flavobacteria and ε-Proteobacteria were observed in disinfected ARs, in terms of relative abundance ($P<0.05$). Disinfection significantly decreased the Shannon diversity index from 1.88 ± 0.05, 1.78 ± 0.06, 1.59 ± 0.02 in

AR1-3 to 1.03±0.03 and 1.23±0.12 in AR 4 and AR5 ($P<0.05$).

Greatest contrast among the AR conditions was observed at the genus-level, with 303 genera identified. Among the α-Proteobacteria, the genera *Sphingobium* (53% of total sequences) and *Sphingomonas* (9.6% of total sequences) were the main components in AR4 while *Sphingomonas* (40% of total sequences) was the sole largest component in AR5. In ARs1-3, α-Proteobacteria were more evenly distributed among different genera (<7 % for each genus), including *Pedomicrobium*, *Rhodobacter*, *Brevundimonas*, *Methylocella*, *Sphingomonas*, *Hyphomicrobium*, *Nitratireductor*, *Rhizobium*, *Brydyrhizobium*, and *Novosphingobium*.

A 3-D MDS plot based on bacterial genus-level dissimilarity matrix is presented in Figure 4.5. A tight cluster of resemblance matrices derived from species, genus, and family levels was noted (Figure S4.4). The most distinct separation was observed between disinfected and undisinfected ARs ($R=1$, $P<0.001$ by ANOSIM analysis). Grouping of samples was also found among ARs receiving different proportions of GAC-filtered water ($R=1$, $P<0.001$ by ANOSIM analysis).

4.6.6 Identification of Opportunistic Pathogens by Pyrosequencing

Legionella spp. were detected by pyrosequencing, with relative abundances varying from 0 to 0.46% in AR biofilm samples. Sequences with highest matches to *L. longbeachae* (41 sequences from AR1 and AR2, I=96.45%-97.21%), *L. lytica* (3 sequences from AR1, I=97.23%) and *L. pneumophila* (3 sequences from AR3, I=97.01%) were retrieved, though most sequences could only be classified at genus level. Consistent with q-PCR,

mycobacteria were detected all biofilm samples, with average relative abundance of 2.2 ±1.7%. Sequences with highest matches with *M. mucogenicum* (I>97%), *M. chelonae* (I>97%), *M. arupense* (I>99%), *M. gordonae* (I=100%), *M. pallens* (I>99%), *M. houstonense* (I=100%) were found, but most sequences were unclassified mycobacteria. Only one sequence from AR3 was classified as *P. aeruginosa* (I=100%). Other potential opportunistic pathogens such as *Acinetobacter*, *Xanthomonas*, *Aeromonas* were also sporadically found in either disinfected or non-disinfected ARs at low relative abundances (<0.5%).

4.7 Discussion

4.7.1 Effect of GAC Pre-treatment on Total Bacteria and Opportunistic Pathogens

GAC biofiltration effectively removed TOC and the blends of treated and untreated water achieved a range of influent TOC. While overall TOC removal efficiency decreased with time as sorptive capacity of the media was gradually exhausted, significant biological treatment was sustained as in previous full- and pilot- scale studies using GAC biofilters to remove natural organic matter (Swietlik et al., 2002; Velten et al., 2011). In this study, there was no apparent trend observed between influent TOC level and 16S rRNA gene copy numbers (proxy for total bacteria) in the ARs. Even though significant differences were observed in some cases among the biofilms of ARs1-3 for 16S rRNA genes, *Mycobacterium* spp., *Acanthamoeba* spp., and *H. vermiformis*, the differences did not correlate with TOC or level of GAC pre-treatment. This suggests either threshold responses of pathogens to these variables, or that GAC pre-treatment contributes to other complexities in the water, such as microbial community composition, which in turn

influences occurrence of opportunistic pathogens.

4.7.2 Effect of Disinfectants on Total Bacteria and Opportunistic Pathogens

The average 16S rRNA gene density in AR4 biofilm was approximately 0.5 log lower than AR5, but the difference was not significant ($P>0.05$). Lower 16S rRNA gene levels were expected in AR4 because chlorine is a stronger oxidant than chloramine and can inactivate microorganisms faster near the biofilm surface (Lee et al., 2011). However, chlorine is less stable, and, as expected, much faster chlorine decay in feed water was observed, requiring frequent amendment of chlorine to the feed reservoir to maintain a target influent of 0.4 mg/L. Lower stability of chlorine likely contributed to a lack of dramatic difference in biofilm 16S rRNA gene densities in AR4 and AR5 biofilm.

Varying effects of upstream chlorine and chloramine disinfection towards *Legionella* spp., *Mycobacterium* spp., *Acanthamoeba* spp. and *H. vermiformis* were observed. Important to note is that total bacterial responses, as indicated by 16S rRNA gene densities, were not generally representative of the responses of specific target opportunistic pathogens and related organisms. This was particularly noted with respect to *Legionella*. Consistent with prior field study trends (Moore et al., 2006; Weintraub et al., 2008), *Legionella* was better controlled by chloramine than chlorine disinfection. However, 16S rRNA genes were reduced to a greater extent by chlorine (though the difference was not significant). This affirms concerns that monitoring of total bacteria may have little direct relevance to quantifying risks from opportunistic pathogens (NRC, 2006).

Mycobacterial densities in AR5 (chloraminated) biofilm were significantly higher than AR4 (chlorinated), which is consistent with prior field data (Pryor et al., 2004). Even

though chlorine was most effective against mycobacteria, the average density was still about $10^{4.5}$ gene copes/cm^2 in AR4. This is typical, given the strong resistance of mycobacteria towards disinfectants, a phenomenon well-acknowledged in other studies (Falkinham, 2003; Le Dantec et al., 2002; Lee et al., 2010; Taylor et al., 2000). For example, the Ct value for 3 log inactivation of *M. fortuitum* is 600 times greater than that of *E. coli* (Lee et al., 2010). In the present study, the presence of amoebae is likely to further increase resistance to disinfectants by serving as hosts and providing shelter (Donlan et al., 2005). Significant disinfection effect on *M. avium* is possibly associated with their higher susceptibilities relative to other mycobacterial strains, such as *M. fortuitum* and *M. chelonae* (Le Dantec et al., 2002).

Stronger response of *Acanthamoeba* spp. to chlorine than chloramine is consistent with the findings of two studies comparing the effect of chlorine, chlorine dioxide and monochloramine towards *Acanthamoeba* (Dupuy et al., 2011; Mogoa et al., 2011). The Ct value of chloramine for a 99.9% inactivation of *A. castellanii* was approximately 18 fold that of chlorine. Chlorine and chloramine can also induce different cell morphology of *A. castellanii* (Mogoa et al., 2011). In contrast, *H. vermiformis* was susceptible to both chlorine and chloramine. Comparable susceptibility of *H. vermiformis* was observed when exposing biofilm to 0.5 mg/L chlorine (1.4 log reduction) and chloramine (1.5 log reduction) for 24 h (Donlan et al., 2005). We also observed stronger vulnerability of *H. vermifomis* towards disinfectants compared to *Acanthamoeba* in a previous simulated DWDS study (Wang et al., 2012b). The different response pattern towards disinfectants between *Acanthamoeba* spp. and *H. vermiformis* was speculated to be associated with their cysts' varying resistance capabilities against disinfectants. *H. vermiformis* cysts

were reported to be only slightly more chlorine resistant than the trophozoite forms (Kuchta et al., 1993). However, the *A. polyphaga* cysts can survive 50 ppm chlorine for 18h and are at least 50 times more resistant to chlorine than trophozoites (Kuchta et al., 1993).

Differential expression of 391 genes involved in stress response, virulence, general metabolism, information pathways and transport have been reported for chlorinate-treated *Legionella pneumophila* (Bodet et al., 2012). It is hypothesized that induction and repression of certain genes by disinfectants account for species-specific responses of target organisms towards chlorine and chloramine. Future studies comparing their transcriptional responses towards chlorine and chloramine will help elucidate different disinfection mechanisms.

4.7.3 Microbial Communities in Annular Reactors

Decrease of Shannon diversity index in disinfected ARs4-5 reveals a selective effect of disinfectants on the microbiome. Shift in DWDS microbial community structure has also been observed in previous studies applying different disinfectants (e.g., chlorine, chlorine dioxide, chloramine) (Roeder et al., 2010, Williams et al., 2005). Different disinfection mechanisms are thought to drive such differences. For example, Williams and colleagues (2004) found that drinking water biofilm exposed to chloramine mainly contained *Mycobacterium* and *Dechloromonas* sequences, while a variety of α- and β-Proteobacteria dominated the clone library of a chlorinated system. Similarly, a recent metagenomic investigation demonstrated different microbial populations in chloraminated (i.e., *Mycobacterium*, *Acidovorax*, *Burkholderia*, *Pseudomonas*,

Dechloromonas) and chlorinated (i.e., *Caulobacter*, *Rhodopseudomonas*, *Synechococcus*, *Bradyrhizobium,* and *Pseudomonas*) drinking water (Gomez-Alvarez et al., 2012). Although the dominant genera/species identities differed, our study also demonstrated distinct differences in biofilm populations subject to upstream chlorination or chloramination, in an otherwise identical upstream water source. Contrary to previous studies, *Mycobacterium* was not the most dominant genus in chloraminated AR biofilm (*Sphingomonas* was the dominant genus), with relative abundance of 4.9%±2.6%, slightly higher than that in chlorinated AR (2.0%±0.4%, P=0.05).

The differences among ARs1-3 indicated that GAC biofiltration can shape the downstream biofilm microbial community structure. Since only slight differences were observed in water chemistry among ARs1-3, it is likely that microorganisms colonizing GAC surfaces contributed to population shifts by interacting with microorganisms in bulk water and/or sloughed biofilm. A seeding effect of filter colonizers was also observed for dual media sand filters in a drinking water treatment plant, where bacterial taxa that colonized the filter were found to persist in the distribution system (Pinto et al., 2012).

4.7.4 Comparison of Target Organisms by q-PCR, Pyrosequencing, and Culturing

Mycobacteria were detected by pyrosequencing in all 15 biofilm samples with relative abundances varying from 0.7%-7.8%. These numbers were consistent with q-PCR estimates of the ratio of *Mycobacterium* spp. gene copy numbers (targeting 16S rRNA) over 16S rRNA gene copy numbers for total bacteria (Range: 4%-17%). Relative abundance for *Legionella* spp. by pyrosequencing were 0-0.46% while the ratio of *Legionella* gene copy numbers [targeting 23S rRNA genes- here we assume equal gene

copies numbers of 23S rRNA and 16S rRNA gene for all *Legionella* based on data of *L. pneumophila* (http://rrndb.mmg.msu.edu/search.php)] over 16S rRNA gene copies were 0.1%-5.7% by q-PCR. Moderate positive associations were found between pyrosequencing relative abundance and q-PCR gene copy ratios for mycobacteria and *Legionella*, respectively (r_s= 0.5571-0.6475, $P<0.05$). However, medium-depth pyrosequencing (about 5000 reads per sample in this study) failed to recover *Legionella* in 47% of samples (7 out of 15), most of which were characterized by *Legionella* /16S rRNA gene copy ratios less than 1% by q-PCR. These results illustrate that q-PCR is a more sensitive and reproducible method for quantitative detection of rare population, relative to medium-depth pyrosequencing.

No cultivable *Legionella* were recovered from biofilm samples, while considerable *Legionella* levels were detected by q-PCR. This phenomenon is consistent with our previous study investigating *Legionella* prevalence in the same distribution system (Wang et al., 2012a) and other field surveys (Wullings et al., 2011; Wullings and van der Kooij, 2006). In this study, the chance that *Legionella* gene copies numbers by q-PCR are all from dead cells is small since the biofilm was developed from scratch during the study period, and also no disinfection was applied to AR1-3. A likely explanation is that *Legionella* in biofilm were in a viable but non-cultivable state (Hwang et al., 2006). Another possibility is that the cultivability of *Legionella* was impaired by sample shipping and processing. Others have observed sample shipping and holding times to be critical factors affecting the cultivability of *Legionella* (McCoy et al., 2012).

A diverse range of mycobacteria were cultivated from biofilm and water samples. All

isolates were known opportunistic pathogens (*M. chelonae*, *M. mucogencum*, *M. gordonae*, *M. abscessus*, and *M. kansasii*) (Falkinham, 2009; Pinho et al., 2009; Vargas et al., 2005), providing direct evidence of the DWDS as a reservoir for pathogen transmission. Pyrosequencing also confirmed the presence of *M. mucogencum*, *M. chelonae*, and *M. gordonae*. Neither culturing nor pyrosequencing recovered *M. avium*, which was consistently detected by q-PCR in ARs1-3. This further illustrated the advantage of q-PCR in detecting rare populations using specific primers. Overall, q-PCR, culturing, and pyrosequencing together provided complementary information in terms of target organism quantification, confirmation of viable strains, and assessment of broader community composition.

4.8 Conclusions

- Blends of GAC-biofiltered and unfiltered water achieved varying influent TOC levels to ARs; however, levels of GAC-pretreatment and TOC were not correlated with 16S rRNA gene or opportunistic pathogen abundance.
- Presence of *Legionella* spp., *Mycobacterium* spp., *M. avium*, *Acanthamoeba* spp., and *H. vermiformis* in AR biofilm and effluent illustrates that the DWDS is an important reservoir for several opportunistic pathogens and related organisms.
- Chlorine was more effective for controlling mycobacteria and *Acanthamoeba*, whereas chloramine was more effective for control of *Legionella*. Both disinfectants strongly inhibited *H. vermiformis* and *M. avium*. Different responses of target bacteria and amoebas compared to total bacteria indicated by 16S rRNA genes and HPC implies that total bacteria is not an ideal indicator for

opportunistic pathogens.

- Pyrosequencing of 16S rRNA genes in the AR biofilms were congruent with q-PCR results and revealed diverse and complex drinking water microbial communities, which were significantly affected by disinfection and GAC pre-treatment.

4.9 Acknowledgements

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4.11 Figures

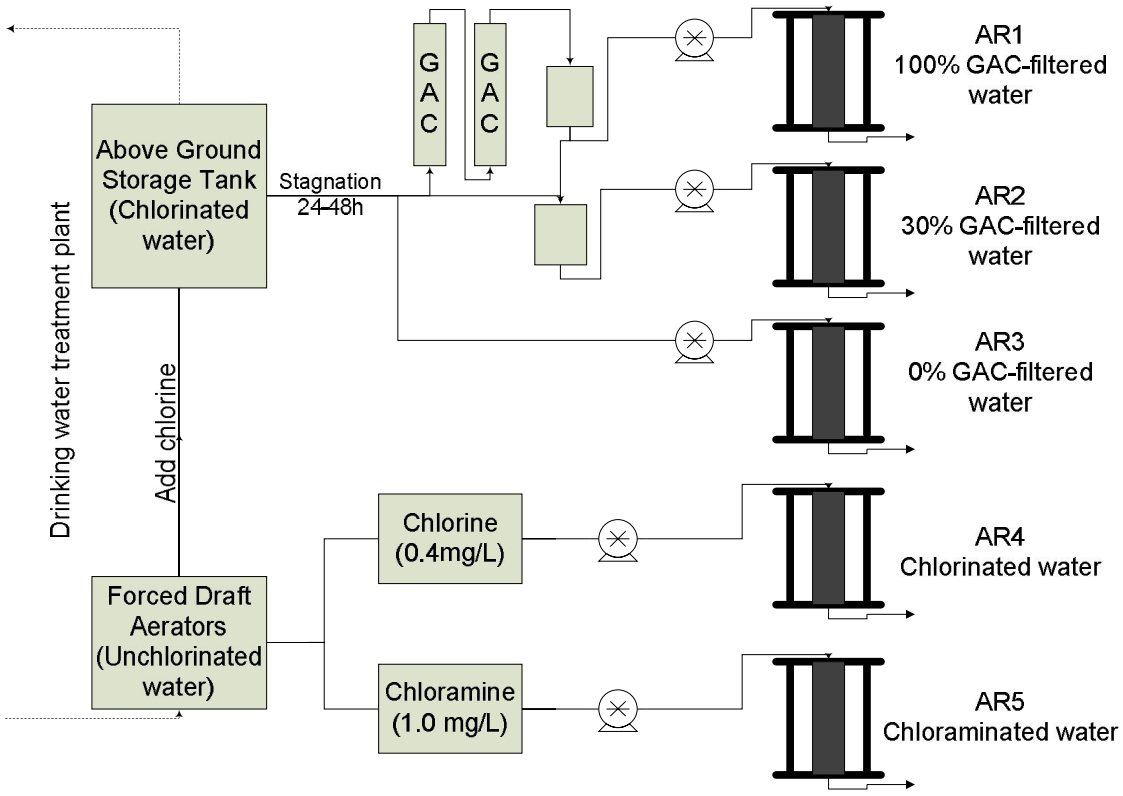


Figure 4.1 Annular reactor set-up, including water sources and target influent disinfectant levels.

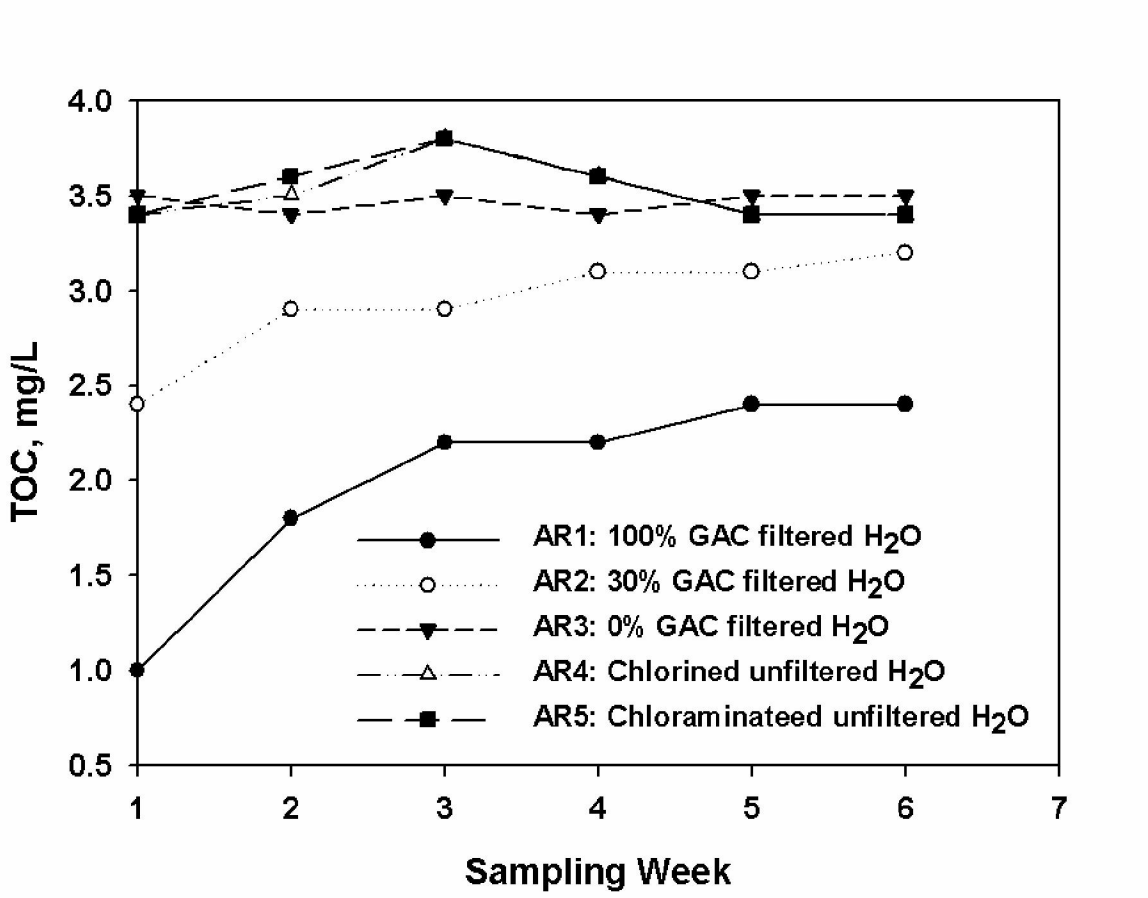


Figure 4.2 TOC concentration in AR feed water with time.

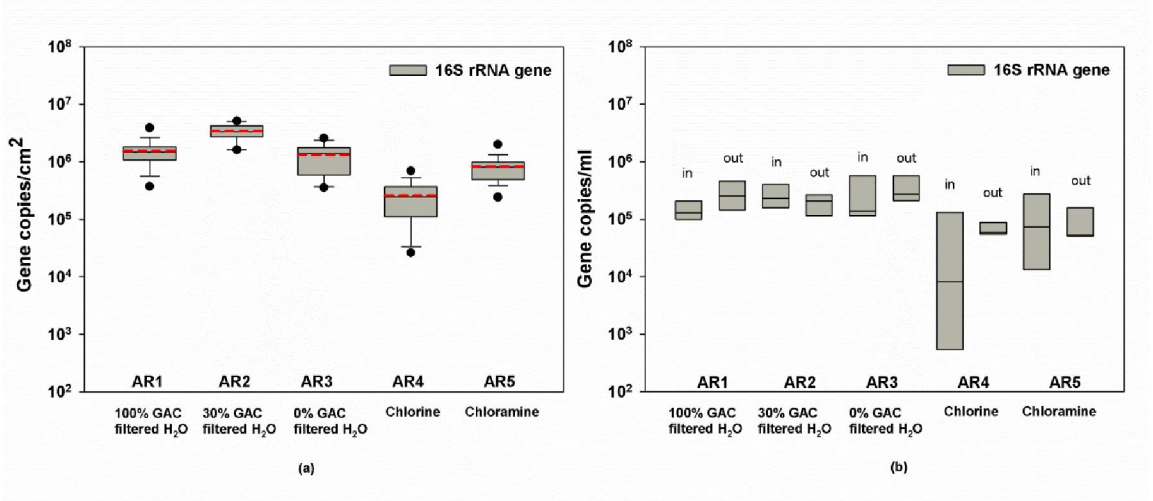
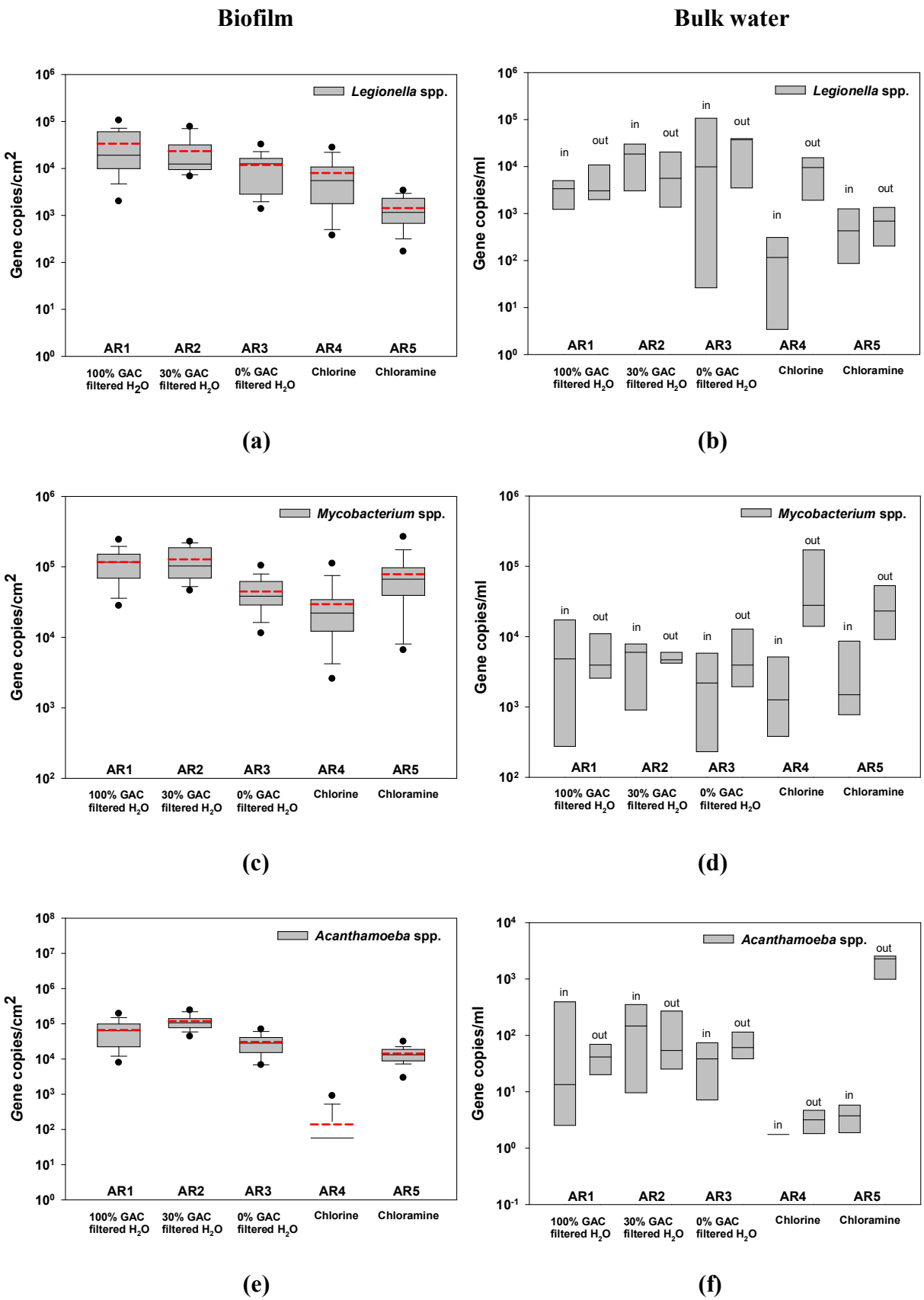
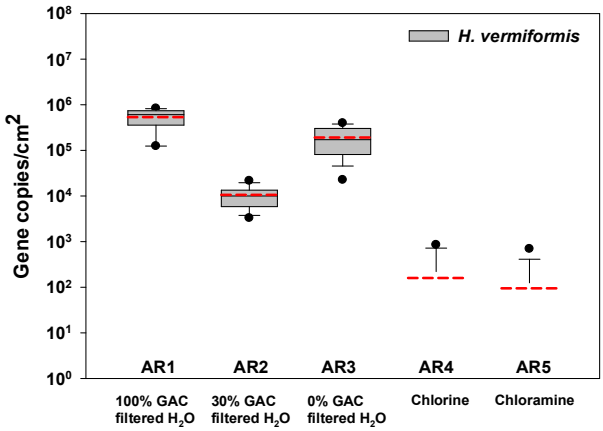
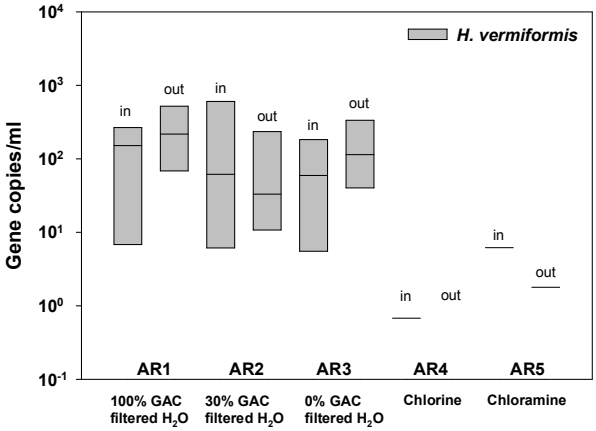


Figure 4.3 Enumeration of 16S rRNA genes in annular reactors by q-PCR. (a) Biofilm 16 rRNA genes on PVC coupons. Each boxplot is based on 18 data points collected from 6 sampling events, except for one sample lost each for AR2 and AR4 in the 5th sampling event. The red dashed line indicates the average concentration of 18 data points; (b) Influent and effluent 16 rRNA genes, in = AR influent, out = AR effluent. Each boxplot is based on 7 data points collected from 7 sampling events.

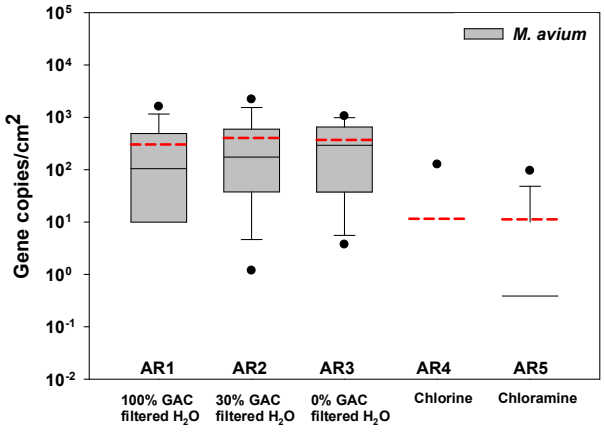

Biofilm
Bulk water
Legionella spp.
Mycobacterium spp.
Acanthamoeba spp.
Gene copies/cm2
Gene copies/ml
AR1
AR2
AR3
AR4
AR5
100% GAC filtered H2O
30% GAC filtered H2O
0% GAC filtered H2O
Chlorine
Chloramine
in
out
(a)
(b)
(c)
(d)
(e)
(f)



(g)



(h)



(i)

Figure 4.4 Enumeration of *Legionella* spp., *Mycobacterium* spp., *Acanthamoeba* spp., *H. vermiformis,* and *M. avium* in annular reactors by q-PCR. For the biofilm (a,c,e,g,i), each boxplot is based on 18 data points collected over 6 sampling events except that one sample was lost for AR2 and AR4 in the 5th sampling event, respectively. For the bulk water (b,d,f,h), each boxplot is based on 7 data points collected from 7 sampling events. In = AR influent, out = AR effluent. Red dashed line indicates the average of 18 data points.

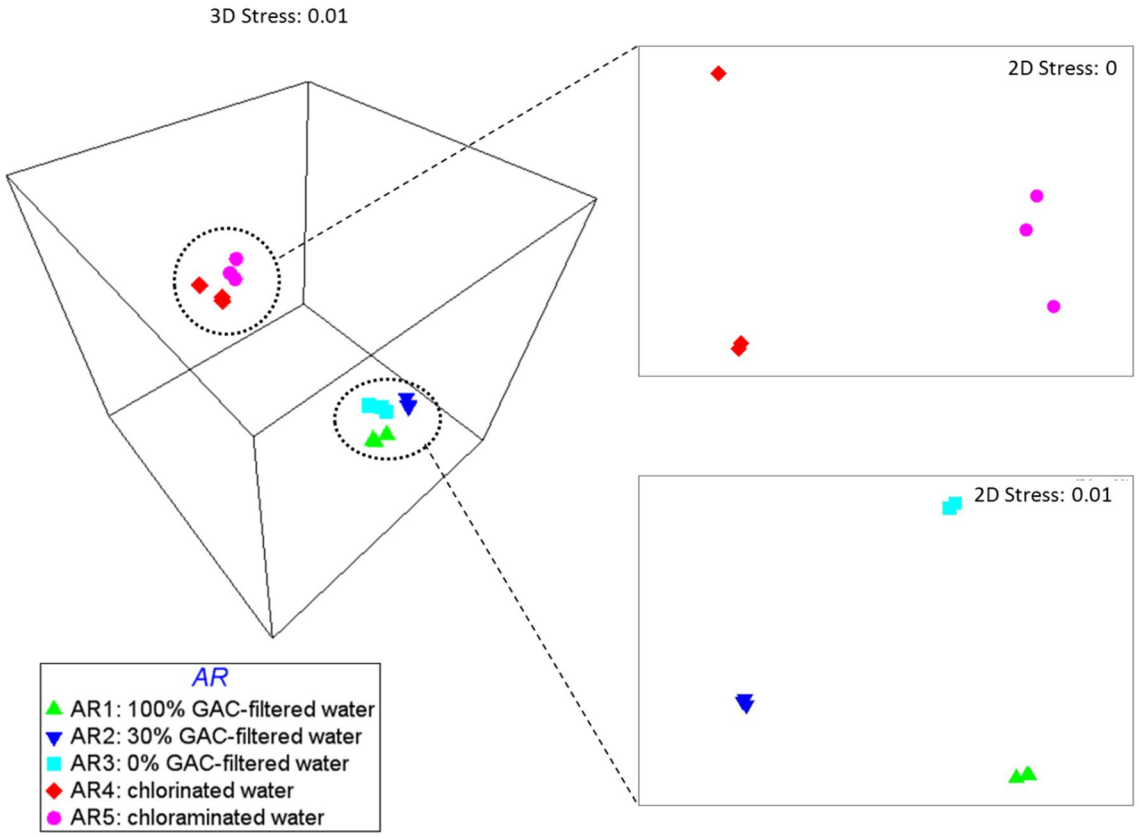


Figure 4.5 Multidimensional scaling analysis (MDS) of annular reactor biofilm bacterial community composition derived from pyrosequencing.

4.12 Supplemental Materials

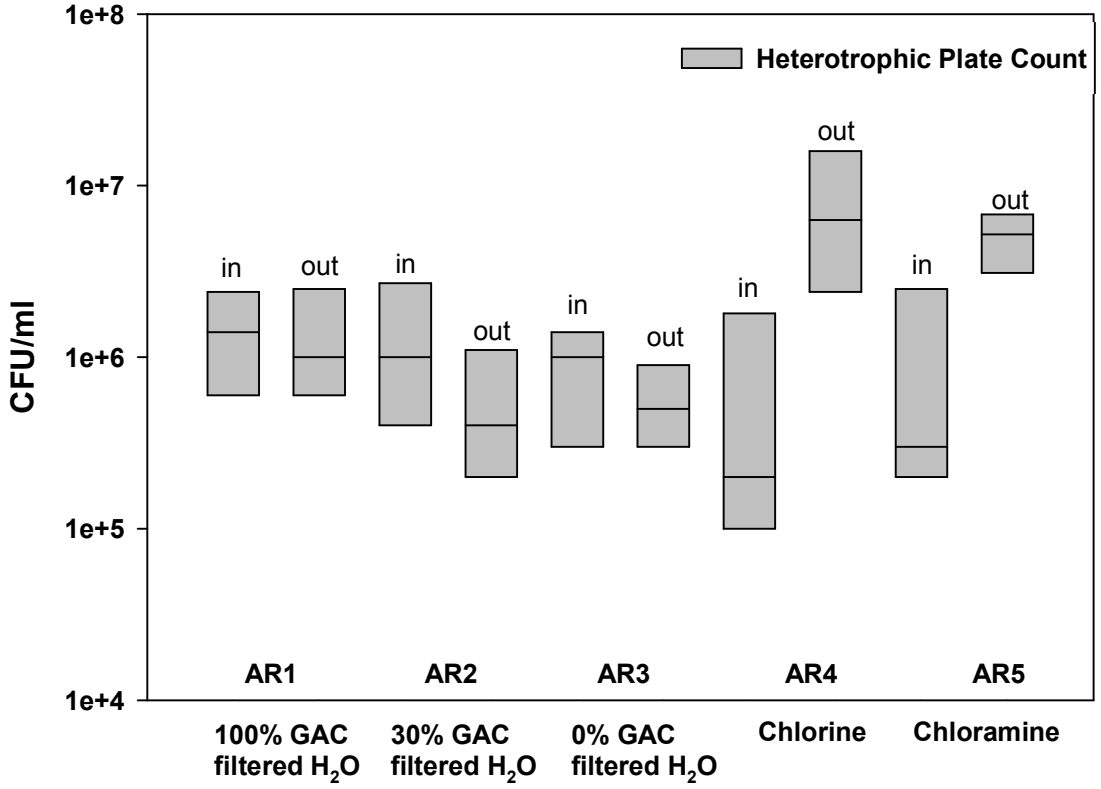


Figure S4.1 Heterotrophic plate count in AR influents and effluents, in = AR influent, out = AR effluent. Each boxplot is based on 7 data points collected from 7 sampling events.

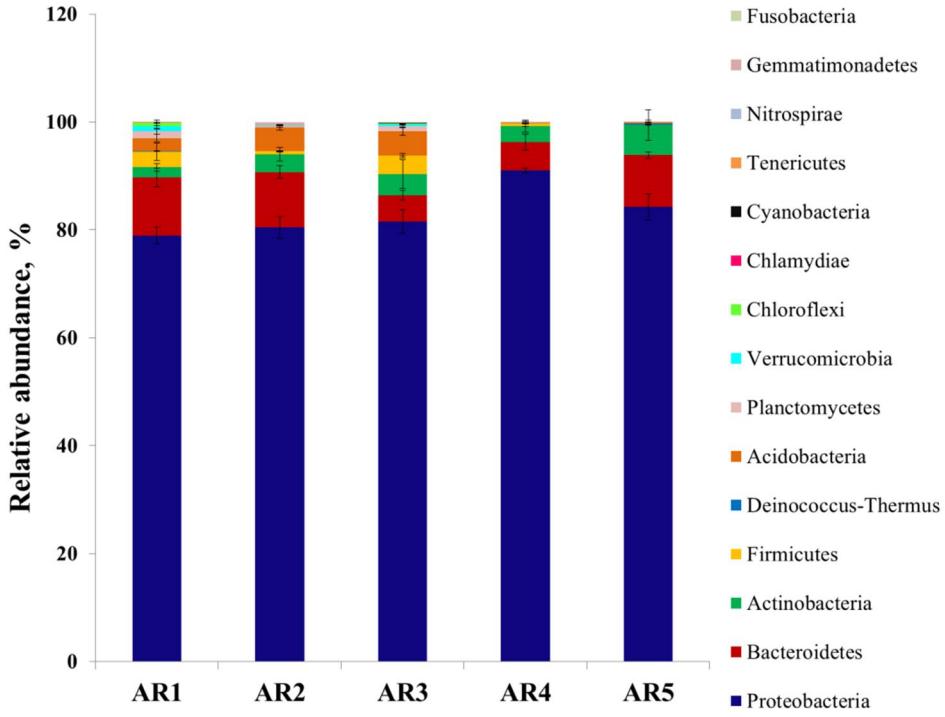


Figure S4.2 Relative abundance of bacterial phyla in annular reactor biofilm. Error bars represent the standard deviation of major bacterial phyla among triplicate biofilm samples. AR1: 100% GAC-filtered water, AR2: 30% GAC-filtered water, AR3: 0% GAC-filtered water, AR4: Chlorinated water, AR5: Chloraminated water.

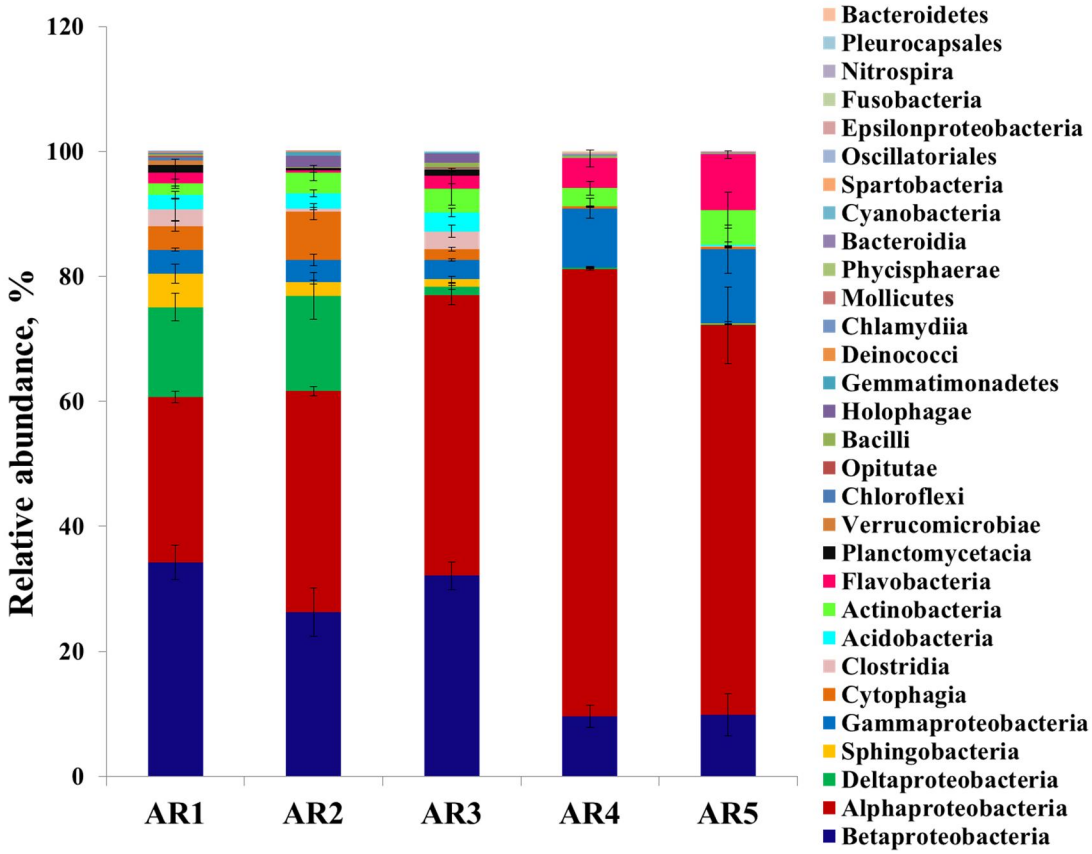


Figure S4.3 Relative bacterial class abundance in annular reactor biofilms. Error bars represent the standard deviations among triplicate biofilm samples. AR1- 100% GAC-filtered water; AR2- 30% GAC-filtered water; AR3- 0% GAC-filtered water; AR4- Chlorinated water, AR5- Chloraminated water.

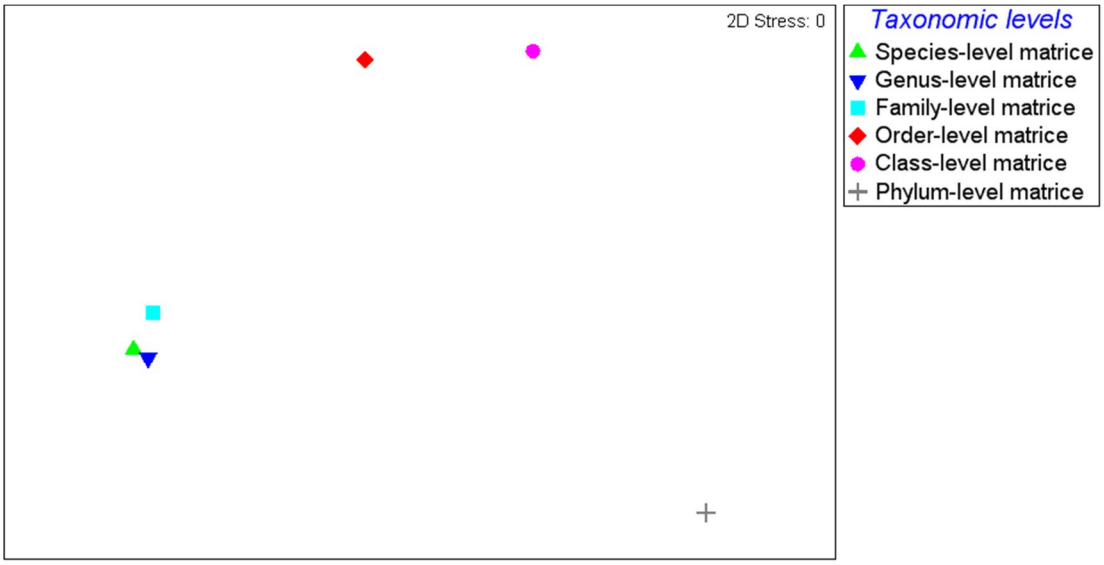


Figure S4.4 2-stage multidimensional scaling analysis comparing resemblance matrices derived from species, genus, family, order, class, and phylum levels

Chapter 5

Conclusions

Waterborne opportunistic pathogens are of increasing public health concern, as evidenced by increased infection cases. Several waterborne opportunistic pathogens are known to establish and grow in drinking water and serve as a source of disease outbreaks. Premise plumbing is of particular interest because it is characterized by ideal growth conditions for opportunistic pathogens and serves as a direct exposure route to humans via aerosol inhalation and direct contact. However, the factors driving the regrowth of opportunistic pathogens in premise plumbing have not been well characterized. This study aimed at advancing understanding of relationships among representative opportunistic pathogens, multiple environmental factors and drinking water microbial ecology via field investigation and lab-scale studies. In summary, the results of this work provided several lines of evidence that premise plumbing is a critical reservoir of opportunistic pathogens, implying potential health risks exposed to drinking water consumers. Abiotic factors such as disinfectant, water age and pipe material and their interactions played an important role in regulating opportunistic pathogens regrowth in drinking water, with species-specific response noted. GAC biofiltration effectively remove organic carbon, but had no apparent effect in controlling opportunistic pathogens. The broader microbial community was suggested to be a potential key factor in governing opportunistic pathogen survival and regrowth, which is in need of further investigation. Specific contributions to understanding of factors triggering opportunistic pathogen growth and drinking water microbial ecology in general by this research include:

A comprehensive and quantitative snapshot of the prevalence of multiple representative opportunistic pathogens and related microorganisms in real-world distribution systems. One novelty of our study lies in part in that multiple opportunistic pathogens were simultaneously investigated. This is critical given the potential for complex ecological relationships among the pathogens that may preclude a common solution to control of all (or most) opportunistic pathogens. Monitoring of key amoebic hosts (*Acanthamoeba* and *Hartmanella vermiformis*) and T-RFLP profiling of microbial community structure provided insight into potential broader biotic factors associated with opportunistic pathogen occurrence. Weak to moderate correlations were also identified between abiotic factors such as chloramine residual, TOC and opportunistic pathogen numbers. Last, this study also indicates that q-PCR provides consistent and quantitative results, which is a critical feature for advancing q-PCR as a drinking water opportunistic pathogen monitoring tool.

Head-to-head comparison of disinfectant, pipe material, and water age on occurrence and persistence of opportunistic pathogens and related organisms provided insight into factors that may trigger proliferation of opportunistic pathogens in the DWDS and potential practical engineering controls. Disinfectant type and dose was demonstrated to exert the strongest influence on the microbial community. It is noted to point out that our study demonstrated a potential link between nitrification and increased prevalence of opportunistic pathogens in drinking water systems for the first time, implying that nitrification control in chloraminated SDSs may be important. Pipe material, and its

interaction with disinfectants, was also observed to be an important factor in governing microbial proliferation.

Direct demonstration of species-specific responses of opportunistic pathogens towards chlorine and chloramine, and illustration of the significant driving force of GAC biofiltration and disinfectant type on the broader bacterial community. Chlorine was more effective for controlling mycobacteria and *Acanthamoeba*, whereas chloramine was more effective for control of *Legionella*. Both disinfectants strongly inhibited *H. vermiformis* and *M. avium*. Compared to previous studies investigating disinfectant effect using lab-cultivated cells under nutrient rich environments, these findings are advantageous for understanding disinfection responses of drinking water opportunistic pathogens by using real-word drinking water with natural occurrence of multiple opportunistic pathogens. Pyrosequencing of 16S rRNA genes in annular reactor further provided a fine-scale understanding of how GAC pre-treatment and disinfectant type can shape the microbial community structure. Further, simultaneous application of q-PCR, culturing, and pyrosequencing in this study also elucidate the advantages and disadvantages of these methods in terms of opportunistic pathogen monitoring.

Overall, the knowledge gained from this study advanced the understanding of drinking water opportunistic pathogens relative to abiotic factors and biotic factors, which can provides practical guidance for water utilities and consumers in drinking water distribution system design and operation, as well as premise plumbing condition optimization with the overall goal of minimizing the health threat posed by opportunistic

pathogens. Further research will include the deep understanding of interaction between opportunistic pathogens and indigenous microbial community in drinking water distribution system, through which a green approach of pathogen control can be achieved by manipulating drinking water plant and premise plumbing conditions to promote beneficial microorganisms' growth and inhibit opportunistic pathogens.