Effect of Various Water Chemistry Factors on *Legionella* Proliferation and the Premise Plumbing Microbiome Composition

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Abstract

Premise plumbing, the pipes and fixtures at the building level, present a unique challenge for maintaining drinking water quality. Of particular concern are opportunistic pathogens, including Legionella pneumophila which can regrow in premise plumbing and cause disease in immunocompromised populations. The goal of this work was to explore engineering methods for control of *L. pneumophila* and total regrowth. The first line of study involved a series of experiments with simulated glass water heaters (SGWHs) to investigate interactions between specific water chemistry factors and *L. pneumophila* regrowth, and the second used laboratory grade purified water to investigate the limits of a nutrient control approach for biological stability.

Several water chemistry factors including assimilable organic carbon (AOC) content, granular activated carbon (GAC) biofiltration, plumbing materials, copper concentrations and temperature were investigated using SGWHs. AOC is the carbon available for bacteria growth in drinking water. Results indicated that AOC reduction may be a promising method for controlling *L. pneumophila* and total bacteria regrowth, but there may be a point at which AOC reduction is no longer effective. Prior GAC biofiltration removed organic carbon and was effective in controlling total bacterial regrowth in SGWHs, but actually encouraged *L. pneumophila* regrowth.

A wide variety of materials typically encountered in premise plumbing was investigated and only had limited effect on proliferation of *L. pneumophila* and total bacteria. The effects were

dynamic, even with long-term studies. Copper pipes held promise for control of *L. pneumophila*, as did copper concentration across a range of pHs. Aqueous copper concentration released from pipes was dependent on temperature, however, and thus this control method may not be applicable in all hot water lines.

The peak temperatures for *L. pneumophila* proliferation fell between 41 and 45 °C, temperatures which could be encountered in a hot water distribution system when the water heater is set to 48 °C, as is often recommended with scalding and energy concerns. A constant temperature of 53 °C seemed to provide control of *L. pneumophila*, but recolonization is possible even at these high temperatures.

Work with laboratory grade water indicated that extreme control of nutrients was not enough to completely control regrowth in premise plumbing. With stagnation in the cleanest conditions, a 2-log increase of a diverse group of bacteria was observed within 10 days. As drinking water can never achieve such nutrient removal, this study presents the limits of nutrient removal as a strategy for regrowth control.

This work explored both the potential and the limitations of several mechanisms for controlling regrowth in premise plumbing. Understanding how these water chemistry factors affect *L. pneumophila* and total bacterial regrowth is critical to identifying the most effective engineering controls.

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Chapter 1

Introduction

1.1 Opportunistic Pathogen and *Legionella pneumophila* Risk

Opportunistic pathogens are infectious microorganisms capable of causing disease in certain risk groups. Immunocompromised populations, including the elderly, children, those taking immunosuppressant drugs and individuals hospitalized with other infections, are especially vulnerable. These microorganisms pose an increasingly significant threat to human health, due to aging population and other risk factors (Pruden 2013).

Of particular concern is *Legionella pneumophila*, which is the causative agent of Legionellosis. This includes two pneumonia like diseases, Legionnaire's disease and Pontiac Fever. Legionnaire's disease hospitalizes 8,000-18,000 people annually in the United States (Center for Disease Control 2011), with a fatality rate of 5-30% of cases (US Department of Health and Human Services 2005). Underreporting is likely an issue, especially as symptoms resemble those of a common cold or pneumonia.

The disease was first identified in 1977 after the microorganism caused 29 deaths and 182 illnesses at a 1976 American Legion convention (Fraser et al. 1977). The disease and the subsequently identified organisms received their name from this convention. Since its discovery, there has been much research on its life cycle, infection mechanisms and possible control mechanisms.

L. pneumophila reproduction is thought to be dependent on a life-cycle of infection of amoeba hosts, such as *Hartmannella vermiformis* and *Acanthamoeba polyphaga* (Declerck et al. 2009; Kuiper et al. 2004; Wang et al. 2012a). *L. pneumophila* enters host cells, reproduces within using amino acids from the host as growth substrate, and bursts free from the cell, lysing it in the process.

L. pneumophila is present in natural surface waters, as might be used for source water for a drinking water treatment plant. *L. pneumophila* and other opportunistic pathogens are not fecal-associated and can be found in the distribution system despite primary and secondary disinfection at the drinking water treatment plant. Exposure to *L. pneumophila* is typically through the inhalation of aerosolized bacteria from various water sources, for example inhalation during showering or hand washing (Schoen and Ashbolt 2011). Exposure can also occur through inhalation of aerosols while swimming, including in hot tubs (Center for Disease Control 2013)

Several outbreaks of Legionnaire's disease have recently captured media attention and public concern. An outbreak at the Veterans Affairs Hospital in Pittsburgh received scrutiny from the Office of the Inspector General (Office of Inspector General 2013). In recognition of these health risks, the U.S. EPA has established a non-enforceable maximum contaminant level goal (MCLG) of zero organisms in drinking water (Environmental Protection Agency 2000). Despite extensive research, the factors which control the proliferation of *L. pneumophila* and other opportunistic pathogens are not fully understood.

1.2 Control at the water treatment plant, AOC

Regrowth within the distribution system after potable water exits the drinking water treatment plant is problematic. Even when opportunistic pathogens are not detected at the drinking water treatment plant, they can regrow in the distribution system (National Research Council 2006). Thus, utilities and water treatment plant operators are concerned with this regrowth and attempt to minimize it.

Two common strategies to target regrowth are provision of disinfectant residual to kill bacteria, and limitation of nutrients required for growth. Disinfectant residual is often lost, especially as water age increases. Temperature and other reactions specific to materials used in the system also result in loss of disinfectant residual (Nguyen et al. 2011). Opportunistic pathogens particularly tend to thrive in niches that have low or no disinfectant residuals and can be especially resistant to traditional disinfection processes (Cooper and Hanlon 2010; Falkinham 2011; Norton et al. 2004; Taylor et al. 2000; Wang et al. 2012b).

In many parts of the world, including Europe, disinfectant is not provided in distributed water. Instead, municipalities aim to remove carbon at the treatment plant so that it is unavailable to support regrowth in the distribution system. As regrowth is a problem in the U.S., even with disinfectant residual (Wang et al. 2012b), carbon removal is an attractive option.

Control of assimilable organic carbon (AOC), or the carbon readily available to drinking water heterotrophs for growth, is widely implemented in some parts of the world in lieu of disinfectants. However, an acceptable AOC limit has not been agreed upon in the literature. It may be dependent on other drinking water factors including the disinfectant residual and

temperature. In the United States, where disinfectant residual is required, a high AOC (> 100 µg/L) was only associated with coliform occurrences when either the temperature was above 15 °C or disinfectant residual had dropped <0.5 mg/L free chlorine, or <1 mg/L Chloramine (Volk and LeChevallier 2000). Another study found that AOC, even in excess of 100 µg/L, was an insignificant factor in predicting regrowth compared with residual of chloramine disinfectant (Zhang et al. 2002).

Where disinfectant residual is limited or not in use, as is common practice in Europe, the acceptable limits of AOC are much lower. A limit of 10 µg/L as C was proposed when no disinfectant residual was used and when carbon, rather than phosphorous or nitrogen, was the limiting nutrient (Kooij 1992). Effectiveness of this threshold was confirmed in cases of low disinfectant residual by a test of batch mode incubations in Japan. With disinfectant residual limited to 0.05µg/L, 10.9µg C/L provided acceptable regrowth limits (Ohkouchi et al. 2013). In Zurich, a slightly higher concentration of AOC, 32 µg C/L, was deemed to provide biostability, or prevention of regrowth, in the distribution system using a modified AOC assay (Hammes et al. 2010).

Even with control mechanisms in place at the drinking water treatment plant, AOC can persist into the distribution system. AOC can also be generated there. Autotrophic microbes can reasonably be found in drinking water and may provide a growth substrate that encourages growth of biofilms (Martin 2012). There is also evidence that significant amounts of TOC can leach from plastic plumbing, such as polyvinyl chloride (Rogers et al. 1994).

The control of regrowth issues may not be possible solely with control AOC or supply of disinfectant residual at the water treatment plant level. Even when regrowth can be controlled in the distribution system, premise plumbing can provide an optimal environment for regrowth.

1.3 Premise Plumbing Risks and Control

Premise plumbing consists of the pipes and fixtures at the individual building level which are the responsibility of the end-user. This differs from the distribution system, which is owned and operated by the water distribution authority. Premise plumbing possesses several key characteristics that likely contribute to biofilm formation and the growth of opportunistic pathogens (Figure 1.1) and which complicate the factors of opportunistic pathogen proliferation and control.

Premise plumbing pipes are smaller than that of the distribution system, and water may sit longer than it would in the distribution system. Smaller pipes contribute to a high surface area to volume ratio. While distribution line water is constantly flushed with use throughout the circuit, water may stagnate in a building for hours, days, or weeks when nobody uses a particular faucet.

Growth within biofilms may have greater impact on water quality in pipes with higher surface area to volume ratio (Zhang et al. 2009). Disinfectant residuals are lost with high water age, extended stagnation (Lautenschlager et al. 2010; National Research Council 2006) and increased temperatures in hot water lines (Bagh et al. 2004). Biofilm formation may be especially important as disinfectant is lost in premise plumbing systems with stagnation, temperature, or other chemical reactions (Nguyen et al. 2012). As opportunistic pathogens thrive in areas of low disinfectant residual, premise plumbing may provide an ideal ground for growth.

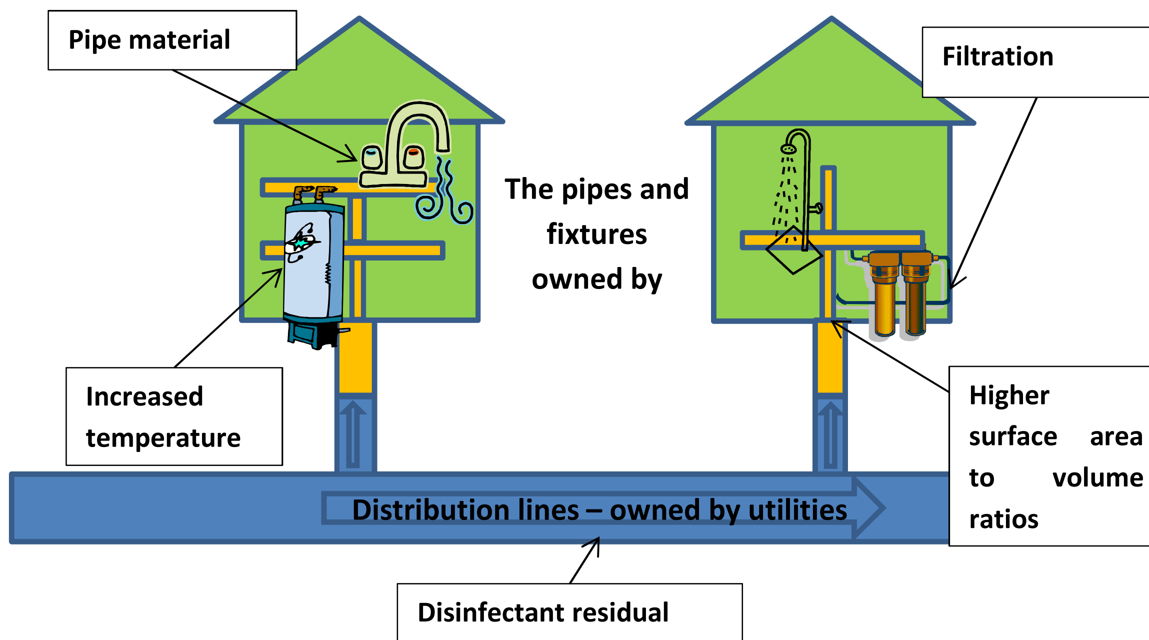

Figure 1.1. Particular risk areas in premise plumbing.

Pipe materials vary greatly from home to home or even within a home. The choice of materials significantly affects both the chemistry and microbiology of the water (National Research Council 2006). Pipes may leach substances that benefit growth conditions. For example, plastic pipes like PEX leach substantial amounts of carbon (Bucheli-Witschel et al. 2012; Inkinen et al. 2014; Rogers et al. 1994; van der Kooij et al. 2005). Magnesium rods used as anodes in electric water heaters release H_2 which could potentially be utilized by H_2-oxidizing autotrophic bacteria (Martin 2012).

Pipe materials may also serve as a detriment to bacterial regrowth. Although copper is a nutrient for production of enzymes, it also has antimicrobial properties (Chaturvedi and Henderson 2014). In fact, copper-silver ionization systems, which take advantage of copper in its ionic form

for disinfection purposes, is known to be useful in control of *L. pneumophila* when used properly (Shih and Lin 2010; States et al. 1998). Literature is conflicting as to whether copper in other forms in real systems is beneficial (Bargellini et al. 2011; Leoni et al. 2005) or detrimental (Mathys et al. 2008; Tiefenbrunner et al. 1993) to *L. pneumophila* control efforts.

Increased temperatures in hot water lines may contribute to optimize growth conditions. While "hot" temperatures, > 60 °C are known to kill bacteria, including opportunistic pathogens (Dennis 1991; Stout et al. 1986), the "warm" temperatures as might be encountered as water is distributed from the water heater may be beneficial for regrowth. Water heaters have been identified as a risk when operated anywhere from 30-54 °C, especially when certain points in the hot water distribution system are allowed to become 'warm' instead of 'hot' (Wadowsky et al. 1982). Recommendations for the operation of water heaters ranges from 48 °C (CDC 2012; Energy Star) to 60 °C (OSHA 1999; World Health Organization 2004) based on concerns for energy, scalding and bacterial regrowth.

.In an effort to improve water taste and odor, some people prefer to use granulated activated carbon (GAC) filters at the point-of-use or at a whole-building level. In trying to improve one aspect of water quality, these individuals may be increasing the likelihood of pathogen regrowth. A particular risk with at home-filters is that bacteria may break through the filter (or slough off) into the water that goes directly to consumers(McCarty and Meyer 2004). As disinfectant residual is also removed with a GAC filter, there is little protection against rampant regrowth downstream in premise plumbing, where high surface to volume ratios offer more growth surface and stagnation may persist longer based on use of the building. While building level filters

should be replaced “periodically” by Department of Health recommendations(Ohio Department of Health 2013), complete colonization of a full-scale GAC biofilter can occur within three months (Servais et al. 1994; Velten et al. 2011), and failure to replace filters within this time frame may result in higher risk to consumers. In addition, several studies suggest that filtration can change the composition of the microbial community downstream (Pinto et al. 2012; Wang et al. 2013). Management of water quality at the premise plumbing level, then, requires regular attention, even for buildings that are not at particularly high risk.

1.4 Consequences and Significance

At particular risk are hospitals, as they house an inherently immunocompromised population and their premise plumbing systems tend to be large. Many hospitals have specific control plans in place to prevent outbreaks which may include the superheating of water, application of extra disinfectant or use of copper-silver ionization, alongside regular monitoring procedures (Allegra et al. 2011; Darelid et al. 2002; Marchesi et al. 2011). These control plans are not always followed, however. Many mechanisms for control require regular extensive maintenance, and when that maintenance is not performed, it can result in death (Office of Inspector General 2013)

The legal responsibility for control of opportunistic pathogens in premise plumbing is debated and varies by country. The building manager or the water consumer may have major responsibility for colonization by harmful microorganisms (Freije 2012), and it will be vital to develop effective, manageable strategies for premise plumbing systems. Better understanding of how factors can affect growth and proliferation of opportunistic pathogens in premise plumbing is necessary to direct engineering control practices.

1.5 Thesis Overview

The goal of this research was to better understand risk factors and engineering control methods for *L. pneumophila* and total bacterial regrowth within premise plumbing systems. In Chapter 2, the effects of both AOC additions and GAC as a method for AOC reduction on the proliferation of *L. pneumophila* are explored with experiments using simulated glass water heaters. In Chapter 3, the same experiments are utilized to analyze the effect of specific plumbing conditions common to premise plumbing on the response of *L. pneumophila*. In Chapter 4, copper is further investigated as a possible control mechanism for *L. pneumophila* using another experiment with similar simulated glass water heaters. In Chapter 5, the limits of temperature as a control mechanism for *L. pneumophila* are considered as a function of AOC and pipe material. In Chapter 6, the extremes of nutrient removal in water are studied with an in-depth characterization of the microbiome in laboratory-grade waters. In Chapter 7, lessons are reviewed and the researcher offers suggestions for future studies.

The reader should gain an understanding for how each of the water chemistry factors investigated – carbon content, pipe material, copper and temperature – can affect the growth patterns of *L. pneumophila* and total bacteria in conditions relevant to premise plumbing.

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Chapter 2

Effects of Assimilable Organic Carbon and Granular Activated Carbon on *Legionella pneumophila* Persistence in Premise Plumbing

2.1 Introduction

Opportunistic Pathogens in Premise Plumbing (OPPPs) pose an increasingly significant threat to human health, due to aging population and other risk factors (Pruden 2013). OPPPs, including *L. pneumophila*, are not fecal-associated and can be found in the distribution system despite primary and secondary disinfection at the drinking water treatment plant. Certain attributes of premise plumbing likely contribute to biofilm formation and growth of OPPPs, including high surface to volume ratio, loss of disinfectant residuals with high water age and extended stagnation (Lautenschlager et al. 2010; National Research Council 2006) and increased temperatures in hot water lines (Bagh et al. 2004). OPPPs particularly tend to thrive in niches that have low or no disinfectant residuals and can be especially resistant to traditional disinfection processes (Cooper and Hanlon 2010; Falkinham 2011; Norton et al. 2004; Taylor et al. 2000; Wang et al. 2012b). Their occurrence can vary widely throughout premise plumbing of buildings and the distribution system (Wang et al. 2012a) and thus the factors that drive their proliferation are still unclear.

Carbon has been targeted in drinking water treatment in order to limit bacterial regrowth in the distribution system, especially where disinfectant is not employed. However, carbon occurs in various forms and not all is bioavailable for microbial growth. Inorganic carbon can be found in ions associated with alkalinity (CO_2, HCO_3^-, H_2CO_3) and is not directly available as a carbon source to heterotrophs, but can be used by autotrophic microbes and fixed into organic forms(Zhang and Edwards 2010). Further, organic carbon can occur in various forms in drinking water, much of it consisting of extremely complex aromatic structures derived from natural organic matter that generally is not bioavailable (Camper 2004; Edwards et al. 1993). Total organic carbon (TOC) is a general laboratory measurement of all organic forms of carbon, including complex biomolecules secreted from living organisms and residuals from dead organisms, as well as simple sugar structures. The portion of organic carbon that is easily assimilated by heterotrophs found in drinking water is referred to as assimilable organic carbon (AOC)(Haddix et al. 2004). AOC generally makes up only a small portion of the TOC (0.1-9%) (Hammes and Egli 2005; Kooij 1990).

Control of AOC is one major strategy for limiting regrowth in the distribution system and is widely implemented in some parts of the world that tend to use lower levels of residual disinfectants. Where disinfectant residual is limited or not in use, as is common practice in Europe, the acceptable limits of AOC are much lower. A limit of 10 μg/L as C was proposed when no disinfectant residual was used and when carbon, rather than phosphorous or nitrogen, was the limiting nutrient (Kooij 1992).

One method for the control of AOC is granular activated carbon (GAC)(Lehtola et al. 2002). In addition to direct removal of organic matter by sorption, GAC also provides a high surface area for attachment of microbes which can biodegrade AOC entering the filter. The resulting effluent has a low and stable concentration of organic carbon. At the treatment plant, GAC filters have been successfully implemented to consume biodegradable organic carbon and provide biological stability (Chien et al. 2008; Chien et al. 2007; Servais et al. 1994; Velten et al. 2011). At the premise plumbing level, GAC filters are used in order to improve taste and odor of drinking water and can be used to treat either an entire building or at a point-of-use. They are commonly employed on individual taps or in pitchers for storage.

A potentially negative aspect of GAC is that it also removes disinfectant residual (Sorlini and Collivignarelli 2005), which leaves the water system downstream vulnerable to microbial colonization. GAC provides a surface for regrowth as well as changing water chemistry. GAC can harbor a high population density of microbes, including pathogens such as *Yersina enterocolitica, Salmonella typhimurium* and *Escherichia coli* (Camper et al. 1985). Thus, GAC filtration has the potential to exacerbate OPPP proliferation, as suggested by a recent high-profile *L. pneumophila* outbreak in Florida (Miami-Dade County Department of Health 2010; Wang et al. 2013). Increase in *Legionella* spp. has been observed after rapid sand filtration in drinking water treatment plants and attributed to biofilm formation on the filter (Wullings et al. 2011). Both the changes in water chemistry affecting AOC and the regrowth on surface are important for understanding how GAC affects water quality.

Much of the research on AOC and AOC removal via methods such as GAC has focused on its effects on total bacterial regrowth or potential for regrowth. There is limited information available about their specific effects on opportunistic pathogens. A positive association between TOC and *L. pneumophila* occurrence was found in a survey of 137 hot water samples from a wide array of locations (Leoni et al. 2005), but many studies on *L. pneumophila* do not quantify organic carbon. There is some evidence that AOC control may provide some benefit for the control of opportunistic pathogens. In a study in the Netherlands, *L. pneumophila* was detected more often when AOC exceeded 10 µg C/L than when it was controlled below 5 µg C/L g C/L (van der Wielen and van der Kooij 2013). Recent work at Virginia Tech has explored the relationships between biodegradable organic carbon and *L. pneumophila* and *M. avium* (Williams 2011; Williams et al. June 2011).

As *L. pneumophila* and other opportunistic pathogens have complex relationships with other organisms and do not feed directly on AOC, the relationship between AOC and opportunistic pathogens may only be indirect. It is thought that *L. pneumophila* survival in drinking water is largely dependent on infection of and replication within host protozoa (Kuiper et al. 2004; Thomas and Ashbolt 2011). There is also evidence that *L. pneumophila* can amplify through necrotrophic growth in the presence of dead biomass, which may provide a readily available source of amino acids, their primary carbon source (Temmerman et al. 2006). Thus control of AOC, while acceptable for controlling regrowth of most heterotrophic bacteria, may not be a suitable tactic for controlling opportunistic pathogens.

The objective of this study was to investigate the effectiveness of AOC control on total bacterial regrowth and the proliferation of *L. pneumophila*. The effect of GAC filtration, a common AOC control strategy, on regrowth potential of *L. pneumophila* was of particular interest. Simulated glass water heaters were operated at laboratory scale in triplicate and over a range of representative premise plumbing conditions that were pooled for specific comparisons of the effect of amended AOC (acetate and glucose) and pre-treatment with biologically active GAC. The results of this study can provide guidance to both municipalities and building owners in the implementation of AOC control as a means of opportunistic pathogen control.

2.2 Materials and Methods

2.2.1 Simulated Glass Water Heaters (SGWHs). SGWHs consisted of 125 mL French square borosilicate glass bottles with polytetrafluoroethylene caps and were equipped with a range of plumbing materials and conditions. All SGWHs were incubated at 32°C, a temperature representative of the bottom of a conventional water heater where certain OPPPs are thought to thrive. SGWHs were originally inoculated with *L. pneumophila* (ATCC 33152, ATCC 33733, ATCC 33734, ATCC 33823) at an approximate initial concentration of 2.03×10^5 gene copies/mL, *Acanthamoeba polyphaga* (ATCC 30871) at 2,079 gene copies/mL, *Mycobacterium avium* A5 at 7.15×10^5 gene copies/mL and *Hartmannella vermiformis* (ATCC50237) at 1.46×10^3 amoeba/mL into each reactor. All SGWHs were operated for 2.5 years prior to commencing the present experiment. Upon commencing the described experiments, cross-inoculation was conducted by pooling effluent from reactors and adding 1 mL to the influent for each reactor in order to achieve a similar baseline microbial community.

2.2.2 Plumbing Conditions. This study incorporated a variety of plumbing conditions in order to observe if GAC and AOC influence was robust across a relevant range of conditions. Plumbing materials and conditions utilized in this study included (1) iron coupon to simulate iron plumbing system components including steel in water heaters, (2) cross-linked polyethylene (PEX) pipe sections, (3) copper pipe sections, (4) magnesium to simulate magnesium anodes used for corrosion control, (5) iron(III) oxide sediment from unlined iron in many distributions systems, (6) ammonium sulfate additions at concentrations 1mg/L free NH_3-N to simulate chloramine systems after residuals disappear, (7) combination of iron coupon, magnesium rod, and ammonium sulfate addition and (8)controls (no additional materials) at pH 7.5 and 10. In addition to the described materials, all SGWHs also contained 1.5 layers of 1mm glass beads, which served to increase the surface area to volume ratio and provide a range of redox zones similar to those found in premise plumbing sediment layers.

2.2.3 Influent Water and Water Changes. Three times a week, an 80% water change was conducted to simulate infrequent use of a water heater. Without mixing and with minimal agitation, 100 mL was discarded and replaced with freshly prepared modified Blacksburg drinking water. Modified Blacksburg drinking water was used rather than modified reverse osmosis or nanopure water in order to continually introduce common inorganics and nutrients into the system.

Town of Blacksburg, VA, tap water is disinfected with chloramines. Water was collected in the lab from the cold water tap and was breakpoint chlorinated (BPC) with addition of a diluted hypochlorite solution. Break-point chlorinated water was then heated to 90 °C for 10 minutes to

remove most disinfectant residual. For corresponding experiments examining the effect of prior GAC filtration, the cooled water was subject to biofiltration with a point of use GAC filter.

All influent water was pre-treated by passage through a 0.45 µm polyvinylidene fluoride (PVDF) filter to remove microbes. Water was adjusted to pH 7.5 ± 0.1, except in the case of the 'high pH control' which is adjusted to pH 10.0 ± 0.1. pH was controlled with additions of 1 M or less NaOH and HCl using an Oakton pH 10 series meter (Oakton Instruments, Vernon Hills, IL).

2.2.4 Experimental Design. SGWHs were divided into two experiments: A (48 SGWHs) and B (36 SGWHs). Experiment A employed all of the pipe and nutrient conditions described in "Plumbing Conditions" above, except for the copper pipe coupon condition. Water for half of the reactors underwent GAC biofiltration to reduce organic carbon ["GAC" Water], while water for the other half did not undergo this step ["BPC" Water].

Table 2.1. Design of Experiment A with each numbered box representing one simulated glass water heater.

Pipe Condition	GAC Water***			BPC Water****		
Control	1	2	3	1	2	3
High pH Control*	1	2	3	1	2	3
Iron Coupon	1	2	3	1	2	3
PEX pipe sections	1	2	3	1	2	3
Magnesium Rod	1	2	3	1	2	3
Iron(III) oxide sediment	1	2	3	1	2	3
Nitrifying bacteria**	1	2	3	1	2	3
Combination**	1	2	3	1	2	3
* pH adjusted to 10.0 ± 0.1 rather than 7.5 ± 0.1						
**Addition of ammonium sulfate at 1mg/L free NH_3-N.						
***GAC water is breakpoint chlorinated, heated to remove residual and treated with a granular activated carbon (GAC) filter						
**** BPC water is breakpoint chlorinated and heated to remove residual						

In Experiment B, the effects of supplemental AOC were investigated. All water for this experiment underwent prior GAC biofiltration, to provide the lowest possible threshold AOC.

Acetate and glucose (equal mass as mg C/L) were added to achieve five different levels of AOC (0 ppb, 5 ppb, 30 ppb, 150 ppb and 700 ppb as added C/L). Triplicate reactors with one of two pipe materials (PEX or copper pipe coupons) were maintained at each level of organic carbon addition, with one extra set of triplicates at the 700 ppb level. All reactors were operated at 32°C.

Table 2.2. Design of Experiment B with each numbered box representing one simulated glass water heater.

Carbon added	PEX pipe			Copper pipe		
0 ppb	1	2	3	1	2	3
5 ppb	1	2	3	1	2	3
30 ppb	1	2	3	1	2	3
150 ppb	1	2	3	1	2	3
700 ppb	1	2	3	1	2	3
700 ppb *	1	2	3	1	2	3
* Additional control for future studies						

2.2.4 Analysis. All samples were collected using aseptic technique in a biosafety cabinet during routine water changes to minimize impact to the reactors.

Trace metal concentrations were measured using inductively coupled plasma mass spectrometry (ICP-MS). 10 mL samples from each SGWH were transferred to a sterile test tube and acidified by adding 2% nitric acid by mass prior to analysis.

TOC was measured with a Sievers 5310 C Laboratory TOC-MS Analyzer using the Data Pro 5310 C Computer Program. Samples were analyzed using 30 mL from each SGWH, or samples were pooled with 10 mL from each triplicate SGWH. Samples were acidified with phosphoric acid and sparged with N_2 gas in order to purge inorganic carbon prior to analysis.

Adenosine triphosphate (ATP) and adenosine mono-phosphate (AMP) concentrations and their ratios, were measured using a LuminUltra® Quench-Gone™ Aqueous Test Kit (LuminUltra, NB, Canada). ATP provides an indicator of viable biomass activity levels, while AMP is an indicator of cell stress. Samples were taken for each condition by pooling 20 mL from each triplicate SGWH for a total volume of 60 mL and cellular contents were captured on a Quench-Gone syringe filter. Cells were lysed to release ATP for analysis by filtering 1 mL of UltraLyse through the syringe. The remaining process followed the LuminUltra® Quench-Gone™ Aqueous Test Kit steps to determine ATP, AMP and the ATP:AMP index.

Effluent water measuring 100 mL from each SGWH was filtered onto sterile 0.22 µm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA). The filter was folded and torn using sterile tweezers and transferred to a Lysing Matrix A tube from the FastDNA® SPIN Kit (MP Biomedicals, Solon, OH). DNA extraction was conducted according to manufacturer instructions.

Quantitative polymerase chain reaction (qPCR) was applied to quantify the macrophage infectivity potentiator (*mip*) gene specific to *L. pneumophila* (Nazarian et al. 2008) and 16S rRNA genes as an indicator of the level of total bacteria(Suzuki et al. 2000). Q-PCR was carried out using a CFX96™ realtime system (Bio-Rad, Hercules, CA). All q-PCR assays were previously been validated for drinking water samples in terms of specificity and limit of quantification (Wang et al. 2012a). For *L. pneumophila*, a Taqman Probe Mix (Bio-Rad, Hercules, CA) assay was used, and for 16S rRNA genes, an Eva Green (Bio-Rad) assay was

used. For each run of q-PCR analysis, a calibration curve was included with at least six (for 16S) or seven (for *L. pneumophila*) points.

Culturing was also performed to confirm *L. pneumophila* viability. 100 µL water samples were heated to 50°C for 30 minutes, then directly plated on buffer charcoal yeast extract agar according to published methods (Leoni et al. 2005).

2.2.5 Statistical Analysis. Statistical analysis was performed using JMP (SAS, Cary, NC). All data was assumed to be normally distributed, except *L. pneumophila*, which was not normally distributed due to non-detects. T-tests and one-way analysis of variance (ANOVA) were used for comparison of conditions. Non-parametric Wilcoxon test was used for *L. pneumophila* analysis to account for non-detects. Statistical significance was set at $p<0.05$.

2.3 Results and Discussion

2.3.1 Effect of Prior GAC Biofiltration Point-of-use or building level GAC biofiltration is widely used to address taste and odor issues, but also has other key effects on water quality including TOC reduction, organic pollutants removal (Chien et al. 2008; Sorial et al. 1993), metals removal and colonization by diverse microbial communities (Wang et al. 2013).

In the first suite of experiments [Experiment A], GAC biofiltration reduced influent TOC by an average of 40% compared to the BPC water. The effect of GAC biofiltration on influent water quality is further characterized in Table 2.3. GAC biofiltration, applied to breakpoint chlorinated

(BPC) influent water, decreases TOC, increases manganese, decreases copper, increases 16S rRNA gene concentrations and has no effect on *L. pneumophila mip* gene concentrations.

Table 2.3. Influent Water Quality

	TOC (ppb)	Manganese (ppb)	Copper (ppb)	16S rRNA (gene copies/mL)	*L. pneumophila mip* (gene copies/mL)
BPC Water	1370	0.557	64.01	$10^{4.9}$	Non-detect
GAC Water	828	2.723	12.61	$10^{5.8}$	Non-detect
% difference	-39.6 %	+388%	-80%	+20%	N/A

2.3.1.2 TOC. TOC in effluent from SGWHs reflected the TOC in the influent. Effluent from SGWHs with GAC treated influent had an average of 280 ppb less TOC than SGWHs that did not have GAC biofiltration applied to the influent (p<0.001, t-test).

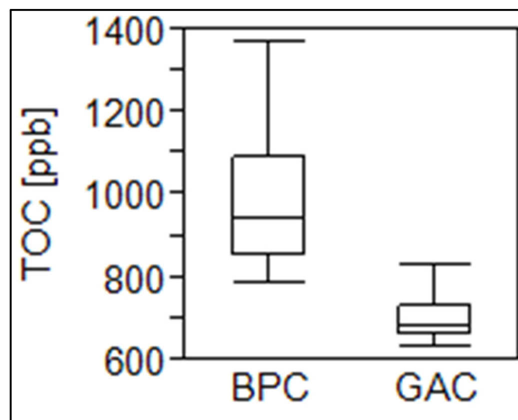

Figure 2.1. TOC [ppb] in Effluent from simulated glass water heaters with influent break-point chlorinated tap water [BPC] and effluent from reactors with similar influent water with additional GAC biofiltration step [GAC]. N = 32, 16 for each influent condition.

2.3.1.2 ATP and AMP Index. While ATP measurement has been proposed as a method of monitoring biological stability, or limit of regrowth in the distribution system (Lautenschlager et al. 2013; van der Wielen and van der Kooij 2010; Vital et al. 2012), ATP did not seem to reflect biological stability in this experiment. Neither ATP nor AMP in effluent from reactors were significantly affected by the GAC filtration step for influent water (p=0.19, t-test for ATP; p =

0.96, t-test, for AMP Index). This is not in congruence with other measures of biological activity (concentration of 16S rRNA) tested from the same reactors.

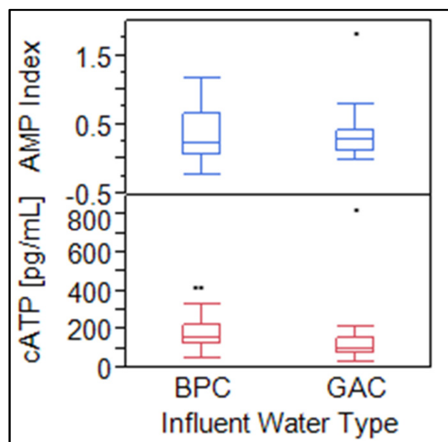

Figure 2.2. Box and Whisker Plots of ATP and AMP Index of Effluent of simulated glass water heaters with Indicated Influent Water Type, with influent break-point chlorinated tap water [BPC] and effluent from simulated glass water heaters with similar influent water with additional GAC biofiltration step [GAC]. Box and Whisker Plots are quartiles. N = 48.

2.3.1.3 Organisms Detected by qPCR. The change in influent water quality corresponds with significant effects on the microbes measured in the effluent in Experiment A (Figure 2.3). The mean level of 16S rRNA genes, an indicator of total bacterial levels, was 162% higher in the effluent from SGWHs receiving BPC water than in those receiving GAC water (p<0.0001, t-test). Conversely, the mean concentration for *L. pneumophila* was 27% higher in effluent of reactors receiving GAC water than in effluent of reactors receiving BPC water (p=0.59, t-test). Although this was an insignificant difference, analysis using non-parametric methods to account for non-detects indicate that this difference becomes significant (p=0.0067, Wilcoxon). The only procedural difference between the influent BPC water and the influent GAC water was the GAC biofiltration step, initially undertaken to reduce TOC.

Thus, GAC treatment to reduce TOC was effective for limiting the growth of the general bacterial population, but actually increased the growth of *L. pneumophila*. Indicators of total bacterial regrowth may not be predictive of *L. pneumophila* concentrations.

While *L. pneumophila* has been known to grow on GAC biofilter and GAC filters may change downstream bacterial communities (Pinto et al. 2012; Wang et al. 2013), slough off is not a factor in this experiment. Although there were occasional detects of *L. pneumophila* in the influent for Experiment B, which also underwent GAC biofiltration, all of the influent water was filtered with a 0.45 µm filter, which should have acted to remove any intact, living bacterial cells. The influence of sloughing of *L. pneumophila* or of an entire community should be limited in this experiment. Rather, the changes in water chemistry drive these differences. Interestingly, activated charcoal, which is similar to activated carbon, is used in culture media for *L. pneumophila* (Leoni et al. 2005) in order to control water chemistry artifacts of autoclaving.

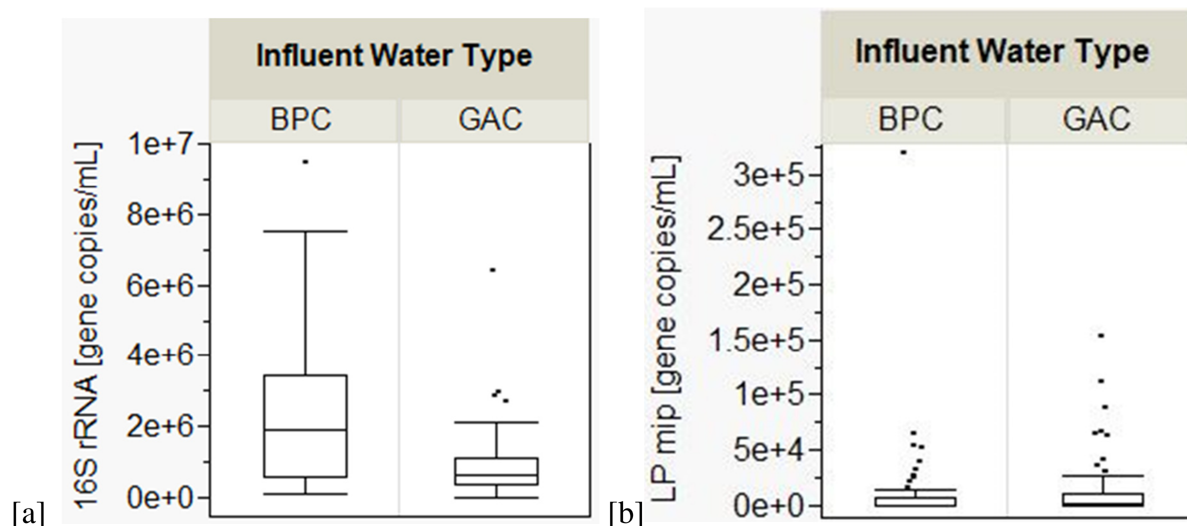

Figure 2.3. Box and whisker plots of [a] total bacterial16S rRNA gene levels in simualated glass water heaters receiving "BPC" water (tap water prepared with breakpoint chlorination and heat to remove disinfectant residual)[n=48] versus "GAC" water (with an additional GAC biofiltration step)[n=48] and [b] *L. pneumophila*-specific *mip* gene levels in simulated glass water heaters receiving "BPC" water [n=98]versus "GAC" water. Box and whisker plots are quartile plots. Data points include up to 4 sampling events from 48 reactors with a suite of plumbing conditions.

GAC biofiltration also alters trace metal concentrations (Table 2.3). Some of the most significant differences were observed with manganese, with an increase of 388% (2.166 ppb) and copper, with a decrease of 80% (51.4 ppb). Previous studies have suggested copper as a control mechanism for *L. pneumophila* colonization (Bargellini et al. 2011), and the effectiveness of copper-silver ionization systems for building disinfection is acknowledged (Shih and Lin 2010). These relationships between trace metals and opportunistic pathogen occurrence are consistent with another study which found positive correlations between manganese and *Legionella spp.* and negative correlations between copper and *Legionella* (Bargellini et al. 2011). The possibility of copper as a control mechanism is further discussed in Chapter 3 (Effects of Premise Plumbing Conditions on Opportunistic Pathogen Persistence) and Chapter 4 (Copper as a Potential Control Mechanism for Opportunistic Pathogens in Premise Plumbing).

2.3.2 Effect of AOC Additions

In the second suite of experiments [Experiment B], acetate and glucose were added directly to the GAC treated and filtered influent, to provide bioavailable forms of TOC. As the carbon added consisted entirely of simple sugars, this directly influences the concentration of AOC.

2.3.2.1 ATP and AMP Index as a Function of added AOC.

The concentration of ATP and AMP are not greatly affected by the concentration of AOC added as acetate and glucose. AMP Indexes were not statistically different at any added level of AOC ($p>0.05$, t-test). Although the concentration of ATP was significantly higher for reactors with 700 ppb added AOC than most other added levels of AOC, it was not significantly higher than those reactors with 0 ppb added AOC ($\alpha=0.05$, t-tests).

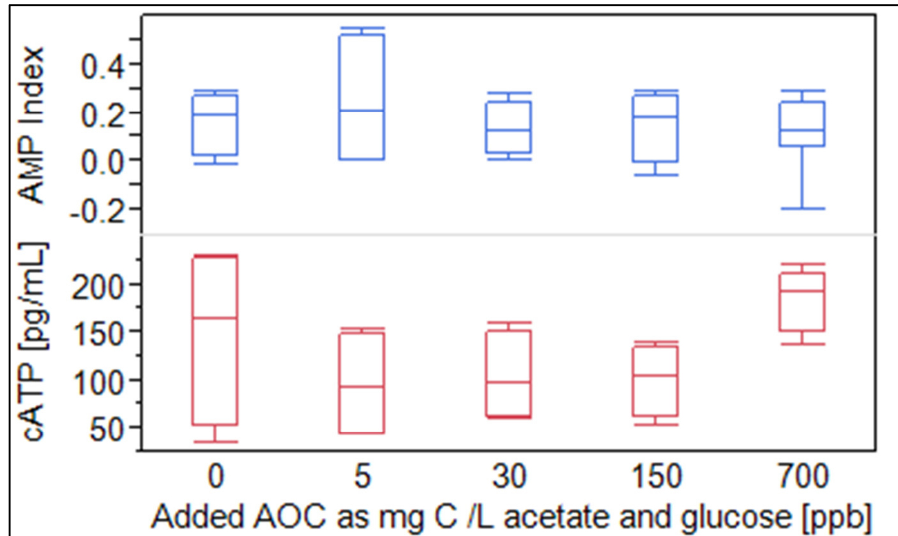

Figure 2.4. Box and Whisker Plots of ATP and AMP index as a function of influent AOC additions in 36 simulated glass water heaters with both PEX and copper pipe coupons at three sampling dates. Box and Whisker Plots are quartiles. For each influent AOC level, n=3, except for 700ppb, n=6. Box and whisker plots are quartile plots.

Although correlations between ATP, AOC and biostability have been observed (van der Wielen and van der Kooij 2010), ATP did not appear to correlate to AOC additions in this experiment. AMP Index was also not affected, although all readings indicated that the reactors were either in low stress (AMP Index <0.1) or moderate stress (0.1 < AMP Index < 3.0). None were under lethal stress (AMP Index >3.0) according to Kit manufacturers (LuminUltra, NB, Canada).

2.3.2.2 Organisms Detected by qPCR. It was observed that acetate and glucose additions had a significant effect on the concentrations of both 16S and *L. pneumophila* (Figure 2.5). The mean concentration of 16S rRNA genes was 2.5 times higher in effluent from the 700 ppb added AOC condition than the mean for all other conditions (p<0.0001, ANOVA). The mean concentration of *L. pneumophila* in the 700 ppb AOC condition was 2 times higher than all conditions (p=0.0023, ANOVA). *L. pneumophila* concentrations in the 700 ppb AOC condition were not significantly different than *L. pneumophila* concentrations in the 150 ppb AOC condition.

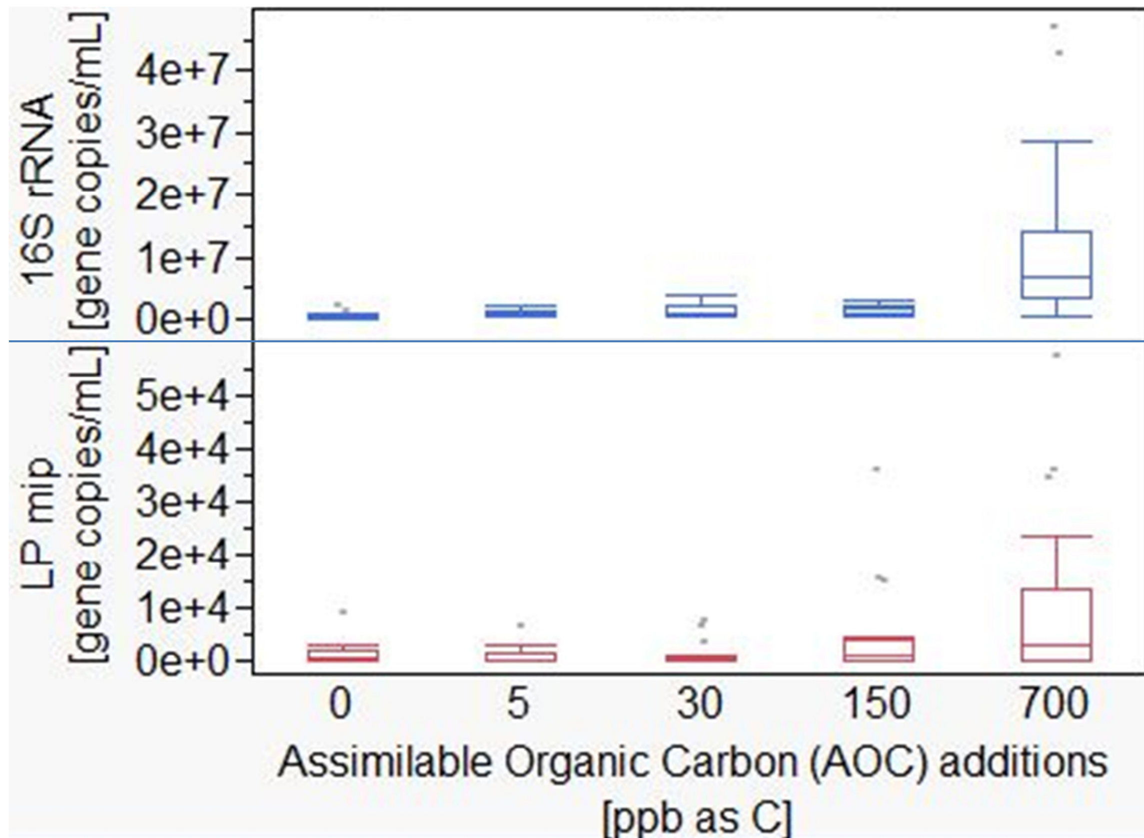

Figure 2.5. Box and whiskers plot of total bacterial 16S rRNA genes (top) and of *L. pneumophila* -specific *mip* genes (bottom) as a function of influent AOC additions in 36 simulated glass water heaters with both PEX and copper pipe coupons at three sampling dates. For each influent AOC level, n=18, except for 700ppb, n=36. Box and whisker plots are quartile plots.

As the form of TOC in the first suite of experiments was likely not as bioavailable, these results suggest that forms of carbon known to be more bioavailable may have a direct influence on both total bacterial population levels and *L. pneumophila*. However, it is important to note that the AOC level at which an effect was observed was at the high end of the spectrum, three times or more what is observed in typical drinking water systems (Lechevallier et al. 1991), and much higher than the proposed limits for regrowth control (Hammes et al. 2010; Kooij 1992; Volk and LeChevallier 2000; Zhang et al. 2002).

In both studies, culturable *L. pneumophila* in SGWH was found in high concentrations. Concentrations often neared or exceeded action limits as directed by the Occupational Safety and Health Administration. Immediate cleaning or biocide treatment is suggested when levels meet or exceed 100 CFU/mL in domestic water (OSHA 2009).

2.4 Conclusions

The results of AOC supplementation and GAC biofiltration from Experiments A and B yielded several conclusions that could be instrumental in directing engineering-based controls of *L. pneumophila* on the utility or building level.

- Controlling overall bacterial population by means of reducing TOC with point-of-use filters may not be effective for targeting specific opportunistic pathogens. SGWHs with GAC biofiltered influent responded to the reduced TOC with decreased overall bacterial population, but supported increased concentrations of *L. pneumophila* -specific *mip* gene. This suggests that GAC biofiltration has the potential to stimulate *L. pneumophila* growth. GAC's influence on trace metals may be important.
- The form of organic carbon is important for the likelihood of *L. pneumophila* proliferation. Additions of acetate and glucose as readily assimilable forms of organic carbon resulted in increases in both *L. pneumophila* -specific *mip* and total bacterial 16S rRNA gene levels. Forms of organic carbon known to be more readily assimilable could have a more direct effect on *L. pneumophila* proliferation than the forms removed by GAC biofiltration.

- Control of AOC in the water may be an effective utility-level or building-level control to consider. However, there may be a point at which reduction is no longer effective. Significant effects were only observed at 700 ppb, seven times recommended levels. Also, unintended consequences of specific methods for AOC removal should be taken into account, as GAC biofilters can harbor and release bacteria into the water (which were removed in this study with a 0.45 um filter), and their influence on trace metals may also be important (See Chapters 3 and 4)

Premise plumbing presents complicated and dynamic issues. With differences in source water, water treatment and distance to the drinking water treatment plant, it is possible that a one-size fits all solution may not be feasible, even on the individual utility level. Point-of-use GAC biofiltration presents a possible risk for *L. pneumophila* colonization.

2.5 Acknowledgments

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Chapter 3

Effect of Plumbing Conditions on the Persistence of *Legionella pneumophila* in Premise Plumbing

3.1 Introduction

Within premise plumbing, the pipe material and plumbing conditions can vary greatly and these conditions can greatly impact both the chemistry and microbiology of the water (National Research Council 2006). Biofilm formation may be especially important as disinfectant is lost in premise plumbing systems with stagnation, temperature, or other chemical reactions (Nguyen et al. 2012). Longer stagnation times within premise plumbing pipe can affect leaching of materials and biofilm formation (Lautenschlager et al. 2010; Lehtola et al. 2007). Higher temperatures encountered in hot water lines may also change the behavior of each material (Inkinen et al. 2014).

As the occurrence of opportunistic pathogens, in particular *Legionella pneumophila*, varies widely within premise plumbing and the distribution system, the factors that drive their proliferation are still unclear (Pruden 2013). Pipe materials and the occurrence of specific niches in premise plumbing are thought to be of importance (van der Kooij et al. 2005; Wang et al. 2012b). Pipe material can impact the likelihood of *L. pneumophila* by leaching substances that are either beneficial, as with nutrients, or harmful, as with metals, to bacterial regrowth.

Plastic pipes, in particular cross-linked polyethylene (PEX), have been shown to release substantial amounts of organic carbon which is used as a substrate for growth of heterotrophic bacteria and amoeba (Bucheli-Witschel et al. 2012; Inkinen et al. 2014; Rogers et al. 1994; van der Kooij et al. 2005). PEX pipes may also release phosphate, nutrients, and light-weight organics especially in extreme circumstances (Lehtola et al. 2004; Lehtola et al. 2001; Skjevrak et al. 2003). These leached materials may have an implication in microbial regrowth (Inkinen et al. 2014).

Copper is known to be toxic to some bacteria, and the metal ion is utilized in building-level copper-silver ionization systems for disinfection (Lin et al. 2011; Shih and Lin 2010). Copper from copper pipes, however, may not be beneficial for controlling regrowth, as it may require a higher disinfectant residual for control of regrowth issues than plastic pipes (Lehtola et al. 2005). Copper pipes explored in model distribution systems seem to control *L. pneumophila* regrowth for a short amount of time, but this effect is lost within pipes used for more than 200 days (van der Kooij et al. 2005). One study found a positive correlation between copper and *L. pneumophila* in field samples (Mathys 2008). Another study investigating the full scale implementation of copper and PEX pipes attributed differences in biofilm formation to operational parameters, wherein the copper pipe system had a dead-end (Inkinen et al. 2014).

Autotrophic bacteria present in the system may also provide a source of AOC for heterotrophic bacteria and amoebae, subsequently encouraging *L. pneumophila* growth. Nitrifying bacteria convert ammonia, which may be present from secondary disinfection with chloramine, to nitrite and nitrate and generate biomass from inorganic carbon. As much as 87 µg organic carbon/mg

NH_3 consumed can be generated by nitrifiers (Zhang et al. 2009), and their presence could greatly impact the regrowth patterns of opportunistic pathogens as well (Wang et al. 2012c). Nitrification is also associated with loss of disinfectant residual, which can allow for greater heterotrophic growth(Zhang and Edwards 2009).

Hydrogen oxidizing bacteria take advantage of molecular H_2 as an electron donor to generate biomass from inorganic carbon (Igarashi 2001). H_2 can be generated from the sacrificial magnesium anode rods in electrical storage water heaters, even leading to explosions in extreme circumstances (Cook 2004). Over a five-year life-time, autotrophs could use the H_2 from a typical magnesium rod could produce up to 160 µg/L·day of organic carbon (Martin 2012).

As iron may be present in the distribution system and may leach from steel components within premise plumbing, it is an important material to consider as a source of nutrients. As iron corrodes, it may provide all macronutrients necessary for bacterial growth, most especially phosphorous (Morton et al. 2005). Humics present from the source water may also bind to iron sediments and become more bioavailable in this form (Butterfield et al. 2002).

Premise plumbing systems are complex and in any one building, many niches that encourage microbial regrowth and proliferation of opportunistic pathogens may be encountered. Thus in situ studies may not be able to discern the contributive effects of each niche. Complex distribution system models may be under the influence of multiple niches, for example with copper heating coils used for systems with plastic piping (van der Kooij et al. 2005).

In this study, the effects of a range of plumbing materials and water chemistry factors on the proliferation of *L. pneumophila* proliferation are investigated with the use of controlled simulated glass water heaters. Each niche investigated, including nitrification-simulating conditions, elevated pH, and a suite of plumbing materials (copper, cross-linked polyethylene (PEX), magnesium anodes, and iron), was evaluated over a range of influent water conditions affecting organic carbon content. The simulated glass water heaters were operated at a low temperature typical of the lower range of water heater operation or premise plumbing distribution (32 °C) in order to support *L. pneumophila* growth and allow comparison of potential for each plumbing condition to control or stimulate *L. pneumophila.*

3.2 Materials and Methods

3.2.1 Simulated Glass Water Heaters. Simulated glass water heaters consisted of 125 mL French square borosilicate glass bottles with polytetrafluoroethylene caps and were equipped with a range of plumbing materials and conditions. All reactors were incubated at 32 °C, a temperature representative of the bottom of a conventional water heater where certain opportunistic pathogens are thought to thrive. Simulated glass water heaters were originally inoculated with *L. pneumophila* at an approximate initial concentration of 2.03×10^5 gene copies/mL, *Acanthamoeba polyphaga* at 2,079 gene copies/mL, *Mycobacterium avium* at 7.15×10^5 gene copies/mL, and *Hartmannella vermiformis* at 1.46×10^3 amoeba/mL into each SGWH as described further in Section 2.2.1. Simulated glass water heaters were operated for 2.5 years prior to commencing the present experiment. Upon commencing the described experiments, cross-inoculation was conducted by pooling effluent from reactors and adding 1 mL to the influent for each reactor in order to achieve a similar baseline microbial community.

3.2.2 Plumbing Conditions. Plumbing materials and conditions utilized in this study included:

1) Iron coupon to simulate iron plumbing system components including steel in water heaters
2) Cross-linked polyethylene (PEX) pipe sections
3) Copper pipe sections
4) Magnesium to simulate magnesium anodes used for corrosion control
5) Iron(III) oxide sediment from unlined iron in many distributions systems
6) Ammonium sulfate additions at concentrations 1mg/L free NH_3-N to simulate chloramine systems after residuals disappear
7) Combination of iron coupon, magnesium rod, and ammonium sulfate addition
8) Controls (no additional materials) at pH 7.5 and 10

In addition to the materials described above, all simulated glass water heaters also contained a 1.5 mm layer of 1mm glass beads, which served to increase the surface area to volume ratio and provide a range of redox zones similar to those found in premise plumbing sediment layers.

3.2.3 Influent Water and Water Changes. Three times a week, an 80% water change was conducted to simulate infrequent use of a water heater. Without shaking or agitation, 100 mL was discarded and replaced with freshly prepared modified Blacksburg drinking water. Modified Blacksburg drinking water was used rather than modified reverse osmosis or nanopure water in order to continually introduce common inorganics and nutrients into the system.

Town of Blacksburg, VA, tap water is disinfected with chloramine. Water was collected in the lab from the cold water tap and was breakpoint chlorinated (BPC) with addition of a diluted hypochlorite solution. Break-point chlorinated water was then heated to 90 °C for 10 minutes in

order to remove most trace disinfectant residual. For corresponding experiments examining the effect of prior GAC filtration, the cooled water was subject to biofiltration with a point of use GAC filter.

All influent water was pre-treated by passage through a 0.45 µm polyvinylidene fluoride (PVDF) filter to remove microbes. Water was adjusted to pH 7.5 ± 0.1, except in the case of the 'high pH control' which is adjusted to pH 10.0 ± 0.1. pH was controlled with additions of 1 M or less NaOH and HCl using an Oakton pH 10 series meter (Oakton Instruments, Vernon Hills, IL)

3.2.4 Experimental Design. Simulated glass water heaters were divided into two experiments: A (48 reactors) and B (36 reactors). Experiment A employed all of the pipe and nutrient conditions described in Section 3.2.2 Plumbing Conditions above, except for the copper pipe coupon condition. Water for half of the reactors underwent GAC biofiltration to reduce organic carbon ["GAC" Water], while water for the other half did not undergo this step ["BPC" Water].

Table 3.1. Experiment A experimental design with each numbered box representing one simulated glass water heater

Pipe Condition	GAC Water***			BPC Water****		
Control	1	2	3	1	2	3
High pH Control*	1	2	3	1	2	3
Iron Coupon	1	2	3	1	2	3
PEX pipe sections	1	2	3	1	2	3
Magnesium Rod	1	2	3	1	2	3
Iron(III) oxide sediment	1	2	3	1	2	3
Nitrifying bacteria **	1	2	3	1	2	3
Combination**	1	2	3	1	2	3
* pH adjusted to 10.0 ± 0.1 rather than 7.5 ± 0.1						
**Addition of ammonium sulfate at 1mg/L free NH_3-N.						
***GAC water is breakpoint chlorinated, heated to remove residual and treated with a granular activated carbon (GAC) filter						
**** BPC water is breakpoint chlorinated and heated to remove residual						

In Experiment B, the effects of supplemental AOC water were investigated. All water for this experiment underwent prior GAC biofiltration to provide the lowest possible threshold AOC. Acetate and glucose (equal mass as mg C/L) were added to achieve five different levels of AOC (0 ppb, 5 ppb, 30 ppb, 150 ppb, and 700 ppb as added C/L). Triplicate reactors with one of two pipe materials (PEX and copper pipe coupons) were maintained at each level of organic carbon addition with one extra set of triplicates operated at 700 ppb. All reactors were operated at 32 °C.

Table 3.2. Design of Experiment B experimental design with each numbered box representing one simulated glass water heater.

Carbon added	PEX pipe sections			Copper pipe sections		
0 ppb	1	2	3	1	2	3
5 ppb	1	2	3	1	2	3
30 ppb	1	2	3	1	2	3
150 ppb	1	2	3	1	2	3
700 ppb	1	2	3	1	2	3
700 ppb *	1	2	3	1	2	3
* Additional set of triplicates						

3.2.5 Analysis. All samples were collected using aseptic techniques in a biosafety cabinet during routine water changes to minimize impact to the reactors.

Total organic carbon (TOC) was measured with a Sievers 5310 C Laboratory TOC-MS Analyzer using the Data Pro 5310 C Computer Program. Samples were analyzed using 30 mL from each simulated glass water heaters, or samples were pooled with 10 mL from each of triplicate simulated glass water heaters. Samples were acidified with phosphoric acid and sparged with N_2 gas in order to purge inorganic carbon prior to analysis.

Adenosine Triphosphate (ATP) and Adenosine mono-phosphate (AMP) concentrations, and their ratios, were measured using a LuminUltra® Quench-Gone™ Aqueous Test Kit (LuminUltra, NB, Canada). ATP provides an indicator of viable biomass activity levels, while AMP is an indicator of cell stress. Samples were taken for each condition by pooling 20 mL from each triplicate simulated glass water heater for a total volume of 60 mL and cellular contents were captured on a Quench-Gone syringe filter. Cells were lysed to release ATP for analysis by filtering 1 mL of UltraLyse through the syringe. The remaining process followed the LuminUltra® Quench-Gone™ Aqueous Test Kit steps to determine ATP, AMP, and the ATP:AMP index.

Effluent water measuring 100 mL from each simulated glass water heater was filtered onto sterile 0.22 µm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA). The filter was folded and torn using sterile tweezers and transferred to a Lysing Matrix A tube from the FastDNA® SPIN Kit (MP Biomedicals, Solon, OH). DNA extraction was conducted according to manufacturer instructions.

Quantitative polymerase chain reaction (qPCR) was applied to quantify the macrophage infectivity potentiator (*mip*) gene specific to *L. pneumophila* (Nazarian et al. 2008) and 16S rRNA genes as an indicator of the level of total bacteria(Suzuki et al. 2000). Q-PCR was carried out using a CFX96™ realtime system (Bio-Rad, Hercules, CA). All q-PCR assays were previously been validated for drinking water samples in terms of specificity and limit of quantification (Wang et al. 2012a). For *L. pneumophila*, a Taqman Probe Mix (Bio-Rad, Hercules, CA) assay was used, and for 16S rRNA genes, an Eva Green (Bio-Rad) assay was

used. For each run of q-PCR analysis, a calibration curve was included with at least six (for 16S) or seven (for *L. pneumophila*) points.

Culturing was also performed to confirm *L. pneumophila* viability. 100 µL water samples were heated to 50 °C for 30 minutes, then directly plated on buffer charcoal yeast extract agar according to published methods (Leoni et al. 2005).

3.2.6 Statistical Analysis. Statistical analysis was performed using JMP (SAS, Cary, NC). T-tests were used to compare all data assumed to be normally distributed. For *L. pneumophila,* the data was not normally distributed due to non-detects, and Wilcoxon non-parametric tests were used to consider this influence. Statistical significance was set at $p<0.05$.

3.3 Results and Discussion

In both of the experiments described, both carbon conditions and materials were investigated. Unless otherwise noted, in analysis multiple influent carbon conditions were considered together in order to determine the effect of the particular pipe material across a wider range of carbon conditions. As influent AOC can vary for different treatment plants (Liu et al. 2002) and for the same treatment plant with different seasons (Polanska et al. 2005), this approach gives insights to the possible interplay between plumbing materials and AOC. Carbon conditions are further discussed in Chapter 2.

3.3.1 TOC. TOC was not significantly affected by plumbing condition in either experiment A or B ($p>0.05$, t-tests). In Experiment A, the additional GAC treatment had a significant effect and in Experiment B, a wide range was driven by acetate and glucose additions [See Chapter 2].

3.3.2 ATP and AMP Index. ATP gives a measure of cell activity, and the ratio between ATP and AMP, or AMP Index, gives an indication of the level of stress in each reactor

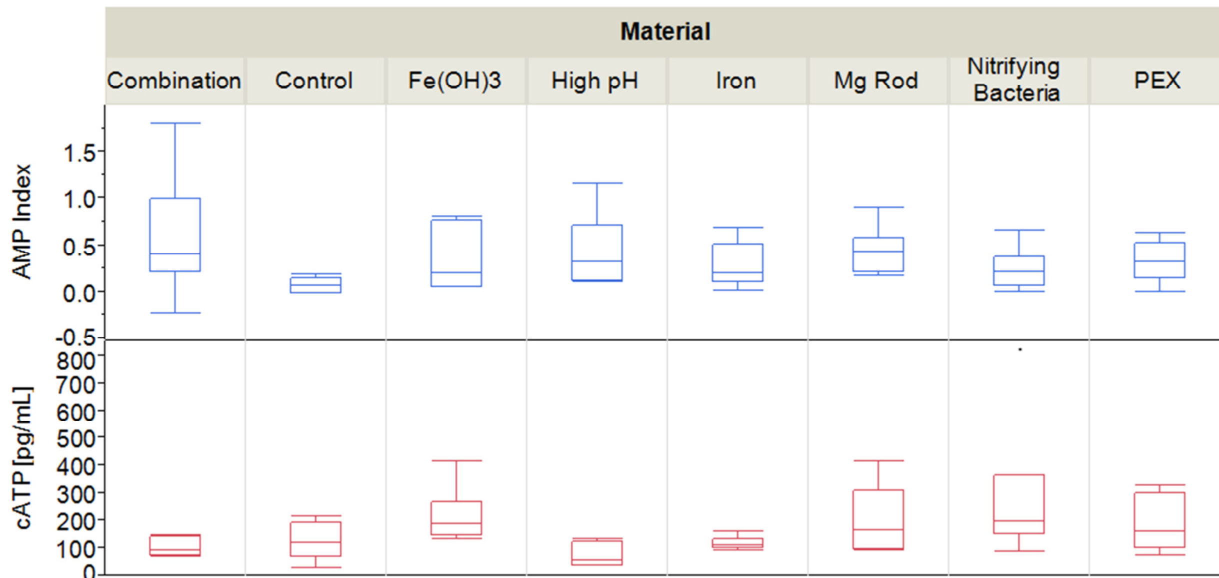

Figure 3.1. Box and whiskers plot of AMP Index and Concentration of ATP [pg/mL]. AMP Index (top) in simulated glass water heater samples (n=6 for each condition) and Concentration of ATP [pg/mL] (bottom) in simulated glass water heater samples (n=6 for each condition). Box and whisker plots are quartile. Includes samples over time (early October 2012 to late July 2013).

The AMP Index was significantly higher for the Combination SGWHs than Control SGWHs (p=0.015, t-test). The stress was higher when multiple materials influenced water chemistry.

The mean concentration of ATP was highest in SGWHs with ammonia additions and lowest in conditions of high pH. SGWHs with ammonia additions are the only SGWHs that had significantly different concentrations of ATP than the Control SGWHs (p=0.0320, t-test). This may be driven by one outlier measurement. However, it is also possible that the addition of ammonia to influent water, in the absence of other stressors, stimulated activity of nitrifying bacteria and other bacteria.

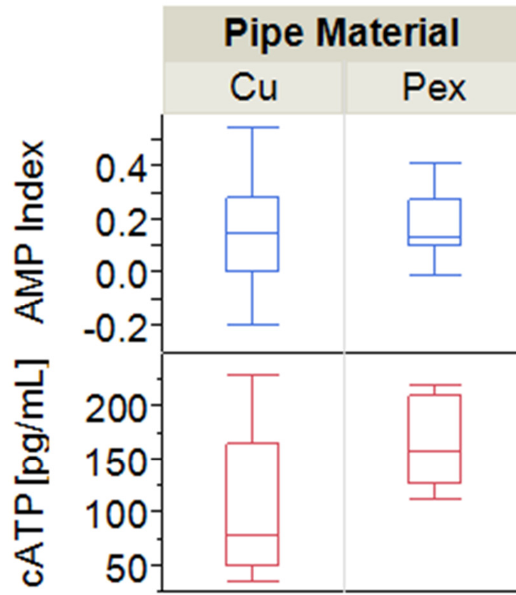

Figure 3.2. Box and whiskers plot of AMP Index and ATP concentration [pg/mL] in simulated glass water heaters containing copper and PEX pipe coupons. AMP Index (top) and ATP concentration [pg/mL] (bottom). Samples were taken from 36 reactors (18 copper, 18 PEX) with varied AOC as acetate and glucose additions on three sampling events. Box and whisker plots are quartile. For each plot n = 12.

AMP Index is not significantly affected by copper or PEX pipe material, comparatively, indicating that all reactors are under approximately the same amount of stress. Concentration of ATP is significantly higher for reactors with PEX material than with copper pipe material, with a decrease of 58 pg/mL for copper pipes (p=0.0222, t-test).This may indicate that there is more viable biomass in reactors with PEX pipe.

3.2.3 *L. pneumophila* and 16S rRNA. In the first suite of experiments [Experiment A], all plumbing conditions previously described except copper were compared (Figure 3.3). While there was some evidence that certain conditions may have had slight impact on *L. pneumophila* and total bacterial growth on individual sampling dates, no significant differences among pipe materials were consistently observed over time on either 16S rRNA (p=0.66, ANOVA) or *L. pneumophila*-specific *mip* (p=0.12, Wilcoxon, nonparametric) gene levels. Effects were higher

on specific dates, and when considering both pipe material and influent water. This indicates that season may affect water quality and that influent water quality may interact with plumbing conditions.

When considering plumbing condition and type of influent water together, the condition with the highest mean concentration of *L. pneumophila* had PEX coupons and GAC treated influent water. These reactors had 1.75 logs more *L. pneumophila* than the glass control condition with the same influent water. At the first sampling event, these differences were much more pronounced, with PEX reactors supporting as much as 4.25 logs more than the Control condition. The condition with iron coupons and BPC influent water had 1.73 logs more *L. pneumophila* than the glass control condition with the same influent water. No other conditions were significantly different than glass controls with the same influent water.

The *L. pneumophila*-specific *mip* gene was detected in all conditions on at least one sampling date, such that none of the plumbing conditions completely controlled against *L. pneumophila* colonization. High concentrations of *L. pneumophila* were found in all conditions, with averages for each plumbing condition ranging from 1.78 – 2.72 log gene copies/mL.

It is somewhat surprising that the array of plumbing and water chemistry conditions tested (Figure 3.3) did not exert a greater impact on 16S rRNA and *L. pneumophila* concentrations in water leaving the simulated glass water heaters. However, these results were collected for conditions in which there was no influent disinfectant. Because prior work with these reactors indicate dramatic difference in rates of chloramine and chlorine decay (Martin et al. 2012), it is

possible and even likely that different results would be obtained if disinfectants were present in the influent. Experiments examining this issue are planned for continued experimentation on the SGWHs used in this experiment.

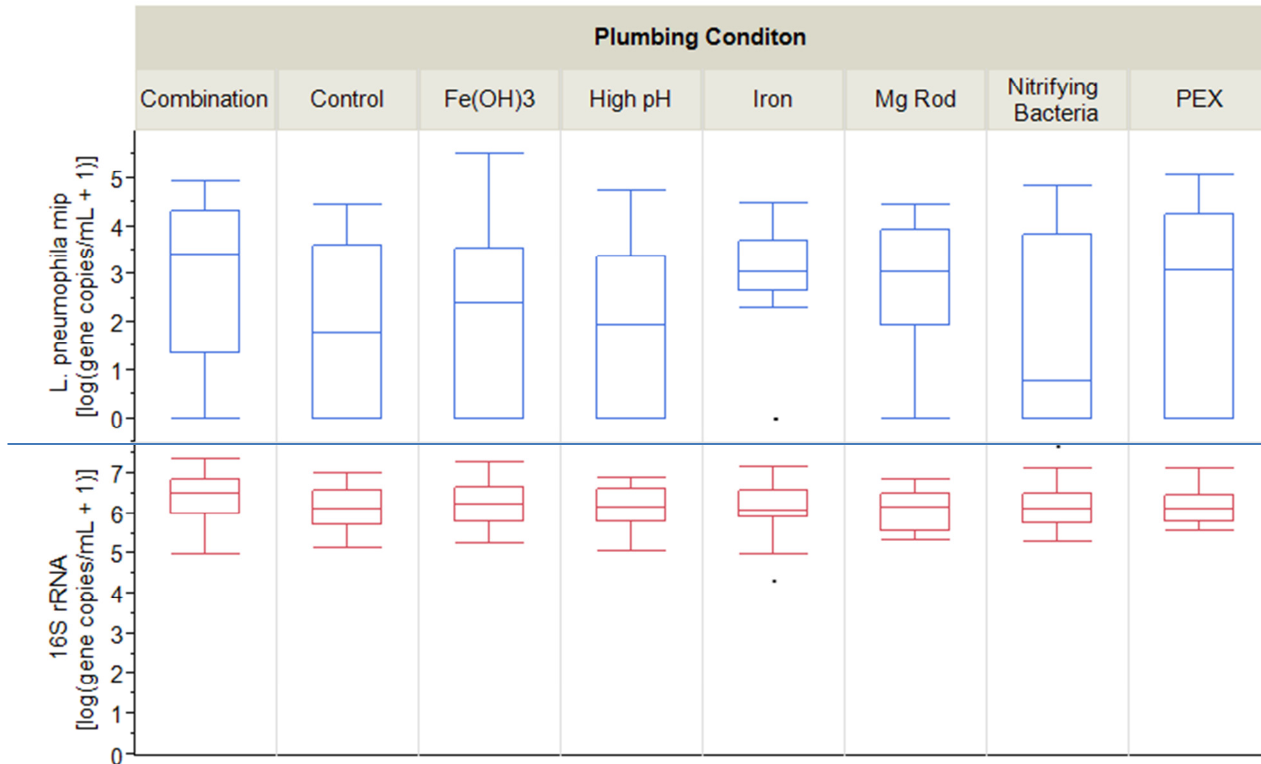

Figure 3.3. Box and whiskers plot of total bacterial 16S rRNA gene levels and *L. pneumophila*-specific *mip* gene levels in simulated glass water heater. 16S rRNA gene levels (top) (n=24 for each condition) and *L. pneumophila*-specific *mip* gene levels (bottom) (n=24 for each condition). Box and whisker plots are quartile. Samples were collected and pooled over time (early October 2012 to late July 2013).

In the second suite of experiments [Experiment B], copper and PEX coupons were directly compared, with equivalent surface area and influent water conditions (Figure 3.4). Copper pipes proved more effective at controlling *L. pneumophila* concentrations than PEX pipes, although total bacterial 16S rRNA gene concentrations were not significantly different. Copper provided an average 2.2-log reduction in *L. pneumophila* -specific *mip* gene copy levels ($P<0.0001$, Wilcoxon nonparametric). Concentrations in SGWHs with PEX coupons were high, with 3.5 log

gene copies/mL detected. Mean 16S rRNA gene levels were less than 0.001 logs different from each other (p=0.526, t-test).

Other studies have compared copper and plastic pipe materials directly, and found that with age of pipes greater than a couple of months, many measures of biological regrowth, including occurrence of *L. pneumophila,* were not significantly different, although initial differences occurred (Lehtola et al. 2004; van der Kooij et al. 2005). Thus, age of the pipe is an important consideration. In this study, pipe materials were in use for 2.5 years before this study, and the benefits of copper in controlling *L. pneumophila* extended far longer than noted by other researchers for different waters.

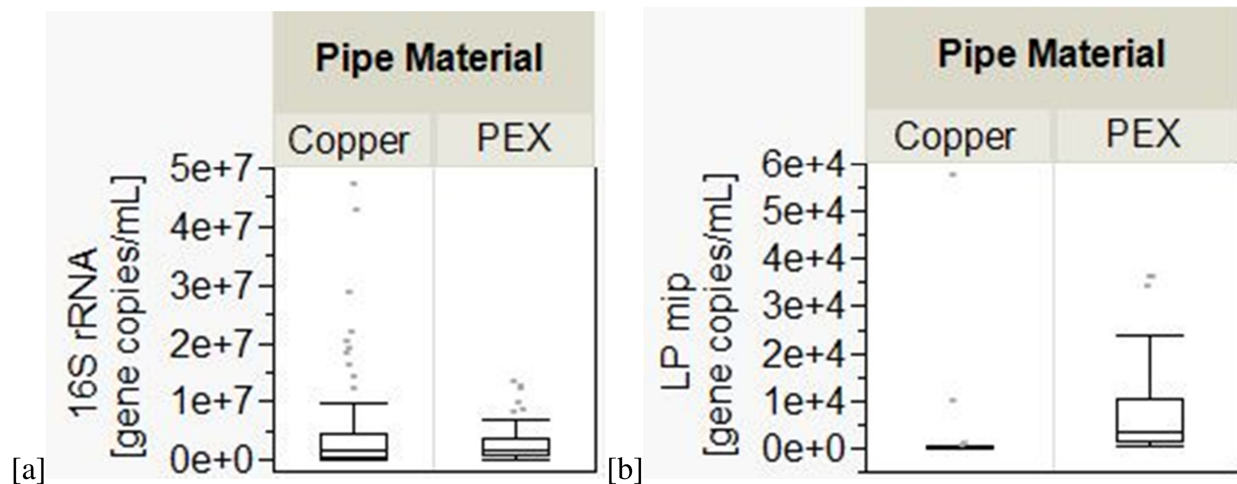

Figure 3.4. Box and whiskers plot of [a]total bacterial 16S rRNA gene levels and [b] *L. pneumophila*-specific mip gene levels. Measured from simulated glass water heaters containing copper (n=54) and PEX (n=54) pipe coupons. Samples were taken from 36 reactors (18 copper, 18 PEX) with varied AOC as acetate and glucose additions on three sampling events. Box and whisker plots are quartile plots.

Thus, copper is sometimes useful as a selective control against *L. pneumophila*. This is also in accordance with data from Experiment B, where an 80% decrease in copper concentration with GAC biofiltration supported a higher mean *L. pneumophila* concentration [Chapter 2]. While

several studies demonstrate that copper is not a permanent long term control mechanism for *L. pneumophila* in all waters (Inkinen et al. 2014; Lehtola et al. 2004; Mathys et al. 2008; van der Kooij et al. 2005), it seems likely that the qualitative and quantitative impact would depend on factors such as pH which control the solubility of copper and its speciation. In one study a copper-silver ionization disinfection system was not effective at a high pH where copper was not available in its toxic form (Lin et al. 2002). Another experiment in this work considers the effects of pH and copper on *L. pneumophila* in simulated glass water heaters [Chapter 4].

In Experiment B, significant differences in *L. pneumophila mip* gene concentrations and concentrations of ATP were found based on pipe material, while these differences were not found in concentrations of 16S rRNA. All measures of total biomass may not be indicative of the behavior of specific pathogens. As suggested by a probiotic approach, the presence of some bacteria or activity may be beneficial for the control of pathogens (Wang et al. 2013).

3.4 Conclusions

The results of these studies emphasize the importance of long-term studies with materials encountered in premise plumbing. Although different plumbing factors strongly influenced colonization and levels of *L. pneumophila* at the beginning of this study, when the study was extended over a year these differences became less significant for the water tested. This occurred although all materials had been aged for 2.5 years prior to the start of the study, and short-term effects may be more pronounced with new materials.

The importance of pipe materials may be dependent on influent water quality. While PEX and iron may have encouraged *L. pneumophila* growth, these effects were limited to specific influent water chemistries.

Use of copper pipes may be an effective control mechanism for *L. pneumophila* in some waters, but research is needed to identify conditions that can clearly explain why copper controls Legionella in some situations while being less effective in others.

3.5 Acknowledgements

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Chapter 4

Inactivation of *Legionella pneumophila* Within Premise Plumbing via Copper Ions

4.1 Introduction

Opportunistic Pathogens in Premise Plumbing (OPPPs) pose an increasingly significant threat to human health, due to aging population and other risk factors (Pruden 2013). Immunocompromised populations including the elderly, children and individuals hospitalized with other infections, are especially vulnerable to OPPP infection. Of particular concern is *Legionella pneumophila,* which is the causative agent of Legionnaire's disease. Legionnaire's disease hospitalizes 8,000-18,000 people annually in the United States (Center for Disease Control 2011), with a fatality rate of 5-30% of cases (US Department of Health and Human Services 2005). Exposure to *L. pneumophila* is typically through inhalation of aerosolized bacteria from water systems, including suspended droplets created during hot showers or hand washing (Schoen and Ashbolt 2011). In recognition of health risks, the U.S. EPA has established a non-enforceable maximum contaminant level goal (MCLG) of zero organisms in drinking water. However, the standard is currently applicable to the effluent of water treatment plans and not in premise plumbing where the microbes are known to amplify and where human exposure occurs (Environmental Protection Agency 2000).

OPPPs, including *L. pneumophila*, are not fecal-associated and can be found in the distribution system even when primary and secondary disinfection are employed at the drinking water treatment plant. Certain attributes of premise plumbing likely contribute to biofilm formation and growth of OPPPs, including high surface to volume ratio, loss of disinfectant residuals with high water age and extended stagnation(National Research Council 2006) (Lautenschlager et al. 2010), and increased temperatures in hot water lines (Bagh et al. 2004). Disinfectant residuals are sometimes lost and likelihood of OPPP growth is increased for microbes thriving under such conditions, although many OPPPs are particularly resistant to residual disinfectants (Cooper and Hanlon 2010). OPPP occurrence can vary widely throughout premise plumbing of buildings and the distribution system (Wang et al. 2012a) and the factors that drive their proliferation are still not clear. Different types and designs of pipe and fixtures are thought to play a major role in OPPP regrowth (Wang et al. 2012b). In addition to materials, flow conditions (i.e., velocity, frequency, and volume), age and maintenance of these systems can vary widely and present a challenge for maintaining low pathogen levels.

In some large-scale buildings, including those with populations at high risk, in-building disinfection methods are used to manage the risk of opportunistic pathogen colonization. Copper-silver ionization systems are often used, which take advantage of the bactericidal properties of metals in their ionic form. This method has been reported to be effective for inactivation of *L. pneumophila* and other pathogens in some cases and proper system operation is thought to be critical (Shih and Lin 2010; States et al. 1998). A dosage of copper ions as low as 0.1 mg/L (100 ppb) was shown to completely inactivate *L. pneumophila* in one study (Lin et al. 1996) conducted at pH 7 in relatively pure water.

The use of copper in various other forms has been explored as a method for controlling colonization and regrowth of *L. pneumophila* with varying degrees of success. Application of copper to point-of-use filters was reported to be ineffective in controlling *L. pneumophila*, whereas application of both copper and silver was reported to be effective (Molloy 2007). In pure culture experiments, copper oxide nanoparticles had a detrimental effect on the expression of certain *L. pneumophila* genes associated with virulence and viability (Lu et al. 2013).

Due to its durability, flexibility and corrosion resistance, copper is a common pipe material in premise plumbing systems. Copper ions are released at varying rates from pipes as a function of age and water chemistry and the aqueous concentration varies widely from system to system, or even within homes of a given system with different ages and water use patterns. pH or addition of phosphate to water at the utility level may control the form copper takes. Thus, the relationship between copper pipes and *L. pneumophila* control may be complex in real systems.

A negative relationship between copper concentrations and *L. pneumophila* colonization was found in a survey of hot water systems in Italy (Leoni et al. 2005). Another survey of a real system indicated a negative relationship between copper concentrations and Legionella spp. (Bargellini et al. 2011). There is some evidence that copper may be necessary or even beneficial for *L. pneumophila* proliferation. A German survey indicated that homes with copper pipe were more likely contaminated by *Legionella* than homes with galvanized iron or plastics (Mathys 2008). In these homes, copper levels were all below 0.1 mg/L and pH was similar for all waters (Mathys 2014). Another field study found *L. pneumophila* contamination only in homes with copper pipes (Tiefenbrunner et al. 1993).

Copper pipe has also been compared to other popular pipe materials, such as PVC and stainless steel, in well-controlled laboratory experiments. In one study coupons of PVC, copper and stainless steel measuring 4.9-6.25 cm^2 were exposed to tap water for 30 days. Biofilm formation was monitored with microscopy on sacrificial coupons approximately every four days. Biofilm formation occurred quickly on PVC and copper pipe coupons compared with stainless steel, but total biofilm formation was lowest on copper pipes for the duration of the 30 day study (Morvay et al. 2011). Control of overall biofilm growth does not necessarily indicate control of pathogens. In another study comparing the same three pipe materials in a hot water system with 5.9 m of pipe connected to full-scale water heaters, copper again did not have significant effects on total biofilm formation compared to stainless steel pipes. Copper was initially shown to have inhibitory effects on cultured Legionella spp. These effects did not continue with long-term exposure to copper pipes with samples collected over 2.25 years, perhaps because of variation in copper levels over the duration of the study (van der Kooij et al. 2005).

Broad control of bacterial growth may not be an achievable or effective control strategy for controlling growth of OPPPs. A more balanced “pro-biotic approach” accepts that many bacteria in water and in premise plumbing biofilms are inevitable and may even help to resist against *L. pneumophila* colonization (Guerrieri 2008; Wang et al. 2013). Previous studies with similar reactors as those used in this study indicated that copper pipe may hold promise for *L. pneumophila* control, even with long-term use of greater than 2.5 years. Results detailed in Chapter 3 suggest that copper may preferentially target *L. pneumophila* over the broader bacterial community [Chapter 3]. Thus, prior research indicates mixed results with respect to the potential for copper to inhibit *L. pneumophila* colonization with some studies indicating no

effect, others indicating significant inhibition and still other studies suggesting increased *L. pneumophila* growth with higher copper levels.

One likely explanation for the discrepancy is the differing chemistry of the waters tested, which in turn, affects the copper solubility, speciation and toxicity. In one study a hospital copper-silver ionization system was rendered ineffective with high pH water (Lin et al. 2002) due to reduced precipitation of the added copper and/or increased complexation of Cu^{+2} (Zhang 2008). Bench testing of this water determined a 6 log inactivation at pH 7 but only a 1 log reduction at pH 9 in response to a fixed dose of copper (Lin et al. 2002). Because temperature, phosphate, inorganic and organic carbon, disinfectant levels and age of plumbing can dramatically influence the speciation and levels of copper that will be observed in premise plumbing systems, it seems likely that water chemistry variations might partly explain the discrepancies in copper impacts described in prior research.

It is also important to note that while copper has antimicrobial properties it is also a micronutrient required for production of many enzymes (Chaturvedi and Henderson 2014), and thus may be beneficial in lower concentrations. Bacteria are also capable of adapting to develop a wide variety of copper-resistance mechanism when exposed to high concentrations of copper, as in agriculture with copper-salt applications (Chaturvedi and Henderson 2014). Thus, the bacterial response to copper may change drastically with both concentration and time of exposure. In this study, both copper concentration and time of bacterial exposure are controlled in order to focus on the contribution of water chemistry factors.

Water chemistry factors, specifically pH, may also have a more direct effect on *L. pneumophila* proliferation. Studies have reported various optimal pHs for *L. pneumophila* growth including 6-8 (Ohno et al. 2003) and 5.5-9.2(Wadowsky et al. 1985) Slightly basic water was found to be favorable for recovery from real systems with successful recovery from hospitals ranging from pH 7.5-9.0 (Dutka et al. 1984). Positive correlations between pH and detection of *Legionella* and *L. pneumophila* were also found in other surveys (Kusnetsov et al. 2003; Leoni et al. 2005; Marrie et al. 1994). Still, pH correlation is conflicting. One study found higher pHs to be detrimental to survival of *L. pneumophila* compared to low to basic pHs (Katz and Hammel 1987). Another survey of hotels found positive relationships with pH for *L. pneumophila* serogroup 1 but negative correlations for all other serogroups tested (2-14) (Mouchtouri et al. 2007).

This research is aimed at better understanding the effectiveness of copper control of *L. pneumophila* under conditions encountered in premise plumbing and over the range of pHs encountered in practice. The results will improve understanding of *L. pneumophila* control by use of copper silver ionization systems or from copper present in the water due to copper pipe or brass alloys.

4.2 Materials and Methods

4.2.1 Simulated Glass Water Heaters (SGWHs). SGWHs consisted of 125 mL French square borosilicate glass bottles with polytetrafluoroethylene caps were acid-washed and baked prior to use. Each was equipped with either chlorinated polyvinyl chloride (CPVC) or cross linked polyethylene (PEX) pipe as noted for specific conditions. New pipes with diameter ¾” were cut

into 1” sections and pre-conditioned with a sodium hypochlorite disinfectant and deionized water for ten days with water replacement every two days, to reduce leaching of organic carbon during the experiment and to simulate commissioning of new plumbing systems (California Builiding Standards Commission 2009; California State Pipe Trades Council 2005). All SGWHs were incubated at 32 °C, a temperature representative of the bottom of conventional electric water heaters which are subject to temperature stratification or for copper pipe in the walls of certain buildings during summer months.

4.2.2 Influent Water and Water Changes. Three times a week, an 80% water change was conducted to simulate low frequency use of a water heater. During each water change, 90 mL was discarded and replaced with freshly prepared modified Blacksburg tap water. Modified Blacksburg tap water was used rather than modified reverse osmosis or nanopure water in order to continually introduce common inorganics and nutrients into the system, a step deemed necessary since years of prior attempts to cultivate *L. pneumophila* under completely synthesized laboratory conditions with oligotrophic water was not successful (Edwards et al. 2012).

The modified Blacksburg water was collected from a lab tap in the Town of Blacksburg, which uses chloramine secondary disinfection. The tap was flushed for 10 minutes before collection. The water was breakpoint chlorinated (BPC) with addition of a diluted hypochlorite solution. Break-point chlorinated water was then heated to 90 °C for 10 minutes in order to remove most disinfectant residual. All water was then subject to biofiltration with a granular activated carbon (GAC) filter. Further water treatment and pH adjustment varied for each condition. Prior to pH adjustment, all influent water was passed through a polyvinylidene fluoride (PVDF) filter with

0.45 µm pore size to remove most microbes. Water was adjusted to the target pH ± 0.1 with drop-wise addition of NaOH and HCl as verified using an Oakton pH 10 series meter (Oakton Instruments, Vernon Hills, IL).

4.2.3 Initial Acclimation Period. During Phase 0 (Table 4.2) each reactor was mixed with 34 mL of effluent from similar reactors that had been colonized by *L. pneumophila* and were in operation for three years prior to commencing this experiment. These reactors were inoculated with *L. pneumophila*, *Acanthamoeba polyphaga*, *Mycobacterium avium*, and *Hartmannella vermiformis* as described in 2.2.1. The remainder of the influent water consisted of modified Blacksburg tap water as described, with pH adjusted to 7.5 ± 0.1. Thereafter Phase 1 of the experiment involved changing the water without purposeful inoculation to allow *L. pneumophila* and biofilms to stabilize.

4.2.4 Experimental Conditions and Phases. After the inoculation and acclimation period (phases 0 and 1), the influent water was changed to one of 6 target conditions (Table 4.1). Ion-exchange treatment to remove trace copper was achieved by filtration through a 3 cm column of Chelex Ion Exchange Resin. pH was adjusted to 7, 8 and 9. Copper dosage (from a stock 0.0014 M copper (II) sulfate solution) to the reactors was incrementally increased every two weeks to a target of with 5, 30, 150 and 1000 ppb in Phases 3, 4, 5 and 6, respectively.

Table 4.1. Experimental conditions for influent water in each reactor

Condition Name (3 reactors/condition)	**Ion Exchange Control**	**Control**	**pH 7 – CPVC**	**pH 7 – PEX**	**pH 8 – PEX**	**pH 9 – PEX**
Pipe Material	PEX	PEX	CPVC	PEX	PEX	PEX
Copper added?	NO	NO	YES	YES	YES	YES
Influent pH	7	7	7	7	8	9
Influent water treatment	GAC & Ion exchange filtered	GAC filtered	GAC filtered	GAC filtered	GAC filtered	GAC filtered

Table 4.2. Experimental Phases for each of 6 conditions

<table>
<tr><th rowspan="2">Phase</th><th rowspan="2">Duration</th><th colspan="6">Condition [pipe material if not otherwise noted]</th></tr>
<tr><th>Ion Exchange Control [PEX]</th><th>Control [PEX]</th><th>pH 7 – CPVC</th><th>pH 7 – PEX</th><th>pH 8 – PEX</th><th>pH 9 – PEX</th></tr>
<tr><td>0</td><td>1 week</td><td colspan="6">Influent is GAC filtered, pH adjusted to 7.5, and spike is added from other reactors for inoculation.</td></tr>
<tr><td>1</td><td>~2 weeks</td><td colspan="6">Influent is GAC filtered, and pH is adjusted to 7.5</td></tr>
<tr><td>2</td><td>2 weeks</td><td rowspan="5">Influent is GAC and ion exchange filtered, and pH is adjusted to 7</td><td colspan="3">Influent is GAC filtered, and pH is adjusted to 7</td><td>GAC, pH 8</td><td>GAC, pH 9</td></tr>
<tr><td>3</td><td>2 weeks</td><td rowspan="4">Influent is GAC filtered, and pH is adjusted to 7</td><td colspan="2">GAC filtered, pH 7, and 5 ppb Cu^{+2} dosed</td><td>GAC, pH 8, 5 ppb Cu^{+2}</td><td>GAC, pH 9, 5 ppb Cu^{+2}</td></tr>
<tr><td>4</td><td>2 weeks</td><td colspan="2">GAC filtered, pH 7, 30 ppb Cu^{+2}</td><td>GAC, pH 8, 30 ppb Cu^{+2}</td><td>GAC, pH 9, 30 ppb Cu^{+2}</td></tr>
<tr><td>5</td><td>2 weeks</td><td colspan="2">GAC filtered, pH 7, 150 ppb Cu^{+2}</td><td>GAC, pH 8, 150 ppb Cu^{+2}</td><td>GAC, pH 9, 150 ppb Cu^{+2}</td></tr>
<tr><td>6</td><td>2 weeks</td><td colspan="2">GAC filtered, pH 7, 1000 ppb Cu^{+2}</td><td>GAC, pH 8, 1000 ppb Cu^{+2}</td><td>GAC, pH 9, 1000 ppb Cu^{+2}</td></tr>
</table>

*All copper levels are in ppb Cu^{+2} dosed as $CuSO_4$. Except for the ion-exchange control which had <3.7 ppb, the initial water also had copper levels ranging from 1-10 ppb naturally present in the influent.
**All influent water passed through PVDF filter with 0.45 µm pore size prior to use.

4.2.5 Analysis. All samples were collected in a BSL II cabinet using standard aseptic techniques during routine water changes to minimize impact to the reactors and possibility of cross-contamination.

Trace metal concentrations were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). 10 mL samples from each SGWH were transferred to a sterile test tube and acidified by adding 2% nitric acid by mass prior to analysis. To distinguish soluble from particulate trace metals, samples were analyzed before and after passage through a PVDF filter with 0.45 µm pore size.

Total organic carbon (TOC) was measured with a Sievers 5310 C Laboratory TOC-MS Analyzer using the Data Pro 5310 C Computer Program. Pooled samples consisting of 10 mL from each of triplicate SGWHs were analyzed. Samples were acidified with phosphoric acid and sparged with N_2 gas in order to purge inorganic carbon prior to analysis.

Adenosine triphosphate (ATP) and adenosine monophosphate (AMP) concentrations and their ratios, were measured using a LuminUltra® Quench-Gone™ Aqueous Test Kit (LuminUltra, NB, Canada). ATP provides an indicator of viable biomass activity levels, while AMP is an indicator of cell stress. Samples were taken for each condition by pooling 20 mL from each triplicate SGWH for a total volume of 60 mL and cellular contents were captured on a Quench-Gone syringe filter. Cells were lysed to release ATP for analysis by filtering 1 mL of UltraLyse through the syringe. The remaining process followed the LuminUltra® Quench-Gone™ Aqueous Test Kit steps to determine ATP, AMP, and the ATP:AMP index.

Effluent water measuring 90 mL from each SGWH was filtered onto sterile 0.22 µm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA). The filter was folded and torn using sterile tweezers and transferred to a Lysing Matrix A tube from the FastDNA® SPIN Kit (MP Biomedicals, Solon, OH). DNA extraction was conducted according to manufacturer instructions.

Quantitative polymerase chain reaction (qPCR) was applied to quantify the macrophage infectivity potentiator (*mip*) gene specific to *L. pneumophila* (Nazarian et al. 2008) and 16S rRNA genes as an indicator of the level of total bacteria (Suzuki et al. 2000). Q-PCR was carried

out using a CFX96™ realtime system (Bio-Rad, Hercules, CA). All q-PCR assays were previously been validated for drinking water samples in terms of specificity and limit of quantification (Wang et al. 2012a). For *L. pneumophila*, a Taqman Probe Mix (Bio-Rad, Hercules, CA) assay was used, and for 16S rRNA genes, an Eva Green (Bio-Rad) assay was used. For each run of q-PCR analysis, a calibration curve was included with at least six (for 16S) or seven (for *L. pneumophila*) points.

Culturing was also performed to assay *L. pneumophila* viability. Water samples measuring 100 µL were heated to 50°C for 30 minutes and were directly plated on buffer charcoal yeast extract agar according to published methods (Leoni et al. 2005). Plating is treated as a semi-quantitative measure as cultivable enumeration differences have been reported to be quite high in different laboratories (Lucas et al. 2011).

4.2.6 Statistical Analysis. Statistical analysis was performed using JMP (SAS, Cary, NC). Data was assumed to be normally distributed. Where means were compared, a t-test was used to compare means. Linear regression was performed with the least-squared regression method and qPCR data was transformed to $\log_{10}$ values. Statistical significance was set at $p<0.05$.

4.3 Results and Discussion

After describing changes in total bacteria and *L. pneumophila* due to changes in the bulk water chemistry, the response to increased copper dosing are described.

4.3.1 Chemical Parameters.

4.3.1.1 pH. During Phase 1, pH was maintained at 7.5 in order to match that of the inoculum from reactors known to support *L. pneumophila*. During Phase 2, pH was adjusted to a wider

range applicable to real water systems and decreased as much as 0.35 units during stagnation (Table 4.3). The condition at pH 7 with CPVC had nearly identical final pH as the PEX.

Table 4.3. pH in reactor influent and effluent during Phase 6.

Condition	Target Influent pH (± 0.1)	Temperature of Effluent	pH in Effluent
Ion-Exchange Control	7.0	32°C	7.04
Control	7.0	32°C	7.06
pH 7 - CPVC	7.0	32°C	7.17
pH 7 - PEX	7.0	32°C	7.04
pH 8 - PEX	8.0	32°C	7.65
pH 9 - PEX	9.0	32°C	8.69

4.3.1.2 Copper Removal. Two controls were established in order to isolate the effects of copper addition. In one, the pH 7 GAC influent is used without any addition of copper. In another, copper was removed with an ion exchange resin (see Methods).

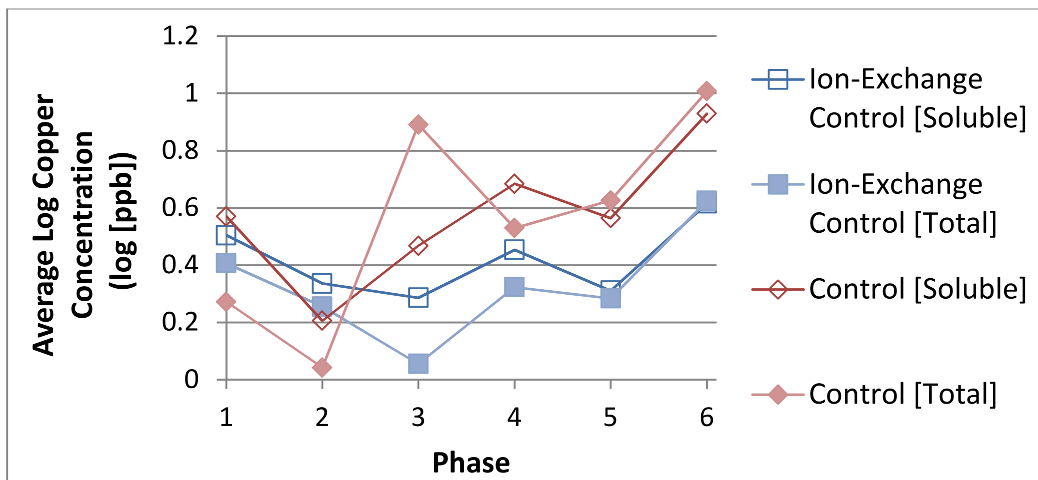

Figure 4.1. Copper concentrations over time in control conditions. The ion exchange control (blue) and the control (red) condition were not dosed with copper. Open shapes indicate the portion that passed through a PVDF filter with 0.45 μm pore size. Closed shapes indicate the total. For each point, n = 3.

The copper levels in the control indicate the base level present in all reactors, and the ion-exchange control indicates the removal with an additional ion-exchange step. The maximum

total copper concentration in control reactors was 10 ppb. Ion-exchange afforded some extra copper removal when the initial concentration was above 0.5 ppb. The ion-exchange step also altered concentrations of other metals (Appendix, Table 4.4).

4.3.1.3 Copper in Reactors with Copper Additions. Copper dosages were increased over 6 phases. The form of copper, whether reduced, Cu^{+}, or oxidized, Cu^{2+}, can significantly affect the toxicity, with Cu^{+} as the more toxic form (Chaturvedi and Henderson 2014). In this study, the least toxic form, Cu^{2+}, was tested. With additions of copper sulfate, it was expected that the pH would have significant effects on the portion of copper which passed through the PVDF filter with 0.45 µm pore-size.

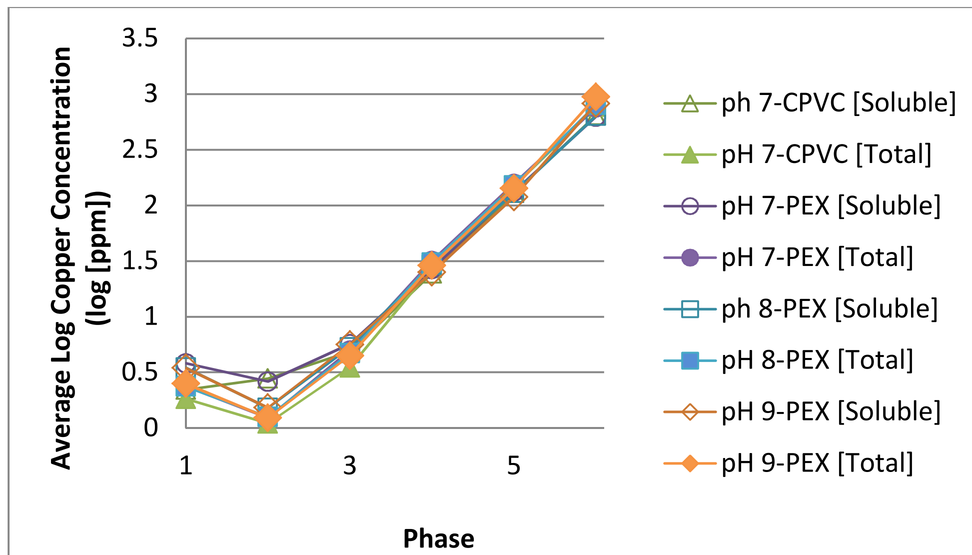

Figure 4.2. Average copper concentrations in effluent from triplicate simulated glass water heaters in each condition with copper dosed. For each point n = 3. Soluble denotes the portion that passed through a PVDF filter with 0.45 µm pore-size. pH was adjusted in Phase 2, and copper dosing began in Phase 3. Copper was dosed at 5, 30, 150 and 1000 ppb in Phases 3-6 respectively. For each dot, n = 3.

Both total and soluble copper increased with increased dosages of copper sulfate solution in Phases 3-6. PVDF filters with 0.45 µm pore-size did not remove more than 20% from the total

concentration of copper throughout the initial phases of the study with copper dosing. This was consistent across all conditions in all pHs. Measurements were taken during regular water changes, at least two days after copper dosing. Copper may not have been uniform in all samples taken as SGWHs were not intentionally shaken prior to sample collection. However, nearly 100% of copper dosed was recovered in samples.

Phase 6 was continued for several months. After 3.5 months, additional measurements were taken to better determine the specific size fraction of colloidal and particulate copper in the water.

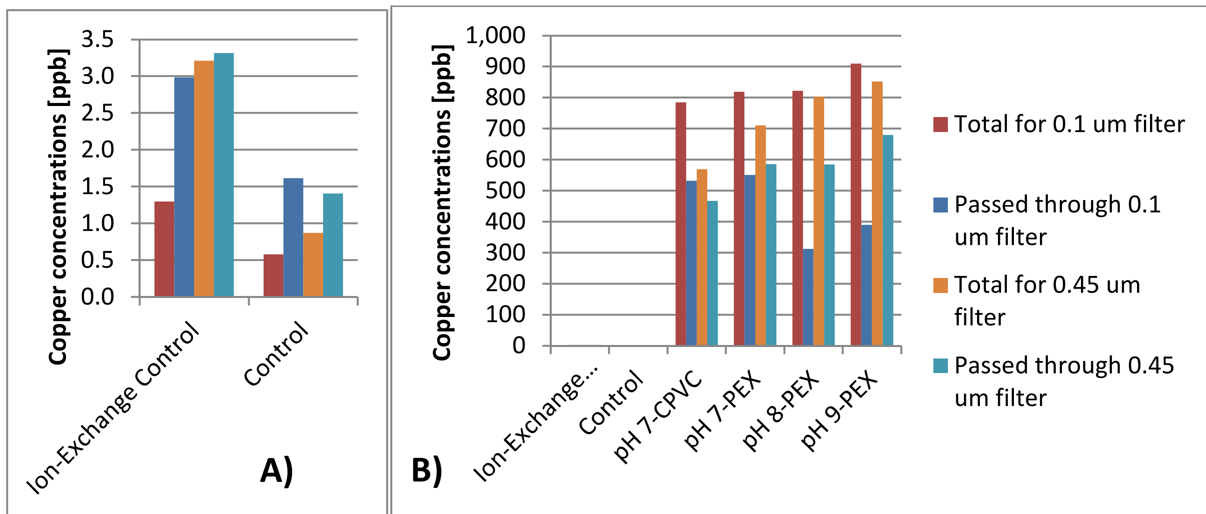

Figure 4.3. Average copper concentrations in effluent from triplicate simulated glass water heaters in each condition [(A) Controls (B) All conditions], total and passed through PVDF filters of varied size. Samples for 0.1 µm filter and 0.45 µm filter taken on different days within a week of each other and analyzed together. Copper was dosed at 1,000 ppb in non-control conditions. For each bar, n=3.

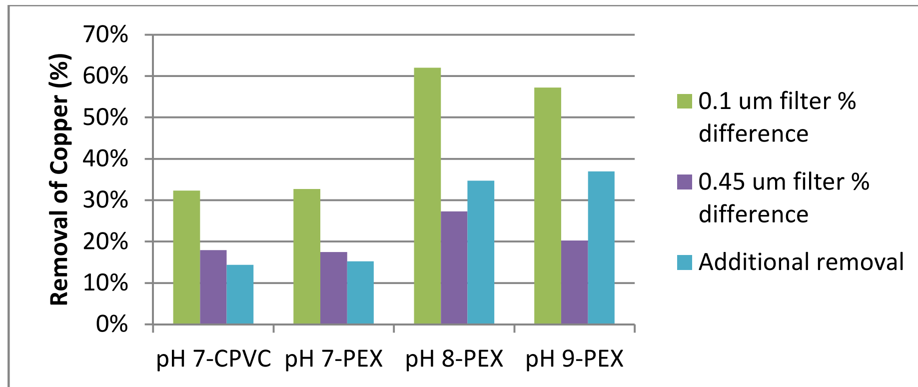

Figure 4.4. Percent removal of copper with PVDF filters in reactors with dosed copper, compared to bulk concentration with filtration through filters with 0.1 µm pore size (green) and 0.45 µm pore size (purple). The additional removal with the 0.1 µm filter compared to the 0.45 µm filter, as percent of total is also indicated (blue). For each bar, n = 3.

Copper speciation was less responsive to change in pH than expected. The difference between total and soluble copper, when measured by passage through a filter with 0.45 µm pore size was less than 30% for all pHs, and was highest for pH 8. When measured by passage through a filter with 0.1 µm pore size, the difference between total and soluble copper greatly increased, and increased much more so for the conditions with elevated pH than the conditions with pH 7. There was little difference between pH 8 and pH 9. The additional removal of copper with the 0.1 µm filter compared to the 0.45 µm filter is 35% and 37% respectively for pH 8 and pH 9.

For the conditions with no added copper sulfate, neither filter consistently removed copper. This was consistent with the expectation that copper would be predominantly soluble at lower concentrations.

4.3.1.4 TOC. TOC measured from reactor effluent followed trends previously established according to the specific plastic material (Tabor et al. 2013; Williams 2011). All reactors with PEX material had the highest TOC during Phase 1 even with pre-conditioning of the samples,

and leaching gradually declined. The CPVC pipe exhibited a different trend without an initial elevation in TOC. After the conditioning step, PEX continued to leach carbon whereas CPVC did not.

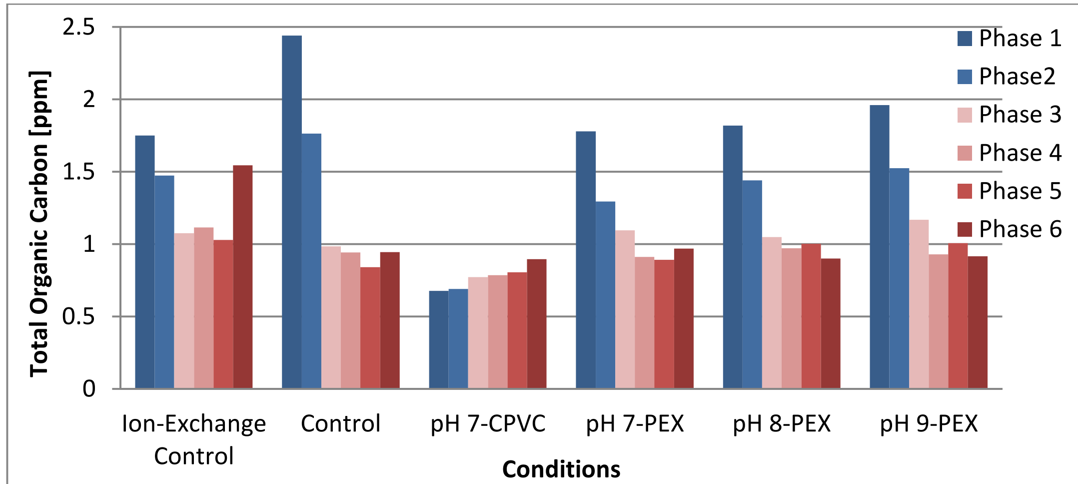

Figure 4.5. Total Organic Carbon [TOC] measured in effluent from triplicate reactors in each condition measured during each phase. Measurements are from samples pooled for each condition. For each bar, n = 1. In Phase 2, pH was adjusted from 7.5 to the target pH. In Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively.

4.3.2 Biological Parameters

ATP, AMP Index, *L. pneumophila* colony formation, and concentrations of both *L. pneumophila* and 16S rRNA genes were measured during each phase of the study. These parameters are best analyzed in three groupings – controls, the different pipe materials at pH 7, and at various pHs.

4.3.2.1 Biological Parameters in Controls. The two control conditions, with GAC water (Control) and ion-exchange water (ion-exchange control) did not react consistently across time. This may be attributed to aging of pipe materials, as with TOC leaching, or varied influent water over time.

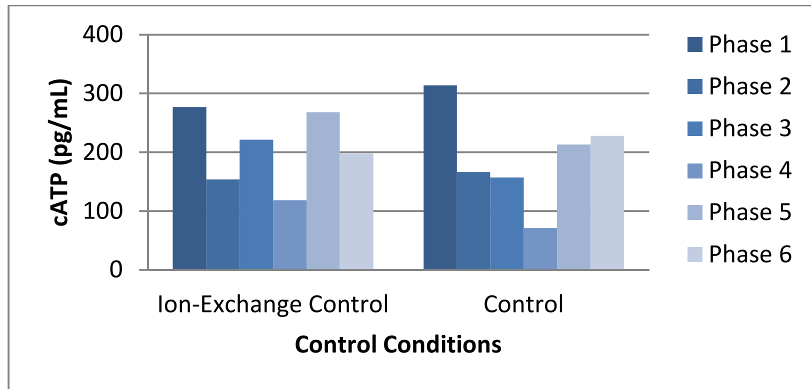

Figure 4.6. ATP concentrations measured for control conditions in each phase. Samples were mixed from triplicate reactors in each condition during each phase. These are control reactors without copper for relative comparisons to reactors with copper. For each bar, n = 1.

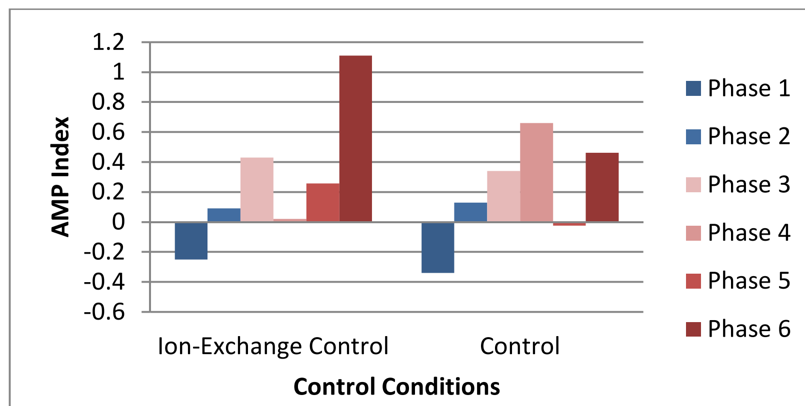

Figure 4.7. AMP Index measured for control conditions in each phase. AMP index is a ratio between AMP and ATP measurements, and is a measure of stress. A higher AMP index indicates a more stressed environment. Samples were mixed from triplicate reactors in each condition during each phase. These are control reactors without copper for relative comparisons to reactors with copper. For each bar, n = 1.

ATP and AMP concentrations indicate that reactors were healthiest in Phase 1, before pH adjustment began. Reactors in the control conditions maintained more consistent concentrations of ATP than of AMP index.

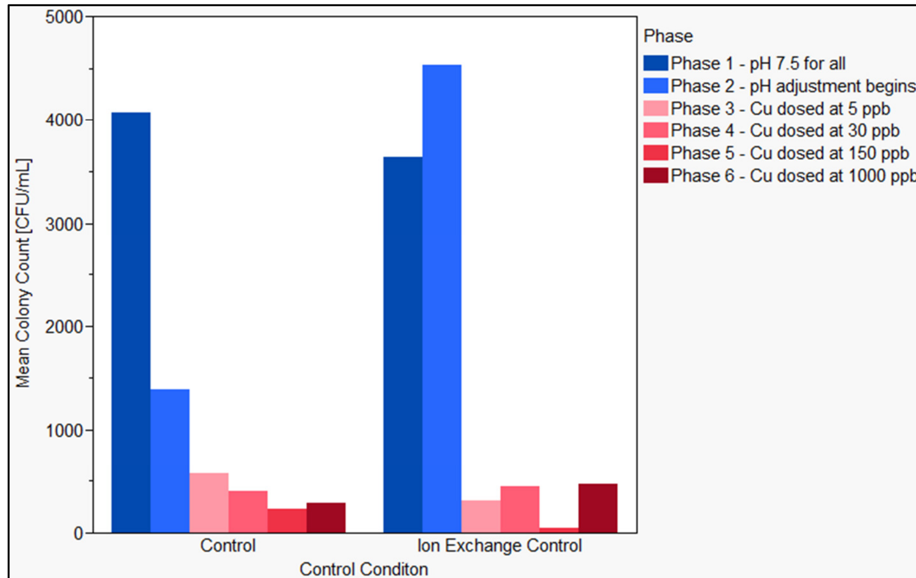

Figure 4.8. Mean colony count [CFU/mL] for triplicate simulated glass water heaters in control conditions across all phases. For each bar, n=3. These are control reactors without copper for relative comparisons to reactors with copper. In Phase 2, pH was adjusted from 7.5 to 7 for control reactors, and ion-exchange water was introduced for the ion-exchange control reactors. For each bar n = 3.

L. pneumophila detected by culture methods (Figure 4.8) was highest in the beginning of the experiment. For the control, there was a significant drop in Phase 2, when pH adjustment began. This effect was not observed in the ion-exchange control, which had an increase in *L. pneumophila* detected in Phase2, when it began receiving ion-exchange water with lowered pH. In phases 3-6, *L. pneumophila* detection varied, but *L. pneumophila* was observed consistently across these control detections. The decrease in *L. pneumophila* detection could also be attributed to TOC reduction. In all reactors with PEX material, including control reactors, leaching of TOC decreased as the experiment progressed.

L. pneumophila concentrations detected by qPCR (Figure 4.9) were also highest in Phase 1, and dropped when pH adjustment began. In control reactors, *L. pneumophila* concentrations remained similar across Phase 2-6.

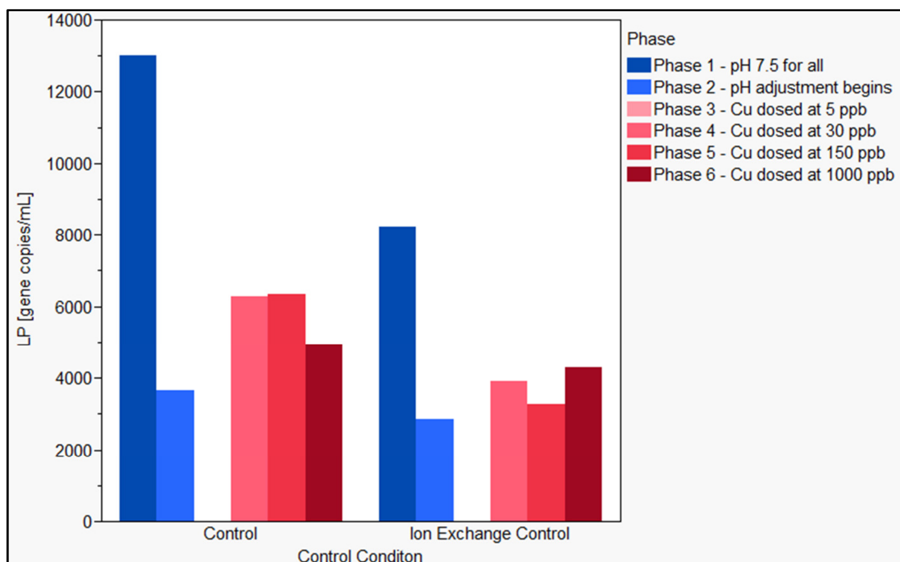

Figure 4.9. Average *L. pneumophila* concentration detected by qPCR for triplicate simulated glass water heaters in control conditions across all phases. For each bar, n=3. These are control reactors without copper for relative comparisons to reactors with copper. In Phase 2, pH was adjusted from 7.5 to 7 for control reactors, and ion-exchange water was introduced for the ion-exchange control reactors. Genetic samples for Phase 3 were lost.

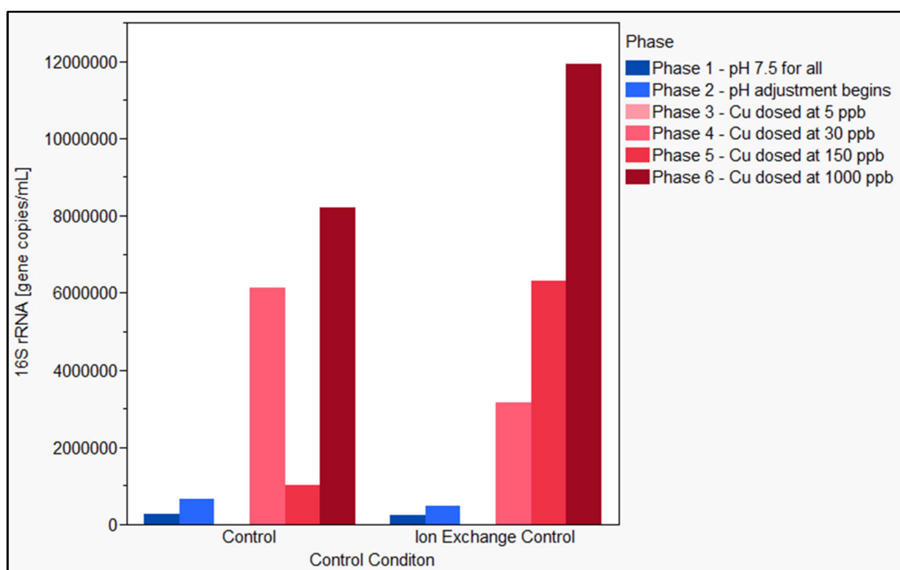

Figure 4.10. Average 16S rRNA concentration detected by qPCR for triplicate simulated glass water heaters in control conditions across all phases. For each bar, n=3. These are control reactors without copper for relative comparisons to reactors with copper. In Phase 2, pH was adjusted from 7.5 to 7 for control reactors, and ion-exchange water was introduced for the ion-exchange control reactors. Genetic samples for Phase 3 were lost.

Concentrations of 16S rRNA increased over the course of the experiment in both control conditions. Concentrations of 16S were lowest in Phase 1, in contrast to trends for *L. pneumophila*, which decreased with pH adjustment then stabilized. It is possible that even though *L. pneumophila* quickly establishes in a system, the overall bacterial community takes more time to establish.

4.3.2.2 Biological Parameters with Different Pipe materials at pH 7. Two conditions, one with PEX and one with CPVC material were operated at pH 7 with copper additions. Comparing these with the Control reactors provides insight into the role both copper additions and pipe-material play on biological parameters.

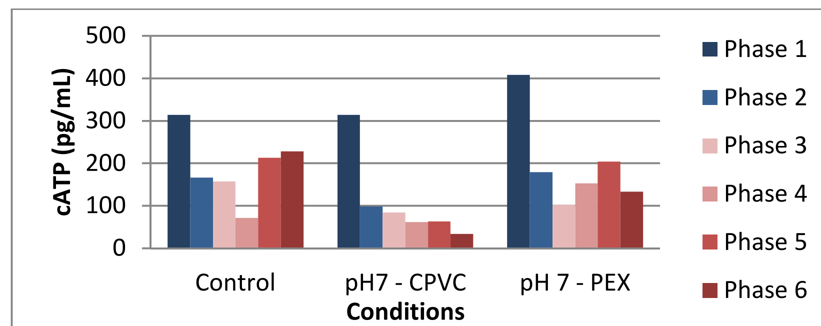

Figure 4.11. ATP concentrations measured for conditions at pH 7 in each phase. Samples were mixed from triplicate reactors in each condition during each Phase. In Phase 2, pH was adjusted from 7.5 to 7. In Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively in the pH7 – CPVC and pH 7 – PEX reactors. The control reactors did not receive copper doses, and influent water was otherwise similar. For each bar, n = 1.

In all conditions at pH 7, there was a noticeable drop in ATP when pH adjustment began in Phase 2. In the reactor with CPVC pipe, ATP steadily declined, whereas ATP was higher with more fluctuation in reactors with PEX pipe. There did not appear to be a strong effect of copper dosing in Phases 3-6. It is possible that ATP is more closely related to TOC. Whereas TOC slowly increased from low levels in CPVC reactors, TOC dramatically dropped then leveled out in reactors with PEX material (Figure 4.5).

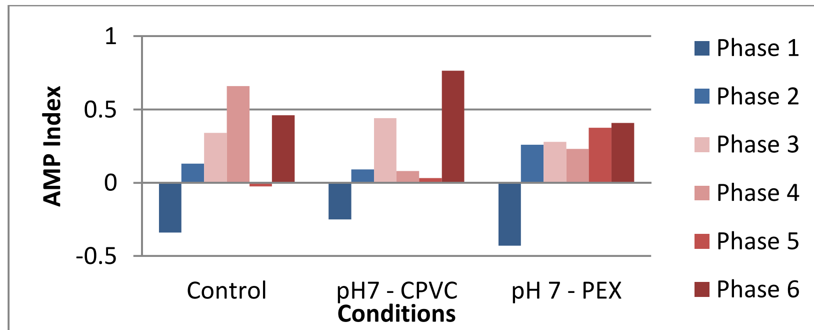

Figure 4.12. AMP Index measured for conditions at pH 7 in each phase. AMP index is a ratio between AMP and ATP measurements, and is a measure of stress. A higher AMP index indicates a more stressed environment. Samples were mixed from triplicate reactors in each condition during each phase. In Phase 2, pH was adjusted from 7.5 to 7. In Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively in the pH7 – CPVC and pH 7 – PEX reactors. The control reactors did not receive copper doses, and influent water was otherwise similar. For each bar, n = 1.

In all conditions at pH 7, AMP index was lowest in Phase 1, indicating an environment with very little stress. There was no clear effect of copper dosing, as AMP index increased and was generally erratic, even in controls. The PEX reactors with copper dosing had more stable AMP index, but still did not have noticeable reaction to increased copper dosing in Phases 3-6.

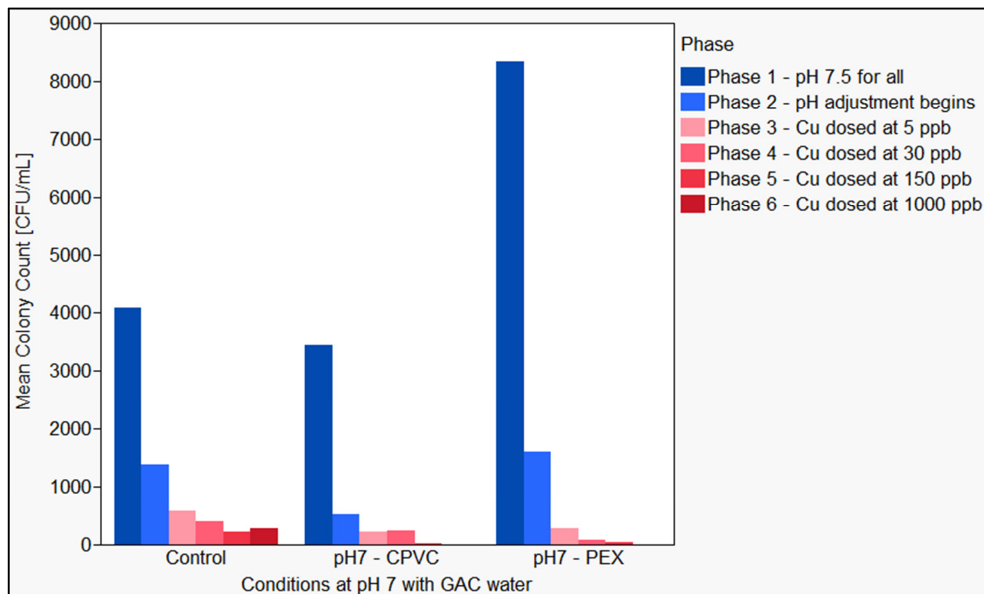

Figure 4.13. Mean colony count [CFU/mL] for triplicate simulated glass water heaters in conditions at pH 7 across all phases. For each bar, n=3. In Phase 2, pH was adjusted from 7.5 to 7. In phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively in the pH7 – CPVC and pH 7 – PEX reactors. The control reactors did not receive copper doses, and influent water was otherwise similar.

In all conditions at pH 7, *L. pneumophila* detected with culture methods (Figure 4.13) was highest before pH adjustment began in Phase 2. Copper dosing had an effect in both CPVC and PEX, with reduced detection in Phases 3-5 and no detection in Phase 6, when copper was dosed at 1000 ppb. The control also had a decrease, but *L. pneumophila* was detected through all phases and at higher levels in Phases 3-6, when copper was dosed in other reactors.

In all conditions at pH 7, *L. pneumophila* concentrations detected with qPCR methods (Figure 4.14) were also highest in Phase 1. There was a decrease in Phase 2, when pH adjustment began. In phases 4-6, the Control reactors had stable levels of *L. pneumophila*. In both sets of reactors with copper dosing, significantly decreased concentrations of *L. pneumophila* were observed in Phases 4-6. A strict decrease with increase copper dosages was not observed.

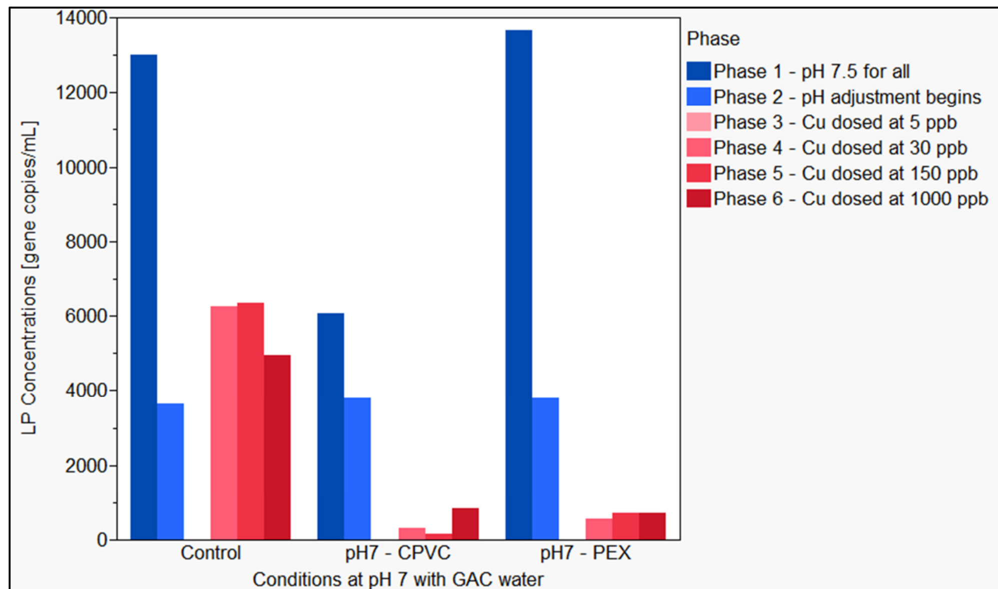

Figure 4.14. *L. pneumophila* concentration [gene copies/mL] for triplicate simulated glass water heaters in each condition at pH 7 across all phases. For each bar, n=3. In Phase 2, pH was adjusted from 7.5 to 7. In phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively in the pH7 – CPVC and pH 7 – PEX reactors. The control reactors did not receive copper doses, and influent water was otherwise similar. Genetic samples for Phase 3 were lost.

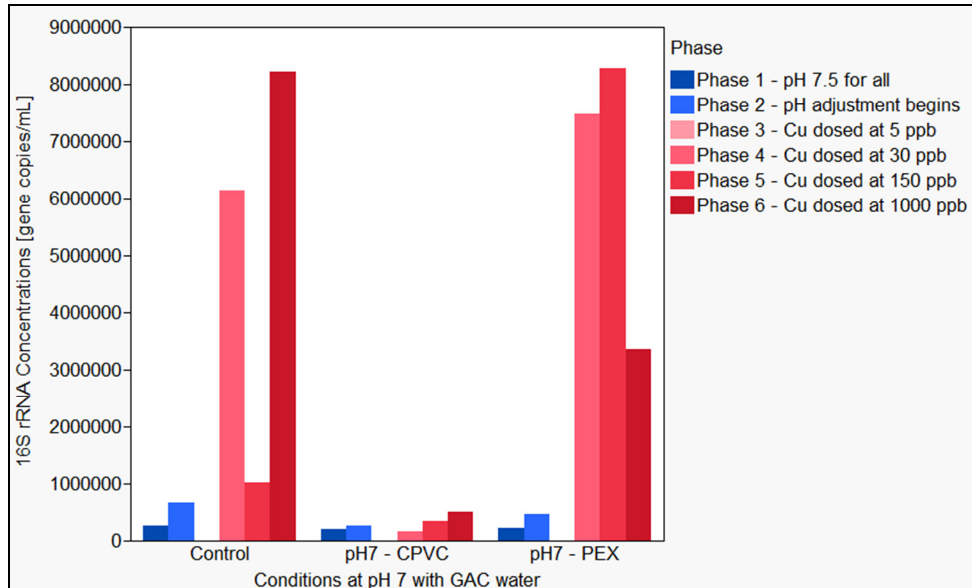

Figure 4.15. 16S rRNA concentration [gene copies/mL] for triplicate simulated glass water heaters in each condition at pH 7 across all phases. For each bar, n=3. In Phase 2, pH was adjusted from 7.5 to 7. In phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively in the pH7 – CPVC and pH 7 – PEX reactors. The control reactors did not receive copper doses, and influent water was otherwise similar. Genetic samples for Phase 3 were lost.

Concentrations of 16S exhibited similar trends based on pipe material. There was a significant increase in 16S concentrations in Phases 4-6 in both the Control and pH7–PEX conditions. This increase was variable, but much greater than that observed in reactors with CPVC material. This could be attributed to different patterns of leaching of organic carbon (Figure 4.5).

4.3.2.3 Biological Parameters at Different pHs. The two remaining conditions had PEX material, copper dosing and influent GAC water at pH 8 and 9. Comparison to both the pH 7 – PEX condition which had copper dosing and GAC water with pH 7 and the Control condition gives insight to possible effects of pH on copper availability and on *L. pneumophila* viability.

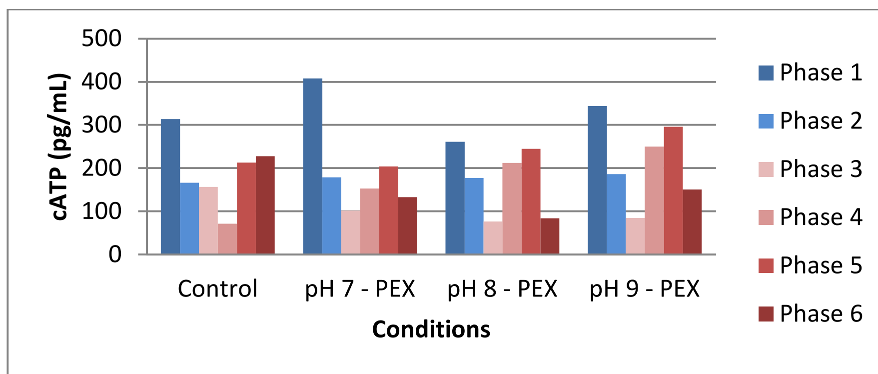

Figure 4.16. ATP concentrations measured for conditions across pHs in each phase. Samples were mixed from triplicate reactors in each condition during each phase. In Phase 2, influent water pH was adjusted from 7.5 to 7, 8, or 9. The control reactors did not receive copper doses. In all other conditions, in Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively. For each bar, n = 1.

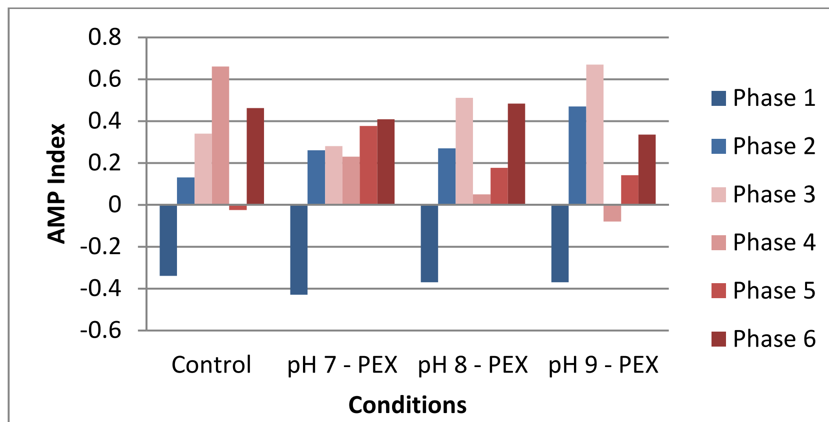

Figure 4.17. AMP Index measured for conditions across pHs in each phase. AMP index is a ratio between AMP and ATP measurements, and is a measure of stress. A higher AMP index indicates a more stressed environment. Samples were mixed from triplicate reactors in each condition during each phase. In Phase 2, influent water pH was adjusted from 7.5 to 7, 8, or 9. The control reactors did not receive copper doses. In all other conditions, in Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively. For each bar, n = 1.

ATP had similar trends across all pHs, with and without copper dosing. There did not appear to be an effect of copper dosing on ATP levels in Phases 3-6.In all conditions, AMP index was lowest, indicating a low-stressed environment before pH adjustment began. There did not appear to be an effect of copper dosing on AMP Index in Phases 3-6.

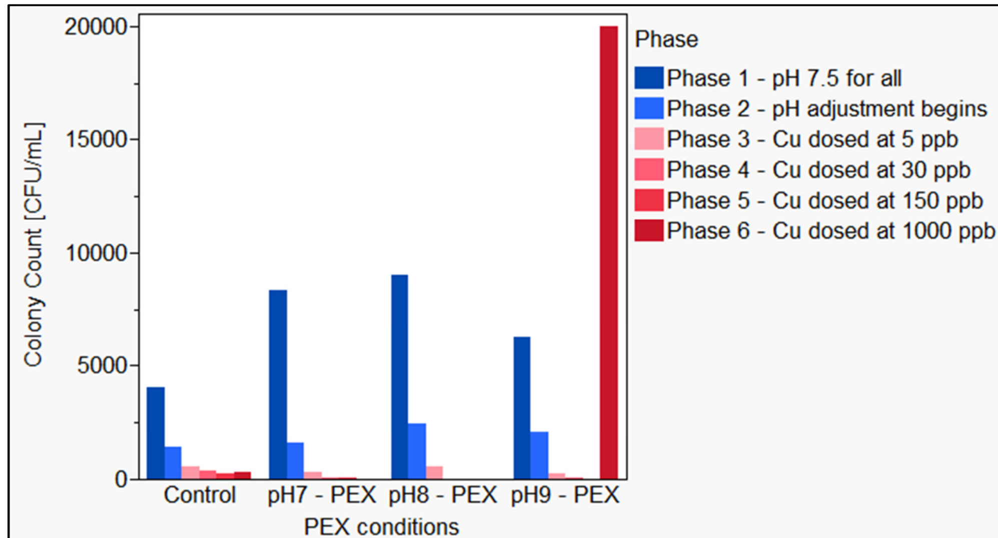

Figure 4.18. *L. pneumophila* concentration [CFU/mL] for triplicate simulated glass water heaters for conditions across pHs across all phases. For each bar, n=3. In Phase 2, pH was adjusted from 7.5 to 7, 8 or 9. The control reactors did not receive copper doses. In remaining conditions, in Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb. Contamination suspected in one sample for pH 9, Phase 6.

In all pH conditions, *L. pneumophila* detection by culture methods (Figure 4.18) was highest in Phase 1 before pH adjustment began. All had a reduction in Phase 2. With copper dosing, another decrease in *L. pneumophila* concentrations occurred, while the Control reactors maintained a stable concentration of *L. pneumophila*. Contamination was suspected in a sample for pH 9, Phase 6. All other samples at pH 9 in Phase 6 were non-detects for *L. pneumophila*.

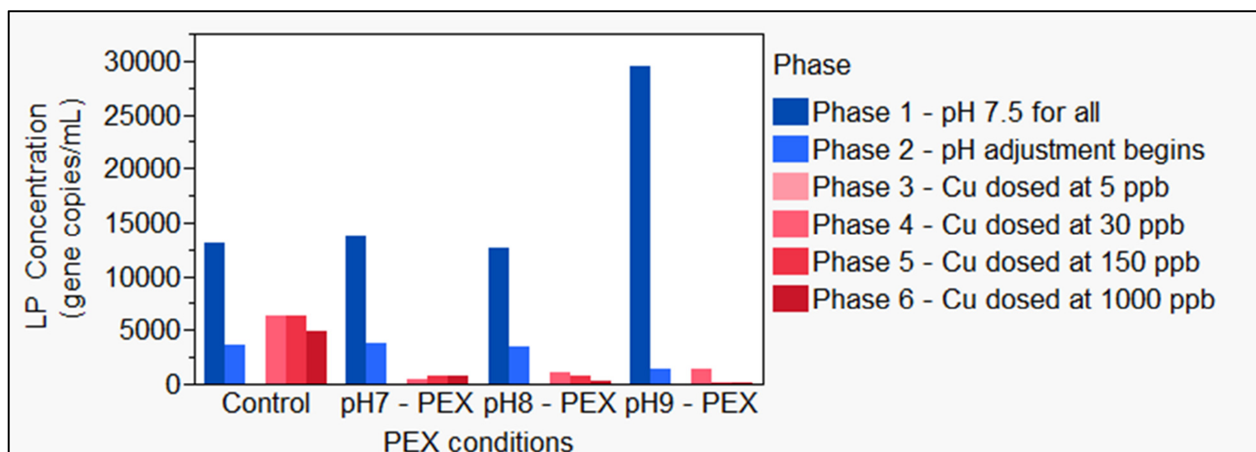

Figure 4.19. *L. pneumophila* concentration [gene copies/mL] for triplicate simulated glass water heaters for conditions across pHs across all phases. For each bar, n=3. In Phase 2, pH was adjusted from 7.5 to 7, 8 or 9. The control reactors did not receive copper doses. In remaining conditions, in Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively. Genetic samples for Phase 3 were lost.

Across the range of tested pHs, *L. pneumophila* detected by qPCR (Figure 4.19) was highest in Phase 1 before pH adjustment began. All had a reduction in Phase 2, and pH 9 reactors had the most significant reduction. With copper dosing, another decrease in *L. pneumophila* concentrations occurred, while the Control reactors maintained a stable concentration of *L. pneumophila*.

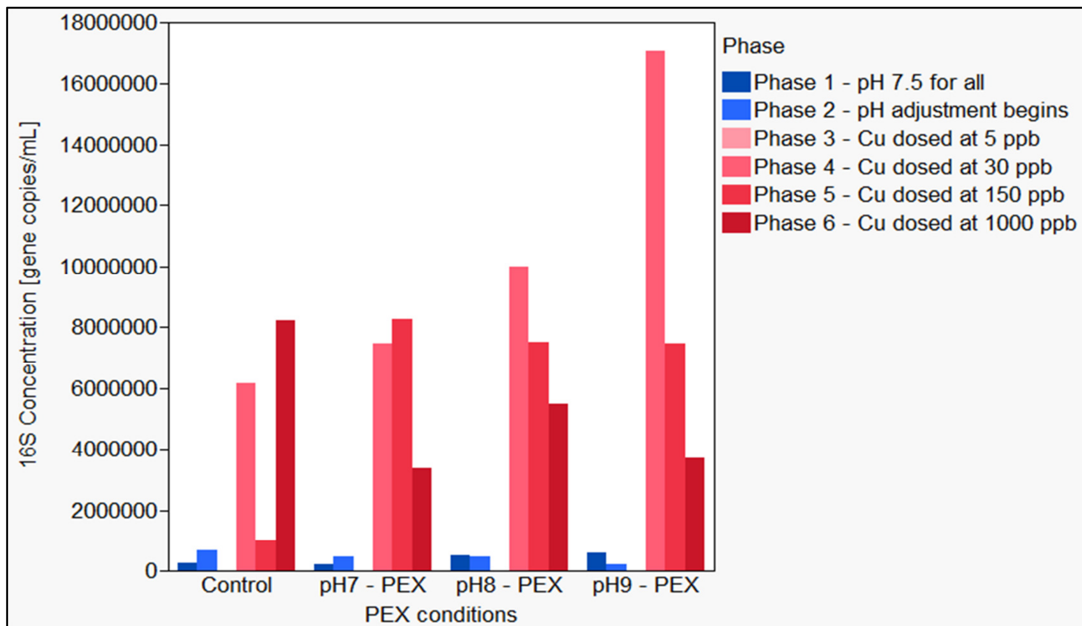

Figure 4.20. 16S rRNA concentration [gene copies/mL] for triplicate simulated glass water heaters for conditions across pHs across all phases. For each bar, n=3. In Phase 2, pH was adjusted from 7.5 to 7, 8 or 9. The control reactors did not receive copper doses. In remaining conditions, in Phases 3-6, copper was dosed at 5, 30, 150, and 1000 ppb respectively. Genetic samples for Phase 3 were lost.

Across all pHs with PEX material, the initial phases exhibited much lower concentrations of 16S rRNA than later phases. In reactors with higher pHs, the concentration of 16S rRNA peaked in Phase 4, with 30 ppb added copper, and decreased afterwards. Other conditions, including the control, had a less stable trend of 16S concentration.

4.3.2.4 Biological Trends in All Reactors with Copper Doses. Data from the reactors with copper added (pH7–CPVC, pH 7–PEX, pH8–PEX and pH9–PEX), indicated that *L. pneumophila* responded to copper dosages negatively while 16S rRNA or total bacteria did not. The 16S concentration does not decrease with increased copper concentration, and even seems to increase. The increase is in part attributed to the delay in overall bacterial colonization also observed in the controls that did not have copper added. The correlation between copper and 16S rRNA concentrations is not statistically significant ($p = 0.0596$). However, it is interesting to note that copper does not seem detrimental to overall concentrations of bacteria. This is consistent with other studies that find that copper does not significantly affect overall bacterial formation (van der Kooij et al. 2005).

There was a weak negative correlation between *L. pneumophila* and 16S rRNA (Figure 4.22), indicating that if there was more total bacteria, there was less *L. pneumophila*. Such a trend could support a probiotic approach to control of pathogens that has been previously suggested (Wang et al. 2013).

L. pneumophila decreases with increased copper concentrations. The negative correlation is statistically significant ($p = 0.0001$). There is also a significant negative correlation between dosed copper and colony counts ($p = 0.0131$) (Figure 4.23).

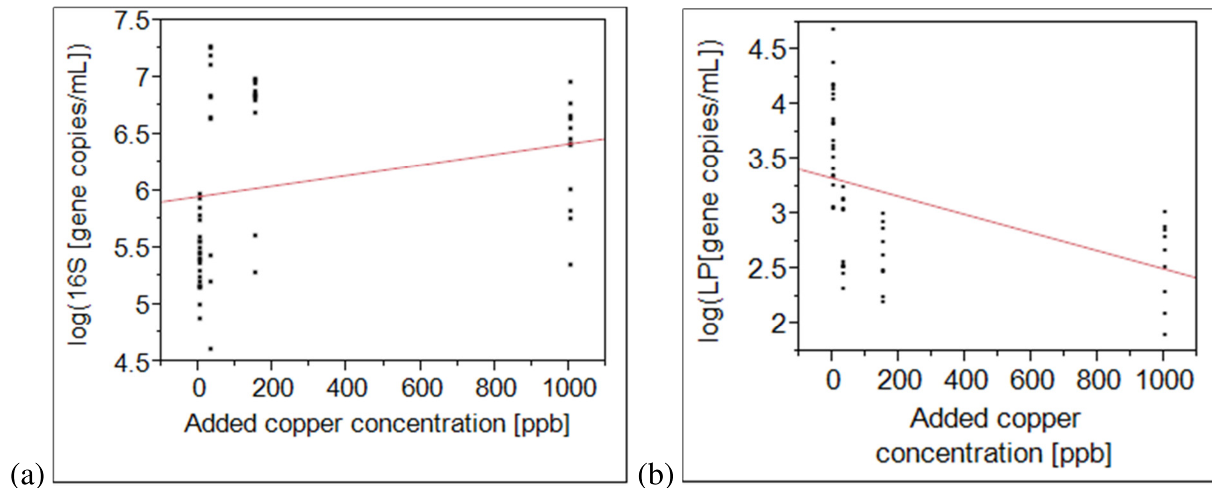

Figure 4.21. (a) log (16S) vs. added copper concentration in reactors receiving copper doses. For each point, n =1, N = 70. Linear line of best fit : Log (16S [gene copies/mL]) =5.9581625 + 0.0004653 * (added copper concentration [ppb]); $R^2 = 0.059831$; p = 0.0596 (b) log (*L. pneumophila*) vs. added copper concentration in reactors receiving copper doses. For each point, n =1, N = 70. Linear line of best fit : Log (*L. pneumophila*) = 3.3337159 – 0.0008248* (added copper concentration); $R^2 = 0.236521$; p = 0.0001.

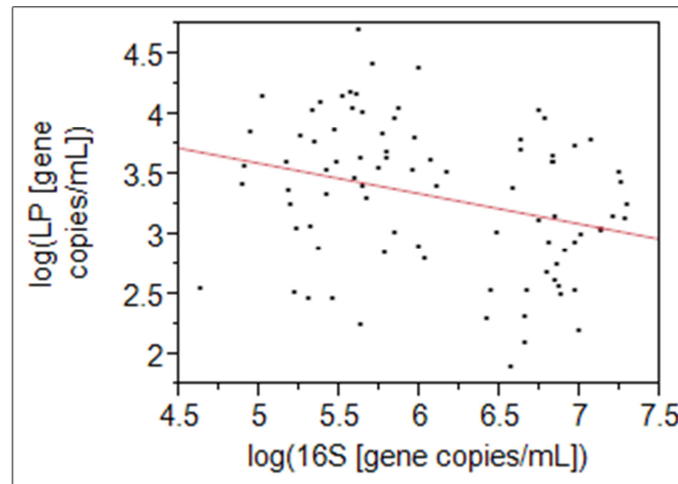

Figure 4.22. (a) log (16S) vs. log (*L. pneumophila*). For each point, n =1, N = 70. For linear line of best fit : $R^2 = 0.089$, p = 0.005, slope -0.25.

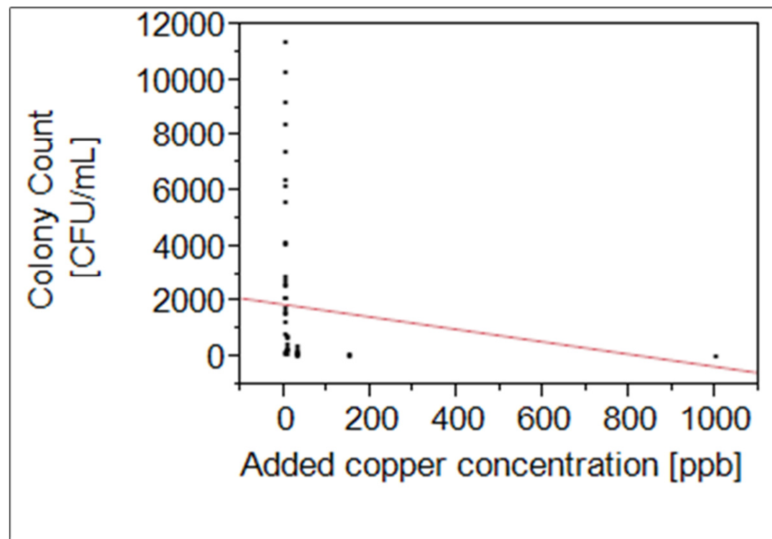

Figure 4.23. Colony Count [CFU/mL] vs. added copper concentration in reactors receiving copper doses. For each point, n =1, N = 82.Colony count = 1917 – 2.239 * (added copper concentration); R^2= 0.086; p = 0.0131

4.4 Conclusions

Contrary to hypothesis, gross speciation of copper as quantified by membrane filtration, did not lead to lower soluble copper at higher pH. Nonetheless, at higher pH much lower levels of Cu^{+2} would be present due to complexation with OH^-.

Establishment of *L. pneumophila* can occur more quickly than establishment of the total bacterial community. While *L. pneumophila* was detected in highest abundance during Phase 1 for all SGWHs, including controls, 16S rRNA was detected in its lowest quantities in early Phases of the experiment.

All types of plastic pipe do not behave the same. With the same commissioning simulation and influent water, SGWHs with CPVC pipe did not establish an overall bacterial community with the same speed as SGWHs with PEX pipe. Carbon leaching from the pipes also showed a

different pattern. Whereas TOC in SGWHs with PEX was highest during Phase 1 and declined, TOC in SGWHs with CPVC started low and gradually increased.

The response of measures reflecting total bacterial regrowth such as ATP and TOC do not necessarily reflect that of specific harmful pathogens of interest. In this study ATP and AMP index did not reflect the reduction of *L. pneumophila* consistently.

This study supports evidence in the literature that copper can be used as a control mechanism for *L. pneumophila* in some waters. Copper dosages as low as 5 ppb disrupted *L. pneumophila* persistence in SGWHs with PEX pipes, and complete reduction in culturable *L. pneumophila* was observed with dosages of 1000 ppb.

4.5 Acknowledgements

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4.7 Appendix

Table 4.4. Concentrations of elements measured by ICP Analysis in GAC influent water, and the same water with an additional ion-exchange filtration step reflecting initial performance of ion-exchange filter. Decrease from total with the additional treatment step and percent decrease also shown.

Element	GAC Influent Water (ppb)	Additional Ion-Exchange (ppb)	Decrease from Total (ppb)	% Decrease from Total (%)
Na - Sodium	14,440.0	31,940.0	-17,500.0	-121.2%
Mg - Magnesium	4,160.0	5.8	4,154.2	99.9%
Al - Aluminum	58.0	1.2	56.8	97.9%
Si - Silicon	3,765.0	3,680.0	85.0	2.3%
P - Phosphorous	227.6	142.4	85.2	37.4%
S - Sulfur	5.0	5.0	0.0	0.4%
Cl - Chlorine	21.6	21.8	-0.2	-0.8%
K - Potassium	1,366.0	315.3	1,050.7	76.9%
Ca - Calcium	11,980.0	16.8	11,963.2	99.9%
V - Vanadium	0.2	0.2	0.0	16.7%
Cr - Chromium	0.2	55.2	-55.0	-28811.0%
Fe - Iron	-4.2	405.8	-410.0	9661.7%
Mn - Manganese	0.2	5.4	-5.2	-2093.5%
Co - Cobalt	0.1	0.1	0.0	-24.6%
Ni - Nickel	0.3	1.0	-0.8	-306.0%
Cu - Copper	1.1	3.7	-2.7	-249.8%
Zn - Zinc	10.9	-1.0	12.0	109.5%
As - Arsenic	1.0	0.4	0.7	64.3%
Se - Selenium	0.0	-0.9	0.9	11175.0%
Mo - Molybdenum	4.1	2.6	1.5	35.9%
Ag - Silver	0.1	0.0	0.0	29.0%
Cd - Cadmium	0.0	0.0	0.0	71.4%
Sn - Tin	2.6	1.4	1.2	46.1%
Ce - Cerium	0.0	0.0	0.0	58.3%
Pb - Lead	0.1	0.1	0.0	24.3%

Ion exchange filtration decreased concentrations of Magnesium, Aluminum, Phosphorous, Potassium, Calcium, Zinc, Arsenic, Molybdenum, and Tin. Ion exchange resulted in increases in the influent for sodium, chromium, Manganese, Nickel, and Fe. The increase in copper was at low levels, and all values were <5 ppb.

Less important changes were found in Si (2%), S (0%), Cl (-1%), V (<0.1ppb), Co (<0.1ppb), Cd (<0.1 ppb), Ce (<0.1 ppb), Pb (<0.1 ppb), Ag (0.1 ppb, 29%). Se had a negative reading with ion exchange, and a 0.0 reading with GAC.

Chapter 5

Effect of Temperature on the Persistence of *Legionella pneumophila* in Premise Plumbing

5.1 Introduction

Outbreaks of Legionnaire's Disease are commonly linked to hot water systems (Berthelot et al. 1998; Meenhorst et al. 1985), and *Legionella pneumophila* occurrence in hot water systems is widespread (Borella et al. 2005). Warmer temperatures increase chemical demand for disinfectant, causing it to decay at a faster rate and thus eliminating its ability to limit regrowth of microorganisms (Ndiongue et al. 2005). Microbial growth rates are also generally directly proportional with temperature up to a certain species-specific optimal growth point, beyond which cellular growth rates typically decline precipitously. Water heaters have been identified as a risk when operated anywhere from 30-54 °C, especially when certain points in the hot water distribution system are allowed to become 'warm' instead of 'hot' (Wadowsky et al. 1982).

In order to control regrowth, several health organizations have recommended setting water heater temperatures to above 60°C (140°C) (OSHA 1999; World Health Organization 2004), as it is generally thought that this temperature is well-beyond the tolerance range of major opportunistic pathogens such as *M. Avium* Complex and *L. pneumophila* (Dennis 1991; Stout et al. 1986). Temperature is often applied for the control of opportunistic pathogen colonization of water systems in hospitals (Exner et al. 2005). One common protocol includes intense heating to 71 -

77 °C for short periods for one half hour or less (Office of Inspector General 2013). When heat is used in short-term temperature spikes, however, recolonization has been known to occur with one-time heating to above 70 °C for two hours(Saby 2005) and heating to 70 °C for 30 minutes twice a week (van der Kooij et al. 2005). *L. pneumophila* has even been detected in hospital systems with automatic preventative 80 °C flushing protection systems in place (Leoni et al. 2005).

While elevated temperatures appear to be inconsistent in their effectiveness for control of L. pneumophila, there are practical drawbacks to maintaining hot water systems at high temperatures. In particular, higher temperature incurs higher energy costs for heating. Energy Star, a US Environmental Protection Agency program, suggests setting water heaters to 120 °F (48 °C) in order to save energy (Energy Star). Scalding is another concern, and the U.S. Centers for Disease Control (CDC) also suggests a setting of 48 °C, or lower, to protect children living in the house (CDC 2012). Ironically, this same measure meant to protect children could actually be increasing their risk with respect to opportunistic pathogens given that children are more susceptible to infections.

The objective of this study was identify an optimal temperature range for control of *L. pneumophila* in hot water systems considerate of covarying factors, such as water chemistry and pipe material. Specifically, the response of *L. pneumophila* in pre-colonized simulated glass water heaters (SGWHs) to incrementally elevated temperatures (32 ºC to 53 ºC increased by 2-5 degrees every 5-7 weeks) was examined over a 12 month study period using quantitative polymerase chain reaction (qPCR). To determine if there were interaction effects with

temperature, SGWHs were equipped with either copper or PEX pipe coupons and operated over a range of assimilable organic carbon (AOC) levels (0-700 ppb). Based on the results of this study, viability of temperature elevation as a practical *L. pneumophila* control strategy is evaluated, with the aim of identifying optimal temperatures that balance energy savings and scalding safety with *L. pneumophila* control.

5.2 Methods

5.2.1 Simulated Glass Water Heaters (SGWHs). SGWHs consisted of 125 mL French square borosilicate glass bottles with polytetrafluoroethylene caps and were equipped with either cross linked polyethylene (PEX) pipe sections or copper pipe sections of equal size. Nine new 1" sections of 3/8" nominal diameter pipe obtained from local hardware stores were placed at the bottom of each water heater. A 1.5 layer of 1mm glass beads served to increase the surface area to volume ratio and provide a range of redox zones similar to those found in premise plumbing sediment layers. All SGWHs were initially incubated at 32 °C, a temperature representative of the bottom of a conventional electric water heater at a sub-optimal temperature setting. SGWHs were originally inoculated with *L. pneumophila*, *Acanthamoeba polyphaga*, *Mycobacterium avium*, and *Hartmannella vermiformis* as described in Section 2.2.1 and were operated for 2.5 years prior to cross- inoculation and commencing the present experiments. Cross-inoculation was conducted 35 days and 9 days before week 0 of the experiment by pooling effluent from reactors and adding 1 mL to the influent for each reactor in order to achieve a similar baseline microbial community.

5.2.2 Influent Water and Water Changes. Three times a week, an 80% water change was conducted to simulate infrequent use of a water heater. With minimal reactor agitation, 100 mL

was decanted and replaced with freshly prepared modified Blacksburg drinking water. Modified Blacksburg drinking water was used rather than modified reverse osmosis or nanopure water in order to continually introduce common inorganics and nutrients into the system.

Town of Blacksburg, VA, tap water originates from a surface water source and is disinfected with chloramine. Water was collected in the lab from the cold water tap and breakpoint chlorinated (BPC) with addition of a diluted hypochlorite solution. BPC water was then heated to 90 °C for 10 minutes in order to remove most trace disinfectant residual. The cooled water was subject to biofiltration with a point of use GAC filter.

Acetate and glucose (equal mass as mg C/L) were amended to the influent water to achieve five different levels of AOC (0 ppb, 5 ppb, 30 ppb, 150 ppb, and 700 ppb as added C/L). Triplicate reactors with two pipe materials (PEX and copper pipe coupons) were maintained at each level of AOC. An additional set of triplicates for each pipe material was operated at 700 ppb to act as a temperature control.

Table 5.1. Experimental design with each numbered box representing one simulated glass water heater

AOC added as acetate and glucose	PEX pipe sections			Copper pipe sections		
0 ppb	1	2	3	1	2	3
5 ppb	1	2	3	1	2	3
30 ppb	1	2	3	1	2	3
150 ppb	1	2	3	1	2	3
700 ppb	1	2	3	1	2	3
700 ppb *	1	2	3	1	2	3
* Remained at 32°C throughout experiment						

All influent water was pre-treated by passage through a 0.45 µm polyvinylidene fluoride (PVDF) filter to remove microbes. Water was adjusted to pH 7.5 ± 0.1, except in the case of the 'high pH control', which is adjusted to pH 10.0 ± 0.1. pH was controlled with additions of 1 M or less NaOH and HCl using an Oakton pH 10 series meter (Oakton Instruments, Vernon Hills, IL).

5.2.3 Temperature Steps. Temperature was elevated in a step-wise fashion (2.5 to 4ºC every 5-7 weeks) in all reactors except the 700 ppb temperature controls, which were maintained at 32 °C for the duration of the experiment. During each temperature step, 5-7 weeks were allowed for acclimation prior to sample collection. Biological sampling for qPCR was done on Wednesdays to minimize the possible effects of weekend stagnation. Temperature was maintained constant with a New Brunswick Scientific Classic Series C24 Incubator Shaker (Edison, NJ, USA). Upon malfunction in week 40 (second week at 49ºC level), reactors were transferred to a New Brunswick Scientific Innova 43 Incubator Shaker (Edison, NJ, USA) for the remainder of the experiment. During the malfunction, the incubator reached 60 °C for up to 24 hours on day 7 and 10 of the first 53 °C phase (week 39-40).

Table 5.2. Incremental temperature elevation schedule

Week	0	4	8	15	20	26	32	38	45	53
Temperature (°C)	32	32	32	34.5	37	41	45	49	53	53

5.2.4 Analysis. All samples were collected using aseptic technique in a biosafety cabinet during routine water changes to minimize impact to the reactors.

Trace metal concentrations were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). 10 mL samples from each SGWH were transferred to a sterile test tube and acidified by adding 2% nitric acid by mass prior to analysis.

Total organic carbon (TOC) was measured with a Sievers 5310 C Laboratory TOC-MS Analyzer using the Data Pro 5310 C Computer Program. Samples were analyzed using 30 mL from each SGWH, or samples were pooled with 10 mL from each of triplicate SGWHs. Samples were acidified with phosphoric acid and sparged with N_2 gas in order to purge inorganic carbon prior to analysis.

Adenosine triphosphate (ATP) and adenosine monophosphate (AMP) concentrations, and their ratios, were measured using a LuminUltra® Quench-Gone™ Aqueous Test Kit (LuminUltra, NB, Canada). ATP provides an indicator of viable biomass activity levels, while AMP is an indicator of cell stress. Samples were collected for each condition by pooling 20 mL from each triplicate SGWH for a total volume of 60 mL and cellular contents were captured on a Quench-Gone syringe filter. Cells were lysed to release ATP for analysis by filtering 1 mL of UltraLyse through the syringe. The remaining process followed the LuminUltra® Quench-Gone™ Aqueous Test Kit steps to determine ATP, AMP, and the ATP:AMP index.

Effluent water measuring 100 mL from each SGWH was filtered onto sterile 0.22 µm-pore-size mixed cellulose ester filters (Millipore, Billerica, MA). Filters were folded and fragmented using sterile tweezers and transferred to a Lysing Matrix A tube (FastDNA® SPIN Kit, MP

Biomedicals, Solon, OH). DNA extraction was conducted according to manufacturer instructions.

Q-PCR was applied to quantify the macrophage infectivity potentiator (*mip*) gene specific to *L. pneumophila* (Nazarian et al. 2008) and 16S rRNA genes as an indicator of the abundance of total bacteria(Suzuki et al. 2000). *H. vermiformis* and Acanthamoeba spp. were also quantified according to established q-PCR methods (Kuiper et al. 2006; Riviere et al. 2006).

Q-PCR was carried out using a CFX96™ realtime system (Bio-Rad, Hercules, CA). All q-PCR assays were previously been validated for drinking water samples in terms of specificity and limit of quantification (Wang et al. 2012). For *L. pneumophila*, a Taqman Probe Mix (Bio-Rad, Hercules, CA) assay was used, and for 16S rRNA genes, an Eva Green (Bio-Rad) assay was used. For each run of q-PCR analysis, a calibration curve was included with at least six (for 16S) or seven (for *L. pneumophila*) points.

Culturing was also performed to confirm *L. pneumophila* viability. 100 µL water samples were heated to 50 °C for 30 minutes, then directly plated on Buffer Charcoal Yeast Extract agar according to published methods (Leoni et al. 2005).

5.2.5 Statistical Analysis. Statistical analysis was performed using JMP (SAS, Cary, NC). Q-PCR data were transformed to $\log_{10}(X+1)$ for statistical analysis. Non-parametric Wilcoxon tests were used for *L. pneumophila* and Acanthamoeba spp., which were not normally distributed due to non-detects. For all other normally distributed data, one-way analysis of Variances (ANOVA)

or t-test was conducted. For linear correlations, least-squared regressions were used. For multivariate methods, the restricted maximum likelihood method was used. Statistical significance was set at $p<0.05$.

5.3 Results and Discussion

This portion of the experiment was conducted after differences based on AOC additions (Chapter 2) and pipe materials (Chapter 3) were established at 32 °C.

5.3.1 Control Reactors. Control reactors were maintained at a constant 32 °C throughout the experiment. They were set up at the highest level of AOC amendment in order to provide a worst-case-scenario at low temperatures for comparison. Although *L. pneumophila* gene copy levels were somewhat dynamic in these reactors, time was not a significant explanatory factor ($p=0.58$, Wilcoxon non-parametric). There were no discernable trends for level of 16S rRNA genes, ATP or AMP index with time (16S $p=0.37$, $R^2 = 0.02$; ATP $p=0.41$, $R^2=0.04$; AMP Index $p=0.59$, $R^2=0.02$).

5.3.2 TOC. TOC was analyzed in the effluents of reactors during the 37 °C and 53 °C phases (Table 5.3). Effluent reflected additional TOC in the influent. Especially at higher temperature, pipe material appeared to have an effect on effluent TOC levels.

5.3.3 Copper Concentrations. Temperature had a significant effect on the copper released from copper pipe coupons (Figure 5.1), even though the pipe age was > 3 years. Copper was a function of temperature with a strong linear correlation ($R^2 = 0.79$, $p<0.001$). Data was collected in weeks 33-45, including data for 32 °C reactors.

Table 5.3. TOC [ppb] measured in effluent from simulated glass water heaters during 37 °C and 53 °C phases.

Condition		Temperature	
Pipe Material	*added AOC (ppb C)*	37 °C	53 °C
PEX	0	574	1195
COPPER	0	553	773
PEX	5	576.5	1175
COPPER	5	564.5	1060
PEX	30	570	1190
COPPER	30	584.5	799
PEX	150	607.5	1450
COPPER	150	613.5	893.5
PEX	700	655	2205
COPPER	700	700.5	1605
Control Reactors, measured at same time as above			
Pipe Material	*added AOC (ppb C)*	32 °C	32 °C
PEX	700	715	556
COPPER	700	638.5	764

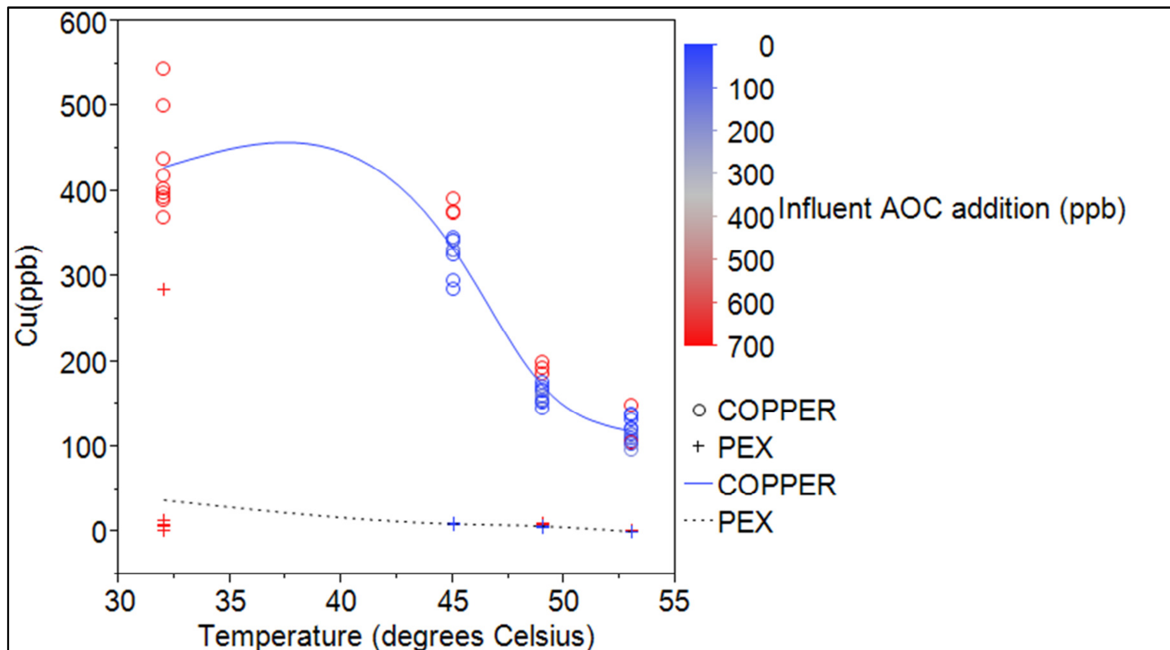

Figure 5.1. Bulk aqueous copper concentrations in reactors with copper and PEX pipes as a function of temperature (°C). All reactors and materials were acclimated/conditioned 3 years prior to collection of these data. N = 108.

5.3.4 ATP and AMP. ATP correlated negatively temperature, while the AMP Index was positively correlated with temperature. This makes sense, as biological activity measured by ATP decreased with temperature, the stress in each environment, measured by AMP Index. The negative correlation of ATP with temperature was stronger in reactors with copper pipe (R^2=0.33) than with PEX pipe (R^2 =0.17), although both were statistically significant ($p<0.05$). The slight resurgence in ATP at 53 °C was primarily due to measurements from eight weeks after the first measurements at 53 °C. At the first sampling date at 53 °C (week 45), average ATP was 5 pg/mL, and this increased to 100 pg/mL at the second sampling date (week 53).

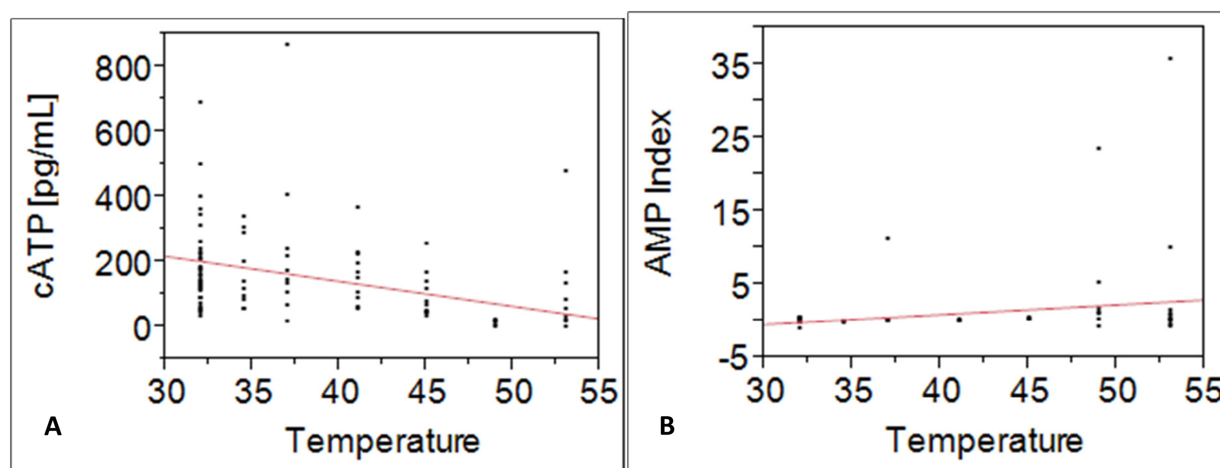

Figure 5.2. ATP (A) and AMP Index (B) in simulated glass water reactors with both PEX and copper pipe materials as a function of temperature (°C). Simulated glass water heaters were incubated at each temperature for 5-7 weeks. Linear relationships, determined with least mean-square method displayed. Equations: cATP [pg/mL] = 447 – 7.65 * temperature, R2 = 0.19, P <0.0001; AMPIndex = -4.4287 + 0.135 * temperature R2 = 0.06, p = 0.0112. N = 108 for each plot.

5.3.5 *L. pneumophila*. The abundance of *L. pneumophila* gene copies was significantly influenced by pipe material at 32 °C (See Materials Chapter). However, at temperatures 45 °C and greater, copper no longer provided a significant advantage for *L. pneumophila* control (α = 0.05, t-test). Although plastic pipes release carbon, a substrate for growth (Bucheli-Witschel et

al. 2012; Rogers et al. 1994), the PEX pipe used in this study was greater than 2.5 years old. Most studies on release of materials from PEX pipes focus on new materials. Thus, the trends in this work are attributed to toxicity of copper rather than the nutrient benefits of PEX [Chapter 3].

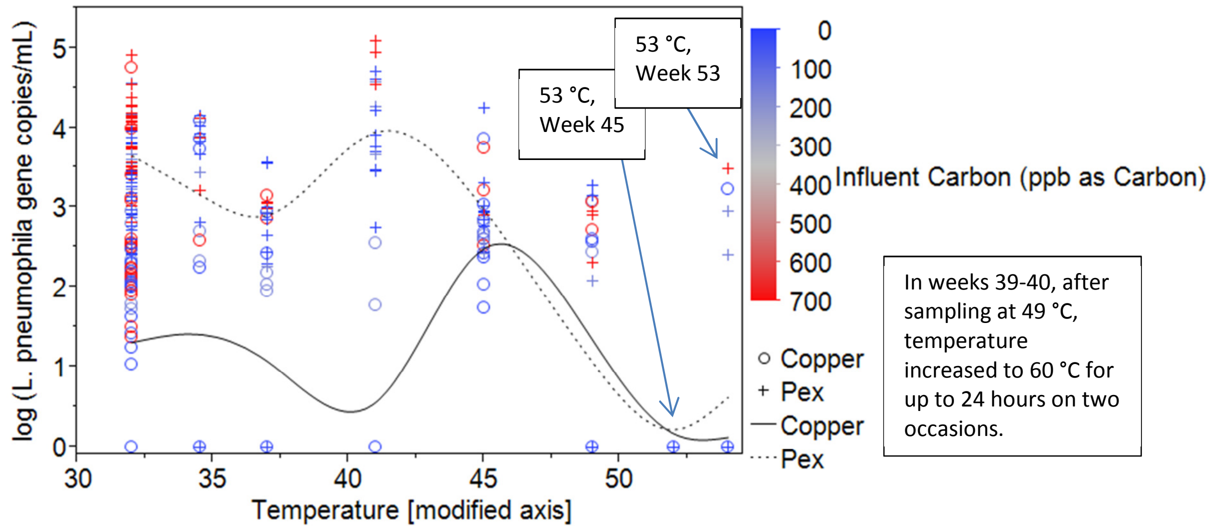

Figure 5.3. Concentration of *L. pneumophila mip* gene as a function of temperature in simulated glass water heaters with copper and PEX pipe coupons. Temperature scale modified and exaggerated to show detail of extended incubation at 53 °C. Temperature malfunction with temperature spike up to 60 °C occurred 2 weeks after 49 °C samples were taken. N = 360.

The highest average levels of *L. pneumophila* ($10^{2.75}$ gene copies/mL) occurred at 45 °C in SGWH with copper pipes. Notably, soluble copper concentrations in the water released from the decreased with elevated temperature (Figure 5.1). Thus, the loss of significance of copper as a control mechanism may be attributable to decreased concentrations of copper in SGWH. Another study found that new copper pipes lost their effectiveness as a control mechanism for *L. pneumophila* regrowth after a year of operation in a hot water system (van der Kooij et al. 2005). While age of pipes is an important factor to consider, the pipes used in this study were 2.5 years old prior to the start of this experiment, thus suggesting that temperature, rather than age, drove this decrease in soluble copper concentration.

In the PEX SGWHs, temperature had a significant effect on the concentration of *L. pneumophila* ($p=0.0001$, Wilcoxon non-parametric). The relationship was not strictly linear, however. The highest levels of *L. pneumophila* occurred at 41 °C, with $10^{4.1}$ gene copies/mL. Beyond 41 °C, levels of *L. pneumophila* decreased with increasing temperature, with an average drop of 0.48 logs (gene copies/mL) / °C.

L. pneumophila remained detectable (average 2.9 log gene copies/mL when detected) through the end of the experiment, regardless of temperature or extended incubation at 53 °C. Although *L. pneumophila* gene copies appeared to plummet below qPCR detection limit after five weeks incubation at 53 °C, gene copies were again detected, just above detection limit, in several of the reactors after extending the incubation an additional 8 weeks. Reactors with PEX material and greater than 30 ppb amended AOC appeared to be especially amenable to *L. pneumophila* regrowth. However, *L. pneumophila* gene copies were also detected in one reactor with copper pipe coupon and 0 ppb added AOC, indicating that the systems were still at risk for recolonization even when the most extreme engineering controls were imposed (lowest possible AOC and antimicrobial copper pipe surface).

5.3.6 Culturable *L. pneumophila*. A recent inter-laboratory evaluation indicated that while standard *Legionella* culturing is generally accurate for presence absence evaluation, quantitation is poor (Lucas et al. 2011). Therefore, *L. pneumophila* were cultured from the reactors in this study for qualitative evaluation of viability. *L. pneumophila* was successfully isolated in culture at temperatures up to 49 °C, but could not be recovered at 53 °C. This is consistent with a prior

study that noted decreased viability at temperatures greater than 50 °C (Hruba 2009). Detection with qPCR also captures the viable-non-culturable portion.

5.3.7 16S rRNA.

Q-PCR of16S rRNA genes, which are present in all bacteria, provided an indicator of the total numbers of bacteria. In both copper and PEX SGWHs, statistically significant negative correlations were found between 16S rRNA genes and temperature (Copper: R^2 = 0.47, p <0.0001; PEX: R^2= 0.26, p<0001).

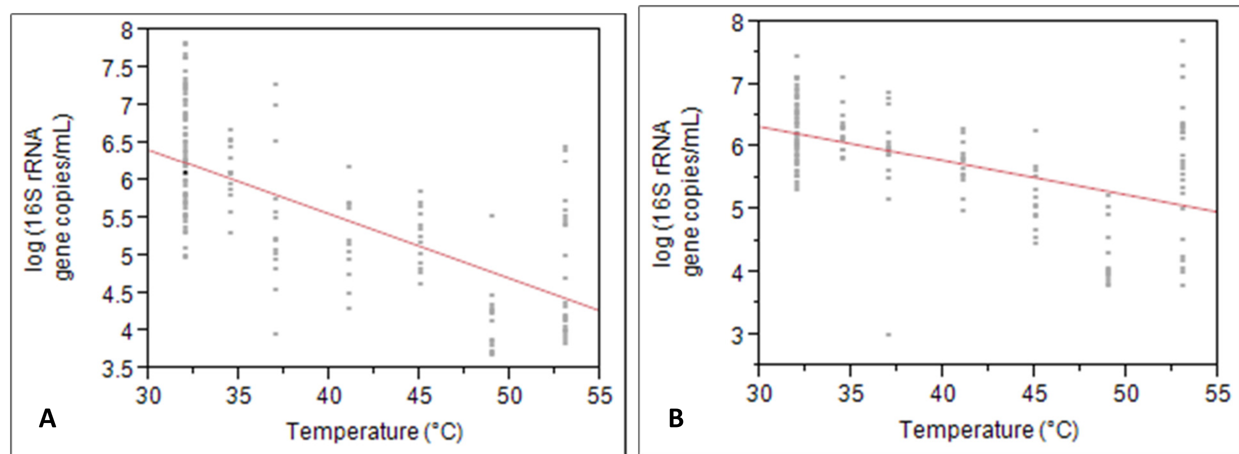

Figure 5.4. Concentration of 16S rRNA genes as a function of temperature in simulated glass water heaters with copper (A) and PEX (B) pipe coupons. Simulated glass water heaters were incubated at each temperature for 5-7 weeks. For copper: log10 (16s+1) = 8.98 - 0.085*Temperature (°C) [R2 = 0.47, p <0.0001]. For PEX: $\log_{10}(16s+1) = 8.0 - 0.055$ *Temperature(°C) [R^2= 0.26, p<0001]. N = 180 for each material.

In both conditions, 16S rRNA genes increased following extended incubation at 53 °C. In copper SGWHs, a 1.1 log increase was observed in this 8-week period, and in SGWH with PEX coupons, a 1.5 log increase was observed (p<0.001, t-test). In SGWH with PEX coupons, the resurgence was higher and not significantly different than during the 32 °C phase. This suggests that with time the general bacterial population was able to acclimate and grow at the highest temperature in this study.

5.3.8 *H. vermiformis* and Acanthamoeba spp.

L. pneumophila reproduction in drinking water is thought to be dependent on a life-cycle of infection of amoeba hosts, such as *H. vermiformis* and *Acanthamoeba polyphaga* (Declerck et al. 2009; Kuiper et al. 2004; Wang et al. 2012). *H. vermiformis* and Acanthamoeba spp. marker genes were detected sporadically in the SGWHs bulk water samples by q-PCR. The two marker genes were not correlated to each other ($p = 0.1351$, Least squares), indicating that the two amoeba have a distinct set of ideal conditions for growth in a mixed community.

Acanthamoeba spp. was significantly affected by temperature ($p < 0.0001$, Wilcoxon non-parametric) and its gene copies were highest in abundance at 45 °C and 49 °C in SGWHs with both PEX and Copper pipes. The correlation with temperature was positive overall ($p < 0.0001$, $R^2 = 0.101$). Unlike other genes, more Acanthamoeba spp. was detected when temperature was increased. This indicates that control of all potential *L. pneumophila* hosts with increased temperature is difficult. Significant differences between pipe materials were only found at 37 °C, with SGWHs with copper coupons harboring 0.75 log more Acanthamoeba spp. gene copies/mL than SGWHs with PEX coupons ($p = 0.04$, Wilcoxon). Influent AOC was only found to be a significant factor at 49 °C ($p < 0.0007$, Wilcoxon), and a negative correlation was found.

It is important to note that Acanthamoeba spp. concentration in control SGWHs was significantly affected by date ($p < 0.0001$, Wilcoxon), indicating that factors other than temperature may have influenced proliferation. It is also important to note that the assay for Acanthamoeba spp. measures concentrations of all species of *Acanthamoeba*, and thus does not necessarily only measure the *Acanthamoeba polyphaga* that was originally inoculated into SGWHs.

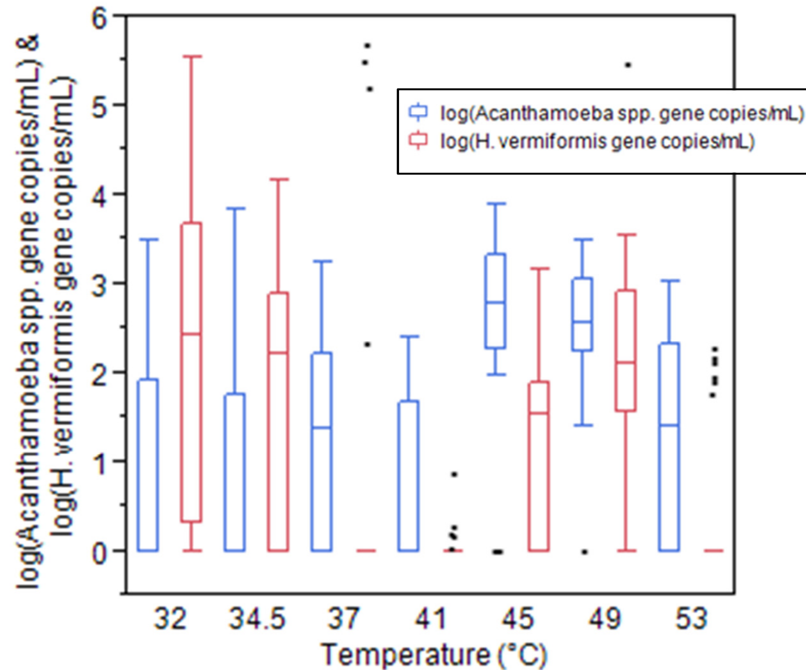

Figure 5.5 Quantification of Acanthamoeba spp. 18S gene and *Hartmannella vermiformis* by q-PCR by as a function of temperature in simulated glass water heaters, with both PEX and Copper pipe reactors pooled. Box and whisker are quartile plots with all points displayed. N = 360 for each gene tested.

H. vermiformis was stable in control SGWHs, indicating that date was not a significant explanatory factor for its growth (p=0.98, Wilcoxon). Throughout the experiment, there was significantly more *H. vermiformis* detected in SGWHs with copper pipe coupons than SGWHs with PEX coupons ($p < 0.0001$, t-test). In SGWHs with copper pipes, the correlation with temperature was negative ($p < 0.0001$, $R^2 = 0.4$), with the highest concentration, 3.5 log gene copies/mL, measured at 32 °C. There were weak positive correlations with *L. pneumophila* and 16S rRNA genes in SGWH with copper pipes ($p = 0.0011$, $R^2 = 0.058$; $p < 0.0001$, $R^2 = 0.37$), perhaps attributed to a similar relationship with temperature. The correlation with added AOC was also significant and weakly positive in SGWHs with copper pipes ($p < 0.0001$; $R^2 = 0.265$).

In SGWH with PEX coupons, *H. vermiformis* and temperature did not have a significant correlation ($p = 0.15$, Least squares), nor did *H. vermiformis* and added AOC ($p = 0.2110$, Least-squares). In SGWH with PEX coupons, the peak occurred at 49 °C, with 2.4 log gene copies/mL.

In both amoebae measured, there is evidence that copper does not control concentrations as it does with *L. pneumophila*, and even results in higher concentrations. This is consistent with evidence that copper-silver ionization systems do not control amoeba and other bacteria, even when effective against *L. pneumophila* (States et al. 1998). The amoebae were also measured from bulk water, which may underestimate the actual concentration present. Kuiper et al. found a high percentage of protozoa in the biofilm rather than the planktonic phase (Kuiper et al. 2004).

For both *H. vermiformis* and Acanthamoeba spp., the concentration of genes was higher during the second sampling at 53 °C, more removed from the malfunction which caused a temperature spike to 60 °C, than the first ($p = 0.01$, $p = 0.0024$). It is likely that amoebae entered a cyst form, which is more resistant to temperature increases. *H. vermiformis* cysts have a low reported survival rate at temperatures of 55 and 60 °C, but were not completely eliminated (Kuchta et al. 1993). *Acanthamoeba* may be resistant to temperatures as high as 65 °C (Coulon et al. 2010). Although *L. pneumophila* reproduction within cysts is only observed at low rates (Kuiper et al. 2004), the resistance of amoeba to high temperatures could explain the resurgence of *L. pneumophila* at 53 °C, further discussed in section 5.3.10.

5.3.9 Multivariate Analysis.

Multivariate analysis was performed in order to determine correlations between total bacterial 16S rRNA genes and *L. pneumophila* and other water chemistry factors. This analysis was performed separately for each pipe material.

Table 5.4. Correlations between measured genes (16S rRNA genes, *L. pneumophila*) and manipulated variables in each pipe material. For all correlations $p < 0.0001$.

	Temperature [° C]	**Added AOC [ppb as C]**
Copper		
$\log_{10}$(*L. pneumophila* +1) [$\log_{10}$(gene copies/mL)]	-0.2026	0.1423
$\log_{10}$(16S+1) [$\log_{10}$(gene copies/mL)]	-0.6866	0.5827
PEX		
$\log_{10}$(*L. pneumophila* +1) [$\log_{10}$(gene copies/mL)]	-0.7467	0.3482
$\log_{10}$(16S+1) [$\log_{10}$(gene copies/mL)]	-0.5114	0.3992

In PEX reactors, *L. pneumophila* and 16S rRNA exhibited strong negative correlations with temperature, indicating that both decreased with increased temperature. Both measured genes had a positive, weaker correlation with amended AOC. This may indicate that temperature is a more important control factor for *L. pneumophila* persistence.

In copper reactors, 16S rRNA genes had a strong negative correlation with temperature, but a strong positive correlation with AOC. The correlation between *L. pneumophila* and temperature was not as significant. This may be due to the relative control of *L. pneumophila* at low temperatures (32 °C) and resurgence at 41 °C.

With both pipe materials, *L. pneumophila* had positive weak correlation with 16S rRNA genes. This relationship was more pronounced in PEX pipes ($R = 0.44$, $p < 0.0001$) than copper pipes

($R = 0.24$, $p < 0.0001$). The positive correlation may indicate that with greater overall regrowth problems, there is a greater abundance of *L. pneumophila*. However, as the relationships are weak, monitoring overall regrowth alone does not give a clear indication of risk for *L. pneumophila*.

5.3.10 Further Discussion. The initial control of *L. pneumophila* may be related to a malfunction in the incubator which caused temperatures to increase to 60 °C for up to twenty four hours on two occasions in the first two weeks of the 53 °C phase. While disturbance was unintended, it did mimic the common mitigation strategy of temporarily elevating the temperature of the lines above 60°C. Interestingly, the *L. pneumophila* quickly recovered, albeit in a non-culturable form. After eight weeks at 53 °C, *L. pneumophila* levels rose again. There was also a slight resurgence in 16S and ATP. The rise in concentrations of *H. vermiformis* and Acanthamoeba is discussed in section 5.3.8.

It has been suggested that *L. pneumophila* may develop heat resistance after exposure to heat for long periods of time. In a study examining several strains of *Legionella* isolated in French hospitals, significant portions of viable but not culturable cells were still present after heat shock treatments of 70 °C of 30 minutes (Allegra et al. 2011). Mechanisms for adaptation and survival in a viable but not culturable state may explain the failure to culture *L. pneumophila* at temperatures greater than 50 °C.

Although inactivation of *Legionella* at temperatures 60 °C or greater has been observed in laboratory settings (Dennis et al. 1984; Stout et al. 1986), it may not be an effective control

mechanism in real systems. Even though a water heater is set at an adequate temperature, water cools as it is distributed. In electric water heaters, with heat stratification, the temperature at the bottom of the heater can drop considerably. Maintaining a constant temperature at 55 °C has been considered a very important and attractive method for control of *L. pneumophila*, but is difficult to maintain in large systems, especially with dead-end lines (Saby 2005). In this work, all temperatures, including the brief spike, were maintained for the entire volume of the SGWH.

It has been hypothesized that *L. pneumophila* may recolonize quickly after temperature reduction because biofilm is killed, rather than removed, in heat-shock treatment (Saby 2005) and *L. pneumophila* has been associated with necrotrophic growth (Temmerman et al. 2006). When the temperature is reduced below levels necessary for inactivation again, recolonization can occur quickly.

In a survey of 18 real hot water systems, *Legionella* gene copies were still detectable and even resurged in samples ranging 55-60 °C, but in much lower frequency of detection for samples > 50 °C. Others have suggested a setting of 55 °C to manage contamination (Hruba 2009). A field survey confirmed that culturable *Legionella* spp. was negatively associated with temperatures > 55 °C (Bargellini et al. 2011).

Maintaining the water heater temperature at 48 °C or lower as recommended by the EPA and CDC may be especially detrimental to *L. pneumophila* risk, especially as the temperature is likely to decrease in the system, even with a recirculating lines (Lobenstein 1993). According to this study, even a constant temperature of 41-45 °C, as might be encountered in a large building

distribution system, may actually increase the concentration of *L. pneumophila* relative to a system maintained with lower building distribution temperatures of 37 °C.

To save on energy, a water heater temperature setting of as low as 53 °C may be appropriate for *L. pneumophila* control if monthly boosts to 60 °C and an efficient recirculating line are in place. When *L. pneumophila* was detected at 53 °C in this study, it was only in a few of the reactors that formerly supported *L. pneumophila* growth and never in a culturable form. Based on clinical and environmental sampling, *L. pneumophila* was kept under control, although not completely eradicated with a setting of 55 °C with recirculating lines (Darelid et al. 2002). However, as maintenance of hot water distribution systems can be neglected even when rigorous protocols and testing in place (Office of Inspector General 2013), a higher constant temperature and higher temperature boosts may be necessary.

5.4 Conclusions

- Temperature does not strictly correlate with abundance of *L. pneumophila* gene copies. In this study, temperatures of 41-45 °C were optimal for *L. pneumophila* growth, while it still persisted and even resurged at the highest temperature tested, 53 °C.
- The concentration of copper released from pipes decreases with elevated temperature, even in the aged pipes employed in this study. Thus, using both antimicrobial metal pipe materials and elevated temperature as control mechanisms for *L. pneumophila* control may not be synergistic, if copper levels consistently drop at higher temperature.
- A system set to a temperature capable of supplying water to taps as low as 53 °C or greater may be appropriate for *L. pneumophila* control if the temperature is increased monthly to 60°C or greater.

The high energy costs incurred with high water heater settings are necessary for control of *L. pneumophila*. A low setting of 48 °C leaves the consumer especially vulnerable when temperatures in building distribution systems decrease to as low as 45 °C or 41 °C. This study indicates that guidance of setting water heater temperatures to 60 °C or greater is likely adequate, given that the temperature at the tap can be maintained at 53 °C or greater.

5.5 Acknowledgements

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Chapter 6

Microbiome of Water Produced from Lab-Grade Water Purification Systems

6.1 Introduction

Pure and ultrapure water systems are core infrastructure in research labs and for many industrial applications, with production scales varying from 1-2 to tens of thousands of L/day. These systems have a range of treatment approaches dependent on the specific type of water that is required(ASTM-D1193 2011; ASTM-D5127 2013; ASTM-D5196 2013).

ASTM classifies three types of "Pure Water", all of which are further classified based on quality. "Ultra-pure water" refers to any water undergoing extra treatment that is used in industrial applications, such as electronics (ASTM-D5127 2013). Two more classifications include "reagent grade" and "bio-application" water. Type 1 reagent grade water is commonly used in labs, with minimum resistivity of 18.0 MΩ·cm (ASTM-D1193 2011). "Bio-application" water is intended for use in clinical, pharmaceutical and biomedical applications, and has more critical standards with respect to colony forming units and TOC (ASTM-D5196 2013). Both laboratory-grade standards advise against any storage of produced water, and suggest periodic monitoring of quality specific to the use of the water in addition to in-line measurements.

Treatment processes for each type of water can vary, but several technologies are common. Reverse Osmosis (RO) uses pressures to pass water through a membrane such that some soluble

ions are removed. Ion-exchange resins (IER) generally remove all dissolved ions and replace them with H+ or OH-. Distillation condenses stream to create water with very low dissolved salts. UV irradiation kills bacteria by disrupting DNA and thus the ability to replicate, and it degrades organic carbon, a substrate for growth (Lehtola et al. 2003). Activated carbon filtration uses the large surface area on carbon treated with oxygen to adsorb organic and non-polar chemical impurities. As discussed in Chapter 2, activated carbon technology is also used in drinking water treatment to remove chlorine and organics and to provide biostability, or prevention of bacterial regrowth in subsequent distribution (Chien et al. 2008; Chien et al. 2007; Servais et al. 1994; Velten et al. 2011). Filtration can use a variety of materials and pore-sizes to remove particles by sieving and other mechanisms, with ultrafiltration removing particles larger than 0.1-0.001µm. One or a combination of these technologies is used to meet standards of each application (ASTM-D1193 2011; Office of Research Facilities 2013).

As with municipal water treatment, survival and regrowth of bacteria is a concern in pure water (Rathod et al. 2013). In pure water systems, the concern may be even higher as even small amounts of growth can be detrimental to intended uses, as when microbial contamination greatly reduces the performance of electronics rinsed with water (Gough et al. 1986) or when water quality impacts scientific results and interpretations (Semião et al. 2013). In order to prevent regrowth in both municipal systems and purified water systems, nutrients necessary for growth are targeted for removal (Lehtola et al. 2002). Many oligotrophic systems can be limited by supply of carbon, and thus, treatment plants often target the removal of assimilable organic carbon (AOC), the type of carbon easily taken up for growth (Chien et al. 2007; Escobar et al.

2001). Reduction of AOC to below 10 µg/L is associated with biostability in the distribution system (Kooij 1992).

Some elements within the purification apparatus may become hotspots for bacterial regrowth. IER may provide a growth medium for bacteria (Flemming 1987), and GAC is a known reservoir for bacterial regrowth in the right circumstances (Velten et al. 2011). However, even in systems with bacterial growth observed on pipelines or membranes (Bhaumik et al. 2004; McFeters et al. 1993), acceptable effluent water can be achieved.

Despite the reduction in carbon and other required nutrients for microbial growth achieved in purified water, certain extreme oligotrophic bacteria still manage to colonize such systems and provide unacceptable effluent (Gough et al. 1986; Governal et al. 1991). A diverse group of bacteria have been observed in these systems (Bohus et al. 2010; Chen et al. 2004; Kawai et al. 2002; Kulakov et al. 2002). Only few studies have focused on contamination issues in laboratory grade water (Matsuda et al. 1996; McFeters et al. 1993), and fewer have attempted to fully characterize the communities observed.

Quantification limits on bacteria focus on culture methods, with a common maximum of 100 CFU/mL (ASTM-D1193 2011). One study found < 10 CFU/mL in laboratory grade waters after use of a final filter (McFeters et al. 1993). However, the low-nutrient conditions in oligotrophic water result in stress for bacteria which may result in a loss of culturability. Studies on indicator bacteria in drinking water found that as much as 10-90 % of these bacteria may not be detected by culture methods(McFeters 1990; McFeters et al. 1986). It is then likely that these prior results

represent a lower bound to total bacteria likely to be present. Although molecular quantification methods may capture both dead and alive cells, they also capture the entire range of bacteria, including viable but non-culturable cells.

Identification of the bacteria in ultra-pure water have traditionally used culture-based techniques (Bohus et al. 2010; Chen et al. 2004; Penna et al. 2002), Molecular methods capturing the non-culturable fraction of bacteria have been used in only a limited number of the studies that characterized the microbial community of ultrapure waters. Characterization methods used include terminal restriction fragment length polymorphism (TRLFP) (Bohus et al. 2010; Chen et al. 2004), sequencing with clone libraries (Kulakov et al. 2002) and gel electrophoresis (Kawai et al. 2002). Next-generation sequencing can offer greater sequence depth which may capture an even greater diversity of bacteria equipped for this ultra-oligotrophic environment (Caporaso et al. 2010), which may provide insights for control of microbial regrowth in pure water systems and possibly certain municipal water systems with very low levels of nutrients.

In this study, a wide range of laboratory grade waters and the effects of often unavoidable storage on this water are explored. The use of molecular methods and next-generation sequencing will offer greater detail than formerly possible and may be revelatory as to what may be considered a typical super-oligotrophic community, indicating which bacteria thrive in these environments. The study also sheds light on the minimum levels of microbes that can be generated with water storage under the best care conditions in bulk water. This study may reveal limits of nutrient limitation as a strategy for preventing regrowth in a drinking water distribution system.

6.2 Methods

Two studies were undertaken to characterize bacterial contamination issues in laboratory grade waters. The first collected samples from several laboratories using different treatment approaches and types of pure water, and the second was designed to track bulk water bacterial growth in pre-sterilized glass lab ware.

6.2.1 Sample Collection and Preservation. Thirteen laboratory grade water purification systems located on the Virginia Tech campus were included in this study. Information about age and maintenance history of the systems was obtained from lab users.

Systems were sampled using pre-sterilized 1 L HDPE Nalgene bottles with polypropylene caps. Two 1 L samples were collected from each system using the highest flow conditions possible. No water was intentionally flushed before sampling and two liters were drawn in order to capture the maximum possible contamination in the system.

After sample collection, 60 mL was collected for adenosine triphosphate (ATP) analysis. Samples were stabilized on site by filtering to capture cellular contents using a Quench-Gone LuminUltra (NB, Canada) syringe filter. Cells were lysed to release and preserve ATP for analysis by filtering 1 mL of UltraLyse (LuminUltra) through the syringe. Stable samples were kept on ice until further analysis.

Water flow rates were also measured after sample collection by recording the time taken to fill containers of measured volume. Blanks consisted of 1 L of nano-pure water sterilized with autoclaving, originating from the system labeled BN_3. Water samples were immediately placed

in a cooler on ice. Upon return to lab, all samples were kept in a 4 °C fridge until filtration. Sample analysis occurred within 12 hours of sample collection.

Trip blanks and field blanks consisted of 1 L of pure water collected from the system named BN_3 which was autoclaved and stored in similar containers. Field blanks were opened at each site, trip blanks were not. Filter blanks were not exposed to any water.

6.2.2 Time Series Study. Two tests were conducted to determine the effects of storage on microbial composition of pure water. The first one was conducted from 1/30/2013 – 2/9/2913[Time Study 1] and the second was conducted from 5/31/2013 – 7/1/2013 [Time Study 2]. Time Study 1 was carried out under exposure to ambient light in order to account for possible phototrophic effects, whereas Time Study 2 was carried out in a closed cabinet shielded from light in order to exclude phototrophic effects.

Water was collected from an in-lab Barnstead Nanopure system [also labeled "BN_3" in the Locations study] into glass Pyrex 10 L media storage bottles with screw caps that had been acid washed and sterilized via autoclaving. Water was thoroughly mixed via manual shaking then distributed into Pyrex 1 L media storage bottles with screw caps that had been acid washed and sterilized via baking at 550°C for 4 hours (glass bottles) or autoclaving (caps). Approximately 1 L was transferred under sterile conditions and subsequently tightly capped.

For each sampling event, the entire liter was sacrificed. Each sample event included a filter blank sample. The time zero samples were taken immediately after transfer to the 1 L incubation bottles. Each storage bottle was shaken manually in the same fashion prior to filtration.

6.2.3 Adenosine Triphosphate (ATP) and Adenosine Monophosphate (AMP) Quantification. ATP provides an indicator of viable biomass activity levels, while adenosine monophosphate (AMP) is an indicator of cell stress. ATP and AMP concentrations, and their ratios, were measured using a LuminUltra® Quench-Gone™ Aqueous Test Kit (LuminUltra). Preserved samples were analyzed within 12 hours, according to manufacturer protocol to determine ATP, AMP, and the ATP:AMP index.

6.2.4 DNA Extraction. Under sterile conditions, samples were concentrated onto sterile mixed cellulose ester filters with 0.22 µm pore-size (Millipore, Billerica, MA) by vacuum filtration. The filter was folded and torn using sterile tweezers and transferred to a Lysing Matrix A tube from the FastDNA® SPIN Kit (MP Biomedicals, Solon, OH). DNA extraction was conducted according to manufacturer instructions.

6.2.5 Quantitative Polymerase Chain Reaction (Q-PCR). All DNA samples were analyzed with quantitative polymerase chain reaction (qPCR), which was applied to quantify 16S rRNA genes as an indicator of the level of total bacteria(Suzuki et al. 2000). Q-PCR was carried out using a CFX96™ realtime system (Bio-Rad, Hercules, CA). Q-PCR assays were previously been validated for drinking water samples in terms of specificity and limit of quantification (Wang et al. 2012). Previous tests (data not shown) indicated that a 1:10 dilution was appropriate for

quantification of pure water samples. For each run of q-PCR analysis, a calibration curve was included with at least seven points.

6.2.6 Illumina Sequencing of 16S rRNA Gene Amplicons. Select samples were also analyzed with amplicon sequencing to analyze biodiversity. 16S rRNA genes were amplified with barcoded primers 515F/806R (Caporaso et al. 2012) using the protocol described (Caporaso et al. 2010). In order to normalize depth of reads/sample, 20 ng of DNA of each amplification product were mixed according to quantification using the Qubit® ds DNA HS Assay Kit (Invitrogen ™) and Qubit ® 2.0 Fluorometer. Combined PCR products were cleaned using QIAGEN PCR Purification Kit. Sequencing was performed on an Illumina Miseq® benchtop sequencer using pair-end 250 bp kits at the Virginia Bioinformatics Institute.

Reads were contigued together using PAired-eND Assembler for DNA Sequences (PandaSeq) (Masella et al. 2012). Qiime (Quantitative Insights Info Microbial Ecology) was used as a pipeline for sequence analysis. Operational Taxonomical Units (OTUs) were assigned using uclust (Edgar 2010) based on 97% similarity to the Greengenes database(DeSantis et al. 2006).

6.2.7 Data Analysis. Statistical analysis for quantitative measures was performed using JMP (SAS, Cary, NC). Data obtained from ATP, AMP and qPCR were assumed to be normal and t-tests were applied to compare conditions. Least-squared regression was applied to determine correlations. Significance was set at $\alpha=0.05$. Weighted and unweighted Unifrac (Lozupone and Knight 2005) distance was computed between all samples using an equal sampling depth of

11,000 sequences/sample. Bootstrapped jackknife trees were produced in Qiime using Unifrac distances. Alpha-diversity was calculated within Qiime.

6.3 Results and Discussion

6.3.1 Resident Microbiome of Water Purification Systems

The operating conditions of the 13 laboratory grade systems are described in Table 6.1. At time of sampling, all were in operational use. All samples were taken within three days in December 2012 with mean outdoor temperatures 51-57°F (11-14° C). This may have limited risk of ambient bacteria contamination.

Table 6.1. System Specifications for Location Study. In the Components column, an X indicates presence of a particular treatment technology.

System Code Name	Flow Rate (L/min)	Resistivity Reading (MΩ·cm)	**Components of System/Feed water***							**Frequency of maintenance/ time since last maintenance**
			Pre-filter	RO	DI	IER	GAC	UF	UV	
Collected 12.3.12										
M_1	0.96	18.2				X	X	X	X	1.5 years
BN_1	1.16	18.2						X	X	2 mo.
BNI_1	1.13	18.0	X	X				X		2 mo.
O_1	1.61	18.2			X			X		Driven by breakdown
O_2	1.62	N/A			X			X		6 mo.
BN_2	0.82	18.3			X			X		6 mo.
Collected 12.4.12										
O_3	1.89	18.2						X	X	1 mo.
O_4	1.76	18.07						X		4 mo.
B_1	1.10	18.2	X	X					X	6mo.
M_2	1.01	19.2		X				X	X	6 mo.
Collected 12.5.12										
B_2	2.25	17.7			X			X		5.5 years
BNI_2	0.29	18.3						X		2 years
BN_3	1.67	18.32			X		X	X	X	6 mo.

*RO = Reverse Osmosis; DI = De-ionized; IER = ion-exchange resin; GAC = granular activated carbon; UF = ultrafiltration; UV = Sterilization with UV light

The systems had varied degrees of treatment and maintenance, different ages, and quality of feed water. Yet, many of these systems had similar resistivity readings (mean 18.24, 95% CI [18.02-18.46], outliers B_2, M_2, BNI_1; N=12) and were used for similar applications in each lab. All systems were advertised to provide Type 1 Reagent Grade Water or better.

Quantification of 16S rRNA genes indicated that these systems still had measurable quantities of 16S rRNA, although these samples registered below the 'blank' samples which had undergone sterilization by autoclaving ($p<0.03$, t-test). As DNA detection methods cannot differentiate between live and dead cells, detection of 16S rRNA does not necessarily indicate that systems were contaminated with live bacteria.

Only field blanks, which were opened and allowed to be contaminated at multiple sites, had significantly higher concentrations of 16S rRNA than any other type of blank or sample ($p<0.03$, t-test). Both field and trip blanks were collected at the same time from one system (BN_3) and underwent autoclaving. Field blanks were opened as much as 6 times in a day, however, compared to trip blanks which were kept tightly capped. Both were exposed to the same changes in temperature.

Filter blanks had significantly lower concentrations of 16S when compared to all other samples, which were exposed to either 1 L (blanks) or 2 L (all sample locations) of pure water ($p= 0.045$, 1-sided t-test), suggesting that DNA contamination persists in many types of laboratory pure water and that the source of DNA contamination was neither the filter itself or the filtering process.

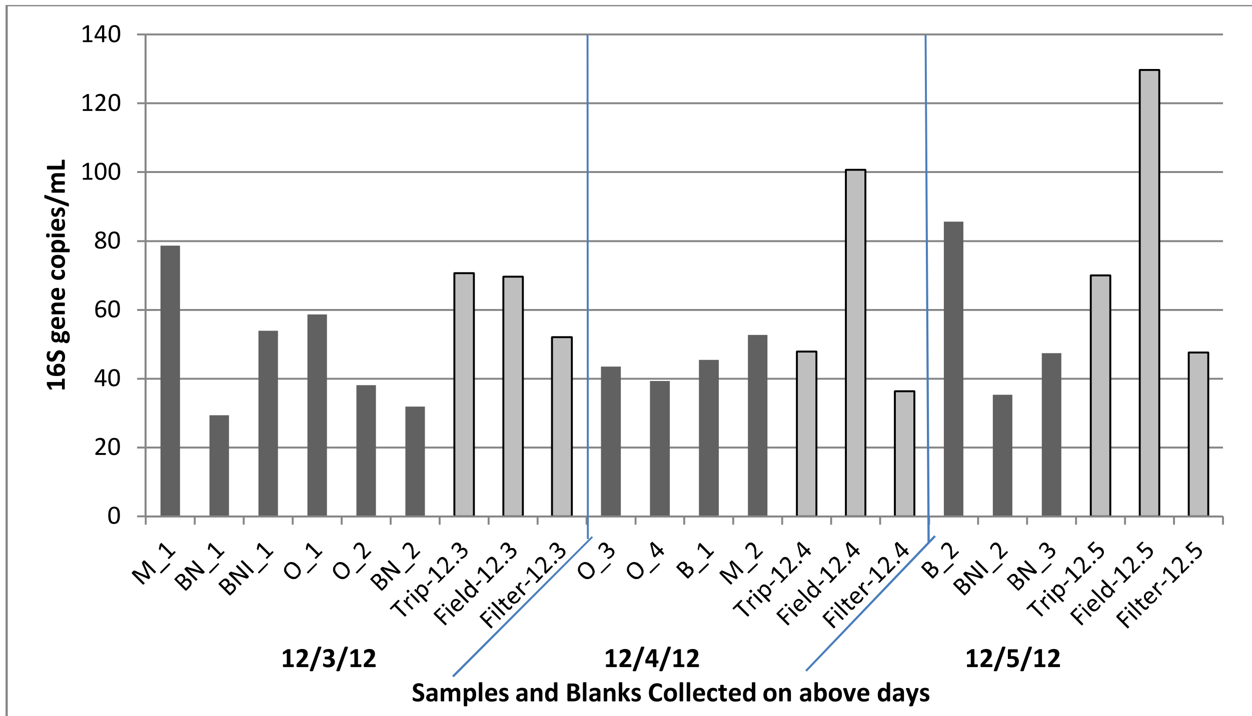

Figure 6.1. Concentration of 16S [gene copies/mL] in 2 L samples of pure water from various locations and Blanks. Trip blanks and field blanks consisted of 1 L of pure water collected from the system named BN_3 which was autoclaved and stored in similar containers. Field blanks were opened at each site, trip blanks were not. Filter blanks were not exposed to any water. For each bar, n=1.

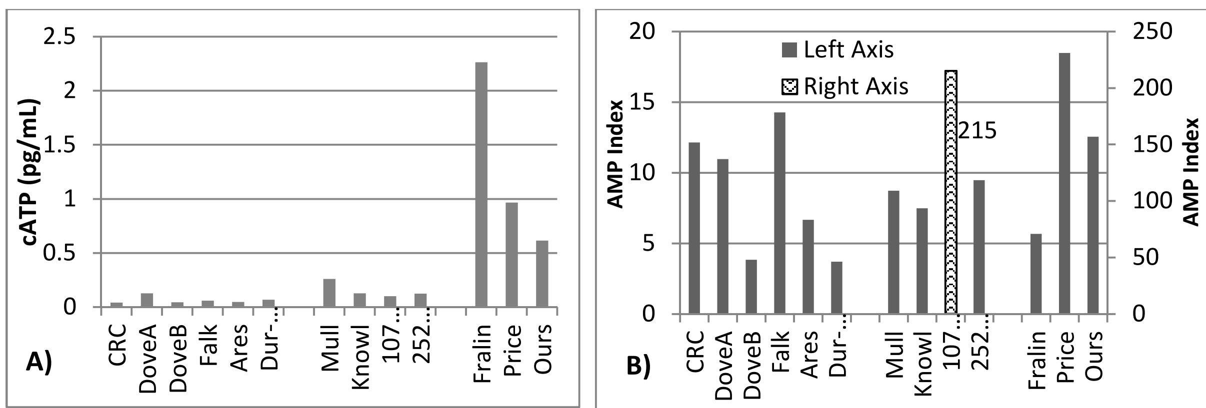

Figure 6.2. A) Concentration of ATP in various pure water Systems. For each bar, n=1. B) AMP Index in various pure water systems. For each bar, n=1.

ATP is a measure of biological activity. Most samples had very low levels of ATP, in the range of < 0.5 pg/mL, which is indicative of "good control" for drinking water by kit manufacturers.

Three samples, all collected on the same day, 12.5.12, were in the range of 0.5-10 which is indicated as "preventative measures needed" for drinking water by kit manufacturers.

AMP Index was above 3.0 for all samples, which is indicated as "Lethal Stress" by kit manufacturers. This is expected, as nutrient limitation and ultrafiltration in pure water treatment systems would put high stress on any surviving bacteria. Neither ATP nor AMP reflected concentrations of 16S rRNA detected ($p > 0.05$, Least Squared Regressions).

6.3.2 Effect of Storage on Bacterial Concentrations

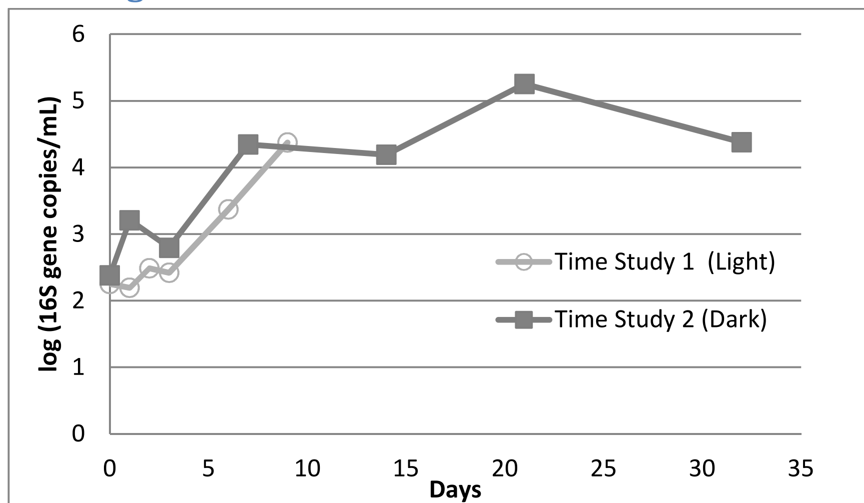

Figure 6.3. Log (16S rRNA Gene Copies) detected by qPCR in two time storage studies of nano-pure water from the same system. In both studies, water was stored in sterilized 1L glass jugs at room temperature after homogenization of all samples for each study. For each point, n = 1. All growth conditions were similar except light conditions and season. In Time Study 1 light infiltration was allowed and in Time Study 2, light infiltration was not allowed. Time Study 1 was conducted in winter and Time Study 2 was conducted in spring.

With storage, a 2 log increase in 16S rRNA occurred within about 10 days in both studies. In Time Study 2, the concentration of 16S rRNA stabilized within 1-log of variation after this point, and remained stable for 2 weeks. DNA capture methods capture both dead and alive bacteria.

The increase in concentration of 16S rRNA collected over time suggested that growth was occurring even if the baseline levels consisted of 16S rRNA from lysed, dead cells.

The conditions were designed to give an idea of the minimum proliferation likely in storage circumstances. With pre-sterilized labware, aseptic techniques and focus on the bulk water rather than biofilm, it is likely that bacterial proliferation is higher in normal storage conditions where these precautions are not taken.

6.3.3 Illumina Sequencing of 16S rRNA Gene Amplicons

Nineteen samples were selected for further characterization of the microbiome by Illumina sequencing of 16S rRNA gene amplicons. From the multiple systems study, 5 of 13 locations (M_1, BN_1, O_2, B_2, and BN_3) and blanks of all three types from the two days encompassed by those samples (12.3.12 and 12.5.12) were chosen. From Time Study 1, samples from Day 0, 6 and 9 were selected. From Time Study 2, samples from Day 0, 7, 14, 21 and 32 were selected.

As described in 6.2 Methods, sequences are identified against a known database to give taxonomical data for each OTU. The abundance of each OTU in a sample is also calculated. With this data, distance matrices between each sample are generated to determine how similar or different samples are. Distance matrices can be constructed based on what unique OTUs are present (unweighted) and based on how often those OTUs are detected (weighted).

Based on weighted Unifrac analysis, or the detection and relative abundance of taxa, all samples allowed to sit in storage more than 1 week cluster together, separated from all samples one week old or less (most are less than one day old) and blanks (Figure 6.4). In the tree, samples from

Days 9-32 of both Time Studies clustered apart from those from Days 0-7. In addition, these initial samples clustered closely with Blanks and samples from other locations. The bacterial communities, then, are dependent on time of stagnation. This mirrors changes in microbial communities observed in drinking water with overnight stagnation (Lautenschlager et al. 2010).

Figure 6.4. Phylogenetic Tree created by weighted Unifrac BootStrapped Jackknife analysis, performed in Qiime for Pure Water Samples. Differences in communities are calculated given both the OTUs present and their relative abundance. Branching indicates a difference in community, length of each branch indicates extent of variation (see scale). Vertical distance holds no significance. Bootstrap values at nodes indicate confidence of clustering for each node, where 1 indicates perfect confidence.

An unweighted UniFrac analysis, which does not take into consideration the relative abundance of each new OTU, giving each unique OTU an equal weight, did not produce similar clustering (Figure 6.6, Appendix). This suggested that certain OTUs that may have been present in initial samples proliferated and grew with stagnation time, thus shifting their relative abundance. While a similar, diverse, microbial community is present immediately after pure water production, stagnation outside the system shifts the community with proliferation of select bacteria.

Samples collected in different studies with different growth conditions on days 6 and 7 of their respective studies also cluster very closely together. This occurs although each had different root communities and developed into different communities at later collection points. This further suggests that certain subsets of the bacteria were able to survive and thrive in the purified water and may have had similar growth times.

Relative abundance data (Figure 6.5) confirmed cluster analysis and gives insight to the bacteria which are able to survive and proliferate in very oligotrophic environments. Profiles of communities with longer storage periods were different than those with very short storage times. Proteobacteria, particularly Alphaproteobacteria and Betaproteobacteria, tended to dominate with greater storage time.

The phyla with high abundance across many samples include Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. This mirrors phylum dominance in US drinking water systems as determined with sampling of 17 drinking water distribution systems (Holinger et al.

2014). In that study, however, Cyanobacteria also dominate. Cyanobacteria were found in this study, but in lower relative abundance than in drinking water.

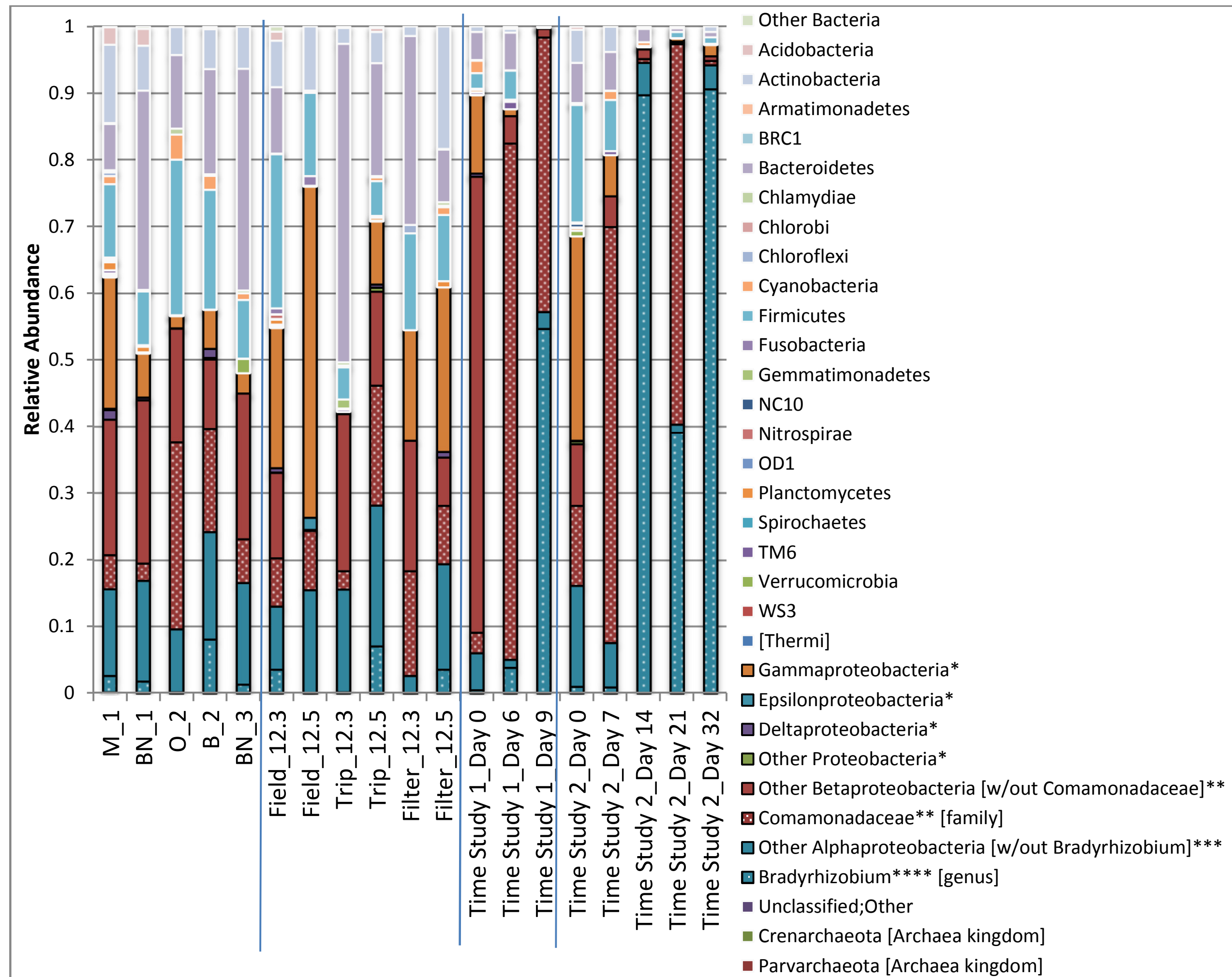

Figure 6.5. Relative Abundance of Phylum in Pure Water Samples. *Proteobacteria broken into classes (Alpha-, Beta-, Gamma-, Delta-, Epsilon-proteobacteria and other) **Betaproteobacteria further divided into the family Comamonadaceae and other ***Alphaproteobacteria further divided into the genus *Bradyrhizobium* and other.

Actinobacteria, Firmicutes and Bacteroidetes were all present in greater relative abundance in the Locations, Day 0, and Blanks samples, than samples with greater storage time. Actinobacteria are gram positive and play an important role in carbon recycling. *Mycobacteria*, which are

prevalent in drinking water(Holinger et al. 2014; Liu et al. 2012), were found at the genus level of classification in all samples, and were found in highest relative abundance in the systems O_2 (3.1%), B_2(3.8%), and Time Study 2.Day 0 (2.7%). In Time Study 2, the relative prevalence of *Mycobacteria* seemed to decrease with time, with Day 7(1.9%) to a low on Day 21 (0.08%). It was also present in the Day 0 sample of Time Study 1, and prevalence also decreased with time. It is important to note that this analysis focuses on bulk water. Although bottles were thoroughly shaken prior to each sampling date, it is possible that biofilms formed on walls of glassware.

Firmicutes are known to produce endospores, which may account for their survival through rigorous treatment processes. Both Clostridia (anaerobic) and Bacilli (obligate or facultative aerobes) were represented in samples.

Bacteroidetes are generally anaerobic bacteria. The most common taxa within Bacteroidetes were Chitinophagaceae. This family has been identified as surviving within free living amoeba in drinking water (Delafont et al. 2013). This, along with the presence of other taxa that were identified within amoeba in drinking water (including *Bacillus*, *Ralstonia*, *Mycobacterium*, *Lactococcus*, and *Legionella*) (Delafont et al. 2013) may indicate that amoeba play an important role in the growth of bacteria in purified water.

The phylum TM6 was ubiquitous to all samples, including filter blanks, although on average it made up only 0.2% of samples. It was in highest concentration on Day 6 of the time study in light (1.2%). TM6 is proposed as a symbiont of another organism and it has been commonly recovered from sinks in hospitals (McLean et al. 2013).

Cyanobacteria, generally phototrophic bacteria, were found in all samples. They were in highest relative abundance (1.2%) in the O_2 system. In Time Study 1, they were found in highest abundance in the Time 0 sample. Their relative abundance decreased with storage time, indicating that growth of these phototrophic bacteria, which were exposed to ambient light during storage in Time Study 1, did not attribute greatly to the observed growth. MLE1-12 was identified in 17 of 19 samples, including blanks. This clade was also identified in drinking water distribution systems (Holinger et al. 2014), and pharmaceutical wastewater (LaPara et al. 2000), both of which are typically not exposed to light, indicating that the clade may not be phototrophic.

Of the phylum Chloroflexi, the greatest relative abundance of phototrophic OTUs (1.2%) was found in the Filter Blank_12.3 sample. Other Chloroflexi include those identified as anaerobic (Yamada et al. 2006), and were found Time.Study 2 – Day 6 (0.048%). Chlorobi, another phototrophic phylum, was found only sporadically, and were not found in Time Study 1, which was exposed to ambient light during storage.

Nitrifying bacteria were sporadically found in low relative abundances. It was found with greatest relative abundances for Nitrospira, a nitrite oxidizer, in Field.Blank1 (0.6%) and for Nitrosomonadaceae, ammonia oxidizers, Time Study 2.Day 0 (1.2%). DNA of ammonia oxidizers could be an artifact of the use of chloramination for secondary disinfection in Blacksburg tap water – which is ultimately used as source water for all systems.

Proteobacteria were detected in greater relative abundance in samples with greater storage time, and may be considered as the primary driver of growth in both time studies. These were able to proliferate in extremely oligotrophic environments. Those that proliferated most include the *Bradyrhizobium* genus and the *Comamonadaceae* family. Their roles in nitrogen fixation and H_2 oxidation may play an important role in oligotrophic bacterial growth.

Proteobacteria have a wide variety of metabolisms. Gammaproteobacteria include many pathogens including *Legionella*, which was detected in this study at the genus level in in two samples with one OTU/sample. Gammaproteobacteria became a less significant class with greater storage time. Alphaproteobacteria and Betaproteobacteria are both diverse metabolically and they both gain relative abundance in samples stored for a longer period of time. The relative dominance of Alpha- and Beta-proteobacteria in relation to each other varies over time (Figure 6.5).

Alphaproteobacteria detected laboratory grade water systems are dominated by the genus *Bradyrhizobium* within the family *Bradyrhizobiaceae* and the class Rhizobiales. *Bradyrhizobium* accounted for up to 90% of OTUs detected in samples collected at Day 14 and Day 32 of Time Study 2, as well as 55% of OTUs detected in samples collected on Day 9 of Time Study 1. *Bradyrhizobium* is commonly associated with nitrogen fixation in soils, and has previously been found in several ultrapure water systems (Chen et al. 2004; Kawai et al. 2002; Kulakov et al. 2002). It is also associated with free living amoeba in drinking water (Delafont et al. 2013).

Among Betaproteobacteria, the order Burkholderiales dominates and is highly variable. Within this order, the *Ralstonia* genus within the *Oxalobacteraceae* family and an unidentified genus in the *Comamonadaceae* family dominated. The *Comamonadaceae* family dominates in samples allowed to stagnate for longer periods of time, accounting for 60% and 57% of OTUs detected in samples collected on Day 7 and 21 of Time Study 2, and 75% and 41% of OTUs detected in samples collected on Day 6 and 9 of Time Study 1. The family is associated with H_2 oxidation(Willems et al. 1991). *Ralstonia* accounts for 60% of OTUs detected in the initial sample for Time Study 1. Some species of this genus are associated with opportunistic pathogens and denitrification (Ryan et al. 2011).

Overall, the taxa observed in laboratory-grade water purification systems are similar to those observed in drinking water, although they vary in abundance. There was surprising diversity, even in filter-blank samples which were not exposed to water.

6.4 Conclusions

Water purification systems allowed for detection of a surprising array of bacteria. Communities are similar for water purification systems of different quality and treatment level at different locations. These water purification systems supported a diverse community of bacteria high in Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria.

A significant portion of bacteria were alive and active, and grew up to two logs during storage of as little as ten days, even in clean and dark conditions. A shift in the bacterial community after about one week indicated that the Proteobacteria phylum was especially important for growth in the extremely oligotrophic environment. Nitrogen fixing (*Bradyrhizobium*) and H_2 oxidizing

(*Comamonadaceae*) bacteria were especially important in purified water allowed to grow in storage for extended periods of time.

This study may have profound implications for use of pure water as controls in laboratories. Although purified water used directly after production will only cause a minimal qPCR increase, storage of the same water may give as much as a 2-3 log increase in 16S rRNA genes detected, and may not be adequate for comparison to experimental conditions.

Under conditions designed to minimize nutrients, even with UV sterilization and destruction of TOC, the lowest level of bacteria achievable after 10 days is 3 log 16S rRNA genes/mL. This may have implications for nutrient control strategies in drinking water.

6.5 Acknowledgements

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6.7 Appendix

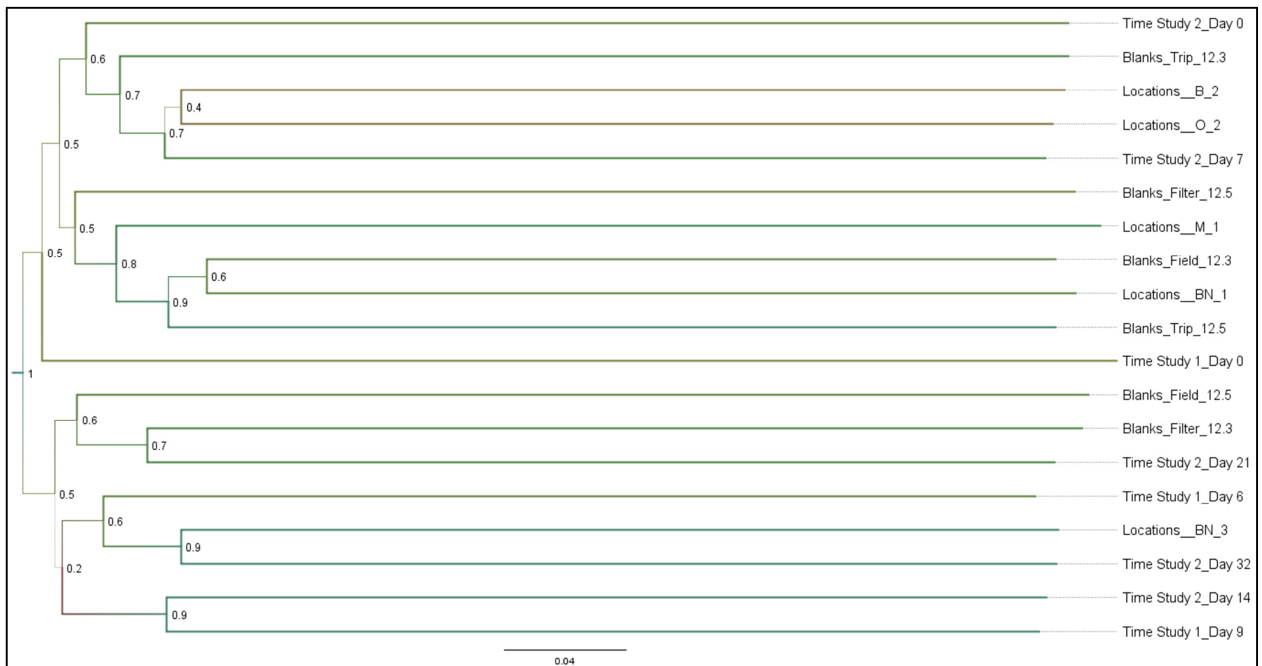

Figure 6.6. Phylogenetic Tree created by unweighted Unifrac BootStrapped Jackknife analysis, performed in Qiime for Pure Water Samples. Differences in communities are calculated given both the OTUs present and their relative abundance. Branching indicates a difference in community, length of each branch indicates extent of variation (see scale). Vertical distance holds no significance. Bootstrap values at nodes indicate confidence of clustering for each node, where 1 indicates perfect confidence.

Chapter 7

Conclusions and Final Thoughts

7.1 Conclusions and Contributions

Premise plumbing varies greatly with materials present and quality of water with respect to biological, chemical and abiotic parameters. In order to advise engineering methods for manipulation of these factors for the control of *Legionella pneumophila* and other opportunistic pathogens, it must first be understand how these factors affect their growth. This study provides valuable insights to the behavior of *L. pneumophila* and total bacterial regrowth in conditions representative of premise plumbing.

Organic carbon is used as a substrate for growth for heterotrophic bacteria, and assimilable organic carbon (AOC) is the portion of organic carbon readily available for use by microorganisms. Although *L. pneumophila* does not use AOC directly for growth, concentration of AOC is somewhat related to proliferation. Total bacterial growth is also related to AOC concentration. Thus, AOC reduction may be a promising method for controlling *L. pneumophila* and total bacteria, but there may be a point at which AOC reduction is no longer effective.

Methods for AOC reduction must be carefully considered. Granular activated carbon (GAC) biofiltration, when applied at a building level, may actually specifically encourage *L. pneumophila* proliferation. Changes in water chemistry, specifically as they relate to copper, may be a driver for this change. In real systems, GAC filtration would also reduce disinfectant

residual and potentially introduce high concentrations of bacteria as they slough off the filter, making GAC filtration an even more unattractive option for building level water treatment.

A single premise plumbing system has a wide variety of materials, specific to pipes, fixtures and water heaters. Each plumbing condition tested may have only a limited effect on the proliferation of *L. pneumophila* and total bacteria. The impact of each plumbing condition tested may also be linked to quality of influent water. In addition, these effects continue to be dynamic, even with long-term studies.

The choice of copper pipes offers promise as an engineering control for *L. pneumophila*, although it does not greatly affect total bacterial regrowth. Increased copper concentration also held promise for *L. pneumophila* control across a range of pHs. In systems with copper pipe, the aqueous copper concentration is dependent on temperature, even with old pipes, and this may diminish the effectiveness of the engineering control at higher temperatures.

The temperature setting on a water heater has profound effects for *L. pneumophila* proliferation. The peak temperatures for *L. pneumophila* proliferation fall between 41 and 45 °C. These temperatures would likely be encountered in the hot water distribution system when the water heater is set to 48 °C, as is often recommended. Higher temperatures of 53 °C seem to provide control of *L. pneumophila*, but recolonization is possible even at these high temperatures.

Extreme control of nutrients, as achieved with laboratory grade waters, may not be enough to completely control regrowth in premise plumbing. With stagnation in the cleanest conditions, a

2-log increase of a diverse group of bacteria was noted within 10 days. As drinking water can never achieve such nutrient removal, this study presents the limits of nutrient removal as a strategy for regrowth control.

Premise plumbing is dynamic, as are the results from this study. Water chemistries may interact to change the effectiveness of engineering controls. Thus, the complete characterization of water chemistry and microbiology is critical in any studies addressing regrowth in premise plumbing.

7.2 Future Studies

This study lends support to a probiotic approach to bacterial control in drinking water. In several experiments, the response of *L. pneumophila* to water chemistry changes did not necessarily reflect that of the total bacterial community. For example, in simulated glass water heaters with copper pipes at 32 °C there were more total bacteria, but less *L. pneumophila*. In simulated glass water heaters receiving GAC biofiltered influent water, there was more *L. pneumophila* and less total bacteria. The presence of some innocuous bacteria may protect against the proliferation of more harmful bacteria. An in-depth community analysis using water samples related to this study may provide valuable insights to support this hypothesis.

The simulated glass water heaters are a valuable tool for examining how water chemistry and microbiology interact. Given the importance of plumbing material age and long-term studies, these water heaters should be maintained for use in future studies. They provide a controlled, yet realistic platform for investigation to premise plumbing. Some chemistries and conditions that were not fully explored in this study include disinfectant residual, pH, and long-term stagnation.

In this study, a critical component of drinking water treatment in the United States, secondary disinfection residual in the system, was removed in order to consider the worst-case scenario in US drinking water. It is possible that the materials and water chemistries explored will have a greater effect when disinfectant residual is considered. If materials or chemistries contribute to loss of residual, then stronger effects on regrowth will be observed.

The literature regarding *L. pneumophila* and pH is somewhat contradictory. As many factors can coincide in real systems, it would be valuable to use this realistic but controlled environment to ascertain the optimal pH for *L. pneumophila*. As *L. pneumophila* proliferation in real systems is dependent on host organisms, it is important to test this with a realistic microbial community. The simulated glass water heater set-up offers such an environment where pH can be isolated as a control factor. Repeating the experiment with other influent waters (i.e. not Blacksburg) would also be a valuable exercise.

Long-term stagnation of a month or so, as might be encountered on schools during a holiday, when a building is under construction or when part of a building goes unoccupied, may have a profound effect on the proliferation of *L. pneumophila* and total regrowth. With frequent water changes, simulated glass water heaters were provided a steady source of nutrients, and biofilm was never disturbed too greatly. The behavior of *L. pneumophila* with extended stagnation may affect recommendations for flushing, i.e. at a building commission.

Another factor not fully explored in this work was the ability of bacteria to adapt and change. Bacteria can grow resistant to the control mechanisms described, including copper and

temperature. Identification of the specific mechanisms bacteria in reactors employed may be possible by comparing the strains of specific bacteria present in reactors to strains originally used for inoculation.

More progress is also dependent on the use of full-scale systems. Field studies on *L. pneumophila* and regrowth problems often lack either full chemical or full biological characterization of the water. The two fields are closely linked in drinking water, however, and should complement one another fully in all studies.