Growth of Opportunistic Pathogens in Domestic Plumbing: Building Standards, System Operation, and Design

William Joseph Rhoads

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Marc A. Edwards, Chair
Amy Jill Pruden-Bagchi
Annie R. Pearce
Joseph O. Falkinham, III

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ABSTRACT

Understanding and limiting public health threats resulting from exposure to opportunistic pathogens (OPs) in domestic water (i.e., hot/cold water for human use) will be one of the grand challenges for water safety in the 21st century. This dissertation anticipates some of the complexities in balancing stakeholder goals and developing building standards to limit OP growth, and advances scientific understanding of OP survival and proliferation in domestic plumbing systems.

In a cross-sectional survey of water- and energy-efficient buildings, domestic water age ranged from 8 days to 6 months and resulted in pH and temperature fluctuations, rapid disinfectant residual decay up to 144 times faster than municipal water delivered to the buildings, and elevated levels of OP gene markers. This motivates future work to determine how to maintain high quality and safe water while preserving the sustainability goals of these cutting-edge buildings.

Head-to-head pilot-scale experiments examining OP growth in recirculating hot water systems revealed that elevated temperature had an overarching inhibitory effect on *L. pneumophila* growth where temperatures were maintained. However, control was undermined in distal branches, especially when density-driven convective mixing gradients maintained ideal growth temperatures and delivered nutrients to the otherwise stagnant branches. These results resolve discrepancies reported in the literature regarding the effects of flow, and identify important system design and operational conditions that facilitate OP growth.

Advancements were also made in understanding how corrosion can trigger OP growth. In Flint, MI, corrosive Flint River water damaged iron pipes, releasing iron nutrients, consuming chlorine residual, and supporting high levels of *L. pneumophila* in large building systems. This likely triggered two unprecedented clusters of Legionnaire's disease.

In pilot-scale systems, copper released from copper pipes, but not dosed as soluble cupric, triggered release of >1,100 times more H_2 into the water due to deposition corrosion. The organic carbon fixed by autotrophic hydrogen oxidation has the potential to facilitate OP growth, but more work is needed to understand the limits of this mechanism.

Finally, well-controlled laboratory experiments confirmed past reports from field surveys that the use of chloramines trigger a trade-off between controlling *Legionella* and allowing non-tuberculous *Mycobacteria* to persist.

Growth of Opportunistic Pathogens in Domestic Plumbing: Building Standards, System Operation, and Design

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GENERAL AUDIENCE ABSTRACT

Understanding and limiting public health threats resulting from exposure to opportunistic pathogens (OPs) in domestic water (i.e., hot and cold water intended for human use) will be one of the grand challenges for water safety in the 21st century. Unlike traditional fecal-based waterborne pathogens that have all but been eliminated through advanced treatment applied at water treatment facilities, OPs are native microbial members of drinking water and tend to proliferate in domestic plumbing. In addition to the complexity and technical nature of engineering controls applied in buildings to limit OP growth, there are many stakeholder groups with varied responsibilities and expertise in preventing, diagnosing, and/or remediating problems. Stakeholders sometimes present additional challenges when their goals have direct or indirect trade-offs with limiting OP growth in buildings. This dissertation anticipates some of the challenges to come, and advances scientific understanding of how OPs survive and proliferate in domestic plumbing systems.

Water- and energy-efficient buildings, while nobly seeking to preserve precious natural resources, potentially create unintended consequences with respect to water quality. In a cross-sectional survey of green building designs, water remained within domestic plumbing for over a week to months before being used by consumers, and resulted in water quality changes that facilitated the growth of OPs. While short-term solutions exist, such as flushing water to decrease water stagnation and introduce "fresh" water into the system, this work motivates future research for how to maintain high quality and safe water while preserving the sustainability goals of these cutting-edge buildings.

Systematic experiments were conducted on water heaters with a recirculating pump, which are marketed as a "green" technology for water and energy savings, to determine the effect of system design and operation on the growth of OPs. Elevated temperature was found to have an overarching inhibitory effect on growth of *L. pneumophila*, the most commonly reported OP. However, when the water heater temperature was not sufficient to completely eliminate *L. pneumophila* (51 °C), higher water temperatures actually supported high levels of *L. pneumophila* growth in infrequently used pipes by periodically disinfecting other microorganisms that are more susceptible to thermal disinfection and decreasing competition for nutrients.

System design also impacted *Legionella* growth. In pipes that slowly mixed with the recirculating line (simulating a pipe running upward to a shower head from a recirculating line in the floor, for instance), *L. pneumophila* were consistently elevated relative to pipes that did not convectively mix (simulating a pipe running downward to a kitchen tap from a recirculating line in the ceiling, for instance). The slow mixing maintained ideal *Legionella* growth temperature in the pipes with mixing, even when water heaters were maintained well above thermal disinfection levels for *Legionella* (i.e., at 60 °C). This is due to continuous delivery of nutrients to the upward pipes with mixing, but not the downward pipes without it. This result is significant because it outlines

scenarios encountered in real buildings where even the most effective thermal disinfection strategy can be undermined in distal branches within the building.

This work also outlines the importance of corrosion in potentially triggering OP proliferation. In Flint, MI, when the water utility began distributing very corrosive Flint River water, the new water source damaged iron water pipes, releasing iron (which is a nutrient for *Legionella*) and eliminating disinfectant residual in the distribution system (which is needed to prevent *Legionella* regrowth). As a result, the corrosion supported increased *Legionella* levels and likely triggered two unprecedented clusters of Legionnaires' disease.

In the pilot-scale systems, corrosion of the water heater anode rod caused trace nutrients to evolve into the water. However, this only occurred when low levels of copper released to the water from the natural corrosion of copper pipes were present. Ionic copper, which is sometimes used to disinfect *Legionella*, did not have the same effect when it was dosed to the experiment at similar concentrations. These trace nutrients generated from copper-enhanced corrosion of the water heater anode rod are a potential source of carbon for OP growth, and may help explain the variable effects of copper that have been reported in the literature. More work is needed to fully understand this potential growth mechanism.

Finally, this work confirmed past field observations that there is a trade-off between controlling *Legionella* and allowing *Mycobacteria*, another OP, to persist when using chloramines disinfectant residual. Reproducing this phenomenon in controlled laboratory settings is an important step in understanding, and ultimately preventing it.

AUTHOR'S PREFACE

The first seven chapters in this dissertation are arranged as individual manuscripts according to Virginia Tech's specifications and are formatted based on the journal to which it was published or submitted. Each manuscript was produced in collaboration with Drs. Edwards and Pruden, and other researchers with intellectual contributions appropriately indicated by authorship or co-authorship.

CHAPTER 1 presents an integrated analysis of several lines of data surrounding the unprecedented Legionniares' disease clusters that occurred in Flint, MI from April 2014-October 2015 during the Flint Water Crisis. It highlights how aging water infrastructure, deficiencies in water treatment and distributions system maintenance, and corrosion, in particular, can contribute to significant public health threats regarding opportunistic pathogens. In this chapter, we specifically link the corrosivisty of the treated Flint River water source to myriad water quality deficiencies experienced in Flint, including higher detection and levels of *Legionella*. We conclude that the two Legionnaires' disease clusters were likely caused by the use of corrosive water.

This chapter will be submitted to Environmental Science & Technology for publication. Chapter Authors include: William J. Rhoads, Emily Garner, Pan Ji, Ni Zhu, Jeff Parks, Otto Schwake, Amy Pruden, Marc A. Edwards.

CHAPTER 2 anticipates how new water infrastructure and building practices that employ energy- and water-efficient designs and technologies may impact drinking water quality. Achieving extreme reductions in water demand are becoming more commonplace while scientific understanding is lagging behind. This chapter illustrates that green plumbing systems present a wide range of challenges for utilities and other stakeholders, and identifies key research knowledge gaps that need to be addressed.

Rhoads, W. J., Pearce, A., Pruden, A., Edwards, M.A. 2015. Anticipating the Effects of Green Buildings on Water Quality and Infrastructure. *JAWWA*, 107(4), 50-61.

CHAPTER 3 presents a cross-section of green buildings that were surveyed and compared to conventional building designs with respect to water retention time (i.e., water age), water chemistry, and levels of opportunistic pathogen genetic markers. From this survey, it appears that elevated water age is inherent to achieving the sustainability goals of many green building plumbing systems, with largely unknown impacts on building water quality. This chapter refines research priorities outlined in Chapter 2.

Rhoads, W. J., Pruden, A., Edwards, M.A. 2016. Survey of green building water systems reveals elevated water age and water quality concerns. Environmental Science: *Water Res. & Technol.*, 2(1), 164-173.

While Chapters 1-3 discuss factors that contribute to opportunistic pathogen growth that are inherent to aging infrastructure, treatment failures, and novel green designs/technologies, **CHAPTER 4** anticipates unintended consequences for applying in-building disinfection at individual buildings. New American Society of Heating, Refrigerating and Air-Conditioning

Engineers (ASHRAE) standard for control of *Legionella* (ASHRAE Standard 188) emphasizes use of in-building disinfection techniques to reduce the exposure of at-risk consumers to opportunistic pathogens. This chapter is a proactive critical review of challenges associated with implementation of Standard 188, where water temperature in building systems emerged as one of the most important individual factors to controlling OPs.

Rhoads, W.J., Pruden, A., Edwards, M. A. 2014. Anticipating challenges with in-building disinfection for control of opportunistic pathogens. *Water Environ. Res.*, 86(6), 540-549.

CHAPTER 5 examines water heater temperature set point in a pilot-scale simulated hot water plumbing system. This chapter provides a new window of understanding into the microbial ecology of potable hot water systems and helps to resolve past discrepancies in the literature regarding the influence of water temperature and stagnation on the growth of *L. pneumophila*. Further, it identifies a potential "sweet spot" for *Legionella* proliferation when temperatures in a hot water reservoir (in this case, the water heater tank and recirculating line) are elevated enough to decrease competition for growth with other microorganisms, but are not hot enough to disinfect *Legionella* in intermittently used distal branches.

Rhoads, W.J., Ji, P., Pruden, A., Edwards, M.A. 2015. Water heater temperature set point and water use patterns influence *Legionella pneumophila* and associated microorganisms at the tap. *Microbiome*, 3(1).

CHAPTER 6 expands upon Chapter 5, using the same experimental apparatus to further investigate how system design impacts the growth of *Legionella pneumophila* at operational extremes. This chapter provides insight to practical limitations to thermal control strategies in distal branches, which can be undermined within distal branches of buildings plumbing systems.

Rhoads, W.J., Pruden, A., Edwards, M.A. 2016. Convective mixing in distal pipes exacerbates *Legionella pneumophila* growth in hot water plumbing. *Pathogens*, 5(1), 29.

CHAPTER 7 augments Chapters 5-6, and examines the interactions between plumbing system materials and disinfection strategies. This chapter gives rise to a mechanistic hypothesis for past discrepancies in the literature regarding the variable effects of copper on *Legionella* growth, and confirms prior reports of trade-offs between *Legionella* and *Mycobacteria* controls when chloramines are applied as secondary disinfectant residual.

This chapter was accepted contingent upon major revisions to Environmental Science & Technology for publication at the time this dissertation was defended. Chapter Authors include: William J. Rhoads, Amy Pruden, and Marc A. Edwards.

CHAPTER 8, which is not a manuscript, describes the major outcomes of this work and highlights directions for future work.

In addition to the five chapters included in this dissertation, the overall body of the PhD research work includes publications not included in this dissertation research as follows:

Published:

Rhoads, W.J, Chambers, B., Pearce, A., Edwards, M.A. Green Building Design: Water Quality Considerations. Water Research Foundation Project 4383 Final Report. Water Research Foundation. Denver, CO, 250 pages.

Rhoads, W.J., Keane, T., Edwards, M.A. 2016. Flint, MI Residential Water Heater Sampling Report. Final Report prepared for the State of Michigan.

Ji, P., Parks, J., Edwards, M. A., & Pruden, A. 2015. Impact of water chemistry, pipe material and stagnation on the building plumbing microbiome. PloS one, 10(10), e0141087.

Lambrinidou, Y., W. Rhoads, S. Roy, E. Heaney, G. Ratajczak, and J. Ratajczak (2014). Ethnography in Engineering Ethics Education: A Pedagogy for Transformational Listening (accepted), 121st American Society for Engineering Education (ASEE) Annual Conference & Exposition, Indianapolis, IN.

Edwards, M.A., A. Pruden, J. Falkingham, III, R. Brazeau, K. Williams, H. Wang, A. Martin and W.J. Rhoads (2013). Relationship Between Biodegradable Organic Matter and Pathogen Concentrations in Premise Plumbing. Denver, CO. 111 pages. Water Research Foundation.

Pruden, A., M.A. Edwards, J. Falkinham, III, M. Arduino, J. Bird, R. Birdnow, E. Bédard, A. Camper, J. Clancy, E. Hilborn, V. Hill, A. Martin, S. Masters, N.R. Pace, M. Prevost, A. Rosenblatt, W.J. Rhoads, J.E. Stout, and Y. Zhang (2013). Research Needs for Opportunistic Pathogens in Premise Plumbing: Methodology, Microbial Ecology, and Epidemiology. Water Research Foundation Project 4379 Final Report. Water Research Foundation. Denver, CO, 188 pages.

Edwards, M.A., Rhoads, W.J., Pruden, A., Pearce, A. Falkinham, J.O. III. 2014. Green Water Systems and Opportunistic Premise Plumbing Pathogens. Plumbing Engineer Online: http://plumbingengineer.com/content/green-water-systems-and-opportunistic-premise-plumbing-pathogens

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CHAPTER 1. DISTRIBUTION SYSTEM OPERATIONAL DEFICIENCIES COINCIDE WITH REPORTED LEGIONNARIES' DISEASE CLUSTERS IN FLINT, MI

William J. Rhoads, Emily Garner, Pan Ji, Ni Zhu, Jeffrey Parks, Otto Schwake, Amy Pruden, Marc A. Edwards

ABSTRACT

We hypothesize that the increase in reported Legionnaires' disease from June 2014-November 2015 in Genesee County, MI (where Flint is located) was directly linked to the switch to corrosive Flint River water from non-corrosive Detroit water in April 2014-October 2015. To address the lack of epidemiological data linking the drinking water supplies to disease incidence, we gather physiochemical and biological water quality data from 2010-2016 to demonstrate why the Flint River water may have been more conducive to *Legionella* growth. First, the treated Flint River water was up to 8.6 times more corrosive than Detroit water, releasing more iron which is a key *Legionella* nutrient, while also directly causing disinfectant to decay more rapidly. The Flint River water source was also 0.8-6.7 °C warmer in the summer than Detroit water, and exceeded the minimum *Legionella* growth temperature of 20 °C 2.4 times more frequently (fraction of days Detroit = 0.17 versus Flint River = 0.40). The corrosive water also led to 1.3-2.2 times more water main breaks in 2014-2015 compared to 2010-2013, where such disruptions have been associated with outbreaks in other locales. Consistent with these changes, *Legionella* spp. and *L. pneumophila* decreased after switching back to Detroit water, both in terms of gene markers and culturablity, when comparing August/October 2015 to November 2016.

INTRODUCTION

When the City of Flint, MI stopped purchasing drinking water from the Detroit Water and Sewer Department (DWSD) in April 2014 and began treating water from the Flint River, the higher chloride in treated Flint River water and absence of corrosion control triggered corrosion of the metallic water infrastructure. This led to a myriad of water quality problems, including leaching of high levels of lead from leaded plumbing, violations of the maximum contaminant level (MCL) for total coliforms (Fonger, 2014a; 2014b; MDEQ, 2014), exceedances of total trihalomethane limits (City of Flint, 2015), and consumer complaints (Smith-Randolph, 2014; Ketchum III, 2015). After public health emergencies were declared (City of Flint, 2015; Williams, 2015; Egan and Spangler, 2016), Flint returned to purchasing treated water from Detroit in October 2015 and began implementing enhanced corrosion control, with steady indication of system recovery since that time (Edwards et al., 2016).

In January 2016, the State of Michigan and Genesee County health departments announced that two unprecedented clusters of Legionnaires' disease had occurred in Genesee County from June 2014-March 2015 (n=45) and May-November 2015 (n=43), with a total of 12 deaths (MDHHS and GCHD, 2015, 2016; MDHHS, 2016; Anderson and Egan, 2016). Of the 88 cases reported during this period, 70% had a known Flint drinking water supply exposure (MDHHS, 2016). However, it has not been possible to establish links of these Legionnaires' disease clusters to the drinking water using standard epidemiological methodology because the necessary culture data

needed to match isolates from patients and tap water were not collected or are not available (Joint Select Committee, 2016). To date, it has only been possible to match one *Legionella pneumophila* isolate collected from a Flint hospital tap water in August 2016 (nearly a year after returning to Detroit water) to three isolates collected from patients, one in November 2016 and two in 2015 (while still using Flint River water) (MDHHS, 2017; Fonger, 2017). Thus, examination of water chemistry data and physicochemical factors known to be conducive to *Legionella* proliferation could provide key lines of evidence linking the water switch to the Legionnaires' disease clusters.

As a genus of bacteria, *Legionella* are commonly found at low levels in potable water systems, even in the absence of outbreak. The most common species associated with disease is *L. pneumophila* serogroup 1, especially those that carry monoclonal antibody group 2 (MAb2 positive), which are associated with 94% of reported outbreaks (Fields et al., 2002; Kozak et al., 2009; Garrison et al., 2016). *Legionella* can proliferate and express increased pathogenicity in building water systems when conditions are suitable (Lau and Ashbolt, 2009). Iron is of particular interest because it an essential nutrient for *Legionella* growth (Pine et al., 1979; Ristroph et al., 1980; Ristroph et al., 1981) and is commonly released from plumbing due to corrosion. Iron also reacts rapidly with free chlorine residual (Frateur et al., 1999; Tuovinen et al., 1984), leaving water systems vulnerable to microbial proliferation. In addition, *Legionella* are sensitive to temperature thresholds, with increasing growth rates as water temperature increases up to 42 °C (Rhoads et al., 2015; Ohno et al., 2008; Kool et al., 1999; Wadowsky et al., 1985; Yee and Wadowsky, 1982). Therefore, certain combinations of conditions in water systems; such as abundant iron, low chlorine residual, and warm temperature, could essentially create a "perfect storm" for *Legionella* proliferation. "Water deficiencies," including water pressure disruptions, flow disturbances, and vibrations experienced during main breaks, are also associated with Legionnaires' disease occurrence (Garrison et al., 2016). For example, all 23 North American outbreaks investigated by the Centers for Disease Control and Prevention from 2010-2014 for which there was sufficient information to evaluate exposure identified at least 1 deficiency, with 48% (n=11) having more than one deficiency.

We recently reported that *Legionella* spp. and *L. pneumophila* gene copy levels were high in large buildings during Flint River water usage relative to buildings that had received only DWSD water and to other wide-scale molecular surveys of tap water in North American hospitals and homes (Donohue et al., 2014; Wang et al., 2012; Schwake et al., 2016; Bedard et al., 2015). We hypothesize that the trigger for high *Legionella* numbers and increase in Legionnaires' disease was the distribution of corrosive treated Flint River water through Flint's municipal water system, which resulted in an elevated number of water main breaks, leaching of excess iron nutrients from iron pipes, instability of free chlorine disinfectant residuals, and elevated water temperatures. Herein, we evaluate the hypothesis that distribution of Flint River water created conditions conducive to *Legionella* growth through an integrated analysis of water quality data from before, during, and after the water switch, including: **1)** monthly water quality reports from 2010-2016; **2)** results from four citizen-led surveys; **3)** distribution system water quality monitoring in 2015 relative to 2016-2017; **4)** a bench-scale chlorine decay experiment; **5)** four molecular field surveys of distribution monitoring stations, individual residences, and large buildings; and **6)** three repeat samplings of one Flint home that had culturable MAb2 positive *L. pneumophila* in June 2016.

EXPERIMENTAL

Monthly Water Quality Reports. Water quality data from the treatment plant and select distribution system monitoring stations were obtained from archived monthly Michigan Department of Environmental Quality (MDEQ) water quality reports from 2010-2016 (**http://www.michigan.gov/flintwater**). Samples were collected from monitoring stations representative of flushed, cold water at the treatment plant or in the water distribution system. Chloride, phosphate, and free chlorine concentrations data were gathered, as well as water demand met by the treatment plant.

Water Chemistry Surveys. We conducted four city-wide, citizen-partnered, inorganic constituent sampling efforts in August 2015, March 2016, July 2016, and November 2016. Cold water samples were collected after at least 6 hours of stagnation (i.e., stagnant, first flush samples; 1 L) and after 3 minutes of high rate flushing (i.e., flushed samples, 125 mL) to collect water representative of the home plumbing and municipal distribution system water, respectively. Of the original 274 homes sampled in August 2015, data from 155 homes passed all QA/QC criteria (i.e., participated in all efforts, properly collected/labelled samples, no water softener) and were included in this study. These samples were subject to a complete inductively coupled plasma mass spectrophotometry (ICP-MS) scan for inorganic constituents, including: iron, chloride, and phosphorus, which can serve as indicators of unlined iron pipe corrosion, corrosivity of the drinking water, and level of corrosion control, respectively.

Overnight Distribution System Monitoring. One Flint home located at an estimated distribution system water age <24 hours from the water treatment plant, referred to as the "ground zero" home of the crisis (Pieper et al., 2017), was subject to an overnight monitoring study. The kitchen tap was continuously flushed from 6:00PM to 6:00AM in August 2015 and one year later in August 2016. Grab samples were collected at regular intervals for measurement of chlorine, pH, and dissolved oxygen (DO). An additional set of chlorine measurements were carried out to assess the effects of automatic water flushers, which were installed by the emergency response team for the purpose of boosting and stabilizing chlorine in the distribution system. These samples were collected in February 2016, prior to installation in April 2016, and in January 2017, after the flushers were removed in November 2016.

Bench-scale Chlorine Decay Test. To assess chlorine stability, a target total chlorine concentration of 1 mg/L and 0.5 mg/L was dosed from a sodium hypochlorite solution to treated Flint River and DWSD water, respectively (to represent typical residuals in each system), in 120 mL glass reactors with and without a new iron nail. Water changes were conducted daily and chlorine and DO were measured as a function of time on day 1 and day 6 of the experiment.

Water Sampling for City-Wide Molecular Surveys. Four molecular surveys were conducted to assess the presence of *Legionella* gene copies in municipal water and track the system over time (Table A1). Non-Flint samples served as control systems that continuously received either DWSD water (Flint township) or well water (Grand Rapids). The surveys include: 1) Sampling of homes and distribution system monitoring stations to assess water quality in August 2015, before the water crisis was widely recognized (Schwake et al., 2016); 2) Sampling two Flint hospitals in high water age zones in October 2015, immediately before the switch back to DWSD water (Schwake et al., 2016), 3) Repeat sampling of the same locations (where feasible) in March 2016 and 4)

August 2016. For all surveys, 1 L stagnant hot and/or cold water samples were collected in sterile polypropylene (PP) bottles pretreated with 24 mg of sodium thiosulfate and 292 mg of ethylenediaminetetraacetic acid (EDTA) (in solution, adjusted to pH 8.5). Thereafter, two 10-50 mL cold water samples were collected in PP tubes, to measure temperature, chlorine, and inorganic constituents. To the extent possible, the same building taps were sampled for each location during repeat surveys.

Remediation study of a Flint Home Colonized with *L. pneumophila*. A June 2016 water quality survey of 30 homes in Flint identified only two homes with culturable *L. pneumophila* positive for serogroup 1 and MAb2 gene markers (Rhoads et al., 2016). Follow-up sampling was conducted for molecular and culture-based analysis at one of these homes in August and November 2016 to track water quality as the emergency response team implemented enhanced flushing, corrosion control, and disinfection and after the homeowner increased her water heater temperature. During each sampling, 1 or 2 L samples were collected in sterile PP bottles with 24 or 48 mg of sodium thiosulfate from the hot and cold kitchen tap, water heater drain valve, stagnant shower head, and a well-flushed (5 minutes) hose bib located outside the home (representative of the distribution system water). This sentinel home provided the opportunity to assess how homes in Flint with elevated *Legionella* may have recovered in the months after the water crisis.

Water Quality Analyses. Total (i.e., dissolved + particulate) iron, phosphate (as phosphorous), and chloride were measured by ICP-MS after acidification with 2% nitric acid (v/v) and >24 hours holding time according to Standard Method 3125B (APHA, 2005). Free and total chlorine were measured in the field using a portable "Pocket HACH" spectrophotometer with a 0.02 mg/L detection limit (HACH, Loveland, CO). Temperature and pH were measured using an Oakton 110 Series pH meter with automatic temperature correction (Cole-Parmer, Count Vernon Hills, IL). DO was monitored using a ThermoScientific Orion 3-star meter. Biological Activity Reaction Tests (BARTs; Hach, Loveland, CO) were used to assess presence/absence of iron-reducing, acid-producing, slime-forming, and sulfate-reducing bacteria.

Legionella was cultured and *Legionella* spp. (23S rRNA gene) and *L. pneumophila* (*mip* gene) gene copies were quantified using established methods (Appendix 1; 25. Rhoads et al., 2015; Rhoads et al., 2016; 31. Wang et al., 2012; ISO, 2015; Nazarian et al., 2008). Briefly, samples for culture were transported at room temperature while samples for molecular analysis were transported on ice. All were filtered through sterile 0.22 μm pore size mixed-cellulose ester membranes (Millipore, Billerica, MA) within ~30 h of sample collection. Exact volume filtered was recorded. For molecular assays, filters were fragmented and stored at −20 °C until extraction using FastDNA SPIN Kits according to manufacturer protocol (MP Biomedicals, Solon, OH). DNA extracts were diluted ten-fold to minimize inhibition. All samples were analyzed in triplicate 10 μl reactions with triplicate ten-fold serial dilutions of template gene standards ranging from 10^7 to 5×10^1 gene copies/μl and a triplicate negative reaction included on each 96-well qPCR plate. The limit of quantification was 50 gene copies/reaction.

Culture was performed with filter concentrated (as before) samples re-suspended in 5 mL of sterile tap water, heat-treated (50 °C, 30 minutes), and spread-plated onto buffered charcoal yeast extract (BCYE) agar base (Remel, Lenexa, KS) supplemented with 0.4 g/L L-cysteine, 3 g/L ammonium-free glycine, 80,000 units/L polymyxin B sulfate, 0.001 g/L vancomycin, and 0.08 g/L

cycloheximide. Presumptive *Legionella* colonies were confirmed via culture on BCYE plates with and without 0.4 g/L L-cysteine. Colonies from confirmed isolates were suspended into 50 uL of molecular-grade water, DNA extracted via freeze-thaw (-20 °C followed by 90 °C for 10 minutes), and further classified using *L. pneumophila* (*mip*), serogroup 1 (*wzm* gene), and MAb2 (*lag-1* gene) end-point PCR primer sets (Appendix 2; Merault et al., 2011; Wullings et al., 2011; Kozak et al., 2009).

Data Analysis. All graphics were produced and data analyzed using R (version 3.2.0) in R Studio (R software). Graphics were generated using packages ggplot2, gtable, cowplot, and RColorBrewer. Summary statistics were calculated using the ddply() function in the plyr package and melt() in reshape2. Correlation analyses were performed using the Kendall Tau Rank option in cor.test(). Two-sided Wilcoxon Rank Sum tests were performed using wilcox.test(). Significance was determined at $p \leq 0.05$.

RESULTS AND DISCUSSION

Corrosive Water Caused Water Quality Deterioration. *Water Treatment –Chloride and Corrosion Control.* There are limited data regarding the corrosivity of Flint drinking water prior to using treated Flint River water, but Flint was purchasing and distributing non-corrosive DWSD drinking water with low chloride concentrations and moderate levels of phosphate-based corrosion inhibitor (on average, 11.4 mg/L Cl^- and 1.07 mg/L PO_4 in 2014; Table A2). However, while using treated Flint River water, chloride spiked to 78 mg/L on average and no corrosion control chemicals were added, resulting in much higher corrosivity and release of lead and iron into the water from April 2014-October 2015. After the switch back to DWSD water, chloride levels reverted to pre-April 2014 concentrations and corrosion control chemicals were re-optimized by the emergency response teams to 3.6 mg/L as PO_4 on average (Fig. 1.1A).

Impact of Water Corrosivity and Released Iron. While the treated Flint River water was much more corrosive than DWSD water, the impacts could have at least partially been mitigated through implementation of phosphate corrosion control. For example, iron nails in a bench-scale corrosion test exposed to treated Flint River water corroded 8.6 times faster than nails exposed to DWSD water, but only 3.5 times faster when 1 mg/L PO_4-P was added (Fig. SI-3 in Pieper et al., 2017). Elevated iron concentrations were also observed in the distribution system due to corrosion of iron mains (Fig. 1.2). By August 2015, median iron concentrations had spiked to 50.8 µg/L at distribution system monitoring stations and to 121 µg/L in well-flushed water from homes, which was likely impacted by galvanized plumbing. For example, galvanized iron contributed an additional 169 µg/L of iron on average to stagnant samples relative to flushed samples (Table S3; t-test, p-value=0.015, $n_{FirstFlush}=n_{Flushed}=156$). Further, 14% (n=22) of stagnant samples in August 2015 exceeded the EPA secondary maximum contaminant level (MCL) of 300 µg/L of iron, relative to 7.7% (n=12) of flushed samples, although median iron was comparable between stagnant and flushed samples (126 vs 121 µg/L). After the switch back to DWSD water, median iron decreased by August 2016 to 10.8 µg/L at monitoring stations and by November 2016 to 10 µg/L in home plumbing (Fig. 1.2; Table A3).

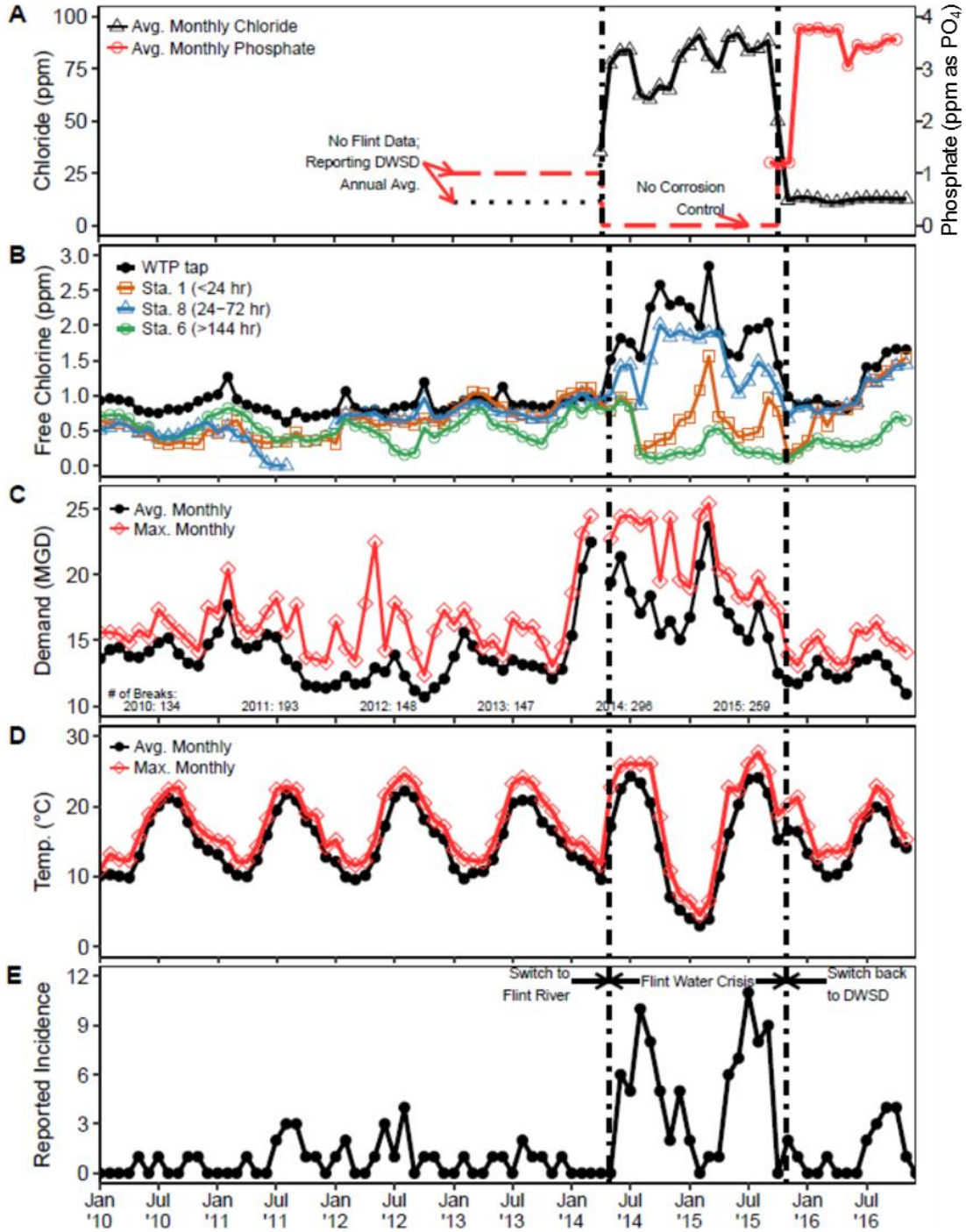

Fig. 1.1. Synthesized data from monthly data.

A) Chloride and phosphate at the treatment plant (reporting Detroit Water and Sanitation District (DWSD) annual average before April 2014; reporting 0 mg/L phosphate April 2014-October 2015 because no inhibitor was dosed during crisis); **B)** Free chlorine residual at the treatment plant and select monitoring stations (approximate water age during crisis reported in legend); **C)** Monthly average and maximum water demand met by the treatment plant (number of water main breaks by year noted); **D)** Monthly average and maximum water temperature at the treatment plant; **E)** Reported incidence of legionellosis (cases per month in Genesee County).

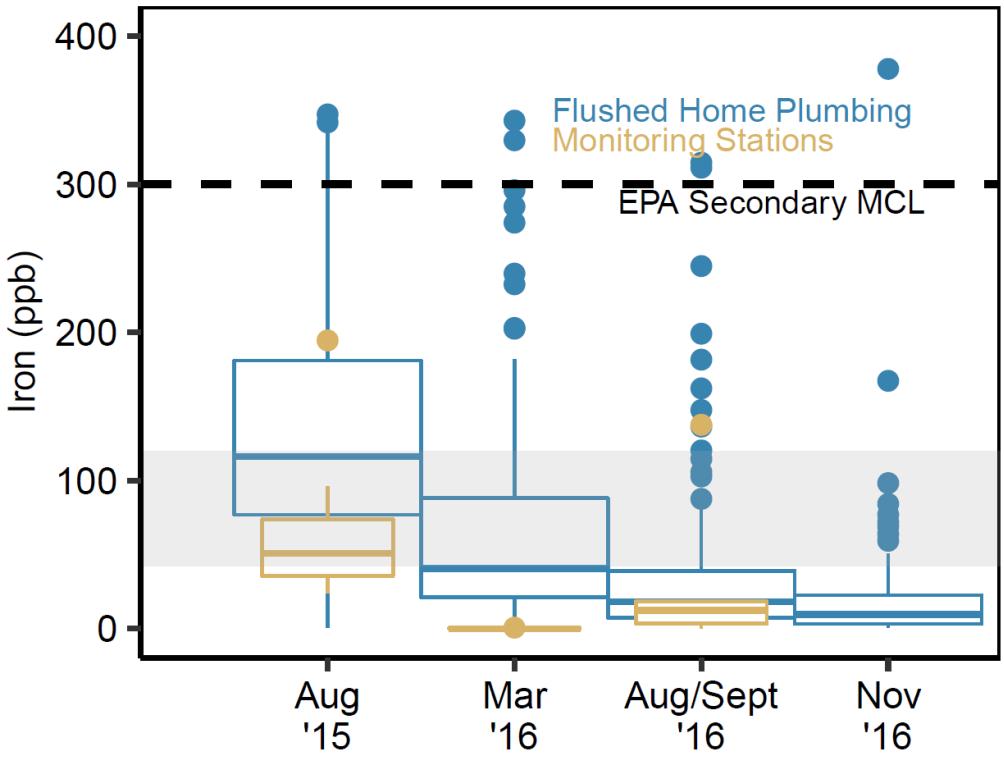

Fig. 1.2. Median and inter-quartile range boxplots of iron levels measured in homes from citizen-led sampling and at distribution system monitoring stations.

Note: April – November 2016 the City of Flint installed automatic water flushers to help boost chlorine and distribute optimized corrosion control in the distribution system (Pressman, 2017); May 2016, City of Flint resident water bills were subsidized to encourage high rate and volume flushing in homes to help deliver the improved corrosion control to home plumbing (DPWU, 2016). Dashed line- EPA secondary MCL for iron; Shaded area- range of iron levels associated with *Legionella* positivity in prior field surveys (Habicht and Muller, 1988; Bargellini et al., 2011; Fields et al., 2002).

Although few samples from the distribution system monitoring exceeded the EPA Secondary MCL of 300 µg/L iron, which is based on aesthetics, excess iron may still have contributed to the growth of *Legionella*. Iron is a key nutrient for *Legionella* growth (Reeves et al., 1981) and increase in virulence (James et al., 1995). Iron has also been noted to be positively associated with the presence of *Legionella* in field studies of domestic (i.e., hot and cold) water (Habicht and Muller, 1988; Bargellini et al., 2011; Fields et al., 2002). While *Legionella* can grow in the presence of >50 mg/L iron in water (States et al., 1987), an upper threshold iron concentration has not been defined. In a few prior field studies, as little as 42-120 µg/L iron has been associated with greater *Legionella* positivity (Bargellini et al., 2011; Rakic et al., 2012), although the relationship is sometimes not statistically significant (Leoni et al., 2005). It is clear that the relationship between iron and *Legionella* is dependent on multiple physicochemical water quality parameters, as well as biological factors such as host-pathogen relationships (Lau and Ashbolt, 2009; Buse and Ashbolt, 2011), life cycles (Garduño et al., 2002), and relationships with other microorganisms responding to physiochemical water quality changes that are not fully understood. Thus, increased iron in Flint drinking could have contributed to enhanced *Legionella* growth, granted that other conditions were not limiting.

Free Chlorine Stability and Concentrations. Iron metal (i.e., zero valent iron) reacts rapidly with free chlorine (Frateur et al., 1999; LeChevellier et al., 1993) and thus contributed to the instability of the chlorine residual during the crisis. In bench-scale tests conducted after 6 days of water changes, 1.1-1.3 mg/L of dosed chlorine residual (as Cl_2) decayed in treated Flint River water in the presence of iron nails in as little as 2.5 hours, while the control with no iron nails remained relatively stable (Fig. 1.3A). Chlorine residual was also less stable in the treated Flint River water than in the DWSD water, even when the initial DWSD water chlorine residual was lower at 0.6-0.7 mg/L (Fig. 1.3A and B).

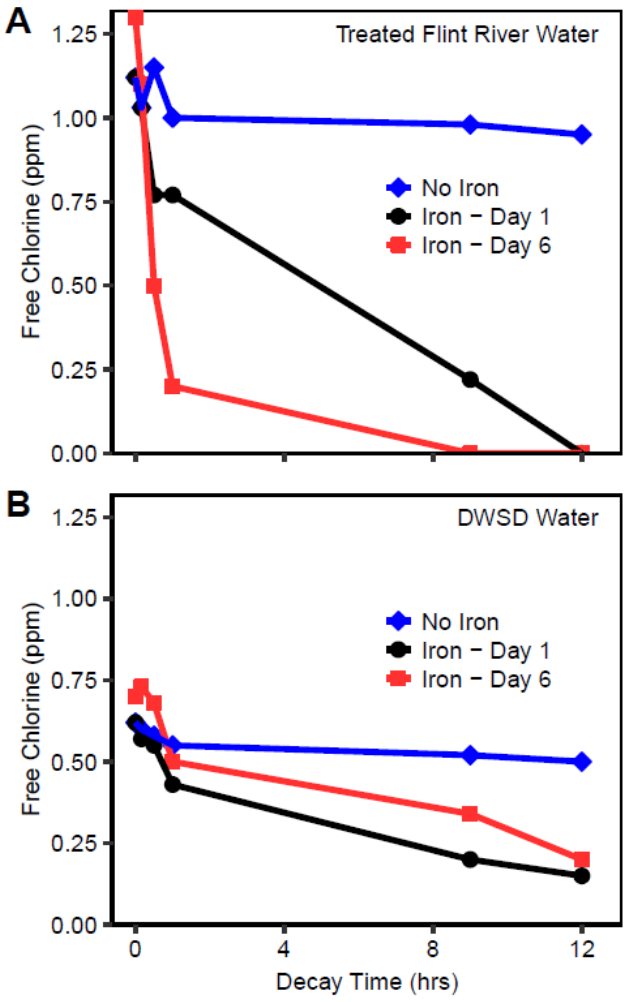

Fig. 1.3. Free chlorine decay in bench-scale reactors (100 mL glass bottles) with and without an iron coupon

A) in treated Flint River Water and **B)** in DWSD water. Tap water was collected directly from well-flushed taps and transported to Virginia Tech for these tests. Tests were performed on day 1 and day 6 after being incubated in reactors with and without an iron nail. Decay tests were conducted in the containers by spiking in the target chlorine residual and monitoring chlorine levels over time in 10 mL aliquots of water. During the tests, the reactors were incubated at room temperature in the dark over 12 hours.

Similar trends were observed in the Flint distribution system (Fig. 1.1B). During the 18 month crisis, the fraction of monitoring station chlorine measurements <0.5 mg/L, a level considered by some to be a threshold limiting incidence of Legionnaires' disease in large buildings (ASHRAE, 2015), increased by a factor of 1.8 relative to the 15 months prior to switching to the treated Flint River water. Even when the residual was increased at the treatment plant from 0.92 mg/L (±0.03 mg/L; n=451) up to 3.3 mg/L during the crisis, elevated free chlorine demand was still observed in seven of the eight monitoring stations during the crisis (Fig. A1 and A2). In addition, significantly higher free chlorine demand remained in two of eight distribution system monitoring stations examined after the switch back to DWSD water (November 2015-October 2016 compared to January 2013-March 2014; Fig. 1C; Wilcoxon Rank Sum, p-value $<2.0\times10^{-6}$ and 4.0×10^{-4}, n=77), suggesting that recovery of the system was affected by lasting damage incurred by the corrosive water.

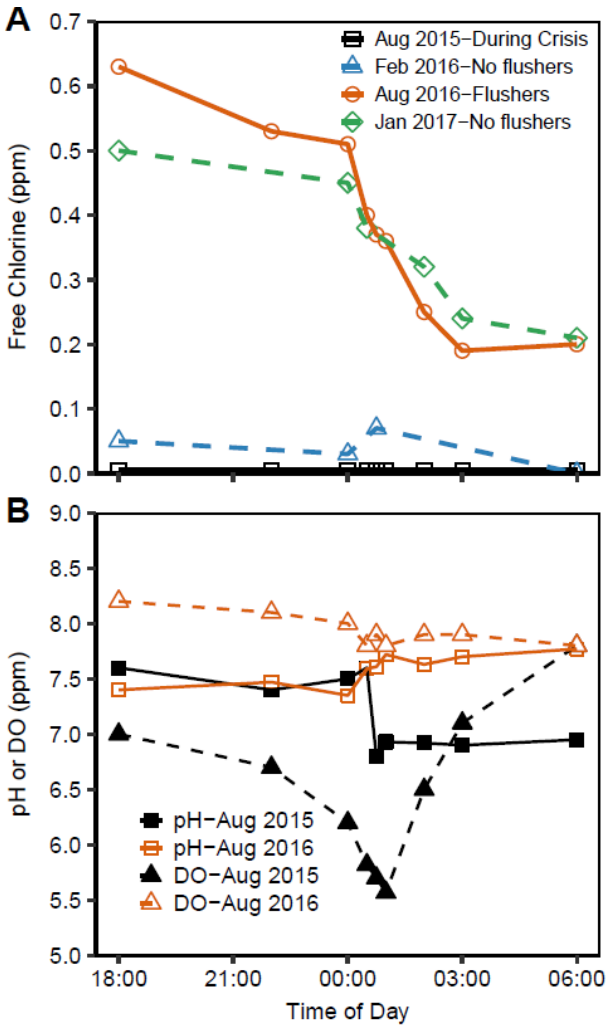

Fig. 1.4. Overnight monitoring of water quality at the "ground zero" house

A) free chlorine residual (solid lines indicate aliquot samples taken from continuously flushing kitchen tap; dashed lines indicate samples collected from stagnant kitchen tap) and **B)** pH and DO at a continuously flushing kitchen sink in one home with an estimated water age of <24 hours (dashed line indicate data from Aug 2015, solid lines indicate data from Aug 2016; no pH and DO was collected in Feb 2016 or Jan 2017).

There were also areas in the distribution system during the crisis that rarely, if ever, received a chlorine residual. At the "ground zero" Flint home in August 2015, where the estimated water age was <24 hours, there was no detectable chlorine residual even after continuous overnight flushing of the kitchen tap (Fig 1.4A). In fact, there was never measureable chlorine in flushed water when this house was monitored daily from August 17-September 3, 2015 (Edwards and Walters, 2015). After the switch back to DWSD, chlorine residual stability gradually increased (Fig. 1.4), especially after automatic water flushers were installed in strategic locations by the emergency response teams, including one about 30 meters from the service line of this home (Pressman, 2017; Fig. 4A). When the same overnight monitoring was conducted in August 2016, with the continuous flushing, 0.63 mg/L Cl_2 was measured in well-flushed water at 6:00PM (Fig. 1.4A). Although chlorine residual and DO decreased during flushing throughout the night, presumably due to increased water age from lower collective consumer demand to the distribution system, detectable residuals were always present (Fig 1.4A). Chlorine remained stable in the distribution system overnight (during decreased consumer demand) in January 2017, two months after the automatic flushers were removed, presumably due to the colder water temperature and gradual passivation of iron pipe in the system by corrosion control. Interestingly, pH also dropped markedly in the distribution system during the night in August 2015 (Fig 1.4B), perhaps due to microbial activity and CO_2 (acid) production, but pH was more stable during summer 2016. In 2015, one of the two distribution system monitoring stations and the "ground zero home" where BARTs were deployed tested positive for iron-reducing, acid-producing, slime-forming, and sulfate-reducing bacteria, all of which consume DO and act to decrease pH, but were negative or lower in relative concentration in 2016 and 2017 when pH and DO were stable overnight (Fig. 1.4B; Table A4).

Main Breaks. Although there were chronic issues with water main breaks and peak monthly demand spiking during winter months before the switch to treated Flint River water, as noted by several high maximum monthly demands from 2010-2013, the corrosivity of the water exacerbated this problem (Fig 1.1B). The number of water main breaks increased by a factor of 1.34-2.2 in 2014-2015 relative to 2010-2013 and maximum monthly water demand remained elevated (>20 MGD) throughout 2014 relative to 2010-2013 (Fig. 1.1C).

"Water deficiencies," such as water pressure disruptions, flow disturbances, and vibrations experienced during a main break, have been associated with Legionnaires' disease outbreaks (Garrison et al., 2016). Main breaks can introduce contaminants into the drinking water supply, disturb existing biofilm/sediment, and release contaminants (e.g., iron rust) that further consume free disinfectant residual and facilitate regrowth of microorganisms (Garrison et al., 2016). In Genesee County, 22 of the 69 case interviews conducted with patients about water quality changes near their home during the legionellosis exposure period were conducted with patients whose residence was served by treated Flint River. Of the Flint resident cases, 31.8% experienced a water main break near their home and 77.3% noticed water quality changes (discoloration, taste, odor, etc.) over the 2 week period prior to diagnosis (typical exposure period) compared to only 2.1% and 12.8%, respectively, of non-Flint resident cases (MDHHS and GCHD, 2015; 2016; MDHHS, 2016).

Water Temperature. While using DWSD water, average and maximum monthly water temperature at the water treatment plan did not exceed 21.5 °C and 24.6 °C, respectively. However, the treated Flint River water had average and maximum summer temperatures ranging from 21.8-24.3 °C and 25.0-27.7 °C, respectively (Fig 2D). The fraction of daily water temperature measurements from 2010-2016 >20 °C increased from 0.17, when previously using DWSD water, to 0.40, after switching to treated Flint River water (Wilcox Rank Sum, p-value 2.14×10^{-6}, n_{DWSD}=1946, n_{FRW}=550). *Legionella* are readily able to survive and multiply above 20 °C (Schulze-Röbbecke et al., 1987), so average distributed water temperatures >20 °C are more likely to support *Legionella* growth. In Genesee County, reported legionellosis incidence significantly correlated with average and maximum water temperature over the period of Jan. 2013-Nov. 2016 (Kendall Correlation, τ = 0.44 and 0.46, p-value = 8.26×10^{-6} and 3.16×10^{-5}, respectively).

***Legionella* Levels Decreased after Water System Remediation.** *City-Wide Survey.* In large Flint buildings, high levels of *Legionella* spp. gene copies were detected immediately before the switch back to DWSD water in October 2015, relative to Flint Township buildings and prior surveys (Table 1.1; Table A5; Donoghue et al., 2012; Wang et al., 2012; Schwake et al 2016). Average *Legionella* spp. gene copies in positive Flint tap water samples subsequently decreased by a factor of 2.6 in March 2016 (Wilcoxon Rank Sum, p-value=1.1×10^{-9}, $n_{Oct\ 2015}$=98, $n_{Mar\ 2016}$=48), after five months of being back on DWSD water. *L. pneumophila* was detected in October 2015 at high levels relative to some survey data reported in the literature (Table 1.1). While the levels of *L. pneumophila* in October 2015 were comparable to reported levels in hospital "System 5" from Bédard et al. (2015; Table 1.1), the point of use temperature in this facility indicated that the system was very conducive to *Legionella* growth. However, levels improved in the March 2016 follow up survey, where *L. pneumophila* was only detectable in one sample (below the quantification limit). Iron levels significantly decreased between the October 2015 and March 2016 large building sampling campaigns (Wilcoxon Rank Sum, p-value=1.1×10^{-12}, $n_{Oct\ 2015}$=46, $n_{Mar\ 2016}$=48), from a median of 33 to less than 1 µg/L. In small buildings, *Legionella* spp. gene copy levels in Flint were generally comparable to other U.S. surveys during and after the crisis, and no *L. pneumophila* was detected in August 2015, March 2016, or August 2016.

Remediation of Flint Home Colonized with L. pneumophila. Legionella spp. gene concentrations were high (>10^4 gene copies/mL) in hot water samples in one home (of 30 homes initially sampled) that had quantifiable *L. pneumophila* gene copies (up to $10^{3.6}$ gene copies/mL) and culturable MAb2-positive *L. pneumophila* in a June 2016 survey of 30 homes (Table 1.2). When the homeowner was informed of her results, she increased her water heater temperature set point from 44.5 °C in July 2016 to 50.7-53.7 °C in August and November 2016. In August 2016, *L. pneumophila* gene levels and detection of MAb2 positive isolates had decreased, and no *L. pneumophila* was detected in November 2016. It is likely that the combination of optimized corrosion control, improved disinfectant residual stability, and enhanced flushing implemented by the emergency response authorities contributed to the improvement. Further, the homeowner's action of elevating the water heater temperature setting also likely posed benefits, while cooler water temperatures (i.e., November vs July) could have also played a role in the decreased gene copy numbers and isolation rate of MAb2 positive *Legionella* in this home.

Table 1.1. Summary of qPCR results for *Legionella* spp. and *L. pneumophila* (log gene copies/mL) from four Flint molecular surveys relative to other U.S. molecular surveys

		Legionella spp. (log 23S rRNA gene copies/mL)			*L. pneumophila* (log *mip* gene copies/mL)		
Source of Samples	n	Range	Mean (± SD)[f]	% positive	Range	Mean (± SD)[f]	% positive
Large Building[a]							
Flint Water (Hosptials)							
Oct 2015[b]	98	BD-5.08	3.3 ± 1.1	79	BD-4.1	1.9 ± 1.5	52
Mar 2016	44	BD-4.9	2.3 ± 0.9	31	BD-BQL	BQL	2
Township (DWSD) Water (School and Hotel)							
Aug 2015[b]	1	BD	0	0	BD	0	0
Mar 2016	13	BD-3.6	2.6 ± 0.6	60	BD	0	0
Aug 2016	2	BD-BQL	BQL	50	BD	0	0
Small Building[c]							
Flint Water (Small Businesses and Homes)							
Aug 2015[b]	31	BD-3.4	2.0 ± 0.8	53	BD	0	0
Mar 2016	17	BD-3.0	2.2 ± 0.8	23	BD	0	0
Aug 2016	40	BD-3.9	1.9 ± 0.7	74	BD	0	0
Township (DWSD) Water (Small Businesses)							
Aug 2015[b]	3	BD	0	0	BD	0	0
Mar 2016	4	BD	0	0	BD	0	0
Aug 2016	2	BD	0	0	BD	0	0
U.S. Cold Water Survey[d]	269	NR	NR	NR	BD-2.6	0.3 ± NR	29
Virginia Survey[e]	90	BD-3.4	1.7± 0.6	30	BD-1.1	1.0 ± 0.3	4.4
Florida Survey[e]	54	BD-2.9	0.9 ± 0.9	83.3	BD-2.3	1.7 ± 0.6	5.6
Hospital (systems 1-3)[f]	30	NR	NR	NR	BD-1.9	BQL ± NR	3
Hospital (system 4)[f]	23	NR	NR	NR	0-3.3[h]	2.4 ± NR	87
Hospital (system 5)[f]	23[i]	NR	NR	NR	2.9-4.5[h]	3.5 ± NR	100

[a] Multistory hospital or hotel, [b] from Schwake et al., 2016, [c] residence or single story business, [d] from Donohue et al., 2014, which only reported *L. pneumophila* serogroup 1, [e] from Wang et al., 2012, which used identical qPCR assays as the present study, [f] from Table S2 in Bedard et al., 2015, [g] mean of positive samples only, [h] minimum and maximum reported values in Table S2 from Bedard et al., 2015, [i] includes 11 first flush samples and 12 repeat samples collected after various stagnation times (1 h - 10 d), BD - below detection, BQL - detected, but below quantification level, NR - not reported, SD - standard deviation

Table 1.2. Summary of *Legionella* qPCR (log gene copies/mL) and culture results from one sentinel Flint home

Sample	*Legionella* spp. (log 23S rRNA gene copies/mL)			*L . pneumophila* (log mip gene copies/mL)			*L. pneumophila* culture (+/- for Lp, LpSG1, & LpSG1MAb2)		
	Jun 2016[a]	Aug 2016[b]	Nov 2016[c]	Jun 2016[a]	Aug 2016[a]	Nov 2016[b]	Jun 2016[a]	Aug 2016[b]	Nov 2016[c]
Stagnant Cold	BD	2.4	3.3	BD	BD	BD	+ + +	- - -	- - -
Flushed Cold	3.9	BQL	2.2	BD	BD	BD	+ + +	- - -	- - -
Stagnant Hot	2.2	NA	1.9	BQL	NA	BD	+ + +	NA	- - -
Flushed Hot	4.0	2.0	1.8	2.9	BD	BD	+ + +	- - -	- - -
Stagnant Shower	4.5	2.9	BQL	1.9	BD	BD	+ - -	+ - -	- - -
Drain Valve	4.7	2.1	BQL	3.6	BD	BD	+ - -	+ - -	- - -

[a] All samples except Flushed Cold collected in 1 L PP bottle with 24 mg $Na_2S_2O_3$, and 250 mL was aliquoted in the field for culture; Flushed Cold sample was collected in 2 L PP bottle with 48 mg $Na_2S_2O_3$ and 250 mL for culture collected immediately afterward, [b] All samples except Flushed Cold collected in 1 L PP bottle with 24 mg $Na_2S_2O_3$, and 500 mL was aliquoted in the field for culture; Flushed Cold sample was collected in 2 L PP bottle with 48 mg $Na_2S_2O_3$ and 250 mL for culture collected immediately afterward, [c] All samples except Flushed Cold collected in 2 L PP bottle with 48 mg $Na_2S_2O_3$, and 1 L for culture aliquoted in the field for culture. BD - Below detection, BQL - Present, but below quantification limit (10-50 gene copies/reaction, applied as appropriate to individual qPCR runs), NA - Not applicable, not sampled.

Implications. This retrospective analysis provides insight into various water quality parameters characterizing the Flint distribution system before, during, and after the crisis as they relate to the potential for *Legionella* to proliferate. It provides additional lines of evidence to support the conclusion that a city-wide switch in the water source and lack of corrosion control was a trigger contributing to the increase in Legionnaires' disease incidence. In particular, this study highlights the potential for corrosive water supplies and unlined iron pipes to trigger growth of *Legionella* through release of iron as a nutrient and consumption of chlorine. These findings are of broad importance as municipalities grapple with aging infrastructure and increasing chloride content (i.e., corrosivity) of surface waters (Strombert, 2014; Corsi et al., 2015). Utilities certainly play a role in preventing Legionnaires' disease through distribution system management and infrastructure upgrades. Even in the absence of federal regulations, increased awareness of this responsibility and improved communication amongst diverse stakeholders (i.e., public health agencies, water utilities, building managers, clinical practitioners, and engineers) will be vital for preventing future disease.

AUTHOR INFORMATION

***Corresponding Author:**
E-mail: edwardsm@vt.edu. Phone: (540) 231-7236. Fax: (540) 231-7916.

Notes:
The authors declare no competing financial interest.

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REFERENCES

American Society of Heating, Refrigeration, and Air-Conditioning Engineers. Standard 188: Legionellosis: Risk Management for Building Water Systems. ISSN 1041-2336. Atlanta, GA.

Anderson, E.; Egan, P. Schuette continues to pursue Legionella deaths. Detroit Free Press. December 20, 2016. http://www.freep.com/story/news/local/michigan/flint-water-crisis/2016/12/20/schuette-continues-pursue-legionella-deaths/95672372/ (Accessed: January 30, 2017).

Bargellini, A., Marchesi, I., Righi, E., Ferrari, A., Cencetti, S., Borella, P., & Rovesti, S. (2011). Parameters predictive of Legionella contamination in hot water systems: association with trace elements and heterotrophic plate counts. *Water research*, *45*(6), 2315-2321.

Bédard, E., Fey, S., Charron, D., Lalancette, C., Cantin, P., Dolcé, P., ... & Prévost, M. (2015). Temperature diagnostic to identify high risk areas and optimize Legionella pneumophila surveillance in hot water distribution systems. *Water research*, *71*, 244-256.

Buse, H. Y., & Ashbolt, N. J. (2011). Differential growth of Legionella pneumophila strains within a range of amoebae at various temperatures associated with in-premise plumbing. *Letters in applied microbiology*, *53*(2), 217-224.

City of Flint. Operation Evaluation Report: Triohalomethane Formation Concern. https://www.cityofflint.com/wp-content/uploads/Flint-TTHM-Operational-Evaluation-Report-August-2015.pdf (Accessed: January 30, 2017).

City of Flint. State of Emergency Declared in the City of Flint. December 14, 2015. https://www.cityofflint.com/state-of-emergency/ (Accessed: January 30, 2017).

Corsi, S. R., De Ciccio, L.A., Lutz, M. A., and R. M Hirsch. River chloride trends in snow-affected urban-watersheds: increasing concentrations outpace urban growth rate and are common among all seasons. (2015), Science of the Total Environment, 508, 488-497.

Department of Human and Health Services. Letter to Genesee County Health Department. January 11, 2017. https://content.govdelivery.com/attachments/MIDHHS/2017/01/11/file_attachments/736554/MDHHSLetterGCHD.pdf (Accessed: January 30, 2017).

Department of Public Works and Utilities (DPWU). Letter to Environmental Protection Agency and Department of Environmental Quality. http://www.michigan.gov/documents/flintwater/Letter_to_EPA_Pollins_and_DEQ_Creagh_from_Flint_McDay_dated_051916_re_Flushing_Recommendations_526815_7.pdf (Accessed: January 30, 2017).

Donohue, M. J., O'Connell, K., Vesper, S. J., Mistry, J. H., King, D., Kostich, M., & Pfaller, S. (2014). Widespread molecular detection of Legionella pneumophila serogroup 1 in cold water taps across the United States. *Environmental science & technology*, *48*(6), 3145-3152.

Edwards, M.A., Tang, M. Rhoads, W.J., Strom, O. Flint Water Press Conference. December 2, 2016. http://flintwaterstudy.org/2016/12/flint-water-study-press-conference-12-2-2016/ (Accessed: January 30, 2017).

Edwards, M.A., Walter, L.A., Chlorine monitoring in resident Ms. Walters' home. http://flintwaterstudy.org/information-for-flint-residents/chlorine-monitoring-in-flint-resident-ms-leeanne-walters-home/ (Accessed: January 30, 2017).

Egan, P.; Spangler, T. President Obama declares emergency in Flint. January 16, 2016. http://www.freep.com/story/news/local/michigan/2016/01/16/president-obama-declares-emergency-flint/78898604/ (Accessed: January 30, 2016).

Fields BS, Benson RF, Besser RE. *Legionella* and Legionnaires' disease: 25 years of investigation. Clin Microbiol Rev 2002;15:506–26.

Fonger, R. Flint expands boil water advisory area after more positive tests for total coliform bacteria. M live. September 7, 2014. http://www.mlive.com/news/flint/index.ssf/2014/09/flint_expands_boil_water.html (Accessed: January 30, 2017).

Fonger, R. Flint issues boil water advisory for section of the city after positive test for total coliform bacteria. M live. September 5, 2014. http://www.mlive.com/news/flint/index.ssf/2014/09/flint_issues_boil_water_adviso.html (Accessed: January 30, 2017)

Frateur, I., Deslouis, C., Kiene, L., Levi, Y., & Tribollet, B. (1999). Free chlorine consumption induced by cast iron corrosion in drinking water distribution systems. Water Research, 33(8), 1781-1790.

Garduño, R. A., Garduño, E., Hiltz, M., & Hoffman, P. S. (2002). Intracellular growth of Legionella pneumophila gives rise to a differentiated form dissimilar to stationary-phase forms. *Infection and immunity*, *70*(11), 6273-6283.

Garrison, L. E., Kunz, J. M., Cooley, L. A., Moore, M. R., Lucas, C., Schrag, S., ... & Whitney, C. G. (2016). Vital Signs: Deficiencies in environmental control identified in outbreaks of Legionnaires' disease—North America, 2000–2014. *American Journal of Transplantation*, *16*(10), 3049-3058.

Habicht, W., & Müller, H. E. (1988). Occurrence and parameters of frequency of Legionella in warm water systems of hospitals and hotels in Lower Saxony. *Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene. Serie B, Umwelthygiene, Krankenhaushygiene, Arbeitshygiene, praventive Medizin*, *186*(1), 79-88.

International Organization for Standardization. Draft International Standard 11731:2015. Water quality-enumeration of Legionella. Geneva, Switzerland.

James, B. W., Mauchline, W. S., Fitzgeorge, R. B., Dennis, P. J., & Keevil, C. W. (1995). Influence of iron-limited continuous culture on physiology and virulence of Legionella pneumophila. *Infection and immunity*, *63*(11), 4224-4230

Joint Committee on the Flint Water Public Health Emergency. Flint Water Crisis: Report of the Joint Select Committee on the Flint Water Emergency. http://www.flintwatercommittee.com/ (Accessed: January 30, 2017).

Ketchum III, W.E. People take to streets to protest Flint water quality. M Live. February 14, 2015. http://www.mlive.com/news/flint/index.ssf/2015/02/flint_residents_protest_citys.html (Accessed: January 30, 2015).

Kool, J. L., Bergmire-Sweat, D., Butler, J. C., Brown, E. W., Peabody, D. J., Massi, D. S., ... & Fields, B. S. (1999). Hospital characteristics associated with colonization of water systems by Legionella and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals. Infection Control & Hospital Epidemiology, 20(12), 798-805.

Kozak NA, Benson RF, Brown E, et al. Distribution of lag-1 alleles and sequence-based types among *Legionella pneumophila* serogroup 1 clinical and environmental isolates in the United States. J Clin Microbiol 2009;47:2525–35

Kozak, N. A., Benson, R. F., Brown, E., Alexander, N. T., Taylor, T. H., Shelton, B. G., & Fields, B. S. (2009). Distribution of lag-1 alleles and sequence-based types among Legionella pneumophila serogroup 1 clinical and environmental isolates in the United States. Journal of clinical microbiology, 47(8), 2525-2535.

Lau, H. Y., & Ashbolt, N. J. (2009). The role of biofilms and protozoa in Legionella pathogenesis: implications for drinking water. *Journal of applied microbiology*, *107*(2), 368-378.

LeChevallier, M. W., Lowry, C. D., Lee, R. G., & Gibbon, D. L. (1993). Examining the relationship between iron corrosion and the disinfection of biofilm bacteria. *Journal (American Water Works Association)*, 111-123.

Leoni, E., De Luca, G., Legnani, P. P., Sacchetti, R., Stampi, S., & Zanetti, F. (2005). Legionella waterline colonization: detection of Legionella species in domestic, hotel and hospital hot water systems. Journal of Applied Microbiology, 98(2), 373-379.

Merault, N., Rusniok, C., Jarraud, S., Gomez-Valero, L., Cazalet, C., Marin, M., ... & Herrmann, J. L. (2011). Specific real-time PCR for simultaneous detection and identification of Legionella pneumophila serogroup 1 in water and clinical samples. Applied and environmental microbiology, 77(5), 1708-1717.

Michigan Department of Environmental Quality (MDEQ). Violation Notice (VN) – Maximum Contaminant Level (MCL) for Total Coliform. https://www.michigan.gov/documents/ag/Boil_Water_Warning_10.7.14_527089_7.pdf (Accessed: January 30, 2017).

Michigan Department of Human and Health Services (MDHHS) and Genesee County Health Department. Legionllosis Outbreak-Genesee County, June, 2014—March, 2015 Full Analysis. https://www.michigan.gov/documents/mdhhs/6-14_to_3-15_Legionellosis_Report_Full_Analysis_Results_511708_7.pdf (Accessed January 30, 2015).

Michigan Department of Human and Health Services (MDHHS) and Genesee County Health Department. Legionellosis Outbreak-Genesee County, May, 2015 – November, 2015 Summary Analysis. https://www.michigan.gov/documents/mdhhs/Updated_5-15_to_11-15_Legionellosis_Analysis_Summary_511707_7.pdf (Accessed January 30, 2017).

Michigan Department of Human and Health Services (MDHHS). MDHHS issues update to 2015 Legionnaires' disease report for Genesee County. http://www.michigan.gov/mdhhs/0,5885,7-339--379334--,00.html (Accessed: January 30, 2017).

Nazarian EJ, Bopp DJ, Saylors A, Limberger RJ, Musser KA. 2008. Design and implementation of a protocol for the detection of Legionella in clinical and environmental samples. Diagn. Microbiol. Infect. Dis. **62**: 125–132.

Ohno, A., Kato, N., Sakamoto, R., Kimura, S., & Yamaguchi, K. (2008). Temperature-dependent parasitic relationship between Legionella pneumophila and a free-living amoeba (Acanthamoeba castellanii). Applied and environmental microbiology, 74(14), 4585-4588.

Personal communication with Jonathan Pressman, PhD, P.E., U.S. Environmental Protection Agency Water Supply & Water Resources Division National Risk Management Research Laboratory to Marc Edwards, PhD, Virginia Tech on 1/24/2017 and 1/30/2017.

Pine, L., George, J. R., Reeves, M. W., & Harrell, W. K. (1979). Development of a chemically defined liquid medium for growth of Legionella pneumophila. Journal of Clinical Microbiology, 9(5), 615-626.

Rakic, A., Peric, J., & Foglar, L. (2012). Influence of temperature, chlorine residual and heavy metals on the presence of Legionella pneumophila in hot water distribution systems. Annals of Agricultural and Environmental Medicine, 19(3).

Reeves, M. W., Pine, L., Hutner, S. H., George, J. R., & Harrell, W. K. (1981). Metal requirements of Legionella pneumophila. *Journal of clinical microbiology*, *13*(4), 688-695.

Rhoads, W. J., Ji, P., Pruden, A., & Edwards, M. A. (2015). Water heater temperature set point and water use patterns influence Legionella pneumophila and associated microorganisms at the tap. *Microbiome*, *3*(1), 67.

Rhoads, W. J., Pruden, A., & Edwards, M. A. (2016). Convective mixing in distal pipes exacerbates Legionella pneumophila growth in hot water plumbing. Pathogens, 5(1), 29.

Ristroph, J. D., Hedlund, K. W., & Allen, R. G. (1980). Liquid medium for growth of Legionella pneumophila. Journal of Clinical Microbiology, 11(1), 19-21.

Ristroph, J. D., Hedlund, K. W., & Gowda, S. (1981). Chemically defined medium for Legionella pneumophila growth. Journal of clinical microbiology, 13(1), 115-119.

Schulze-Röbbecke, R., Rödder, M., & Exner, M. (1987). Multiplication and killing temperatures of naturally occurring legionellas. *Zentralblatt fur Bakteriologie, Mikrobiologie und*

Hygiene. Serie B, Umwelthygiene, Krankenhaushygiene, Arbeitshygiene, praventive Medizin, *184*(6), 495-500.

Schwake, D. O., Garner, E., Strom, O. R., Pruden, A., & Edwards, M. A. (2016). Legionella DNA Markers in Tap Water Coincident with a Spike in Legionnaires' Disease in Flint, MI. *Environmental Science & Technology Letters*, *3*(9), 311-315.

Smith-Randolph, W. Flint officials flushing water system amid complaints of brown water. Chanel 25 News. September 25, 2014. http://nbc25news.com/news/local/flint-officials-flushing-water-system-amid-complaints-of-brown-water (Accessed: January 30, 2017)

States, S. J., Conley, L. F., Kuchta, J. M., Oleck, B. M., Lipovich, M. J., Wolford, R. S., Keleti, G. (1987). Survival and multiplication of Legionella pneumophila in municipal drinking water systems. Applied and Environmental Microbiology, 53(5), 979-986.

Strombert, J. What Happens to All the Salt We Dump on the Roads. Smithsonian.com. (2014). Accessed 07-26-2015 at http://www.smithsonianmag.com/ist/?next=/science-nature/what-happens-toall-the-salt-we-dump-on-the-roads-180948079/

Tuovinen, O. H., Mair, D. M., & Banovic, J. (1984). Chlorine demand and trihalomethane formation by tubercles from cast iron water mains. Environmental Technology, 5(1-11), 97-108.

Wadowsky, R. M., Wolford, R., McNamara, A. M., & Yee, R. B. (1985). Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring Legionella pneumophila in potable water. Applied and environmental microbiology, 49(5), 1197-1205.

Wang, H., Edwards, M., Falkinham, J. O., & Pruden, A. (2012). Molecular survey of the occurrence of Legionella spp., Mycobacterium spp., Pseudomonas aeruginosa, and amoeba hosts in two chloraminated drinking water distribution systems. *Applied and environmental microbiology*, *78*(17), 6285-6294.

Williams, C. Public health emergency declared over lead in Flint water after children show elevated levels. U.S. News. October 2, 2015. http://www.usnews.com/news/us/articles/2015/10/01/public-health-emergency-declared-due-to-lead-in-flint-water (Accessed: January 30, 2015)

Wullings, B. A., Bakker, G., & van der Kooij, D. (2011). Concentration and diversity of uncultured Legionella spp. in two unchlorinated drinking water supplies with different concentrations of natural organic matter. Applied and environmental microbiology, 77(2), 634-641.

Yee, R. B., & Wadowsky, R. M. (1982). Multiplication of Legionella pneumophila in unsterilized tap water. Applied and Environmental Microbiology, 43(6), 1330-1334.

APPENDIX A - SUPPLEMENTAL INFORMATION FOR CHAPTER 1.

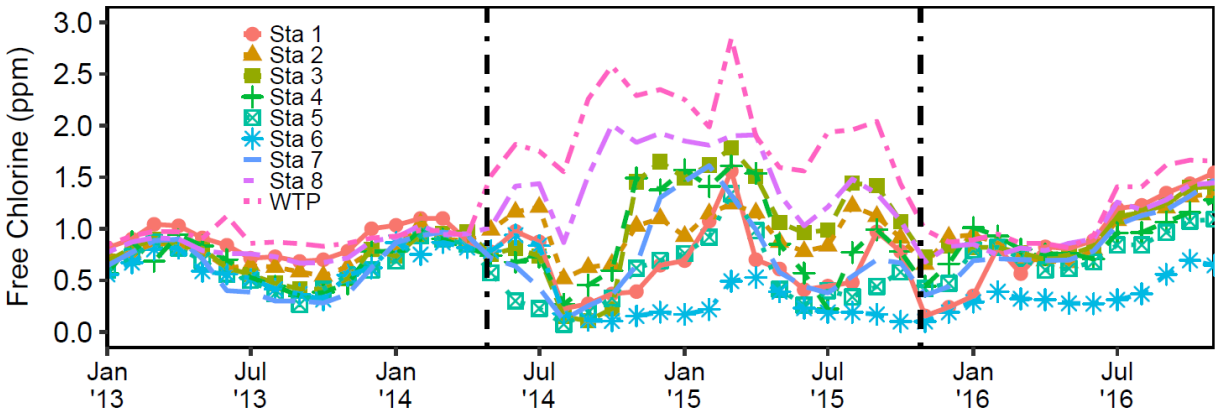

Fig. A1. Free chlorine data from all monitoring stations + WTP tap 2013-2016. WTP- Water treatment plant tap; Sta- Distributions system monitoring station number; Dash-dotted vertical lines indicate the switch to Flint River water (April 2014) and the switch back DWSD water (October 2015). Note: April – November 2016 the City of Flint operated automatic water flushers to help boost chlorine and distribute optimized corrosion control in the distribution system (52. Pressman, 2017); May 2016, City of Flint resident water bills were subsidized to encourage high rate and volume flushing in homes to help deliver the improved corrosion control to home plumbing (53. DPWU, 2016).

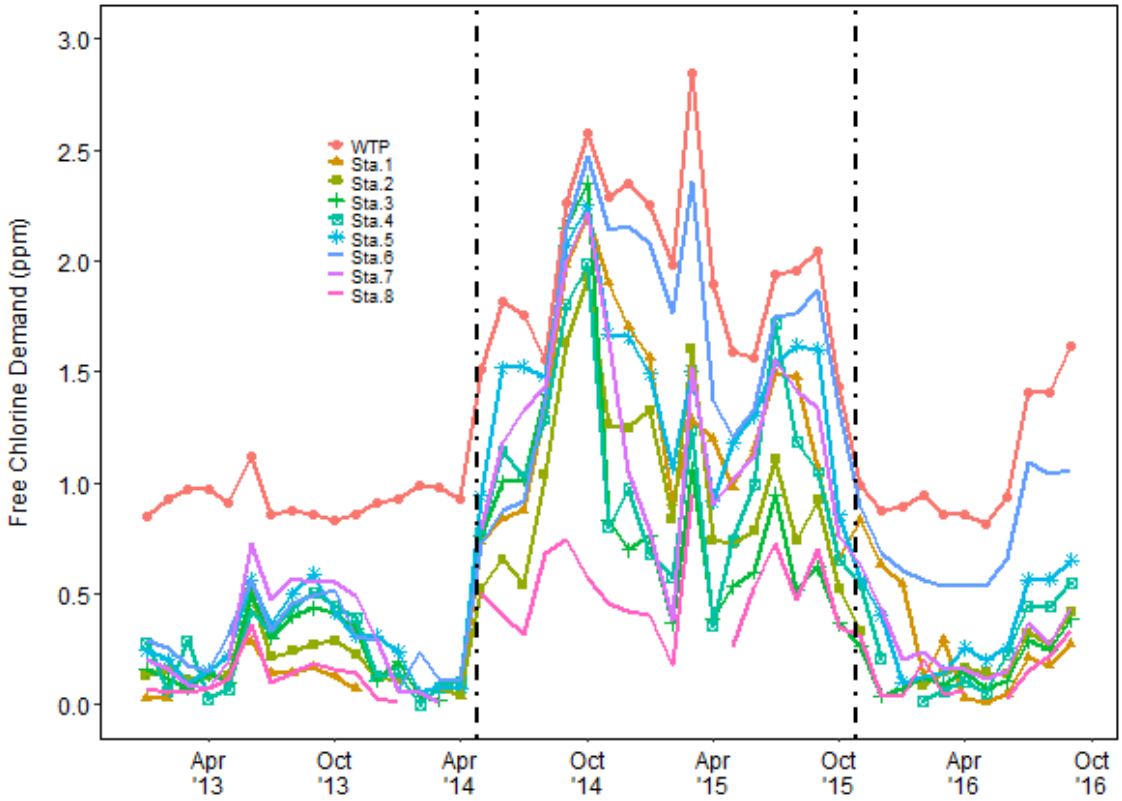

Fig. A2. Free chlorine demand (calculated by subtracting chlorine concentration at the monitoring stations from chlorine concentration at the water treatment plant) 2013-2016. Sta- Station number; Dash-dotted vertical lines indicate the switch to Flint River water (April 2014) and the switch back DWSD water (October 2015). Note: April – November 2016 the City of Flint operated automatic water flushers to help boost chlorine and distribute optimized corrosion control in the distribution system (52. Pressman, 2017); May 2016, City of Flint resident water bills were subsidized to encourage high rate and volume flushing in homes to help deliver the improved corrosion control to home plumbing (53. DPWU, 2016).

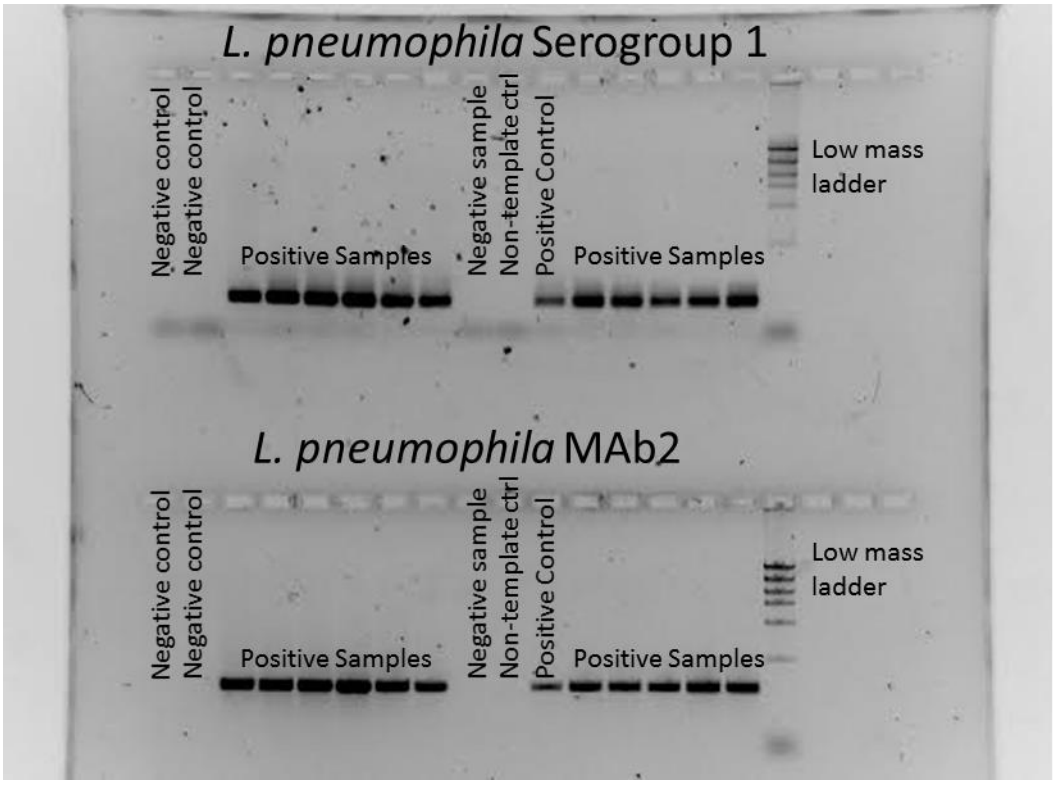

Fig. A3. Example gel-image from end-point PCR assays for serogroup 1 and monoclonal antibody group 2 (MAb2).

Negative controls were two unidentified bacteria that showed growth on the L-cysteine negative culture confirmation plate, indicating it is not *Legionella*. Non-template control was microbiological grade water. The positive control was *L. pneumophila* 130b (ATCC BAA-74) altered to express a green fluorescent protein (M. Swanson, University of Michigan).

Table A1. Summary of four molecular surveys conducted in Flint

Survey		n	Description of Sample Types Collected	Description of Samples Collected
1)	15-Aug	35	Distribution system monitoring stations, private residences, and small one-story businesses in Flint and Flint Township	Stagnant cold water Hot water only from three residences
2)	15-Oct	98	Two Flint hospitals	Stagnant cold water Flushed hot water
3)	16-Mar	78	Distribution system monitoring stations, private residences, small one-story buildings, and health-care facilities in Flint and Flint Township	Stagnant cold water[a] Flushed hot water[a] One water heater sample
4)	16-Aug	42	Distribution system monitoring stations, private residences, and small one-story businesses in Flint and Flint Township	Stagnant cold water[a] Flushed hot water[a] Two water heater samples, one fire hydrant sample

[a] except when sampling the home as part of the section "**Remediation study of a Flint Home Colonized with *L. pneumophila*"** where cold and hot stagnant and flushed samples were collected along with water heater and shower head samples.

Table A2. Water quality parameters for drinking water supplied in Flint, MI before and after the April 2014 switch (source: http://flintwaterstudy.org/2015/09/our-virginia-tech-research-team-wins-a-50000-grant-from-the-national-science-foundation-to-study-flint-water/)

Parameter	Before Switch[a]	After Switch[b]
pH	7.4	7.61
Hardness (mg/L as $CaCO_3$)	101	183
Alkalinity (mg/L as $CaCO_3$)	78	77
Chloride (mg/L)	**11.4**	**92**
Sulfate (mg/L)	25.2	41
CSMR[c]	0.45	**1.6**
Inhibitor (mg/L as P)	0.35	**NONE**
Larson Ratio[d]	0.5	**2.3**

[a] Source: DWSD 2014 Water Quality Report, Available: www.dwsd.org, [b] Source: City of Flint Monthly Operation Report, June 2015, [c] Chloride to Sulfate Mass Ratio - a measure of corrosivity to lead; CSMR > 0.5 is a critical trigger (Edwards et al., 1999), [d] A measure of corrosivity to mild steel and iron; indicates that corrosion rates increase linearly with Larson Ration (Larson and Skold, 1958).

Edwards, M.A.; Jacobs, S; Dodrill, D. Desktop guidance for mitigating Pb and Cu corrosion by-products. *Journal American Water Works Association*, **1999**, 91(5), 66.

Larson, T. E.; Skold, R. V. Laboratory studies relating mineral quality of water to corrosion of steel and cast iron. *Corrosion*, **1958,** 14(6), 43-46.

Table A3. Iron concentrations (µg/L) in first flush and 3 min flushed samples from the four citizen-led, city-wide sampling campaigns for inorganic constituents.

156 homes met all QAQC criteria. Note: April – November 2016 the City of Flint installed automatic water flushers to help boost chlorine and distribute optimized corrosion control in the distribution system (52. Pressman, 2017); May 2016, City of Flint resident water bills were subsidized to encourage high rate and volume flushing in homes to help deliver the improved corrosion control to home plumbing (53. DPWU, 2016).

	First Draw Samples (>6 hr stagnation)				Flushed Samples (~3 min at kitchen tap)			
Parameter	Aug-15	Mar-16	Jul-16	Nov-16	Aug-15	Mar-16	Jul-16	Nov-16
N	156	156	156	156	156	156	156	156
N > 300 µg/L	22	22	14	7	12	6	6	2
Median	126	54	24	7	121	42	19	10
Average	324	313	155	83	155	86	84	48
StdDev	902	1275	579	328	175	157	443	369

Table A4. Biological Activity Reaction Test (BART) results.

Numbers indicate approximate cfu/mL based on the number of days passed until reaction occurred. BARTs are semi-quantitative: cfu/mL is estimated based on how quickly the reaction occurs (measured in days). "-" indicates the test was negative for the presence of the test bacteria. Sta 1 and 6 are two City of Flint Distribution system monitoring stations. The "Ground Zero" home from Pieper et al.(2017) had an estimate water age of <24 hours.

Location	Date	Iron Related Bacteria	Heterotrophic Aerobic Bacteria	Acid Producing Bacteria	Sulfate Reducing Bacteria	Slime Producing Bacteria
Sta 1	Aug 2015	140,000	575,000	-	18,000	105,000
	Aug 2016	-	6,500	-	-	-
	Jan 2017	-	-	-	-	-
Sta 6	Aug 2015	-	-	1,500	-	-
	Aug 2016	-	-	-	-	-
	Jan 2017	-	-	-	-	-
Ground Zero Home	Aug 2015	140,000	575,000	-	100,000	105,000
	Aug 2016	-	700	-	-	-
	Jan 2017	35,000	6,500	-	500	50,000

Table A5. Molecular survey meta-data from March and August 2016 (August and October 2015 data are included in Schwake et al., 2016).

Year	Month	Flint/ Non-Flint	Building Class	Sample	Volume Filtered (mL)	Temp (°C)	pH	Cl2 (ppm)	P (ppb)	Cl (ppb)	Fe (ppb)	Cu (ppb)	Zn (ppb)	Pb (ppb)	Leg. spp. (gc/mL)	L. pneum. (gc/mL)
2016	March	Flint	LRG BLD	COLD	560	6.2	7.32		1,272.0	11.4	1.0	5.6	52.4	0.0	0	0
2016	March	Flint	LRG BLD	COLD	610	12.3	7.13		1,182.0	10.5	0.9	203.9	32.0	0.0	73051	BQL
2016	March	Flint	LRG BLD	COLD	580	14.2	7.61		1,279.0	11.7	-1.3	25.6	20.8	0.0	0	0
2016	March	Flint	LRG BLD	COLD	578	7.4	7.3		1,209.0	11.4	23.9	5.1	108.0	0.0	0	0
2016	March	Flint	LRG BLD	COLD	602	6.5	7.25		1,284.0	11.6	2.3	5.1	20.4	0.0	0	0
2016	March	Flint	LRG BLD	COLD	606	6	7.3		1,130.0	11.0	0.1	3.5	72.1	0.2	0	0
2016	March	Flint	LRG BLD	COLD	588	45.1	7.25		1,221.0	10.7	0.0	170.5	45.2	0.0	2528	0
2016	March	Flint	LRG BLD	COLD	562	8	7.6		1,258.0	11.9	1.3	49.4	30.4	0.1	0	0
2016	March	Flint	LRG BLD	COLD	586	6.6	7.29		1,281.0	11.8	-0.2	11.6	56.1	0.0	315	0
2016	March	Flint	LRG BLD	COLD	534	7.3	7.33		1,241.0	11.6	0.7	16.5	39.4	0.1	0	0
2016	March	Flint	LRG BLD	COLD	570	14	7.3		1,251.0	11.7	0.4	47.5	35.8	0.1	0	0
2016	March	Flint	LRG BLD	COLD	616	23	7.15		1,220.0	11.1	-1.8	234.1	59.4	0.0	65	0
2016	March	Flint	LRG BLD	COLD	568	7	7.25		1,292.0	11.5	-1.3	5.5	103.7	0.0	0	0
2016	March	Flint	LRG BLD	COLD	578	8.9	7.4		1,301.0	10.1	-0.4	8.8	125.3	0.1	1945	0
2016	March	Flint	LRG BLD	COLD	540	9	7.2		1,170.0	10.2	4.4	25.1	81.9	0.0	0	0
2016	March	Flint	LRG BLD	COLD	590	11.3	7.25		1,190.0	10.4	2.3	4.1	101.6	0.1	0	0
2016	March	Flint	LRG BLD	COLD	576	8.6	7.3		1,139.0	10.7	26.5	103.5	40.6	0.4	0	0
2016	March	Flint	LRG BLD	COLD	546	12.1	7.47		1,130.0	10.1	34.6	15.9	37.4	0.6	250	0
2016	March	Flint	LRG BLD	COLD	554	18.8	7.4		1,186.0	10.0	3.6	37.4	57.0	0.5	BQL	0
2016	March	Flint	LRG BLD	COLD	560	12.6	7.16		1,083.0	10.5	3.7	106.3	72.7	1.2	0	0
2016	March	Flint	LRG BLD	COLD	574	12.3	7.25		1,178.0	10.2	37.2	56.1	9.2	0.3	0	0
2016	March	Flint	LRG BLD	COLD	530	11.8	7.25		1,174.0	10.3	21.8	17.3	30.9	0.1	0	0
2016	March	Flint	LRG BLD	HOT	566	41.5	7.09		1,220.0	12.8	-0.4	168.5	56.4	0.4	BQL	0
2016	March	Flint	LRG BLD	HOT	608	40.5	7.09		1,217.0	10.8	-3.2	156.2	19.3	0.2	118	0
2016	March	Flint	LRG BLD	HOT	576	46.5	7.22		1,078.0	10.8	-1.8	153.2	27.9	0.0	0	0
2016	March	Flint	LRG BLD	HOT	614	37.4	7.11		1,097.0	11.4	-1.0	165.7	5,631.0	0.9	BQL	0
2016	March	Flint	LRG BLD	HOT	632	46.7	7.4		1,200.0	10.7	-1.0	132.9	23.2	0.0	0	0
2016	March	Flint	LRG BLD	HOT	512	39	7.08		1,220.0	10.7	-1.9	165.3	18.7	0.0	0	0
2016	March	Flint	LRG BLD	HOT	512	13	7.35		1,249.0	11.8	4.7	3.6	65.0	0.0	78	0
2016	March	Flint	LRG BLD	HOT	562	47.8	7.3		1,191.0	10.6	1.1	131.8	27.4	0.0	0	0
2016	March	Flint	LRG BLD	HOT	502	45	7.19		1,212.0	11.0	-1.7	140.1	26.6	0.0	0	0
2016	March	Flint	LRG BLD	HOT	620	42.4	7.19		1,219.0	10.9	-1.4	158.4	49.8	0.1	0	0
2016	March	Flint	LRG BLD	HOT	610	40	7.07		1,082.0	11.1	-1.9	160.9	82.9	0.0	0	0
2016	March	Flint	LRG BLD	HOT	628	40.5	7.19		1,265.0	10.7	54.5	256.5	134.6	0.5	148	0
2016	March	Flint	LRG BLD	HOT	546	23.4	7.25		1,194.0	11.5	-0.9	100.7	80.5	0.0	0	0
2016	March	Flint	LRG BLD	HOT	576	46.6	7.3		1,079.0	10.5	0.2	121.4	38.7	0.2	0	0
2016	March	Flint	LRG BLD	HOT	586	33.6	7.2		1,057.0	10.4	1.7	93.8	30.6	0.0	0	0
2016	March	Flint	LRG BLD	HOT	598	20.1	7.17		1,062.0	10.5	3.0	150.1	22.1	0.0	0	0
2016	March	Flint	LRG BLD	HOT	566	23	7.36		1,073.0	10.0	0.5	17.0	21.4	0.0	0	0
2016	March	Flint	LRG BLD	HOT	550	34.8	7.18		1,067.0	10.5	-1.5	161.9	22.7	0.1	0	0
2016	March	Flint	LRG BLD	HOT	614	34.8	7.24		1,134.0	11.1	1.2	185.0	32.2	0.2	BQL	0
2016	March	Flint	LRG BLD	HOT	528	44	7.07		990.5	10.9	1.0	169.3	28.0	0.1	163	0
2016	March	Flint	LRG BLD	HOT	574	54.5	7.19		1,037.0	11.1	-2.7	174.8	35.6	0.1	BQL	0
2016	March	Flint	LRG BLD	HOT	582	51.3	7.2		990.3	11.0	55.7	232.3	40.2	0.8	531	0
2016	March	Flint	RES	COLD	640	9	7.08		1,180.0	10.1	-0.4	11.6	12.7	0.0	1044	0
2016	March	Flint	RES	COLD	618	16.1	7.3		1,149.0	11.3	1.3	28.8	46.7	0.5	0	0
2016	March	Flint	RES	COLD	598	7.4	7.37		670.3	9.9	-0.5	14.4	158.8	0.1	134	0
2016	March	Flint	RES	COLD	572	7.5	7.45		1,145.0	9.8	9.2	18.3	19.0	0.6	0	0
2016	March	Flint	RES	HOT	644	50	7.26		1,074.0	11.0	-3.4	7.6	7.4	0.1	0	0
2016	March	Flint	RES	HOT	576	64	7.36		1,080.0	10.2	-1.4	14.8	7.2	0.0	0	0
2016	March	Flint	RES	HOT	626	56	7.25		1,012.0	10.4	-2.2	47.2	2,512.0	0.2	0	0
2016	March	Flint	RES	HOT	404	69.8	7.76		656.9	9.7	-1.1	8.7	18.4	0.0	0	0
2016	March	Flint	SML BLD	COLD	544	7.1	7.8		1,190.0	9.9	-2.2	77.3	9.9	0.1	0	0
2016	March	Flint	SML BLD	COLD	544	18	7.1		1,171.0	9.9	0.6	45.7	38.9	0.2	816	0
2016	March	Flint	SML BLD	COLD	594	19.5	7.3		1,099.0	9.7	-0.4	64.4	31.8	0.0	0	0
2016	March	Flint	SML BLD	COLD	656	9	7.15		1,238.0	9.9	-0.4	136.0	11.8	0.0	0	0
2016	March	Flint	SML BLD	COLD	562	8.9	7.25		1,213.0	10.6	0.3	39.2	21.0	0.3	BQL	0
2016	March	Flint	SML BLD	HOT	600	49	7.1		1,078.0	10.1	-3.0	40.5	17.9	0.0	0	0
2016	March	Flint	SML BLD	HOT	554	40	7.15		1,207.0	10.0	-2.9	59.8	8.4	0.0	0	0
2016	March	Flint	SML BLD	HOT	556	42.1	7.23		1,157.0	10.3	-2.7	133.9	11.9	0.0	0	0
2016	March	Flint	SML BLD	HOT	582	43	7.13		1,195.0	10.6	1,110.0	1,064.0	94.7	17.3	BQL	0

Table A5 cont'd

Year	Month	Flint/ Non-Flint	Building Class	Sample	Volume Filtered (mL)	Temp (°C)	pH	Cl2 (ppm)	P (ppb)	Cl (ppb)	Fe (ppb)	Cu (ppb)	Zn (ppb)	Pb (ppb)	Leg. spp. (gc/mL)	L. pneum. (gc/mL)
2016	March	Non-Flint	LRG BLD	COLD	1102										357	0
2016	March	Non-Flint	LRG BLD	COLD	908										98	0
2016	March	Non-Flint	LRG BLD	COLD	468	47	7.04		17.9	32.3	0.7	50.7	13.8	0.3	0	0
2016	March	Non-Flint	LRG BLD	COLD	1122										4151	0
2016	March	Non-Flint	LRG BLD	COLD	1130										123	0
2016	March	Non-Flint	LRG BLD	COLD	1036										84	0
2016	March	Non-Flint	LRG BLD	COLD	474	17	7.84		9.6	34.1	0.0	23.0	5.3	0.0	0	0
2016	March	Non-Flint	LRG BLD	COLD	584	18.1	7.84		9.0	35.1	-0.3	44.3	17.3	0.6	1632	0
2016	March	Non-Flint	LRG BLD	COLD	526	15.8	7.8		4.5	34.7	-0.9	14.7	4.9	0.0	646	0
2016	March	Non-Flint	LRG BLD	COLD	608	7.7	7.18		365.9	10.2	-3.1	4.6	2.1	0.0	0	0
2016	March	Non-Flint	LRG BLD	HOT	602	45.7	7.5		2.3	35.1	-2.5	13.2	5.4	0.2	572	0
2016	March	Non-Flint	LRG BLD	HOT	512		7.72		5.1	35.6	-1.8	62.6	9.7	0.0	0	0
2016	March	Non-Flint	LRG BLD	HOT	476	29	7.75		14.3	36.0	-2.8	46.7	12.4	0.5	506	0
2016	March	Non-Flint	RES	COLD	472	52	7.26		286.2	10.3	-3.3	4.9	3.5	0.1	0	0
2016	March	Non-Flint	RES	COLD	502	17	7.45		379.9	9.9	-0.5	3.5	4.2	0.0	0	0
2016	March	Non-Flint	SML BLD	COLD	498	19	7		363.6	10.1	-1.9	10.7	4.3	0.0	0	0
2016	March	Non-Flint	SML BLD	HOT	548	43	7.3		373.2	9.9	-2.1	15.4	5.3	0.1	0	0
2016	Aug	Flint	RES	COLD	302	24.9	6.75	0.5	1,064.0	11.7	31.7	6.2	59.4	1.2	0	0
2016	Aug	Flint	RES	HOT	510	50.3	6.8	0.08	863.1	10.92	4.759	7.317	51.6	0.592	0	0
2016	Aug	Flint	RES	COLD	472	24	7.01	0.02	982.7	11.12	14.92	37.4	-1.671	0.04	0	0
2016	Aug	Flint	RES	HOT	484	52	7.08	0.13	1016	11.86	5.15	26.22	3.715	-0.002	116	0
2016	Aug	Flint	RES	COLD	562	29.1	7.05	0.1	888.9	10.81	17.24	9.34	38.05	0.401	81	0
2016	Aug	Flint	RES	HOT	448	36.1	7.1	0	761.3	10.87	51.83	7.491	51.34	0.294	75	0
2016	Aug	Flint	RES	COLD	460	21.5	7.08	0.84	957.8	10.72	7.769	27.73	16.1	2.055	0	0
2016	Aug	Flint	RES	HOT	540	35.5	7.26	0.02	840.3	10.78	14.05	23.34	13.44	1.127	25	0
2016	Aug	Flint	RES	COLD	510		7.43	0.03	963	11.6	15.51	2.516	-3.328	0.036	25	0
2016	Aug	Flint	RES	COLD	468	21.4	7.31	0.08	882.1	11.4	94	45.15	24.67	3.302	204	0
2016	Aug	Flint	RES	HOT	490	28	7.49	0.04	807.4	11.46	32.57	94.38	45.86	0.733	524	0
2016	Aug	Flint	RES	HOT	488	53.7	7.78	0.06	823.7	11.26	30.58	36.03	72.88	0.242	81	0
2016	Aug	Flint	RES	HOT	466	40.7	7.54	0.08	861.5	11.11	-3.172	60.93	17.35	0.059	630	0
2016	Aug	Flint	RES	WH	444	49.4	7.73	0.04	904	11.43	23.84	22.67	25.07	0.136	89	0
2016	Aug	Flint	RES	COLD	560	21.2	6.55	0.15	916.2	11.44	17.59	9.355	131.7	1.007	0	0
2016	Aug	Flint	RES	HOT	518	50.7	7	0.1	871	11.47	23.5	33.27	35.72	1.55	113	0
2016	Aug	Flint	RES	COLD	522	33.3	7.41	0.8	948.1	10.77	16.57	29.78	16.09	2.979	25	0
2016	Aug	Flint	RES	HOT	540	23.8	7.41	0.03	819.9	10.9	47.3	49.83	31.92	19.56	0	0
2016	Aug	Flint	RES	COLD	532	24.9	6.95	0.1	926.9	11.67	8.244	40.1	5.817	0.002	342	0
2016	Aug	Flint	RES	HOT	582	54	7.16	0	500.5	10.83	28.61	14.28	7.83	-0.011	46	0
2016	Aug	Flint	RES	WH		40.01	6.9	0							25	0
2016	Aug	Flint	RES	COLD	510	23.5	7.12	0.35	894.4	10.64	159.2	53.78	11.64	13.29	17	0
2016	Aug	Flint	RES	HOT	474	37.5	7.38	0	693.7	10.93	80.03	41.2	14.75	1.275	577	0
2016	Aug	Flint	RES	COLD	566	20.7	6.71	0.38	692.8	11.17	-2.906	31.51	-0.879	0.052	0	0
2016	Aug	Flint	RES	HOT	532	68.4	7.17	0.03	297.8	11.52	-2.625	47.36	4.933	0.055	25	0
2016	Aug	Flint	SML BLD	COLD	538	24.5	6.72	0.31	1058	11.68	137.6	29.74	11.84	0.738	25	0
2016	Aug	Flint	SML BLD	HOT	574	48.5	6.87	0.03	897.1	11.51	55.54	38.18	9.216	0.411	25	0
2016	Aug	Flint	SML BLD	COLD	498	21.9	7.13	0.53	1134	11.9	-1.145	4.889	49.82	0.659	25	0
2016	Aug	Flint	SML BLD	HOT	494	35.2	7.07	0.53	914.7	11.73	10.93	18.74	10.48	1.933	25	0
2016	Aug	Flint	SML BLD	COLD	474	22.2	7.09	1.18	1041	10.69	12.29	54.39	10.27	0.192	25	0
2016	Aug	Flint	SML BLD	HOT	516	59	7.25	0	674.2	10.77	29.27	49.34	76.08	0.183	0	0
2016	Aug	Flint	SML BLD	COLD	546	24.3	7.18	0.65	947.9	10.91	19.69	327.7	14.44	0.078	0	0
2016	Aug	Flint	SML BLD	HOT	522	33.6	7.22	0.33	973.5	10.82	7.941	47.24	2.702	0.251	25	0
2016	Aug	Flint	SML BLD	COLD	504	21	6.83	0.51	1149	11.77	1.272	20.28	0.967	-0.017	0	0
2016	Aug	Flint	SML BLD	HOT	506	43.2	7.1	0.51	1048	11.6	4.164	39.92	29.12	0.284	25	0
2016	Aug	Flint	SML BLD	COLD	602	26.5	6.85	0.16							1406	0
2016	Aug	Flint	SML BLD	HOT	596	25.5	6.9	0.25	1181	11.27	293.8	347.3	81.16	3.898	7363	0
2016	Aug	Flint	SML BLD	COLD	518	21.5	7	0.32	998.8	10.79	17.15	42.64	319.4	0.643	0	0
2016	Aug	Flint	SML BLD	COLD	544	25.5	6.7	0.26	1052	11.74	5.358	78.52	76.26	0.241	25	0
2016	Aug	Flint	SML BLD	HOT	496	46.5	6.84	0	936.6	11.88	29.43	59.23	191.7	0.378	40	0
2016	Aug	Flint	DIST	HYD	510	20.1	6.94	0.39							0	0
2016	Aug	Township	LARGE BU	COLD	514	21.5	7	0.31	359.8	11.74	247.2	10.55	7.584	-0.012	25	0
2016	Aug	Township	LARGE BU	HOT		52.3	7.25	0.09	329.4	10.55	9.786	34.31	1.631	0.199	0	0
2016	Aug	Township	SMALL BU	COLD	476	21.7	7.27	0.87	435.2	10.03	7.983	6.019	6.301	0.062	25	0
2016	Aug	Township	SMALL BU	COLD	524	22.3	6.98	1.04	1019	10.91	32.46	3.979	58.45	0.057	25	0

CHAPTER 2. ANTICIPATING THE EFFECTS OF GREEN BUILDINGS ON WATER QUALITY AND INFRASTRUCTURE

William J. Rhoads, Annie Pearce, Amy Pruden, and Marc A. Edwards

INTRODUCTION

Widespread adoption of green/sustainable plumbing designs is driving a paradigm shift in the relationship between water utilities and their customers. In the past half century, consumers in the United States have relied on drinking water utilities to collect, treat, and convey safe and aesthetically satisfactory water to their taps. Conventional building plumbing systems had relatively little variation from one building to another, and most consumer concerns have historically been resolved through relatively simple "decision tree" approaches with water rate-setting directly tied to water use. However, a green building revolution is underway, with over 74,000 Leadership in Environmental Engineering Design (LEED)–certified projects worldwide and up to 48% of all new US non-residential construction projects (by value) poised to "go green" by 2015 (USGBC, 2014a; McGraw Hill Construction, 2010). As a result, a wide variety of atypical plumbing systems are being installed, which will likely increase the scope and complexity of problems that water consumers will experience and report to utilities.

The green building revolution that is currently underway calls for a proactive response from water utilities and all stakeholders in terms of anticipating water quality changes that can occur in building and community water systems. This article is focused on water age as a factor that will surely increase as a function of water conservation efforts and discusses its ramifications. Water age is the water residence time, measured from the entry point of a system to the time it is used. Total water age, therefore, is the summation of the distribution system age (i.e., residence time from the treatment facility to the water meter of a building) and premise plumbing water age (i.e., residence time from the water meter to the point of use). It is well-known that increased water age can compromise water quality and cause a host of other plumbing-related issues (USEPA, 2002a). This article (1) describes how water age is increased as a result of conservation and green buildings, (2) summarizes expected changes in water quality as a result of high water age, (3) describes secondary impacts of high water age, (4) presents some solutions and a shared responsibility model for addressing concerns, and (5) highlights key knowledge gaps that will need to be addressed to ensure future success of green water systems.

HOW WATER AGE IS INCREASED BY GREEN PLUMBING

A higher premise plumbing water age will result whenever there are significant reductions in the amount of potable water demand without proportionally reducing the total plumbing system volume. Reducing water demand is generally standard practice in green buildings. For example, water conservation practices using low-flow fixtures, reducing potable water demand for applications such as landscaping, and occupant education encouraging less water use markedly decrease demand for individual buildings. The problem of elevated water age can be further

exacerbated by increases in total plumbing volume incurred by certain types of systems. Using new water sources such as rainwater/reclaimed water for landscaping, toilet flushing, or even as potable water also decreases overall utility water demand and often requires large storage volumes to endure drought (Fig. 2.1A). Thus, total plumbing system volume and overall water age can be increased far beyond historical practice. In solar and other water heating applications, large water heater tank sizes are often installed to store sufficient thermal energy to meet demand when it is not sunny, resulting in increased system volume and water age in hot water systems as well (Fig. 2.1B).

The typical reduction in potable water use as a result of green designs via LEED certification is on the order of 20–50% (USGBC, 2014b), but more advanced designs using alternative water sources to meet potable water go far beyond these targets. One field investigation of buildings using rainwater to flush toilets documented a 58–80% reduction in potable water use depending on the type of building, with premise plumbing water age exceeding three weeks to some taps (Nguyen et al, 2012). Limited data suggest that water age for modern green homes averages about 250% higher than in conventional residences (Fig. 2.2), assuming that pipe sizes and water volumes are unchanged, that no potable water is used for landscaping in green houses, and that "other" demands and leak rates are unchanged (Energy Star, 2014; USEPA, 2014b; Mayer et al, 1999).

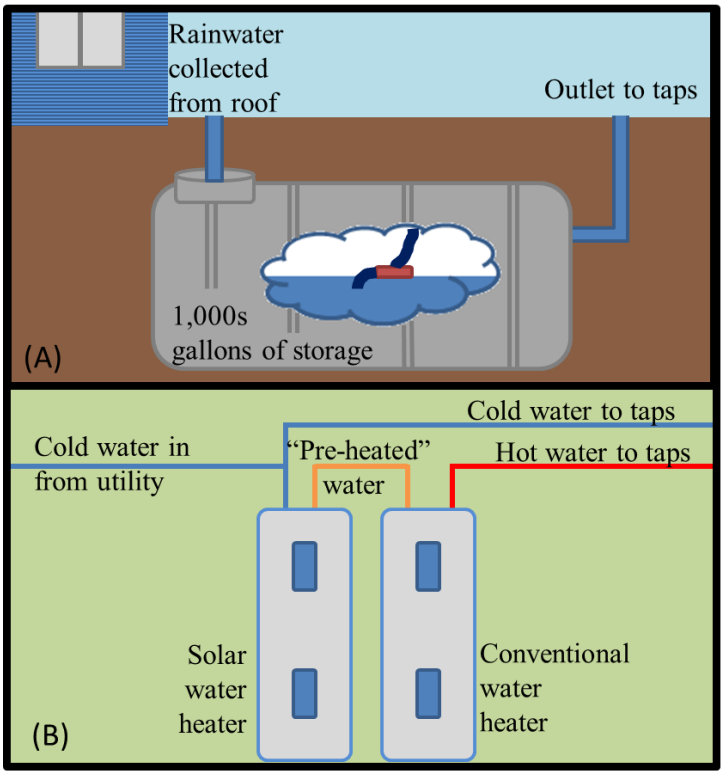

Fig. 2.1. Green strategies that require large water storage tanks
An underground rainwater cistern storing thousands of gallons of water at a time (A) and a solar water heater that reduces energy demand by "pre-heating" the water before it enters a conventional water heater (B).

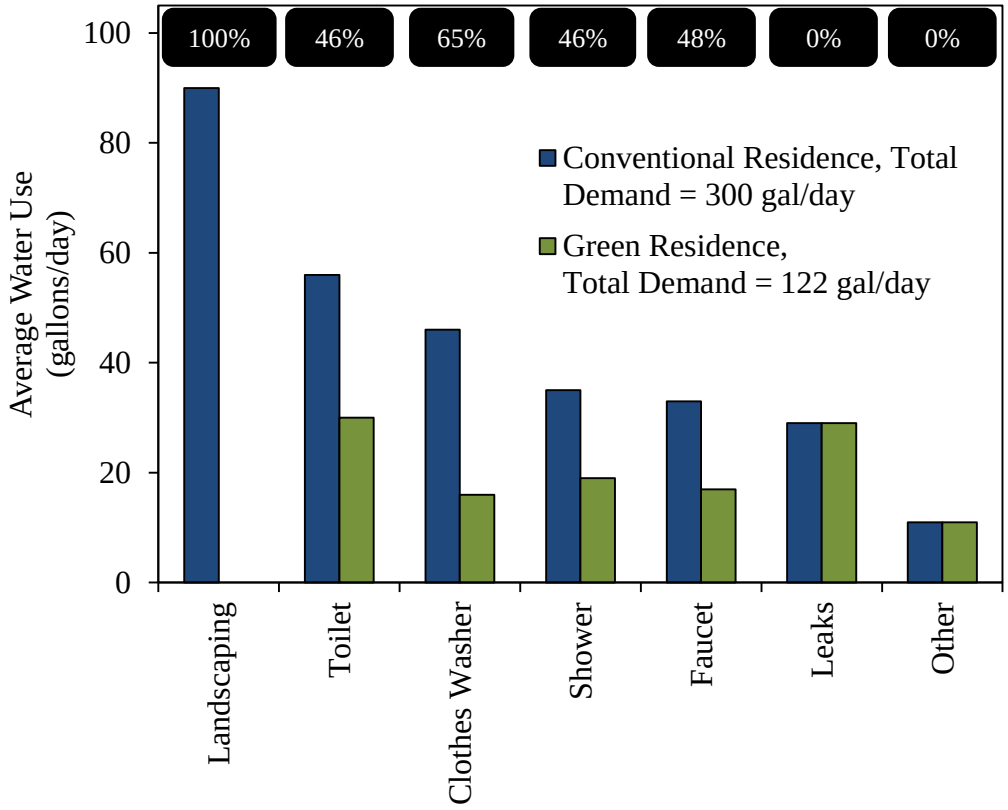

Fig. 2.2. Average potable water use in green residences and conventional residences (percent decrease in green buildings noted for each use category at the top of the figure).

EXPECTED CHANGES IN WATER QUALITY RESULING FROM ELEVATED WATER AGE

High water age has been documented as a key cause of main water distribution system issues such as corrosion, development of taste and odors (T&Os), and regrowth of microorganisms (USEPA, 2002a). The characteristics of premise plumbing relative to main distribution systems—such as higher surface area to volume ratios, intermittent use patterns, water temperature fluctuations, and the use of different plumbing materials—adds further complexity relative to utility experiences (NRC, 2006; Edwards et al, 2003). Our preliminary experiences with consumer complaints in green buildings has revealed that higher water age contributes to problems such as unprecedented loss rates of disinfectant residuals, warmer water temperatures encountered in cold water systems, more temperate water temperatures in hot water systems, enhanced corrosion of plumbing infrastructure, and increased microbial regrowth problems relative to conventional plumbing system designs (Table 2.1).

Microbiological and chemical ramifications. Issues summarized in Table 2.1 are interrelated and can result from a sequence of biological and chemical reactions initiated by the unique environment in premise plumbing. For example, as monochloramine decays within a system, ammonia is released (Vikesland et al., 2001; Morris & Isaac, 1981), which enhances growth of autotrophic nitrifying bacteria. This process consumes 8.62 mg alkalinity (HCO_3^-) for every mg of

Table 2.1. Summary of issues related to green building plumbing designs

Issue	Concept and link to Green Buildings	Selected References
Rapid loss of disinfectants	Chemical, physical, and biological reactions that consume disinfectant residual at a rate much faster than what occurs in the distribution system, especially at high water age.	LeChevallier et al., 1990; Zhang and Edwards, 2009; Nguyen et al., 2011; Hua et al., 1999; Thomas, 1987
Thermal targets	Consumers report that cold water is too warm, and hot water is too cold. Water warms (and cools) to room temperature in stagnant, unused lines with high water age; Water heater temperature may be reduced to save heating energy.	Brazeau and Edwards, 2012; Rhoads et al., 2014
Blue water	The natural aging process of copper pipes can be inhibited by microbial activity (decrease in pH, absence of disinfectant residual, microbial growth), causing high levels of copper in the water resulting in blue staining of plumbing fixtures or blue colored water.	Edwards et al., 2000; Schock et al., 1994, 1995; Edwards et al., 1996; Dodrill and Edwards, 1995; Zhang et al., 2009; Boulay and Edwards, 2001; Edwards et al., 2001; Schock and Sandvig, 2009
Pin hole leaks	Small (i.e., pinhole) leaks develop in some copper pipes as a result of changes in water chemistry. Sulfate reducing bacteria (SRB), proliferating in iron distribution mains, are thought to colonize copper pipes, creating tubercules under which SRB proliferate and cause non-uniform corrosion on the copper pipe, especially in parts of distribution systems with high water age.	Scardina et al., 2008; Edwards et al., 2009; Marshall, 2004
Lead leaching	Lead can leach from pre-2014 brass. The new NSF/ANSI standard for "lead-free" brass of 0.25% Pb by weight will likely prevent issues in the future; however, older brass devices are at risk especially at pH below ~8 and low alkalinity (<30 mg/L as $CaCO_3$). Rainwater is especially prone to lead leaching.	Lytle and Schock, 2000; Zhang et al., 2009; Elfland et al., 2010; Nguyen et al., 2011; Dodrill and Edwards, 1995; Triantefyllidou and Edwards, 2007; Gardels and Sorg1989; Abhijeet et al., 2005
Taste and odor development	Premise plumbing is already a major cause of T&O, and its importance will probably increase in green buildings due to higher temperatures and high water age,	Burlingame and Anselme, 1995; Lin, 1977; Rigal and Danjou, 1999; Tomboulian et al., 2004; Dietrich, 2006; Durand and Dietrich, 2007; Khiari et al., 2002
Microbial regrowth	Growth of microorganisms is typically limited in main distributions systems by frequent flow, presence of disinfectant residuals, low surface: volume ratios, and cooler temperatures. To the extent water age is increased, there is increased likelihood for growth. In addition, some "green" devices, such as low-flow metered and electronic faucets have been directly associated with increased pathogen growth.	Lautenschlager et al., 2010; LeChevallier et al., 1987; Ciesielski et al., 1984; Harper, 1988; Durand and Dietrich, 2007; Heim and Dietrich, 2007; Whelton et al., 2004; 2007
Low flow rate	More flushing time is required to obtain adequate cold and hot water temperatures. More laminar flow in pipes may facilitate pathogen growth within biofilms. Lower flows on the waste side may be inadequate to remove toilet and urinal wastes, causing foul odors within buildings.	Chambers et al., 2014; Liu et al., 2006;

NH_4^+-N that is oxidized, and will reduce pH in poorly buffered waters (Zhang et al., 2009). In general, lower pH and low alkalinity (< 30 mg/L as $CaCO_3$) waters are more corrosive, and can cause higher levels of lead and copper leaching from building plumbing (Elfland et al., 2010; Lytle & Schock, 2000, 1996; Edwards et al., 1999,). In some instances, this can lead to discoloration of water or staining of fixtures (Fig. 2.3). The removal of oxygen by nitrifiers can also enable growth of anaerobic bacteria in biofilms, which has been associated with serious damage to plumbing infrastructure (Fig. 2.4) (Masters et al, 2014; Kleczyk & Bosch, 2008; Scardina et al, 2008).

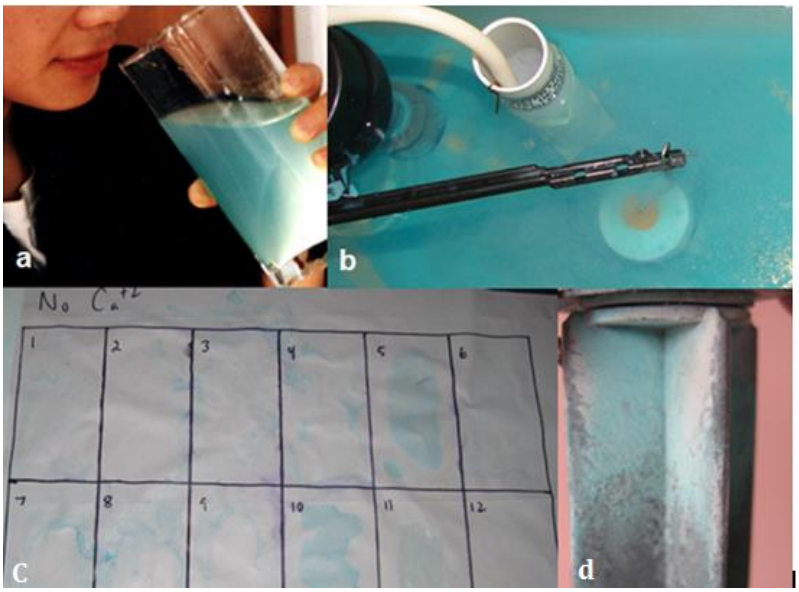

Fig. 2.3. **Blue water phenomenon.**

Blue water and blue water staining: a.) Blue water observed in drinking water; b.) Blue water in toilet tank; c.) Blue water staining on shower curtain materials with different soaps and cleaning products applied; d.) Blue water staining on a drain plug. Source of 3A: Edwards et al., 2000

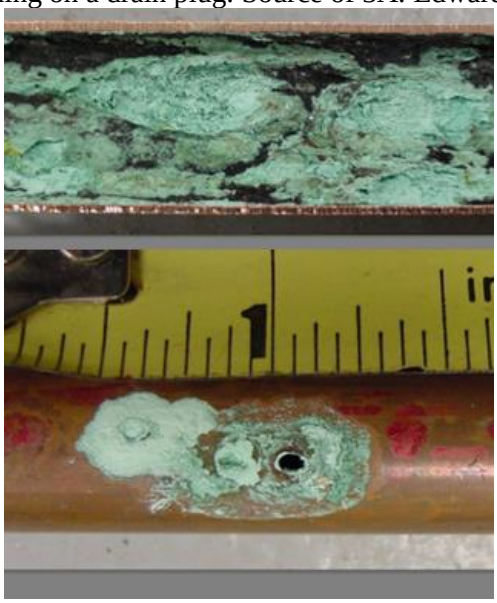

Fig. 2.4. Non-uniform (i.e., pitting) corrosion on copper tubing from a residence in a community with high water age.

Source: Scardina et al., 2008

Rapid disinfectant loss. The first critical link in the above chain reaction is often the rapid loss of chloramine or chlorine residual. In one green building, chloramine was observed to dissipate in pipes with a 1st order decay rate of 0.21–0.52 1/hour causing essentially complete loss of a 3.0 mg/L as Cl_2 residual in pipes in as little as 5.2 hours (Nguyen et al, 2012). An aliquot of the same water taken from inside the plumbing and placed into a glass container with no headspace had a decay rate of just 0.025 1/hour, which would maintain a chloramine residual greater than 0.2 mg/L as Cl_2 for about 110 hours. Therefore, from the perspective of chloramine residual loss, water in contact with surfaces of premise plumbing pipes can effectively "age" as much as 20 times faster compared to utility experience in water mains.

Taste and odors. Many T&O-causing compounds originate in parts of the distribution system where microbial growth is increased or when water stagnates in prolonged contact with plumbing materials (USEPA, 2002a; Burlingame & Anselme, 1995). Most materials used within water main distribution and premise plumbing systems leach T&O-causing compounds at some level (Tomboulian et al, 2004). When this water enters premise plumbing and is warmed, any off-flavors are likely to become more pronounced and are further exacerbated by T&O-causing compounds leached from premise plumbing (Khiari et al, 1999; Payment et al, 1997). Flushing is often suggested as a temporary solution to T&O problems, but this solution would require much longer flush durations in green buildings, given that low-flow fixtures and high volumes of stored water are often present. Flushing also presents capacity problems in systems relying on collected water as a primary source. It is widely recognized that more research is needed to assess the exact role water age plays in producing off-flavors that have been associated with plumbing materials and devices (Dietrich, 2006).

Limitations in utility influence. Utilities have limited control over changes in water quality that occur in premise plumbing systems as a result of increased premise plumbing water age. In some instances, problems are not considered to be very serious or widespread enough to merit changes in water treatment or distribution system practices. On the other hand, identifying factors that cause rapid disinfectant losses or installing individual premise plumbing chlorine booster stations could dramatically reduce the likelihood of problems with microbial regrowth, T&Os, and corrosion. Moreover, some consumers seek completely off-grid water systems, which generally require no water from utilities except during droughts or emergencies, are transitioning to independence from water utilities. In some situations there is very little that utilities can do to ensure high quality water in buildings. Current strategies and future research needs for utility-based strategies to help mitigate the effects of water age are discussed in the "Seeking Solutions" section of this article.

OTHER EMERGING ISSUES WITH HIGH WATER AGE

Metered faucets and other new devices. Most of the green plumbing devices routinely installed in green buildings have not been studied for possible health effects on consumers, despite numerous studies suggesting problems. For example, a growing body of literature has shown that metered/electronic hands-free faucets, which were designed to save water and prevent the spread of germs, sometimes support growth of much higher concentrations of some opportunistic pathogens, such as *Pseudomonas aeruginosa* and *Legionella* spp., compared with conventional, manually operated faucets for reasons that are yet unknown (Sydnor et al, 2012; Yapicioglu et al,

2011; Berthelot et al, 2006; Chaberny & Gastmeier, 2004; Halabi et al, 2001). Thus, in an attempt to reduce water waste and improve hygiene, another serious potential human health problem may have been created. Despite 10 years of reported problems with these faucets, not one laboratory study has ever been published to verify field observations or to determine exactly why these devices are sometimes problematic (Edwards et al, 2014). This example illustrates the extent to which green plumbing design is not receiving the attention it deserves with respect to considerations of water quality and public health, both in practice and in the actions of potentially responsible regulatory agencies such as the U.S. Environmental Protection Agency.

Rain water and cisterns providing a supplemental or sole potable water source. The quality and safety of rainwater used for potable water uses is relatively unstudied. Rainwater typically has negligible alkalinity and low pH and can be further acidified as a result of acid rain and contaminated by atmospheric lead, airborne herbicides/pesticides, or other contaminants from the roof collection/conveyance system, including fecal contamination from wildlife. As with potable water, microbes can grow in onsite rainwater storage tanks and plumbing (Fig. 2.5) (Fujioka, 1993; Krishna, 1993; Thomas & Greene, 1993; Lye, 1992; Waller, 1989; Yaziz et al, 1989; Body, 1986). These tanks are often sized to hold weeks or months of building water demand. In some instances, water is recirculated to avoid complete stagnation, creating a completely mixed system as opposed to plug flow typically encountered in water systems.

Fig. 2.5. Completely off-grid rainwater system

A) roof collection surface, B) buried rain barrels, C) new cross-linked polyethylene (PEX), and D) biofilm and debris buildup on interior of rainwater system PEX pipe.

Buildings as part stand-alone and part consecutive systems. The movement toward net zero water may result in more onsite treatment systems, which can trigger costly requirements to comply with all the national primary drinking water standards (USEPA, 2014a). It is widely acknowledged that installation of similar treatment systems in hospitals for *Legionella* control is often not reported to avoid financial and regulatory burdens. The authors anticipate that consumers in many green buildings will follow a similar path. Further, the efficacy of some in-building disinfection systems has not been established (Lin et al, 2011, 2002,1998; Muraca et al, 1987), requirements for adequate disinfection design in buildings have not been vetted (i.e., "CT" requirements at utilities), nor has the impact of disinfectants on the corrosion of building plumbing infrastructure been assessed (Table 2.2) (Rhoads et al, 2014).

Conservation conundrum. The term "conservation conundrum" was first applied to describe the loss of revenue for power utilities that resulted from reduced power use across a service region (Gordon & Olson, 2004); it was later applied to drinking water utilities that are encouraging customers reduce water use while maintaining dated pricing models that lead to significant losses in revenue when customers reduce their water consumption (Hughes et al, 2014). In some instances, total water sales have decreased by as much as 25% (Hughes et al, 2014). This decrease in revenue for utilities comes at a time when the physical state of treatment facilities and distribution networks is in urgent need of upgrades (ASCE, 2014), with a projected total funding gap of more than $300 billion by 2019 for capital treatment costs, operations, and maintenance of water treatment facilities and infrastructure (USEPA, 2002b). Seasonal customers (e.g., "snowbirds") in many drought-prone areas, and customers requiring only emergency standby service from water utilities, will present additional rate-setting challenges. Utilities are expected to maintain service connections in these cases but earn relatively little revenue from each customer. Although there are emerging solutions for these very low-end and seasonal users as explored elsewhere (Hughes et al, 2014), the amount charged for water will have to increase to cover the widening funding gap.

A secondary impact of the conservation conundrum described by Hughes et al (2014) that has not yet been explored is the potential for water quality to be degraded as a result of conservation. Specifically, utility distribution system water age and its associated problems can be increased when water demand decreases with time. One drinking water utility in Virginia anticipated a water demand of 60 mgd by 2012; however, actual demand was only 36 mgd (Ramaley, 2013). Consequently, not only was the water utility selling 40% less water than planned by 2012, but water age at the far reaches of the system were roughly twice the intended design. Thus, total water age was increased markedly, even in homes that did not adopt water conversation, and both dimensions of total water age (distribution system + premise plumbing) were increased in green buildings.

Table 2.2. Potential impacts of disinfectants on the corrosion of building plumbing infrastructure

Disinfectant/ Pipe Material	Copper	Iron	Polyvinyl chloride (PVC)	Polyethylene (HDPE)	Cross-linked Polyethylene (PEX)	Selected References
Chlorine	Corrosive; Rapid rxns with scale; pitting	Corrosive; rapid rxns with scale	Temporary leaching of T&O compounds	Consumes residual; Reduces OIT; Repeat shock treatments damage structure	Consume residual; High concentration changes surface chemistry of PEX-B; Reduces OIT	Ando & Sayto, 1984; Heim & Dietrich, 2007; Durand & Dietrich, 2006
Chloramine	Corrosive; Rapid rxn with scale	Corrosive; Rapid rxn with scale	Unknown	Unknown	Consumes residual; Reduces OIT	Chung et al., 2006
Chlorine dioxide	Corrosive; rapid rxn with scale	Corrosive; rapid rxn with scale	Unknown	Unknown	Consumes residual; Reduces OIT; Failure when exposed to high concentrations	Chung et al., 2006
Ozone	Corrosive	Corrosive	Causes brittleness	Unknown	Unknown	Chung et al., 2006
Copper-Silver	Ag possibly corrosive?	Deposition corrosion on galvanized	Unknown	Unknown	Unknown	Clark et al., 2011
UV	None likely	None likely	None likely	None likely	None likely	

Increased importance of plumbing codes and regulation of green water systems. For consumers who achieve partly or completely off-grid status with respect to their water supply, plumbing codes and regulations are needed to ensure safe plumbing design, installation, maintenance, and monitoring guidelines. The primary focus of green building plumbing design has been on energy or water savings, and virtually no attention has been given to the quality of water that results from water conservation. Organizations such as the International Code Council (ICC), International Association of Plumbing and Mechanical Officials, and the American Society for Heating, Refrigeration, and Air-conditioning Engineers have put forth construction codes for green plumbing and/or building features, but these codes do not address water quality concerns outlined in this article. One recent code has limited the length of pipe in hot water recirculating and non-recirculating systems (ICC, 2012), which reduces both energy (i.e., heat) loss and water age and which could serve as a basis for similar guidelines in cold water systems.

In addition to construction requirements, there is a need for regulation of completely off-grid water systems. An Internet survey for off-grid rainwater system designs was completed by 222 members of the American Rainwater Catchment Systems Association, representing approximately 2,700 rainwater harvesting systems across the United States (Thomas et al, 2013). It showed that 32% of these systems were used for potable water supplies, and of the respondents who reported on the water quality testing, only 12% of homes and 3% of businesses tested their rainwater quarterly. While this survey represents a first step to identifying gaps in the safety of off-grid water systems, there is a need for water quality monitoring guidance.

SEEKING SOLUTIONS

Past experiences. For systems using water from a drinking water utility, flushing the plumbing system has successfully mitigated serious problems in the past. In one case study, problems caused by lead leaching (> 100 ppb) elevated bacterial growth (heterotrophic plate count > 300,000 colony forming units per mL), and T&O complaints were ultimately remediated by installing an automatic flushing device at the end of the plumbing system that used less than 1% of the total daily flow of the building (Nguyen et al, 2012; Elfland et al, 2010). Another successful flushing protocol was implemented in response to an outbreak of Legionnaires' disease in Florida. The hotel conducted extensive daily flushing throughout the building to maintain chlorine residuals in its plumbing system at all times (Miami-Dade, 2010). Flushing is currently considered an adequate solution to lower the water age (and therefore increase the disinfectant residual and maintain corrosion control applied at the utility); however, the misconception that this is "wasting" water could impede implementation and this strategy might not improve water quality and would put additional stress on the amount of water stored in off-grid systems with storage tanks.

Shared responsibility model. Because of the complexity of issues that develop in premise plumbing systems and the increasing use of green/sustainable design, a "shared responsibility" model for provision of safe and aesthetically pleasing water to consumers has been proposed (Edwards et al, 2014; Pruden et al, 2012). That is, a range of stakeholders including water utilities; consumers; green building organizations, manufacturers, and commercial entities; building designers; plumbers; code setting organizations; and plumbing device manufacturers now have critical roles to play in preventing and solving water quality problems in buildings. While

knowledge of these problems and strategies to avoid them are still developing, recommendations for different stakeholders to assist in reducing concerns associated with high water age are provided in the following sections.

Water utilities. Temporary or seasonal problems might be resolved by periodic flushing of the water distribution system at hydrants to bring fresh cold water with a disinfectant residual into the system and clean sediment from pipes. Long-term changes that can enhance the quality of water for consumers include installation of booster chlorination, eliminating "dead ends," lining or replacing unlined iron or cement pipes, and considering improvements to maintenance and operation of water storage facilities. While utilities cannot be solely responsible for water quality beyond the property line, utilities can take steps to minimize and compensate for higher water age in their system. Furthermore, investigations into water chemistry that provide more stable disinfectant residuals within premise plumbing would likely help to counteract some of the effects of high water age, especially given that preliminary data show very large variations in disinfectant decay rates from one building to another (e.g., Nguyen et al, 2012). In addition, complaints about water quality should be tracked by aggressively soliciting, documenting, mapping, and investigating consumer concerns. Such tracking can provide insights to the origin of problems and the portions of water distribution systems that might be impacted.

Building or home owner/operators. Proposed standards for control of disease-causing bacteria such as Legionella (the cause of Legionnaires' disease) have outlined responsibilities for building owners and operators to protect health. Building owners and operators can reduce problems by responding to, diagnosing, documenting, remediating, and reporting problems (e.g., to utilities, health-care organizations, device manufacturers) with tastes and odors, inadequate hot water, and elevated cold water temperatures. Long periods of stagnation or vacancies might initiate more complex problems that can be avoided by scheduled flushing of taps or flushing immediately before occupancy or water use is resumed. Building owners and operators also should realize that problems associated with new water conservation technologies and strategies are not yet fully understood. Building owner/operators must be alert to aesthetic and health complaints that might be associated with these new devices or strategies.

Plumbing system designers. System designers should minimize the volume of the plumbing system to the extent possible by providing the minimum storage that can meet demands in both hot and cold water systems. Pipe diameters also should be minimized to the extent possible in systems with high levels of conservation. Placing multiple water fixtures on the same line and avoiding long runs of pipe with little or no demand that create "dead ends" will help reduce water age.

Plumbers. Plumbers can serve as important sentinels for problems occurring throughout a city or in specific neighborhoods, including frequent complaints about T&Os in hot and cold water systems, plumbing failures, or failures of specific plumbing devices. Their accumulated wisdom and observations can be tracked through plumber surveys and outreach to help guide consumers and water utilities to identify and resolve problems efficiently as has been done at the local and national level for pinhole leaks in premise plumbing (Scardina et al., 2008). In addition, online resources such as plumbing forums and blogs are a relatively untapped resources.

Building occupants/consumers. For employees or residents occupying green facilities, it is important to report problems with water quality (e.g., taste, odor, health) to building managers and/or water utilities. Sometimes utilities and building operators are not aware of their responsibilities or the potential dangers of ignoring problems.

Code organizations/regulatory agencies. As consumers seek to make their water systems more efficient, they will move beyond installation of efficient faucets, toilets, and urinals and begin exploring more innovative (and untested) strategies, such as rainwater harvesting or reclaimed water systems. Building plumbing codes should provide guidelines to help avoid selection of a water system that is too difficult to maintain or which results in long water ages. Regulatory agencies should provide clear recommendations to consumers about proper and adequate water quality testing procedures.

Green building organizations/manufacturers/commercial entities. As new technologies and consumer products become available to increase the water efficiency of green buildings, green building accreditation organizations should create a reward system that allows green building designers to select products that are proven to work for their specific application and in their specific geographical location. Given differences in water and climate geographically, regional differences can be very important in success of devices and consumer satisfaction. Manufacturers and commercial entities selling "off-the-shelf" conservation technologies should have extensive and clear information on their product and the risks and benefits involved.

KEY KNOWLEDGE GAPS

As this article illustrates, green plumbing systems present a wide range of challenges for utilities and other stakeholders. We are at the earliest stages of a green building revolution, where achieving extreme reductions in water demand are becoming more commonplace while scientific understanding is lagging behind. Sufficient information is available to cause legitimate concern, and there are several specific areas that are in need of research:

- Determining which water chemistries and approaches minimize effects of higher distribution system and premise plumbing water age;
- Identifying pipes, fittings, and devices that are most compatible with very high water age;
- Formulating design guidelines that go beyond simply reducing water use but also maintain good water quality based on scientific evidence;
- Exploring the emerging impacts of in-building disinfection systems on public health and corrosion of plumbing;
- Evaluating appropriate and effective utility responses to consumer complaints, rate setting, and issues regarding supplemental and emergency stand-by connections for buildings;
- Developing equitable and sustainable rate systems for supplemental and emergency connections, given the uncertainty of risk taken on by the utility to maintain such connections and that they would most likely be used in times of higher water demand across the community;
- Encouraging checks and balances on manufacturer responsibility with respect to devices that could cause problems with water quality;

- Developing sustainable future rate structures that reward water conservation while also fairly compensating water utilities, given the reduced sales of water across the United States and widening funding gap.

While challenging, addressing these knowledge gaps will be essential to truly advancing sustainable water efforts and supporting innovation in green building plumbing systems that truly meet the needs of their occupants while maintaining resource efficiency, minimal ecological impact, aesthetically satisfactory water, and safety.

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REFERENCES

Abhijeet, D.; Schock, M.; Murray, N.; & Edwards, M.A., 2005. Lead Leaching From In-Line Brass Devices: A Critical Evaluation of the Existing Standard. *Journal AWWA*, 97:8:66.

Ando, M. & Sayato, Y., 1984. Studies on Vinyl Chloride Migrating into Drinking Water From Polyvinyl Chloride Pipe and Reaction Between Vinyl Chloride and Chlorine. *Water Research*, 18:3:315. http://dx.doi.org/10.1016/0043-1354(84)90105-2.

ASCE (American Society of Civil Engineers), 2014. Drinking Water. 2013 Report Card for America's Infrastructure. www.infrastructurereportcard.org/a/#p/drinking-water/ (accessed Oct. 16, 2014).

Berthelot, P.; Chord, F.; Mallaval, F.; Grattard, F.; Brajon, D.; & Pozzetto, B., 2006. Magnetic Valves as a Source of Faucet Contamination With *Pseudomonas aeruginosa*? *Intensive Care Medicine*, 32:8:1271. http://dx.doi.org/10.1007/s00134-006-0206-6.

Body, P., 1986. *The Contamination of Rainwater Tanks in Port Pirie, Report No. 8*. Department of Environment and Planning, Adelaide, South Australia.

Boulay, N. & Edwards, M.A., 2001. Role of Temperature, Chlorine, and Organic Matter in Copper Corrosion By-product Release in Soft Water. *Water Research*, 35:3:683. http://dx.doi.org/10.1016/S0043-1354(00)00320-1.

Brazeau, R.H. & Edwards, M.A., 2012. A Review of the Sustainability of Residential Hot Water Heater Infrastructure: Public Health, Environmental Impacts, and Consumer Drivers. *Journal of Green Buildings*, 6:4:77. http://dx.doi.org/10.3992/jgb.6.4.77.

Burlingame, G.A. & Anselme, C., 1995. Distribution System Tastes and Odors. Advances in Taste-and-Odor Treatment and Control. Awwa Research Foundation, Denver, Colo.

Chaberny, I.F. & Gastmeier, P., 2004. Should Electronic Faucets Be Recommended in Hospitals? *Infection Control and Hospital Epidemiology*, 25:11:997. http://dx.doi.org/10.1086/502333.

Chung, S.; Oliphant, K.; Vibien, P.; & Zhang, J., 2007. An Examination of the Relative Impact of Common Potable Water Disinfectants (Chlorine, Chloramines and Chlorine Dioxide) on

Plastic Piping System Components. Plastics Pipes XIII, 5. Proc. ANTEC 2007, Cincinnati, Ohio.

Ciesielski, C.A.; Blaser, M.J.; & Wang, W.L., 1984. Role of Stagnation and Obstruction of Water Flow in Isolation of *Legionella pneumophila* From Hospital Plumbing. *Applied and Environmental Microbiology*, 48:5:984.

Clark, B.; Hernandez, A.L. & Edwards, M.A., 2011. Deposition Corrosion of Water Distribution System Materials. Water Quality Technology Conference, Phoenix, Ariz.

Dietrich, A., 2006. Aesthetic Issues for Drinking Water. *Journal of Water and Health*, 2006;4:Suppl 1:11. 10.2166/wh.2005.034.

Dodrill, D.M. & Edwards, M.A., 1995. Corrosion Control on the Basis of Utility Experience. *Journal AWWA*, 87:7:74.

Durand, M.L. & Dietrich, A.M., 2007. Contributions of Silane Cross-Linked PEX Pipe to Chemical/Solvent Odours in Drinking Water. *Water Science and Technology: A Journal of the International Association on Water Pollution Research*, 55:5:153.

Edwards, M.A.; Bosch, D.; Loganathan, G.V.; & Dietrich, A.M., 2003. The Future Challenge of Controlling Distribution System Water Quality and Protecting Plumbing Infrastructure: Focusing on Consumers. Proc. IWA Leading Edge Conference, Noordwijk, Netherlands.

Edwards, M.A.; Elfland, C.; & Pearce, A., 2009. Optimization of Urban Water Systems. Proc. Athens International Symposium on Water. Sustainable Development and Waster: A Global Challenge for Local Actions, Athens, Greece.

Edwards, M.A.; Jacobs, S.; & Dodrill, D., 1999. Desktop Guidance for Mitigating Pb and Cu Corrosion By-products. *Journal AWWA*, 91:5:66.

Edwards, M.A.; Jacobs, S.; & Taylor, R.J., 2000. The Blue Water Phenomenon. *Journal AWWA*, 92:7:72.

Edwards, M.A.; Powers, K.; Hidmi, L.; & Schock, M., 2001. The Role of Pipe Ageing in Copper Corrosion By-product Release. *Water Science & Technology: Water Supply*, 1:3:25.

Edwards, M.A.; Rhoads, W.J.; Pruden, A.; Pearce, A; & Falkinham III, J.O., 2014. "Green" Water Systems and Opportunistic Premise Plumbing Pathogens: Grand Challenge of Meeting Water and Energy Sustainability Goals without Endangering Public Health. *Plumbing Engineer*, Nov:63. http://www.plumbingengineer.com/content/green-water-systems-and-opportunistic-premise-plumbing-pathogens

Edwards, M.A.; Schock, M.R.; & Meyer, T.E., 1996. Alkalinity, pH, and Copper Corrosion By-product Release. *Journal AWWA*, 88:3:81.

Elfland, C.; Scardina, P.; & Edwards, M.A., 2010. Lead-Contaminated Water From Brass Plumbing Devices in New Buildings. *Journal AWWA*, 102:11:66.

Energy Star, 2014. Certified Products. www.energystar.gov/products/certified-products (accessed Oct. 15, 2014).

Fujioka, R.S., 1993. Guidelines and Microbial Standards for Cistern Waters. Proc. 6th International Conference on Rainwater Catchment Systems, Nairobi, Kenya.

Gardels, M.C. & Sorg, T.J., 1989. A Laboratory Study of the Leaching of Lead From Water Faucets. *Journal AWWA*, 81:7:101.

Gordon, K. & Olson, W., 2004. Retail Cost Recovery and Rate Design in a Restructure Environment. Edison Electric Institute, Washington. www.hks.harvard.edu/hepg/Papers/Gordon.Olson.Retail.Cost.Recovery.pdf (accessed Oct. 27, 2014).

Halabi, M.; Wiesholzer-Pittl, M.: Schoberl, J.; & Mittermayer, H., 2001. Non-touch Fittings in Hospitals: A Possible Source of *Pseudomonas aeruginosa* and *Legionella* spp. *Journal of Hospital Infection*, 49:2:117. http://dx.doi.org/10.1053/jhin.2001.1060.

Harper, D., 1988. Legionnaires' Disease Outbreaks—The Engineering Implications. *Journal of Hospital Infection*, 11:Suppl A:201. http://dx.doi.org/10.1016/0195-6701(88)90188-0.

Heim, T.H. & Dietrich, A.M., 2007. Sensory Aspects and Water Quality Impacts of Chlorinated and Chloraminated Drinking Water in Contact with HDPE and cPVC Pipe. *Water Research*, 41:4:757. http://dx.doi.org/10.1016/j.watres.2006.11.028.

Hua, F.; West, J.R.; Barker, R.A.; & Forster, C.F.R., 1999. Modeling of Chlorine Decay in Municipal Water Supplies. *Water Research*, 33:12:2735. http://dx.doi.org/10.1016/S0043-1354(98)00519-3.

Hughes, J.; Tiger, M.; Eskaf, S.; Berahzer, S.I.; Royster, S.; Boyle, C.; Batten, D.; Brandt, P.; & Noyes, C., 2014. Defining a Resilient Business Model for Water Utilities. Water Research Foundation Project #4366, Denver, Colo.

ICC (International Code Council), 2012. *International Green Construction Code*. ICC, Washington.

Khiari, D.; Chinn, R.; Barrett, S.; Matia, L.; Ventura, F.; Suffet, I.; Gittelman, R.; & Leutweiler, R., 2002. Distribution Generated Taste-and-Odor Phenomena. Water Research Foundation & American Water Works Association, Denver, Colo.

Khiari, D.; Bruchet, A.; Gittelman, T.; Matia, L.; Barrett, S.; Suffet, I.H.; & Hund, R., 1999. Distribution-Generated Taste-and-Odor Phenomena. *Water Science and Technology*, 40:6:129. http://dx.doi.org/10.1016/S0273-1223(99)00548-X.

Kleczyk, E.J. & Bosch, D.J., 2008. Incidence and Costs of Home Plumbing Corrosion. *Journal AWWA*, 100:12:122.

Krishna, J., 1993. Water Quality Standards for Rainwater Cistern Systems. Proc. 6th International Conference on Rain Water Cistern Systems, Nairobi, Kenya.

Lautenschlager, K.; Boon, N.; Wang, Y.; Egli., T.; & Hammes, F., 2010. Overnight Stagnation of Drinking Water in Household Taps Induces Microbial Growth and Changes in Community Composition. *Water Research*, 44:17:4868. http://dx.doi.org/10.1016/j.watres.2010.07.032.

LeChevallier, M.W.; Babcock, T.M.; & Lee, R.G., 1987. Examination and Characterization of Distribution System Biofilms. *Applied and Environmental Microbiology*, 53:12:2714.

LeChevallier, M.W.; Lowry, C.D.; & Lee, R.G., 1990. Disinfecting Biofilms in a Model Distribution System. *Journal AWWA*, 82:7:87.

Lin, S., 1977. Tastes and Odors in Water Supplies: A Review: Illinois State Water Survey. www.isws.illinois.edu/pubdoc/C/ISWSC-127.pdf (accessed Nov. 11, 2013).

Lin, Y.; Stout, J.E.; & Yu, V., 2011. Controlling *Legionella* in Hospital Drinking Water: An Evidence-Based Review of Disinfection Methods. *Infection Control and Hospital Epidemiology*, 32:2:166. http://dx.doi.org/10.1086/657934.

Lin, Y.S.E.; Vidic, R.D.; Stout, J.E.; & Yu, V.L., 2002. Negative Effect of High pH on Biocidal Efficacy of Copper and Silver Ions in Controlling *Legionella pneumophila*. *Applied and Environmental Microbiology*, 68:6:2711. http://dx.doi.org/10.1128/AEM.68.6.2711-2715.2002.

Lin, Y.; Stout, J.E.; Yu, V.; & Vidic, R.D., 1998. Disinfection of Water Distribution Systems for *Legionella*. *Seminars in Respiratory Infections*, 13:2:147.

Liu, Z.; Lin, Y. E.; Stout, J. E.; Hwang, C. C.; Vidic, R. D.; & Yu, V. L., 2006. Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. *Journal of Applied Microbiology*, 101(2), 437-442.

Lye, D., 1992. Microbiology of Rainwater Cistern Systems: A Review. *Journal of Environmental Science and Health*, 27:8:2123. http://dx.doi.org/10.1080/10934529209375845.

Lytle, D.A. & Schock, M.R., 1996. *Stagnation Time, Composition, pH, and Orthophosphate Effects on Metal Leaching From Brass* (Vol. 96). National Risk Management Research Laboratory, Office of Research and Development, US Environmental Protection Agency, Washington.

Lytle, D.A. & Schock, M.R., 2000. Impact of Stagnation Time on Metal Dissolution From Plumbing Materials in Drinking Water. *Journal of Water Supply: Research and Technology – AQUA*, 49:5:243.

Marshall, B.J., 2004. Initiation, Propagation, and Mitigation of Aluminum and Chlorine Induced Pitting Corrosion. Master of Science Thesis, Virginia Tech, Blacksburg, Va. http://scholar.lib.vt.edu/theses/available/etd-11222004-195901/unrestricted/msthesis2.PDF (accessed Oct. 27, 2014).

Masters, S.; Wang, H.; Pruden, A.; Edwards, M.A., 2014. Redox Gradients in Distribution Systems Influence Water Quality, Corrosion, and Microbial Ecology [Epub ahead of print]. *Water Research*, 68C:140.

Mayer, P.W.; DeOreo, W.B.; Opitz, E.M; Kiefer, J.C.; Davis, W.Y.; Dxiegielewski, B.; Nelson, J.O., 1999. Residential End Uses of Water. Awwa Research Foundation, Denver, Colo.

McGraw Hill Construction, 2010. *Green Outlook 2011: Green Trends Driving Growth*. McGraw Hill Construction, New York. http://aiacc.org/wp-content/uploads/2011/06/greenoutlook2011.pdf (accessed Oct. 15, 2014).

Miami-Dade (Miami-Dade County Health Department), 2010. *Outbreak of Legionnaires' Disease: 2009-2010 Final Report*. www.dadehealth.org/search.asp?q=legionnaires+disease (accessed Oct. 17, 2014).

Morris J.C. & Isaac R.A., 1981. A Critical Review of Kinetic and Thermodynamic Constants for the Aqueous Chlorine-Ammonia System. *Water Chlorination: Environmental Impact and Health Effects* (Vol 4) (R.L. Jolley, W.A. Brungs, J. A. Cotruvo, R.B. Cumming, J.S. Mattice, & V.A. Jacobs, editors). Ann Arbor Science, Ann Arbor, Mich.

Muraca, P.; Stout, J.E.; & Yu, V.L., 1987. Comparative Assessment of Chlorine, Heat, Ozone, and UV Light for Killing *Legionella pneumophila* Within a Model Plumbing System. *Applied and Environmental Microbiology*, 53:2:447.

Nguyen, C.K., Powers, K.A.; Raetz, M.A.; Parks, J.L.; & Edwards, M.A., 2011. Rapid Free Chlorine Decay in the Presence of Cu (OH)2: Chemistry and Practical Implications. *Water Research*, 45:16:5302. http://dx.doi.org/10.1016/j.watres.2011.07.039.

Nguyen, C.; Elfland, C.; & Edwards, M.A., 2012. Impact of Advanced Water Conservation Features and New Copper Pipe on Rapid Chloramine Decay and Microbial Regrowth. *Water Research*, 46:3:611. http://dx.doi.org/10.1016/j.watres.2011.11.006.

NRC (National Research Council), 2006. *Drinking Water Distributions Systems: Assessing and Reducing Risks*. National Academy of Sciences, Washington.

Payment, P.; Siemiatycki, J.; Richardson, L.; Renaud, G.; Franco, E.; & Prevost, M., 1997. A Prospective Epidemiological Study of Gastrointestinal Health Effects Due to the Consumption of Drinking Water. *International Journal of Environmental Health Research*, 7:1:5. http://dx.doi.org/10.1080/09603129773977.

Pruden, A.; Edwards, M.A.; Falkinham, III, J.O, 2012. Research Needs for Opportunistic Pathogens in Premise Plumbing: Experimental Methodology, Microbial Ecology and Epidemiology. Water Research Foundation Project #4379, Denver, Colo.

Ramaley, B., 2013. The Changing Water Utility. Environmental and Water Resources Seminar, Virginia Tech, Blacksburg, Va..

Reynolds, T.D. & Richards, P.A., 1996. Preliminary Unit Operations and Processes. *Unit Operations and Processesses in Environmental Engineering* (2nd ed.) Cengage Learning, Independence, Ky.

Rhoads, W.J.; Pruden, A.; & Edwards, M.A., 2014. Anticipating Challenges Associated with In-Building Disinfection for Control of Opportunistic Pathogens in Premise Plumbing. *Water Environment Federation*, 86:6:540. http://dx.doi.org/10.2175/106143014X13975035524989.

Rigal, S. & Danjou, J., 1999. Tastes and Odors in Drinking Water Distribution Systems Related to the Use of Synthetic Materials. *Water Science and Technology*, 40:6:203. http://dx.doi.org/10.1016/S0273-1223(99)00558-2.

Scardina, P.; Edwards, M.A.; Bosch, D.; Loganathan, G.; & Dwyer, S., 2008. Assessment of Non-Uniform Corrosion in Copper Piping. Water Research Foundation, Denver, Colo.

Schock, M.R & Sandvig, A.M., 2009. Long-Term Effects of Orthophosphate Treatment on Copper Concentration. *Journal AWWA*, 101:7: 71.

Schock, M.R.; Lytle, D.A.; & Clement, J.A., 1994. Modeling Issues of Copper Solubility in Drinking Water in Critical Issues in Water and Wastewater Treatment. Proc. National Conference on Environmental Engineering, Boulder, Colo.

Schock, M.R.; Lytle, D.A.; & Clement, J.A., 1995. *Effect of pH, DIC, Orthophosphate and Sulfate on Drinking Water Cuprosolvency*. National Risk Management Research Laboratory, Cincinnati, Ohio.

Sydnor, E.R.; Bova, G.; Gimburg, A.; Cosgrove, S.E.; Perl, T.M.; & Maragakis, L.L., 2012. Electronic-Eye Faucets: *Legionella* Species Contamination in Healthcare Settings. *Infection Control and Hospital Epidemiology*, 33:3:235. http://dx.doi.org/10.1086/664047.

Thomas, M.R.B.; Kirisits, M.J; Lye, D.; & Kinney, K., 2013. Rainwater Harvesting in the United States: A Survey of Common System Practices. Poster Presentation, Association of Environmental Engineering & Science Professors Conference, Golden, Colo.

Thomas, P. & Greene, G., 1993. Rainwater Quality From Different Roof Catchments. *Water, Science and Technology*, 28:3:291.

Thomas, P.M., 1987. Formation and Decay of Monochloramine in South Australian Water Supply Systems. Proc. 12th Federal Convention, Australian Water and Wastewater Association, Adelaide, Australia.

Tomboulian, P.; Schweitzer, L.; Mullin, L.; Wilson, J.; & Khiari, D., 2004. Materials Used in Drinking Water Distribution Systems: Contribution to Taste-and-Odor. *Water Science and Technology*, 49:9:219.

Triantafyllidou, S. & Edwards, M.A., 2007. Critical Evaluation of the NSF 61 Section 9 Test Water for Lead. *Journal AWWA*, 99:9:133.

USGBC, 2014b. Water Efficiency. www.usgbc.org/credits/new-construction/v4/water-efficiency (accessed Oct. 15, 2014).

USEPA, 2014a. Public Drinking Water Systems Programs. http://water.epa.gov/infrastructure/drinkingwater/pws/index.cfm (accessed Oct. 15, 2014).

USEPA, 2014b. Water Use Today. www.epa.gov/watersense/our_water/water_use_today.html (accessed Oct. 15, 2014).

USEPA (US Environmental Protection Agency), 2002a. Effects of Water Age on Distribution System Water Quality. Office of Water and Office of Ground Water and Drinking Water, Cincinnati, Ohio.

USEPA, 2002b. The Clean Water and Drinking Water Infrastructure Gap Analysis. Office of Water – EPA816-R-02-020, Cincinnati, Ohio.

USGBC (United States Green Building Council), 2014a. Leadership in Energy and Environmental Design – Building Design + Construction (Version 4.0). www.usgbc.org/leed (accessed Oct. 15, 2014).

Vikesland, P.J., Ozekin, K., & Valentine, R.L., 2001. Monochloramine Decay in Model and Distribution System Waters. *Water Research*, 35:7:1766. http://dx.doi.org/10.1016/S0043-1354(00)00406-1.

Waller, D., 1989. Rain Water: An Alternative Source in Developing and Developed Countries. *Water International*, 14:1:27. http://dx.doi.org/10.1080/02508068908692031.

Whelton, A.J.; Dietrich, A.M.; Gallagher, D.L.; & Roberson, A.J., 2007. Using Customer Feedback for Improved Water Quality and Infrastructure Monitoring. *Journal AWWA*, 99:11:62.

Whelton, AJ.; Dietrich, A.M.; Burlingame, G.A.; & Cooney, M.F., 2004. Detecting Contaminated Drinking Water: Harnessing Consumer Complaints. Proc. 2004 AWWA Water Quality and Technology Conference, San Antonio, Tex.

Yapicioglu, H.; Gokmen, T.G.; Yildizdas, D.; Koksal, F.; Ozlu, F.; Kale-Cekinmez, E.; & Candevir, A., 2011. *Pseudomonas aeruginosa* Infections Due to Electronic Faucets in a Neonatal Intensive Care Unit. *Journal of Pediatrics and Child Health*, 48:5:430. http://dx.doi.org/10.1111/j.1440-1754.2011.02248.x.

Yaziz, M.; Gunting, H.; Sapiari, N.; & Ghazali, A., 1989. Variations in Rainwater Quality From Roof Catchments. *Water Research*, 23:6:761-765. http://dx.doi.org/10.1016/0043-1354(89)90211-X.

Zhang, Y. & Edwards, M.A., 2009. Accelerated Chloramine Decay and Microbial Growth by Nitrification in Premise Plumbing. *Journal AWWA*, 101:11:51.

Zhang, Y. & Edwards, M.A., 2007. Anticipating Effects of Water Quality Changes on Iron Corrosion and Red Water. *Journal of Water Supply: Research & Technology*, 56:1:55. http://dx.doi.org/10.2166/aqua.2007.048.

Zhang, Y.; Love, N.; & Edwards, M.A., 2009. Nitrification in Drinking Water Systems. *Critical Reviews in Environmental Science and Technology*, 39:3:153. http://dx.doi.org/10.1080/10643380701631739.

CHAPTER 3. SURVEY OF GREEN BUILDING WATER SYSTEMS REVEALS ELEVATED WATER AGE AND WATER QUALITY CONCERNS

William J. Rhoads, Amy Pruden, Marc A. Edwards

ABSTRACT

Widespread adoption of innovative water conservation strategies has potential unintended consequences for aesthetics and public health. A cross-section of green buildings were surveyed and compared to typical conventional buildings in terms of water retention time (i.e., water age), water chemistry, and levels of opportunistic pathogen genetic markers. Water age was estimated to be 2-6.7 months in an off-grid office, an average of 8 days in a Leadership in Environmental Engineering Design certified healthcare suite, and was increased by 1.7 days from <1 day due to installation of a solar "pre-heat" water tank in a net-zero energy house. Chlorine and chloramine residuals were often completely absent in the green building systems, decaying up to 144 times faster in premise plumbing with high water age when compared to distribution system water. Concentration of 16S rRNA and opportunistic pathogen genus level genetic markers were 1-4 orders of magnitude higher in green versus conventional buildings. This study raises concerns with respect to current green water system practices and the importance of considering potential public health impacts in the design of sustainable water systems.

KEY WORDS: Water age; Green building; Water quality; Disinfectant residual; Opportunistic pathogens; *Legionella*, *Mycobacterium*

INTRODUCTION

The number of buildings marketed as "green" is increasing exponentially, with >75,000 certified Leadership in Energy and Environmental Design (LEED) projects in 2014 versus <7,500 in 2010 (USGBC, 2014). Within 10 years the annual global market for energy efficient building products is predicted to exceed $620 billion, with 40-48% of new non-residential construction classified as green by 2015 (Navigant Research, 2001; McGraw Hill, 2011). Green potable water systems are operationally defined as those achieving at least a 20% overall decrease in potable water use compared to designs that implement no water conservation/efficiency strategies while also meeting applicable plumbing codes. For instance, LEED offers "points" toward certification for reducing potable water demand by 20-50% through use of water efficient landscapes and low flow fixtures (USGBC, 2015). However, consequences to public health that may result from increased stagnation in premise plumbing (i.e., increased premise plumbing water age) due to reduced water demand is a generally overlooked aspect of water conservation.

It is well established that high water age is problematic in main distribution systems, contributing to problems with corrosion, taste and odors, and microbial regrowth (EPA, 2002). High water age in premise plumbing can further amplify such problems and also instigate proliferation of human pathogens (Nguyen et al., 2012; Pruden et al., 2012; NRC, 2006; Kelley et al., 2014). Synergistic effects can be incurred when both distribution and premise plumbing system water ages are high, as demonstrated in a few copper pitting corrosion case studies (Scardina et al., 2008).

Water age is likely to increase in both distribution and premise plumbing systems as water conservation practices are adopted at the community level. One study reported total utility system water demand decreased between 1997 and 2010 in 70% of municipalities surveyed (n=436), with 15% of those systems decreasing by at least 25% between 2007 and 2010 (Hughes et al., 2014). These trends have given rise to concerns that widespread adoption of green building and water conservation practices will create another "conservation conundrum" and further detract from potable water quality in buildings (Hughes et al., 2014; Gordon and Olson, 2004; Rhoads et al., 2015; Edwards et al., 2014).

Higher water age also has possible implications for opportunistic pathogens in premise plumbing (OPPPs), including *Legionella* spp. (especially *L. pneumophila*), *Mycobacterium avium* complex (Non-tuberculosis mycobacteria), *Pseudomonas aeruginosa*, *Acanthamoeba* spp., and *Naegleria fowleri*, which are now the waterborne pathogens of highest concern in the U.S (NRC, 2006; CDC, 2011; Bartrand et al., 2014). OPPPs can thrive under conditions created by premise plumbing, including the presence of materials that rapidly react with disinfectants or leach nutrients to water, inherently high plumbing surface area to volume ratios, widely variable flow conditions, numerous dead ends, impaired ability to achieve and maintain thermal targets that discourage microbial growth, and high water age (Nguyen et al., 2012; Pruden et al., 2012; NRC, 2006; Nguyen et al., 2011; Brazeau and Edwards, 2011a; Edwards et al., 2003; Bedard et al., 2015). OPPPs are integral members of potable water microbial communities and have complex life cycles, including amplification and enhanced virulence when hosted by free-living amoebae such as *Vermamoeba vermiformis* (Thomas and Ashbolt, 2011). *L. pneumophila* causes about 90% of reported premise plumbing related disease outbreaks, with other ecologically-related *Legionella* spp. also a cause for health concern (Pruden et al., 2012; NRC, 2006; CDC, 2011; WHO, 2014).

Only a few prior studies have touched upon the potential for OPPPs to proliferate in green water systems. In two conventional plumbing systems fitted with electronic-eye or hands-free metered faucets, higher colonization rates of *Legionella* spp. and *P. aeruginosa* were observed relative to conventional faucets (Yapicioglu et al., 2012; Sydnor et al., 2012). Another study showed higher colonization rates in faucets with larger hot and cold mixing volumes (Charron et al., 2014). In off-grid rainwater buildings, the occurrence of OPPPs, in particular *L. pneumophila*, has been documented and roof-collected rainwater system contamination has been serologically linked to Legionnaires' disease (Ahmed et al., 2008; Ahmed et al., 2010; Albrechtsen, 2002; Simmons et al., 2008; Schlech et al., 1985). However, prior studies did not investigate or report basic system features expected to influence OPPPs such as storage methods, plumbing materials, water chemistry, water demand, or water treatment in such facilities. Further, pathogen occurrence data is needed to inform quantitative microbial risk assessment models and raise awareness if a health concern is justified (Ahmed et al., 2010; Wang et al., 2012).

The purpose of this study was to conduct the first survey of a cross-section of cutting-edge green buildings in order to establish a baseline understanding of water age, water quality characteristics, and design differences relative to conventional buildings. Water systems were characterized in terms of disinfectant residual, disinfectant decay rates, and levels of genetic markers corresponding to total bacteria, *Legionella* spp., *L. pneumophila*, *M. avium*, and *V. vermiformis* using quantitative polymerase chain reaction (qPCR).

EXPERIMENTAL

Description of Field Sites. Four buildings with a cross-section of energy and water conservation strategies were surveyed and sampled to explore effects of green plumbing systems on water quality (Table 3.1). The buildings included a conventional house with no conservation features (Fig. 3.1A), a healthcare suite (Fig. 3.1B), a net-zero energy house (Fig. 3.1C), and a net-zero energy and net-zero water office building (Fig. 3.1D).

The net-zero energy home was an unoccupied single-family dwelling laboratory, simulating typical U.S. residential hot water demand of 245-300 L/day (65-80 gallons/day) to demonstrate the feasibility of achieving net-zero energy in a residential setting. Water was supplied by a public utility with free chlorine residual. A solar-powered water heater was installed upstream of an electric water heater to "pre-heat" water and reduce the amount of electricity required by the electric tank. Both hot and cold water plumbing was $^{3}/_{8}$" diameter flexible cross-linked polyethylene (PEX) tubing, with a maximum pipe volume of 1.1 L (0.3 gallons) to distal taps (equivalent to 12 seconds of flow with a 5.7 L/min (1.5 gpm) showerhead). The conventional single family home was sampled as a control and had ¾" copper plumbing, four residents, was served by the same public utility at the net-zero house, and no water conservation features.

The healthcare suite occupied the first floor of a LEED-Gold building. Water was supplied by a public utility with monochloramine residual. The suite had 20 exam rooms, each with a manually operated faucet for hand-washing (≤5.7 L/min; ≤1.5 gpm flow rate), five bathrooms with electronic faucets (1.9 L/min; 0.5 gpm flow rate), and one kitchenette sink. The minimum pipe diameters (½-1") specified in the plumbing code ensured adequate supply capacity for the large number of

fixtures, but resulted in a high volume of water storage relative to the demand in this facility, which was essentially limited to bathroom use and hand-washing. All plumbing in this facility was copper.

The small net-zero energy and net-zero water office building collected, treated, and stored rainwater for all potable and non-potable uses. This building minimized water demand by using composting toilets, installing 1.0 gpm fixtures on all taps, and having no outdoor irrigation. Supply lines were ½ or ¾" copper pipe.

Sampling Routine. A sampling plan was developed for each field site based on building performance data, investigation of building plumbing blueprints, and field observations. Samples were designed to capture profiles of water quality as it was flushed from taps. The net-zero energy house was visited twice: once before the start of the net-zero energy experiment when the water had been relatively stagnant for two months and again after it was used regularly for two months. During the first visit to the net-zero energy house, water was flushed extensively until it was representative of water from the main distribution system, as indicated by steady cold water temperatures and chlorine residuals, but this was not possible during the second visit because of an energy experiment that was then in progress. Therefore, water from three showering events, which were part of the energy study, were sampled instead.

At the healthcare suite, hot and cold water was sampled in four exam rooms at the end of the plumbing system. Starting with the first flush, grab samples were collected until a disinfectant residual consistent with the main distribution system was obtained. Afterwards, the taps were shut off and water was allowed to sit stagnant and low volume samples (~35 mL) were collected at regular intervals during stagnation for physical and chemical parameters.

At the net-zero rainwater office, only stagnant and moderately flushed (3 minutes) water samples were collected from the taps because the facility was not connected to a water main. Therefore, additional flushing would not have a beneficial impact on water quality typically expected in systems connected to water utilities.

Water Quality Analysis. Temperature, pH, ammonia, nitrite, and total chlorine were measured in the field at the time of collection using a pH 110-Series meter (Oakton Research, Vernon Hills, Il) or a DR2700 spectrophotometer (Hach, Loveland, CO) according to Standard Methods. Aliquots were transported to the lab on ice and analyzed using USEPA Method 300.1 for anions. Cations were measured by inductively coupled plasma mass spectrometry after acidification with 2% nitric acid (v/v) and >24 hours holding time. Samples of unacidified water were passed through a 0.45 µm pore size filter in the field to differentiate "soluble" metals and later acidified with 2% nitric acid (% v/v). Biological activity reaction tests (BARTs; Hach, Loveland, CO) indicating the presence/absence of viable nitrifying bacteria were used for select samples.

Biological Sample Processing. Water samples were transported on ice and filtered through a 0.22 µm pore-size mixed cellulose ester filters (Millipore, Billerica, MA) within 36 hours of collection. Bottles were pre-dosed with filter-sterilized sodium thiosulfate to remove disinfectant residuals where applicable. Stagnant water samples (first draw) were 250-500 mL while flushed samples

Table 3.1. Summary of key factors regarding water age in three field sites and a baseline comparison [T]

Parameter		Healthcare suite	Net-zero Energy House	Net-zero Office	Conventional Home in FS#2 Distribution System
Building Type		10,000 sqft LEED-Gold outpatient healthcare facility	Net-zero energy single family household	Small net-zero water and net-zero energy office	Conventional household, no green features
Total storage volume		80 gallon water heater	160 gallons hot water due to solar water heater	3,000 gallon rainwater cistern	80 gallons hot water
Approximate Demand		2,627 gallons in 2013 (7.2 gallons/day on average)	26,000 gallons/yr (65-80 gallons/day)	5,000 - 8,000 gallons/yr[†]	110,000 gallons/yr[‡] (>300 gallons/day)
Water Age Estimate		8 days on average	Hydraulic retention time = 2.7 days in hot water storage tanks; higher in infrequently used lines	1-6 months, dependent on rainfall and use	Hydraulic retention time ~1 day in water heater
Water system		Cold line: plug flow Hot lines: continuous recirculation	Plug flow	Continuously mixed, Air-water interface storage	Plug flow
Disinfectant Residual		Chloramine	Chlorine	N/A	Chlorine
Primary Plumbing Material		Copper	Copper to manifold; PEX after manifold	Copper	Copper
In-building treatment		N/A	N/A	20 µm + 5 µm + GAC filter + UV inactivation	N/A
Residual (as Cl_2) 1st draw	Cold	0.01 -0 .04 mg/L	0.04 mg/L	N/A	0.71 mg/L
	Hot	0.0 - 0.03 mg/L	0.02 mg/L	N/A	0.06 mg/L
Residual (as Cl_2) flushed	Cold	<0.25 mg/L 3 min; 1.12 mg/L 80 min	0.17 mg/L 3 min; 1.15 mg/L 30 min	N/A	1.04 mg/L 3 min
	Hot	0.02 -0.03 mg/L 3 min	0.41 mg/L 5 min; 0.96 mg/L 50 min	N/A	0.06 mg/L 3 min
Cause of high water age		Long service pipe within building, high number of fixtures, infrequent use	Solar water heater with storage to pre-heat water before heat pump	Storage in cistern necessary, water age dependent on rainfall, use, and maintenance	Normal water age

[†] Estimated based on discussions with employees and observations made in the field
[‡] Average annual use for American Families

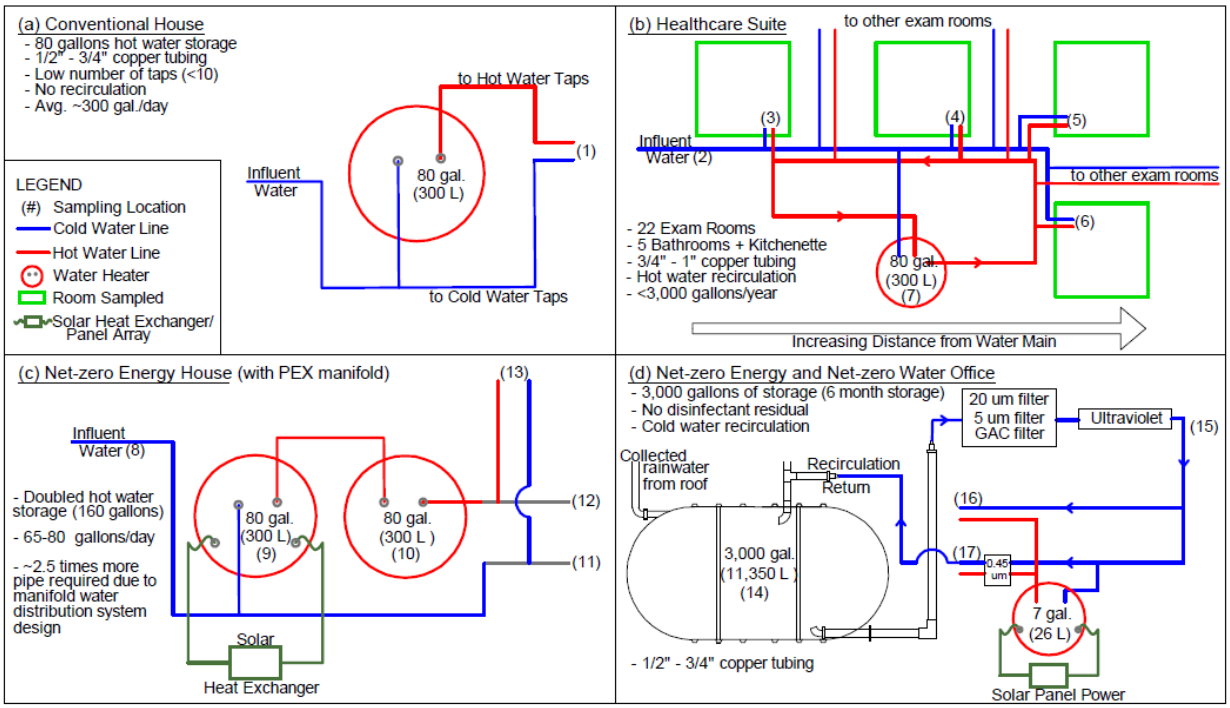

Fig. 3.1. Plumbing schematics and sampling locations for each field site
Note: Numbers 1-17 in parentheses indicate sampling locations referred to in Table 2.

(following a three minute flush) were 1 L. FastSpin DNA extraction kits (MP Biomedicals, LLC) were used, and protocols are described elsewhere (Wang et al., 2012).

Quantitative Polymerase Chain Reaction (qPCR). *Legionella* spp., *L. pneumophila*, *V. vermiformis*, *M. avium*, and total bacteria (16S rRNA) were quantified using previously developed and reported methods, and assays used are described elsewhere (Wang et al., 2012). Deviation from Wang et al. include a 1:10 dilution, optimized to minimize qPCR inhibition. All values are reported as log(gene copies/mL + 1). Samples with detectable but non-quantifiable numbers were recorded as 0.5 log gene copies/mL.

Statistical Analysis. Non-parametric Mann-Whitney U-tests were performed on non-transformed qPCR data to identify statistically significant differences in the concentrations of bacterial genes where applicable (p-value < 0.05).

RESULTS

This study provided detailed documentation of the potable water systems in three innovative green buildings (Fig. 3.1). After reviewing the characteristics of each water system and closely examining trends in disinfectant residuals within the buildings, we provides an overview of microbial sampling from each building.

Survey of Building Water Systems. *Water Age.* A survey of the three green buildings revealed extremely high water age associated with innovative features intended to conserve water and energy. The net-zero rainwater office building offered on-site storage of up to 11,350 L (3,000

gallons) to provide sufficient water through regular periods without rain. Demand was estimated to be 1,700 L/month (450 gallons/month) during fall/spring and up to 5,500 L/month (1,450 gallons/month) during the summer, resulting in water age ranging from 2-6.7 months. The healthcare suite uses only 10.6 L/m^2/year (0.26 gallons/ft^2/year), which is 60 times lower than typical commercial buildings (BOMA BEst, 2014). Very low use at each tap in patient exam rooms, coupled with large diameter pipes stipulated by plumbing code, resulted in an average overall premise plumbing water age of 8 days. At the net-zero energy house, the solar water heater increases the hot water storage and hot water age from < 1 day to ~2.7 days. The conventional home used more than 4 times more water than the net-zero energy house studied.

Temperature Profiles. Temperature profiling of the green buildings generally revealed the need for very extensive flushing to draw fresh distribution system water to the point of use. In general, temperatures were in the ideal growth range for OPPPs (35-42° C; WHO, 2014). In the healthcare facility, with 17° C water in the distribution main, 70 minutes of continuous cold water flushing was required for water temperatures to drop below maximum cold water recommendations for OPPPs control (20° C; ASHRAE, 2000). The cold water lines at the net-zero energy house reached lower temperatures much faster (<5 minutes) than the healthcare facility, likely owing to the fact that it is a much smaller building. However, after thoroughly flushing the hot water system, the temperature in the solar "pre-heat" tank did not increase (on a partly cloudy day), whereas the electric water heater downstream recovered to its set point of 49 °C within an hour. Clearly, in the solar water heater there were long periods during which the water temperature was not sufficient to control regrowth (i.e., > 55 °C; Darelid et al., 2002). The net-zero rainwater office building was characterized by warm temperatures, even in the cold water, and flushing did not lower the temperature for this building. During the summer sampling, water temperatures were consistently >23° C, whereas groundwater systems are typically < 20 °C (EPA, 2013).

In-Building Treatment at the Net-Zero Rainwater Office. The net-zero rainwater office provided an opportunity to examine the effects of in-building treatment, which is a common strategy for managing water quality in green buildings. During normal operation, rainwater at the net-zero rainwater office was collected and passed through a 20 µm filter, a 5 µm filter, a granular activated carbon (GAC) filter, and then irradiated with ultra violet light at 253 nm at a dose of 30 mJ/cm^2. An additional 0.45 µm filter was installed at each potable tap (bathroom and kitchenette sinks). Water was stored in an 11,350 L (3,000 gallon) cistern that was recirculated at 19 L/min (5 gpm) through the treatment sequence twice a day for two hours (2,270 L (600 gallons) recirculated each time). This configuration created a completely mixed water system as opposed to the approximate plug flow that exists in conventional buildings. Twice a year, the water was treated with a high concentration of bleach (>50 ppm) for 24 hours, after which time the water was completely drained and primed with 5,680 L (1,500 gallons) of groundwater from an on-grid building nearby. Thus, the office building was not truly "net-zero" water, as advertised, due to extensive water demands for maintenance procedures.

Water Sources in the Net Zero Office. Water quality analysis revealed that alkalinity, calcium, and magnesium (~110 ppm as $CaCO_3$, ~12 ppm Mg^{2+}, and ~30 ppm Ca^{2+}) were much higher than expected for rainwater and were actually more representative of the groundwater used for maintenance. This validated the estimation that groundwater, rather than rainwater, made up 38-

58% of total water use in the facility. This estimate was based on interviews with occupants and observations made during sampling (Table B.1).

Disinfectant Residual Decay. A disinfectant residual was generally not maintained in the plumbing of the green buildings. At the healthcare suite, >80 minutes of flushing was necessary to establish a chloramine residual similar to that present in the main distribution system (Fig. 3.2a). Taps at these locations are typically used for 15-30 seconds at a time; therefore, it is unlikely that a chloramine residual is ever present in portions of this plumbing system. Approximately 30 minutes of flushing was required at the net-zero energy house to establish a residual in the cold water system during the first site visit. In contrast, the conventional home had a residual of 0.71 mg/L chlorine as Cl_2 in stagnant cold water. The water heaters at the net-zero house had <0.15 mg/L chlorine as Cl_2 during a 20 minute flush due to the added storage volume and water age associated with the solar heater (Fig. 3.1c).

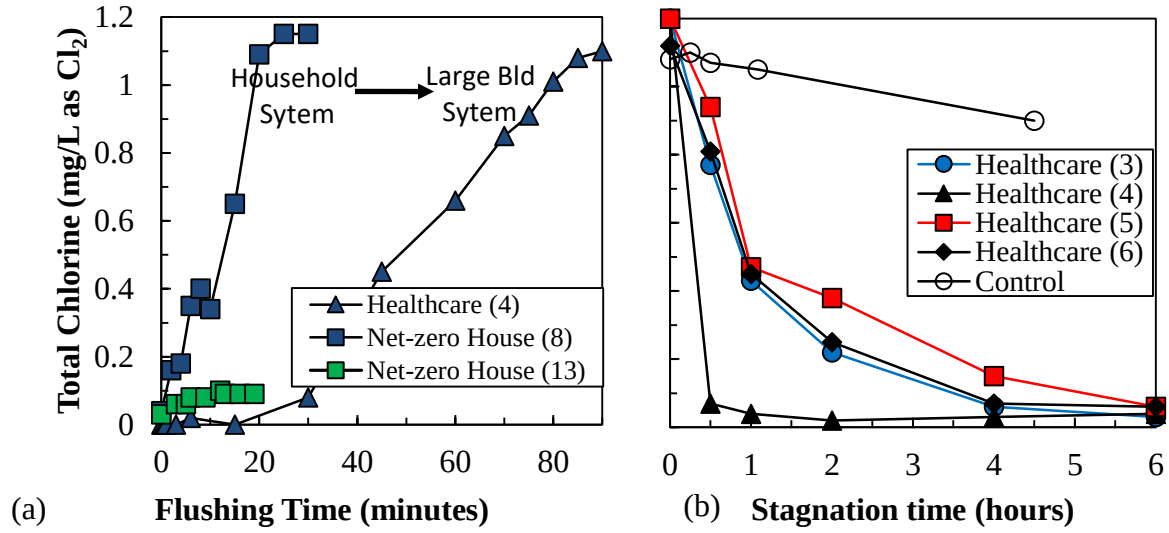

Fig. 3.2. Total chlorine residual concentrations as a function of flushing time and stagnation. (a) as a function of stagnation in one room at the healthcare suite (Large Building System above), a tap near the head of the building at the net-zero house (Household System above), and during three showering events at net-zero house, and (b) as a function of stagnation in several rooms at FS#1 (control represents identical water placed in a glass container with no head space). Sampling locations indicated in parenthesis in the figure legends correspond to sample locations depicted in Fig. 3.1.

During the second site visit at the net-zero house, influent cold water had a residual of 0.95 mg/L as Cl_2, indicating enough water was being used to maintain a residual during regular periods of low use such as during the night; however, this did not translate to chlorine residuals in the hot water system or at the point of use. Disinfectant residual never increased during the three showering events monitored (Net-zero House – Location 13 in Fig. 3.2A). Assuming the hot water had no residual, the 30 gallons of 40° C water used in the shower (of approximately 10 gallons of cold water at 20° C + 20 gallons of hot water at 49° C) is predicted to have a disinfectant residual of 0.31 mg/L as Cl_2. Instead, the highest concentration observed was 0.10 mg/L as Cl_2, which is 68% lower than expected. Therefore, the chlorine residual was either rapidly decaying as it flowed through the short pipes to the shower (via reactions with the pipe material or biofilms) or an instantaneous chlorine demand was present in water from the hot water system.

The healthcare suite also showed evidence of very rapid disinfectant residual decay. In all four exam rooms monitored, the first-order decay rate of chloramine in the plumbing was 20-144 times greater than in well-flushed (>80 minutes) water collected from the same tap and placed into a glass container with no headspace (i.e., "Control," Fig. 3.2B). The highest rate of decay (sample location 4) was observed in a sink that had been recently replaced, where the newer plumbing material may have been more reactive than older materials (Nguyen et al., 2012).

Microbial Constituents. 16S rRNA genes, *V. vermiformis*, *Legionella* spp., *and M. avium* were detectable at all green building field sites (Table 3.2). Total bacterial levels, as represented by 16S rRNA genes, ranged from 400 gene copies/mL in well-flushed water at the conventional house to 6.46×10^7 gene copies/mL (5-log higher) in a stagnant cold water sample at the net-zero energy house. *V. vermiformis*, *Legionella* spp., and *M. avium* ranged from below detection in well-flushed samples to 2.57×10^5, 9.33×10^5, and 4.90×10^5 gene copies/mL, respectively, in stagnant or hot water samples. *L. pneumophila* was detected in nine samples but was never above the quantification limit.

Increased OPPP Gene Marker Detection during Stagnation. At all field sites, there were higher total bacterial genetic markers in stagnant samples than flushed samples. The median increase in 16S rRNA gene copies/mL was 2.8 log (p-value 0.007; $n_{stagnant}$ =18, $n_{flushed}$=10). At the healthcare suite, there was also a median increase of 1.64 log in 16S rRNA gene copies/mL in distal hot water sample taps (sample sites 3-6) compared to water collected directly from the water heater (sampling site 7; Table 3.2), but the number of samples was insufficient to conduct statistical confidence testing.

The effects of prolonged stagnation in newly commissioned plumbing systems are effectively illustrated by comparing the first (prolonged stagnation before regular use) and second (regular use) site visits at the net-zero energy house. Overall levels of bacterial genetic markers decreased by up to 1.13 logs with regular water use at sample locations 9-11, but not location 12 (the hot water manifold). The conventional house receiving the same municipal water with a 2-fold reduction in water age yielded >20X less 16S rRNA gene copies/mL in stagnant cold water samples relative to the net-zero energy house during regular use. In addition, levels of *Legionella* spp., *M. avium* and *V. vermiformis* gene copies in the conventional house were below the quantification limit in stagnant samples and below the detection limit in flushed samples, whereas detectable levels of these genetic markers were frequently present in hot and cold stagnant and flushed shower samples in the net-zero energy house (Table 3.2). Concentrations of target markers decreased multiple orders of magnitude (1.84-3.11 log gene copies/mL) during the shower event flushing at the net-zero energy house (Table 3.2).

In contrast, changes in median bacterial 16S rRNA genes between stagnant and flushed samples at the net-zero rainwater office were small in magnitude (0.41 log gene copies/mL), indicating that this universal marker for bacteria was more evenly spread throughout the system (sample sites 14-17; Table 3.2). It should be noted that UV treatment systems can damage DNA such that it is no-longer detectable by qPCR, but such damage is can be repaired by viable bacteria (McKinney and Pruden, 2012).

Table 3.2. Log of qPCR results (log of gene copies/mL) for samples at all field locations

Location	Sample Description		Sample Location[†]	16S rRNA	V. vermiformis	Legionella spp.	M. avium
Healthcare	Cold Water	Influent	2	4.00	BD[¶]	BD	BD
	Cold Water	Stagnant	3-6	7.33 (7.08)[‡]	4.87 (4.30)	5.20 (5.29)	BQL
		Flushed	3-6	6.84 (4.25)	3.59 (1.82)	4.68 (4.46)	BQL
	Hot Water	Top of Heater	7	4.10	BD	BQL	BQL
		Bottom of Heater	7	3.07	0.78	3.16	3.43
	Hot Water	Stagnant	3-6	6.74 (6.66)	4.61 (4.44)	5.13 (5.00)	3.22 (3.31)
Net-zero House	Influent	Flushed	8	3.30	BD	BD	BD
	Solar Heater	Visit #1	9	5.08	BQL[*]	3.81	2.98
		Visit #2	9	4.18	BD	BQL	BQL
	Water Heater	Visit #1	10	5.40	BQL	3.54	3.11
		Visit #2	10	5.03	BD	BQL	BQL
	Cold Manifold	Stagnant, Visit #1	11	7.81	BQL	4.67	BQL
		Stagnant, Visit #2	11	6.68	3.44	3.73	BQL
	Hot Manifold	Stagnant, Visit #1	12	7.59	2.88	4.30	3.16
		Stagnant, Visit #2	12	7.69	BD	5.97	BQL
	Simulated Shower	Stagnant	13	5.44	2.86	3.11	BQL
		Flushed (10 gallons)	13	4.16	2.40	2.57	BQL
		Flushed (30 gallons)	13	3.60	BQL	BQL	BQL
Net-zero Office	Cistern (storage)	N/A	14	6.96	2.60	4.36	2.00
	Post-Treatment	Flushed 3 min	15	6.36	3.43	4.26	2.61
	Cold Potable	Stagnant	17	6.77	4.49	4.58	2.73
		Flushed 3 min	17	6.51	3.00	3.48	BQL
	Hot Potable	Stagnant	17	6.90	4.20	4.28	2.65
		Flushed 3 min	17	6.52	2.26	3.34	3.32
	Cold Non-potable	Stagnant	16	6.95	2.93	3.32	3.69
Conventional House	Cold Water	Stagnant	1	5.32	BQL	BQL	BQL
		Flushed 3 min	1	2.61	BD	BD	BD
	Hot Water	Stagnant	1	2.91	BQL	BQL	BQL
		Flushed 3 min	1	2.83	BD	BD	BD

[†] Sampling Location numbers refer to locations depicted in Fig. 3.1.
[‡] Average (and median) of the four exam rooms is reported.
[¶] BD = Below Detection
[*] BQL = Below Quantification Limit

Targeted Sampling of Water Heater at the Healthcare Facility. The top and bottom of the water heater at the healthcare suite was sampled, but such samples were not accessible at the other facilities. Interestingly, samples from the bottom of the water heater had high concentrations of genetic markers of both *M. avium* (>2,600 gene copies/mL) and *Legionella* spp. (>1,500 gene copies/mL), while concentrations were below the quantification limit in water at the top of the water heater. Although this was not confirmed by sampling in this building, one hypothesis for elevated levels of *Legionella* spp. and *M. avium* in the bottom of the water heater is that this environment is conducive to regrowth due to warm (not hot) temperatures and high levels of nutrients (Alary and Joly, 1991; Brazeau and Edwards, 2011b).

High Prevalence of OPPPs at the Net-zero Rainwater Office. Quantifiable levels of *V. vermiformis* and *Legionella* spp. gene copies were detectable in all samples and *M. avium* was above the quantification limit in 86% (n= 7) of samples at the net-zero rainwater office. Collectively across the other sites, only 55%, 68%, and 26% (n=31) of samples were above the quantification limit for gene copies corresponding to *V. vermiformis, Legionella* spp., and *M. avium*, respectively.

DISCUSSION

This study reveals cause for concern about the public health implications of green building water systems, particularly with respect to potential creation of conditions ideal for the proliferation of OPPPs. Given the rapid expansion of green building construction at a time when OPPPs are now the primary source of waterborne disease outbreaks, fundamental research is needed to guide green building science down a path that protects public health while also conserving water and energy.

Rapid residual decay. Absence of disinfectant residuals and its rapid decay in green buildings was a major finding of this study. Several factors might have contributed to the high rate of chloramine decay in the healthcare suite in particular, including increased abiotic decay due to elevated temperatures, reactions with cupric ions, plumbing materials, or biofilm and biotic decay due to microbial growth (Nguyen et al., 2012; Kelley et al., 2014; Sathasivan et al., 2009; Zhang and Edwards, 2009; Vikesland et al., 2001; Margerum et al., 1994; LeChevallier et al., 1990 Hallam et al., 1999; Powers, 2001). In the healthcare suite, temperatures at cold taps were sometimes elevated beyond room temperature (>5 °C) and there was evidence of low levels of nitrification (Table B.2). Although no direct quantification of biofilms was made, there were sometimes over 10,000 times more 16S rRNA genes in stagnant versus flushed samples, confirming significant levels of microbial activity in the stagnant pipes serving the taps compared to the water main (Lautenschlager et al., 2010).

It was previously thought that rapid residual decay is only rarely observed in buildings (Zhang and Edwards, 2009; Edwards et al., 2005); however, these results, in concert with Nguyen et al. (2012), give rise to the concern it may be commonplace in premise plumbing with high water age. Overall, disinfectant residual decay rates in these buildings were 2-16 times greater than reported in previous work in a green plumbing system with a chloramine residual, for which serious problems with lead and consumer complaints of the taste and odor of the water had been reported.[6] It is clear that the reputation chloramine has earned in providing a stable residual for main distribution systems does not always extend to building plumbing systems (Zhang and Edwards, 2009).

Total Bacterial Levels. Levels of 16S rRNA genes in stagnant samples from green buildings were orders of magnitude higher than what is typically observed in premise plumbing. A companion survey of distal taps in conventional residences across two chloramine distribution systems using the same qPCR assay yielded levels that were 2-4 orders of magnitude lower than those measured in the green buildings (Wang et al., 2012). A study comparing total bacteria via flow cytometry in stagnant to flushed water samples showed up to a 2 log reduction from 8×10^5 cell counts/mL in total bacteria over a 40 L flushing volume in cold water taps (Lipphaus et al., 2014). The cold water in the conventional home, sampled herein as a control, also harbored about three orders of magnitude less overall 16S rRNA genes than the green buildings. It is apparent that as water age approaches that of conventional buildings overall bacterial regrowth approaches the same low levels. Building designers should be aware of designs that increase water age and water storage, and minimize their effect where possible.

Occurrence of Legionella markers. The levels of *Legionella* spp. gene markers detected at all three green building sites were high. The average *Legionella* spp. level in this work was 8.91×10^4 gene copies/mL compared to an average of 100 (n=54) and 2.3×10^3 (n=90) gene copies/mL in samples with quantifiable levels across sampling conventional homes in two distribution systems reported in the companion survey (Wang et al., 2012). This trend is expected, given the higher water age and lack of disinfectant residuals in the green buildings compared to the conventional residences (<0.15 mg/L as Cl_2 in this work compared to >2 mg/L as Cl_2 in the survey of conventional homes; Wang et al., 2012).

The higher prevalence of *Legionella* detected in the rainwater system is of particular concern, as only a small number of studies on the presence of OPPPs in rainwater have been conducted (Ahmed et al., 2010). The focus of these studies has been *L. pneumophila*, and concentrations are typically very low (<10 cells/mL) in comparison to those of *Legionella* spp. observed in this study. Considering there are up to 25 infectious species of *Legionella*, and given serologic links of disease outbreak to rainwater cisterns, the potential for rainwater storage to contribute to *Legionella* infections merits further examination (WHO, 2014; Simmons et al., 2008; Schlech et al., 1985). Extensive colonization of the rainwater system with *V. vermiformis* is also of concern, as it is an established host for *Legionella* amplification (Thomas and Ashbolt, 2011). Even if pathogenic microbes are inactivated by the UV treatment in this facility, the amoeba downstream could serve as a means for re-amplification.

Temperature and Aesthetics. Temperature is also a critical overarching factor that influences the growth rates of microorganisms; for example, *Legionella* growth is reportedly inhibited below 20 °C and above 52 °C (ASHRAE, 2000). Temperature profiles in buildings can fail to achieve their targeted settings; therefore, characterizing a system's temperature profile is suggested as an informative first step in identifying areas of high risk (Bedard et al., 2015). Risks associated with lower temperature settings and storing warm/hot water must be assessed and balanced with conservation goals, in recognition that there are some necessary compromises at the nexus of public health, sustainable water, and sustainable energy. International plumbing codes have recently put limits on the amount of hot water volume in pipes to distal taps, which might help to reduce water age and associated problems in the future, especially if similar considerations are made for cold water lines as well (IgCC, 2012; IAPMO, 2012). However, smaller diameter pipes

have higher surface area to volume ratios, which might create another set of problems relative to biofilm formation and disinfectant residual demand.

Aesthetics can also be a critical factor in the ultimate success of green building water systems. For example, waterless urinals have received poor consumer reviews and, in many cases, are replaced well before reaching their design life (Chambers, 2013). Other aesthetic factors could also hinder the success of green building water systems if not considered carefully in advance. In this study, aesthetics of the warm (23-29 °C) ambient temperature of the cold water could be undesirable to some users who are accustomed to cooler water for drinking, especially compared to the lower temperatures provided from conventional on-demand well systems (Whelton and Dietrich, 2004). In addition, there is increased likelihood to develop taste- and odor-causing compounds due to increased water age and microbial growth at elevated temperatures (Lautenschlager, 2010; Malleville and Suffet, 1987).

A Temporary Practical Solution and Limitations. Regular flushing of water is one practical approach to addressing water quality and public health concerns in green buildings connected to water mains. Prior work in a LEED-Gold certified building with very high water age solved problems with elevated lead and microbial growth by regularly flushing a small volume of water (3 minutes of flushing every 6 hours, < 1% of the total daily flow in the green building) to regularly introduce some fresh water with disinfectant into the system (Nguyen et al., 2012; Elfland et al., 2010). While flushing and wasting water may be viewed to conflict with the conservation goals of green buildings, it may serve as a temporary solution to maintaining a disinfectant residual within buildings and assist microbial regrowth control. In the prior case study, temporary flushing provided semi-permanent benefits. Consistently maintaining a disinfectant residual is a known master variable governing microbial quality of drinking water and microbes recover quickly after the residual decays (Bédard et al., 2014).

Microbial regrowth control in off-grid net-zero water facilities, on the other hand, may prove to be an even greater challenge because simple flushing will not introduce a disinfectant residual from the water main. Intermittent/continuous recirculation in off-grid systems, which would decrease water stagnation but not water age, is not expected to improve water quality since microbes can accumulate and are not as readily washed from the system compared to conventional plug-flow configurations. Increased *Legionella* spp. growth has been observed in continuously recirculating versus completely stagnant conditions in a controlled pipe loop at room temperature with no disinfectant residual, conditions that are similar to the system layout observed at the net-zero rainwater office (Liu et al., 2006). Other work also suggests that recirculation can be a determining factor associated with increased detection of *Legionella* (Pryor et al., 2014). In such circumstances, maintaining higher hot water temperatures and dosing a disinfectant within the building may be necessary to control OPPP regrowth, though there are concerns related to efficacy and practicality of in-building disinfection systems (Muraca et al., 1990; Lin et al., 1998; Kim et al., 2002; Rhoads et al., 2014).

CONCLUSIONS

This work raises concerns with respect to the chemical and microbiological stability of water quality in systems with high water age. The green buildings sampled had exceptionally high water

age, and it appears that elevated water age is inherent to achieving sustainability goals of many green building plumbing systems.

The rapid rate of disinfectant loss in buildings with high water age needs to be better understood and addressed. A temporary solution to water quality problems in green buildings connected to drinking water mains involves routine flushing to maintain disinfectant residuals, improve corrosion control, and achieve temperature targets. Long-term solutions include determining if specific water chemistries employed by the utility can retain chemical and microbial stability of water in the distribution and premise plumbing system. Further, research is needed to determine if there are specific premise plumbing devices or plumbing materials that should be avoided.

Conservation strategies employed at each green building created both hot and cold water temperature profiles in plumbing that are conducive to OPPP growth during stagnation. Therefore, strategies for effectively maintaining target temperature profiles in buildings need to be explored. Design of green buildings with water conservation features should minimize overall water age and eliminate unnecessary water storage and should give special attention to avoiding conditions conducive to OPPPs. Determining how severe these anticipated problems are, and how widespread they are likely to become, is a high research priority given the massive investment society is making in green buildings.

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REFERENCES

A. Pruden, M.A. Edwards, J.O. Falkinham, III. Research Needs for Opportunistic Pathogens in Premise Plumbing: Experimental Methodology, Microbial Ecology and Epidemiology. Water Research Foundation Project 4379, Denver, CO, 2012, http://www.waterrf.org/PublicReportLibrary/4379.pdf (accessed September, 2015).

A. Sathasivan, J. Chiang, P. Nolan. Temperature dependence of chemical and microbiological chloramine decay in bulk waters of distribution system. *Water Science & Technology,* 2009, **9**(5), 493-499.

A.J. Whelton, A.M. Dietrich. Relationship between intensity, concentration, and temperature for drinking water odorants. *Water Research,* 2004, **38**(6), 1604-1614.

B. Chambers. Understanding the Selection and Use of Water Related Innovations in Green Buildings, Master's Thesis, Virginia Tech, Blacksburg, VA, December 2013. Available: https://vtechworks.lib.vt.edu/handle/10919/25298 (accessed May 2015).

B.R. Kim, J.E. Anderson, S.A. Mueller, W.A. Gaines, A.M. Kendall. Literature review—efficacy of various disinfectants against *Legionella* in water systems. *Water Research*, 2002, **36**(18), 4433-4444.

Building Owners and Managers Association Building Environment Standards (BOMA BEst). BOMA BEst Energy and Environment Report – The National Green Building Report, Toronto, ON, 2014. http://www.bomabest.com/wp-content/uploads/BBEER-2014-Full-Report.pdf (accessed December 2014).

C. Elfland, P. Scardina, M.A. Edwards. Lead-Contaminated Water from Brass Plumbing Devices in New Buildings (PDF). *Journal of the American Water Works Association.* 2010, **102**(11), 66-76.

C. Nguyen, C. Elfland, and M.A. Edwards. Impact of advanced water conservation features and new copper pipe on rapid chloramine decay and microbial regrowth. *Water Res.,* 2012**,** **46**(3), *611-621*; DOI:10.1016/j.watres.2011.11.006

C.K. Nguyen, K.A. Powers, M.A. Raetz, J.L Parks, M.A. Edwards. Rapid free chlorine decay in the presence of Cu $(OH)_2$: Chemistry and practical implications. *Water Res.,* 2011, **45**(16), 5302-5312.

C.W. McKinney, A. Pruden, A. Ultraviolet Disinfection of Antibiotic Resistant Bacteria and Their Antibiotic Resistance Genes in Water and Wastewater. Environmental Science & Technology, 2012, **46**(24), 13393-13400. DOI:10.1021/es303652q.

Centers for Disease Control (CDC). Legionellosis -United States, 2000-2009. *Morbidity and Mortality Weekly Report.* 2011 60(32), 1083-1086.

D. Charron, E. Bédard, C. Lalancette, C. Laferriere, M. Prevost. Impact of Electronic Faucets and Water Quality on the Occurrence of *Pseudomonas aeruginosa* in Water: A Multi-Hospital Study. *Infection Control & Hospital Epidemiology*, 2014, **36**(3), 311-319.

D.W. Margerum, L.M. Schurter, J. Hobson, E.E. Moore. Water chlorination chemistry: nonmetal redox kinetics of chloramine and nitrite ion. *Environmental Science & Technology,* 1994, **28**(2), 331-337.

E. Bédard, D. Charron, C. Lalancette, E. Déziel, M. Prévost, M. Recovery of *Pseudomonas aeruginosa* culturability following copper-and chlorine-induced stress. *Federation of European Microbiological Societies Microbiology Letters*, 2014, **356**, 226-234. DOI: 10.1111/1574-6968.12494

E. Bédard, S. Fey, D. Charron, C. Lalancette, P. Cantin, P. Dolcé, C. Laferrière, E. Déziel, M. Prévost. Temperature diagnostic to identify high risk areas and optimize *Legionella pneumophila* surveillance in hot water distribution systems. *Water Res.,* 2015, **71**, 244-256. DOI:10.1016/j.watres.2015.01.006

E.R. Sydnor, G. Bova, A. Gimburg, S.E. Cosgrove, T.M. Perl, L.L. Maragakis,. Electronic-eye faucets: *Legionella* species contamination in healthcare settings. *Infection Control and Hospital Epidemiology*, 2012, **33**(3), 235-240.

G. Simmons, S. Jury, C. Thornley, D. Harte. J. Mohiuddin, M.A. Taylor. Legionnaires' disease outbreak: A water blaster and roof-collected rainwater systems. *Water Res.,* 2008, **42**(6), 1449-1458.

H. Albrechtsen. Microbiological investigations of rainwater and graywater collected for toilet flushing. *Water Science and Technology,* 2002, **46**(6-7), 311-316.

H. Wang, M.A. Edwards, J.O. Falkinham, III, A. Pruden, A. Molecular survey of the occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa*, and amoeba hosts in

two chloraminated drinking water distribution systems. *Applied Environmental Microbiology,* 2012, **78**(17), 6285-6294.

H. Yapicioglu, T.G. Gokmen, D. Yildizdas, F. Koksal, F. Ozlu, E. Kale-Cekinmez, K. Mert, B. Mutlu, M. Satar, N. Narli, A. Candevir. *Pseudomonas aeruginosa* infections due to electronic faucets in a neonatal intensive care unit. *Journal of Pediatrics and Child Health,* 2012, **48**(5), 430-434.

International Association of Plumbing and Mechanical Officials (IAPMO). 2012 Green Plumbing & Mechanical Code Supplement, 2nd Edition.Ontario, CA. Available: www.iapmo.org (accessed May 2015).

International Code Council, Inc. 2012 International Green Construction Code (IgCC). Club Hills, IL. ISBN: 978-1-60983-059-5.

J. Darelid, S. Löfgren, B.E. Malmvall. Control of nosocomial Legionnaires' disease by keeping the circulating hot water temperature above 55° C: experience from a 10-year surveillance programme in a district general hospital. *Journal of Hospital Infection,* 2002, **50**(3), 213-219.

J. Hughes, M. Tiger, S. Eskaf, S.I. Berahzer, S. Royster, C. Boyle, D. Batten, P. Brandt, C. Noyes. Defining a Resilient Business Model For Water Utilities. Water Research Foundation Project 4366, Denver, CO, 2014.

J. Malleville, I.Suffet. Identification and Treatment of Tastes and Odours in Drinking Water. American Water Works Association Cooperative Report. AWWA, Denver, CO, 1987.

J.M. Thomas, N.J. Ashbolt. Do Free-Living Amoeba in Treated Drinking Water Systems Present an Emerging Health Risk? *Environ. Sci. Technol.,* 2011, **45**, 860-869.

K. Gordon, W. Olson. Retail Cost Recovery and Rate Design in a Restructure Environment. Technical Report for Edison Electric Institute: Washington, D.C., 2004, www.eei.org (accessed May 19, 2015).

K. Lautenschlager,N. Boon, Y. Wang, T. Egli, F. Hammes. Overnight stagnation of drinking water in household taps induces microbial growth and changes in community composition. *Water Research,* 2010, **44**(17), 4868-4877.

K.A. Powers. Aging and copper corrosion by-product release: role of common anions, impact of silica and chlorine and Mitigating release in New pipe. Master's Thesis, Virginia Tech, Blacksburg, VA, 2001.

K.M. Kelley, A. Stenson, R. Dey, A.J. Whelton. Release of drinking water contaminants and odor impacts caused by green building cross-linked polyethylene (PEX) plumbing systems. *Water Res.*, 2014, **67**, 19-32.

Legionella Control Guideline. American Society for Heating, Refrigeration, and Air-Conditioning Engineers (ASHRAE), Atlanta, GA, 2000 Available: http://legionella.org/publications/non-visible/ashrae-guideline-12-2000/ (accessed December 2014).

M. Alary, J.R. Joly. Risk factors for contamination of domestic hot water systems by legionellae. *Applied Environmental Microbiology,* 1991, **57**(8), 2360-2367.

M. Pryor, S. Springthorpe, S., Riffard, T. Brooks, Y. Huo, G. Davis, and S.A. Sattar. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. *Water Science & Technology*, 2004, **50**(1), 83-90.

M.A. Edwards, B. Marshall, Y. Zhang, Y.J. Lee. Unintended consequences of chloramine hit home. In proceedings of the *Water Environment Federation*, 2005(1), 240-256.

M.A. Edwards, D. Bosch, G.V. Loganathan, A.M. Dietrich. The future challenge of controlling distribution system water quality and protecting plumbing infrastructure: focusing on consumers. In Proceedings of the International Water Association Leading Edge Conference, Noordwijk, Netherlands, 2003.

M.A. Edwards, W.J. Rhoads, A. Pruden, A. Pearce, J.O. Falkinham, III. Green water systems and opportunistic premise plumbing pathogens. *Plumbing Engineer*, 2014. Available: http://plumbingengineer.com/nov_14/edwards_feature.php (accessed May 2015).

M.W. LeChevallier, C.D. Lowry, R.G. Lee. Disinfecting biofilms in a model distribution system. *Journal of the American Water Works Association*, 1990, **82**(7) 87-99.

McGraw Hill Construction. Green Outlook: Green Trends Driving Growth. McGraw Hill Construction Outlook 2011: Industry Forecasts and Trends. **2011,** http://aiacc.org/wp-content/uploads/2011/06/greenoutlook2011.pdf (accessed December 2014).

N.B. Hallam, J.R. West, C.F. Forster, J. Simms. The potential for biofilm growth in water distribution systems. *Water Research*, 2001, **35**(17), 4063-4071.

National Research Council (NRC). Drinking water distribution systems: assessing and reducing risk, Washington, DC, 2006.

Navigant Research. Energy Efficient Buildings: Global Outlook Advanced HVAC, Lighting, Water Efficiency, Building Controls, Energy Management Systems, and Energy Service Companies: Global Market Analysis and Forecasts, 201, http://www.prnewswire.com/news-releases/energy-efficient-buildings-global-outlook-advanced-hvac-lighting-water-efficiency-building-controls-energy-management-systems-and-energy-service-companies-global-market-analysis-and-forecasts-300013046.html (accessed May 19, 2015).

P. Lipphaus, F. Hammes, S. Kötzsch, J. Green, S. Gillespie, A. Nocker. Microbiological tap water profile of a medium-sized building and effect of water stagnation. *Environmental Technology*, 2014, **35**(5), 620-628.

P. Scardina, M.A. Edwards, D. Bosch, G. Loganathan, S. Dwyer, S. Assessment of Non-Uniform Corrosion in Copper Piping. AWWA Research Foundation, Denver, CO, 2008.

P.J. Vikesland, K. Ozekin, R.L. Valentine. Monochloramine decay in model and distribution system waters. *Water Research,* 2001**, 35**(7), 1766-1776.

P.W. Muraca, V.L. Yu, A. Goetz. Disinfection of water distribution systems for *Legionella*: a review of application procedures and methodologies. *Infection Control and Hospital Epidemiology.* 1990, **11**(2), 79-88.

R.H. Brazeau, M.A. Edwards. A review of the sustainability of residential hot water infrastructure: Public health, environmental impacts, and consumer drivers. *Journal of Green Buildings.* 2011a, **6**(4), 77-95.

R.H. Brazeau, M.A. Edwards. Role of Hot Water System Design on Factors Influential to Pathogen Regrowth: Temperature, Chlorine Residual, Hydrogen Evolution, and Sediment. *Environmental Engineering Science,* 2011b, **30**(10), 617-627.

T.A. Bartrand, J.J. Causey, J.L. Clancy. *Naegleria fowleri*: An emerging drinking water pathogen. *J. - Am. Water Works Assoc.*, 2014, **106**(10), 418-432.

United States Environmental Protection Agency (USEPA). Average Temperature of Shallow Ground Water. U.S. Environmental Protection Agency Ecosystems Research Webpage. 2013. Available: http://www.epa.gov/athens/learn2model/part-two/onsite/ex/jne_henrys_map.html (accessed December 2014).

United States Environmental Protection Agency (USEPA). Effects of Water Age on Distribution System Water Quality. Office of Ground Water and Drinking Water: Cincinnati, OH, 2002, http://www.epa.gov/ogwdw/disinfection/tcr/pdfs/whitepaper_tcr_waterdistribution.pdf. (accessed May 2015).

United States Green Build Council (USGBC). Directory –Projects, http://www.usgbc.org/projects (accessed December 2014).

United States Green Build Council (USGBC). Guide to LEED Certification, http://www.usgbc.org/cert-guide (accessed January 2015).

W. Ahmed, A. Viertiz, A. Goonetilleke, T. Gardner. Health Risk from the Use of Roof-Harvested Rainwater in Southeast Queensland, Austrailia, as Potable or Nonpotable Water, Determined Using Quantitative Microbial Risk Assessment. *Applied Environmental Microbiology*, 2010, ***76****(22), 7382-7391*. DOI: 10.1128/AEM.00944-10.

W. Ahmed, F. Huygens, A. Goonetilleke, T. Gardner. Real-time PCR detection of pathogenic microorganisms in roof-harvested rainwater in Southeast Queensland, Australia. *Applied Environmental Microbiology*, 2008, **74**(17), 5490-5496.

W.F. Schlech, G.W. Gorman, M.C. Payne, C.V. Broome. Legionnaires' Disease in the Caribbean: An Outbreak Associated With a Resort Hotel. *Archives of Internal Medicine*, 1985, **145**, 2076-2079.

W.J. Rhoads, A. Pruden, A. Pearce, M.A. Edwards. Anticipating Water Quality and Infrastructure Impacts due to Green Buildings. *J. - Am. Water Works Assoc.*, 2015, **107**(4), *50-6.* DOI**:** http://dx.doi.org/10.5942/jawwa.2015.107.0058

W.J. Rhoads, A. Pruden, M.A. Edwards. Anticipating Challenges Associated with In-Building Disinfection for Control of Opportunistic Pathogens in Premise Plumbing. *Water Environment Research*, 2014, **86**(6), 540-549. **DOI:** http://dx.doi.org/10.2175/106143014X13975035524989

World Health Organization (WHO). *Legionella* and the Prevention of Legionellosis. ISBN 92 4 156297 8. Available: http://www.who.int/water_sanitation_health/emerging/legionella.pdf (accessed December 19, 2014).

Y. Lin, J. Stout, V.L. Yu, R. Vidic. Disinfection of water distribution systems for *Legionella*. *Seminars in Respiratory Infections.* 1998, **13**(2), 147-159.

Y. Zhang, M.A. Edwards. Accelerated Chloramine Decay and Microbial Growth by Nitrification in Premise Plumbing. *Journal of the American Water Works Association*, 2009, **101**(11), 51-62.

Y. Zhang, N. Love, M.A. Edwards. Nitrification in drinking water systems. *Critical Reviews in Environmental Science & Technology*, 2009**, 39**(3), 153-208.

Z. Liu, Y.E. Lin, J.E. Stout, C.C. Hwang, R.D. Vidic, V.L. Yu. Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. *Journal of Applied Microbiology*, 2006, **101**(2), 437-442.

APPENDIX B - SUPPLEMENTAL INFORMATION FOR CHAPTER 3

Table B.1. Calculation of percentage of water used for maintenance relative to total annual water demand

Total Volume of Water Used for Maintenance	3000	gallons/yr	(using 1500 gallons to prime system 2X/yr after disinfection)
Total Volume of Water Used by Consumers	5200	gallons/yr	(Calculation based on reported use)
	7950	gallons/yr	(Calculation based on estimated use)
Percent Used for Maintenance	58%		(Reported Use)
	38%		(Estimated Use)

Notes about Calculation based on reported use:
1. Facility reports to have 10 full time employees, that each use 520 gallons/year
2. 10 employees * 520 gallons/yr-employee = 5200 gallons/yr

Notes about Calculation based on estimated use:
1. Inquiry revealed that upwards of 20 additional people are on-site during the summer months, to conduct research
2. Each full-time employee was assumed to use the 520 gallons/year, so again 5200 gallons.
3. To estimate the use due to the extra people on-site, the 520 gallons/employee-year was divided into gallons/work day (520 gallons/employee-year / 50 work weeks/year / 5 work days/week = 2.08 gallons/employee/day.
4. For the additional 20 people during the summer months, 2.08 gallons/employee-work day was used to estimate additional use (2.08 gallons/employee-work day * 20 additional employees * 66 work days = 2750 gallons/summer)
5. Total use = 2750 + 5200 = 7950

Table B.2 Evidence of mild nitrification at the LEED healthcare suite

Parameter	Influent	Exam Rm1 - Stagnant	Exam Rm1 - Flushed	Exam Rm2 - Stagnant	Exam Rm2 - Flushed	Exam Rm3 - Stagnant	Exam Rm3 - Flushed	Exam Rm4 - Stagnant	Exam Rm4 - Flushed
pH	7.1	6.8	7.0	6.5	6.6	6.5	6.7	6.5	6.7
Temp. (°C)	18	22	24	23	24	23	22	24	24
Total Chlorine (ppm Cl_2)	1.2	0.04	0.08	0.01	0.19	0.02	0.22	0.01	0.15
NH_3 (ppm as N)	0.36	0.1	0.35	0.34	0.37	0.26	0.38	0.2	0.39
NO_2^- (ppm as N)	0.009	0.002	0.008	0.015	0.006	0.025	0.005	0.024	0.009
NO_3^- (ppm as N)	1.21	1.47	1.35	1.21	0.97	1.40	1.06	1.67	1.03
Alkalinity (ppm as $CaCO^3$)	50	56	49	57	49	59	48	57	48

1. Slight drop in pH in stagnant vs flushed samples
2. Change in nitrogen speciation; decrease in NH3in stagnant vs flushed samples
3. Increase in alkalinity of 6-9 mg/L as CaCO3
4. Biological Activity Reaction Tests (BARTs) positive for nitrifying bacteria
5. Absence of chlorine and elevated temperatures give the opportunity for growth

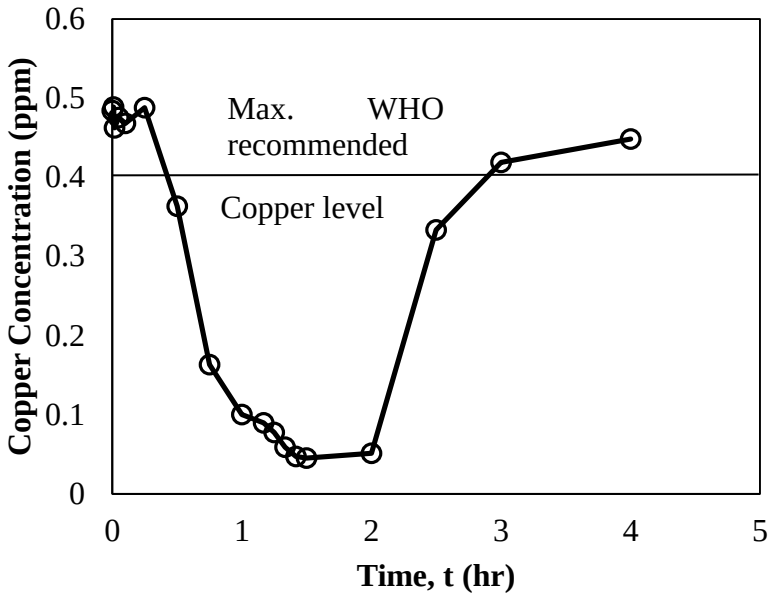


Fig. B.1. Copper concentration at a tap as a function of flushing ($0 < t < 2$ hours), followed by stagnation ($t > 2$ hours)

Notes: Further evidence of nitrification at the tap as opposed to in well-flushed distribution system water.

Below: Water quality data from the field sites.

Net-Zero House Water Use and Quality Data:

1. Details of total water use during water draws in simulated net-zero energy building

Day of Week	Total Use (gal)
Monday	65.21
Tuesday	65.21
Wednesday	86.94
Thursday	65.21
Friday	65.21
Saturday	86.64
Sunday	65.21
TOTAL	499.63

Note: the only cold water used was to temper the hot water to the desired temperature during simulated use. Typical household use in the U.S. is about 300 gallons/day

2. Water quality data as a function of hot water flushing during the first site visit

Hot Flushing Time (min)	Solar Water Heater Temperature (° C)	Electric Water Heater Temp (° C)	Chlorine (ppm Cl_2)	pH	TOC (ppm)
0	59.4	50.6	0.02	8.9	1.26
5	54.4	36.7	0.33	8.3	1.81
10	43.9	37.8	0.36	8.3	1.56
15	37.8	37.8	0.36	8.1	1.69
20	31.1	33.3	0.37	8.0	1.55
25	24.4	28.9	0.48	7.9	1.50
30	21.1	26.7	0.63	7.9	1.51
35	20.0	22.2	0.76	7.9	1.42
40	15.6	20.6	0.83	7.9	1.36
50	15.6	17.8	0.96	7.9	1.43

*TOC=Total Organic Carbon

3. Water quality data as a function of cold water flushing during the first site visit

Cold Flushing Time (min)	Cold Water Temperature (° C)	Chlorine (ppm Cl_2)	pH	TOC (ppm)
0	21.2	0.04	8.4	1.44
2	10.9	0.16	8.8	1.44
4	11.3	0.18	8.9	1.41
6	10.8	0.35	8.9	1.42
8	9.8	0.40	8.7	1.30
10	9.5	0.34	8.7	1.34
15	9.6	0.65	8.5	1.38
20	9.8	1.09	8.0	1.29
25	9.9	1.15	7.4	1.79
30	9.9	1.15	7.5	1.35

4. Water heater temperature recovery in the solar water heater and electric water heater as a function of stagnation time after both tanks had been thoroughly flushed during the first site visit

Recovery Time (min)	Solar Water Heater Temperature (° C)	Electric Water Heater Temp (° C)
0	15.6	17.8
13	16.7	25.6
36	17.8	37.8
60	17.8	48.8

5. Water quality of the showering water as a function of flushing during the second site visit; All water was collected from three consecutive simulating showing events from the same showerhead.

Shower Number	Approximate Volume Flushed (gallons)	Temperature (° C)	pH	Total Chlorine (ppm Cl_2)
1	0	27.6	7.5	0.03
	4	42.6	7.6	0.06
	8	43.5	7.6	0.06
2	8.5	38.3	7.7	0.08
	12.5	42.3	7.7	0.08
	16.5	41.8	7.7	0.1
3	17	37.8	7.5	0.09
	21	39.1	8.0	0.09
	25	42.8	7.6	0.09

6. Water quality from the hot and cold tap manifold and water heaters during the second site visit

Sample	Temperature (° C)	pH	Total Chlorine (ppm Cl_2)
Solar Water Heater	26.1	7.33	0.11
Electric Water Heater	26.2	7.25	0.02
Hot water manifold (stagnant)	30.1	7.44	0.05
Hot water manifold (flushed)	48.1	7.35	0.08
Cold water manifold (stagnant)	23.2	7.39	0.33
Cold water manifold (flushed)	22.2	7.62	0.95

Conventional House Water Quality Data:

1. Water quality data as a function of cold tap flushing

COLD TAP flushing time (min)	Temperature (° C)	pH	Total Chlorine (ppm Cl_2)
0	23.6	6.85	0.71
3	9.9	6.8	1.04
5	10.1	6.9	1.01
10	10.5	7.02	1.01
15	11.7	6.95	1.01
After 15 minutes stagnation	15.6	6.79	0.99

Notes: chlorine concentration was stable after overnight stagnation

2. Water quality data as a function of hot tap flushing

HOT TAP flushing time (min)	Temperature (° C)	pH	Total Chlorine (ppm Cl_2)
0	42.4	6.92	0.06
3	44.4	7.12	0.06
5	44.9	7.17	0.08
10	45.9	7.2	0.11
20	42.6	7.19	0.16
30	33	7.05	0.68
40	29.4	7.03	0.89
50	28.1	6.96	0.92
60	27.5	7.13	0.96
70	27.2	7.11	0.98
80	27.1	7.08	0.99
90	26.7	7.07	1.01
After 15 minutes stagnation	28.4	7.1	0.95

Notes: Chlorine residual was stable in hot tap during short-term stagnation, but did decay after overnight stagnation

Net-zero Rainwater Office Water Quality Data:

1. Temperature and pH by sampling location

Sample	Temperature (° C)	pH
Cistern	25	7.6
Post-Treatment	28	7.7
Men's Cold Stagnant	29	7.2
Men's Cold Flushed	26	7.6
Kitchen Cold Stagnant	26	7.3
Kitchen Cold Flushed	26	7.6
Janitor Cold Stagnant	26	7.6
Janitor Cold Flushed	26	7.6
Kitchen Hot Stagnant	45	7.3
Kitchen Hot Flushed	47	7.6
Men's Hot Flushed	49	

2. Alkalinity by sampling location

Sample Name	Alkalinity (mg/L as $CaCO_3$)
Cistern	104
Post Treatment	112
Men's Cold Stagnant	113
Men's Cold Flushed	113
Well Water	303

Table B.4. Additional information on qPCR assays used
The supplementary information from Wang et al., 2012 has all relevant assay information. Please see:
http://aem.asm.org/content/suppl/2012/08/08/AEM.01492-12.DCSupplemental/zam999103617so1.pdf
qPCR assay information:

Targeted Organisms	Targeted Genes	Sequences (5'-3')	Program		Amplicon (bp)	Refs
			Denaturation / Enzyme Activation	Denaturing / Annealing/ Extension		
Legionella spp.	23S rRNA	Leg23SF: CCCATGAAGCCCGTTGAA Leg23SR:ACAATCAGCCAATTAGTACGAGTTAGC Probe: HEX-TCCACACCTCGCCTATCAACGTCGTAGT	95 ºC for 2 min	40 cycles of 95 ºC for 5 s and 58.5 ºC for 10 s	92	1
M. avium	16S rRNA	MycavF: AGAGTTTGATCCTGGCTCAG MycavR: ACCAGAAGACATGCGTCTTG	98 ºC for 2 min	40 cycles of 98 ºC for 5 s and 68 ºC for 18 s	180	2
V. vermiformis	18S rRNA	Vv1227F: TTACGAGGTCAGGACACTGT Vv1728R: GACCATCCGGAGTTCTCG	98 ºC for 2 min	40 cycles of 98 ºC for 5 s and 72 ºC for 18 s	502	3
Total Bacteria	16S rRNA	BACT1369F: CGGTGAATACGTTCYCGG PROK: GGWTACCTTGTTACGACTT	98 ºC for 2 min	40 cycles of 98 ºC for 5 s and 55 ºC for 5 s	124	4

CHAPTER 4. ANTICIPATING CHALLENGES WITH IN-BUILDING DISINFECTION FOR CONTROL OF OPPORTUNISTIC PATHOGENS

William J. Rhoads, Amy Pruden, Marc A. Edwards

ABSTRACT

A new American Society of Heating, Refrigerating and Air-Conditioning Engineers (ASHRAE) standard for control of *Legionella* (ASHRAE Standard 188) emphasizes use of in-building disinfection techniques to reduce the exposure of at-risk consumers to opportunistic pathogens in premise plumbing (OPPPs). This standard and other recommendations for OPPP control have implications for scaling in and corrosion of plumbing systems, which can sometimes adversely affect the efficacy of the disinfection method and physical integrity of the plumbing system, prompting this proactive critical review of challenges associated with implementation of Standard 188.

KEY WORDS: In-building disinfection, thermal disinfection, pipe scaling, corrosion, ASHRAE 188, *Legionella*

INTRODUCTION

Waterborne disease originating from premise (i.e. building) plumbing is a growing concern (CDC, 2011; NRC, 2006). New standards have been developed to protect consumers against disease via thermal and chemical disinfection of building plumbing systems (ASHRAE, 2013). To avoid unanticipated negative consequences, proposed regulations must carefully consider practical impacts in terms of corrosion control, water scaling, operation/maintenance practices, energy conservation, and scalding. Although several reviews of the efficacy and application of in-building disinfection systems exist (Muraca et al., 1987; Lin et al., 2011; Lin et al., 1998), a comprehensive synthesis of possible secondary impacts of building-level treatments on premise plumbing infrastructure and efficacy of each treatment is needed.

Protection of premise plumbing assets, which are valued on the order of one trillion dollars (Edwards, 2004), while meeting intended public health goals of OPPP control is important (NRC, 2006; Brazeau and Edwards, 2012). New standards are necessary because there are limitations to utility control of pathogens within the far reaches of premise plumbing. Even if a utility employs very high levels of secondary disinfectant residuals, the community-wide effectiveness can be undermined either passively (e.g., normal disinfectant decay) or actively (e.g., granular activated carbon treatment that removes chlorine) in buildings. Ultimately, several stakeholders have a shared responsibility for addressing this growing public health challenge, and the new American Society of Heating, Refrigeration, and Air-Conditioning Engineers (ASHRAE) standard therefore addresses an important gap in responsibility by documenting building owner responsibilities for prevention of waterborne disease outbreaks.

Premise plumbing has unique properties relative to main distribution lines. Differing premise plumbing pipe materials vary in their propensity to form corrosion scale, and pipes and scale can directly consume chlorine depending on various factors, including water chemistry, disinfection method(s), consumer water use patterns, and plumbing system design and operation (Nguyen et al., 2011; 2012; Zhang et al., 2009). In some situations, there is little a water utility can do to cost-effectively control OPPPs for the entire range of situations encountered within individual buildings in the distribution system.

In-building disinfection approaches that have demonstrated effectiveness include both remedial (i.e., one-time treatments) and continuous disinfection regimes. Each approach can be effective for some OPPPs in some situations, but for reasons not well understood they are less effective in other situations (e.g., Stout and Yu, 2003). A recent report synthesized the state of the knowledge of OPPPs and ways in which they are controlled (Pruden et al., 2012). In particular, the inherent complexity and variability in premise plumbing systems often makes it difficult to predict, diagnose, and remediate outbreaks of waterborne disease originating from premise plumbing.

Several publications present comprehensive overviews of the different methods of building-level disinfection systems (Best et al., 1983; Muraca et al., 1990; Lin et al., 1998; Lin et al., 2011). The focus of these reviews is on the proven efficacy of each method toward *Legionella* disinfection in hospitals, and only touches on the inter-relationship between the disinfection method and the plumbing system. While it is important to maintain safe drinking water, some of the in-building techniques suggested in OPPP control guidelines and peer-reviewed literature have potentially

deleterious effects on premise plumbing assets and limited efficacy of disinfection across application scenarios.

The purpose of this review is to identify potential challenges associated with in-building disinfection for control of OPPPs in potable water systems in light of recent recommendations (e.g., ASHRAE 188), drawing from the state-of-the-knowledge of the efficacy of in-building disinfection methods. Legionnaires' disease (LD) is used as an exemplar because it is the most common source of waterborne disease outbreak in the United States (CDC, 2011) and strategies for mitigating *L. pneumophila* growth will establish important precedents for other OPPPs.

GUIDELINES FOR CONTROL OF LEGIONELLA

Several government and private entities have developed guidelines for building owners/operators to follow in the event of a LD outbreak (WHO, 2007; Florida DoH, 2013 AWT, 2003; ACHD, 1997; OSHA, 1999; ASHRAE, 2000; 2013). The focus of these guidelines, until recently, has been on remedial actions to be taken after an outbreak occurs. They often cover basic disinfection procedures, requirements for epidemiological studies, and how to maintain a safe plumbing system after disinfection. Because detection of *Legionella* does not necessarily predict the occurrence of disease and the relationship between levels of *L. pneumophila* and incidence of disease is unclear (Stout et al., 2007), standardized sampling and monitoring guidelines in these recommendations aim to achieve engineering operational targets and not achieving specific levels of *Legionella*.

ASHRAE Standard 188. ASHRAE Standard 188 is the most recent and first legally enforceable standard dealing with control of *Legionella*. The basic requirement of ASHRAE 188 is a written Hazard Analysis Critical Control Point (HACCP) plan that addresses all aspects of prevention of, response to, and long-term control of *Legionella* outbreaks. Building owners and operators are required to have preventative and reactive measures in place before an outbreak occurs.

The first protective step reduces risk associated with the entire water system and the second step addresses specific plumbing devices (Fig. 4.1). The HACCP plan developed in Section 5.2 of the standard invokes ASHRAE Guideline 12-2000 to prevent LD through use of in-building disinfection systems. If a building has devices that are historically linked with LD outbreaks (Cordes et al., 1980; Dondero et al., 1980; Nguyen et al., 2006), then a Water Treatment Plan must be developed for that specific device according to Section 5.3 of the standard (Fig. 4.1).

Although ASHRAE 188 is a step toward assigning enforceable responsibilities for preventing outbreaks, it is also unrealistic in some situations. For instance, a requirement to measure influent chlorine residual to a building is a quick and simple first check on delivery of disinfectant to the premise plumbing system. But, in practice, disinfectant residuals will vary markedly seasonally and with daily system water demand. In some systems, residual disinfectants may be present only after extensive flushing in the summer, but could be present at nearly all times during the winter. It is possible that building operators could collect samples in a manner that incorrectly classifies the disinfectant in water to the building, depending on where, when, and how they take measurements. At this time, it is unclear how this ambiguity impacts implementation and legal responsibility under the standard. At a minimum, there should be a specified procedure for measuring the disinfectant residual that encompasses the range of scenarios exhibited within a

given building, including samples taken at the end of the distribution system at distal taps after several hours of system stagnation. Moreover, the standard refers to a comprehensive guideline for preventing LD outbreaks (ASHRAE, 2000), yet neither the standard nor the guideline provide a basic framework for determining which remedial action(s) or continuous disinfection system(s) are optimal for different water qualities, system design features, or intended uses. Some of the key questions that need to be addressed to properly consider these important factors are outlined below.

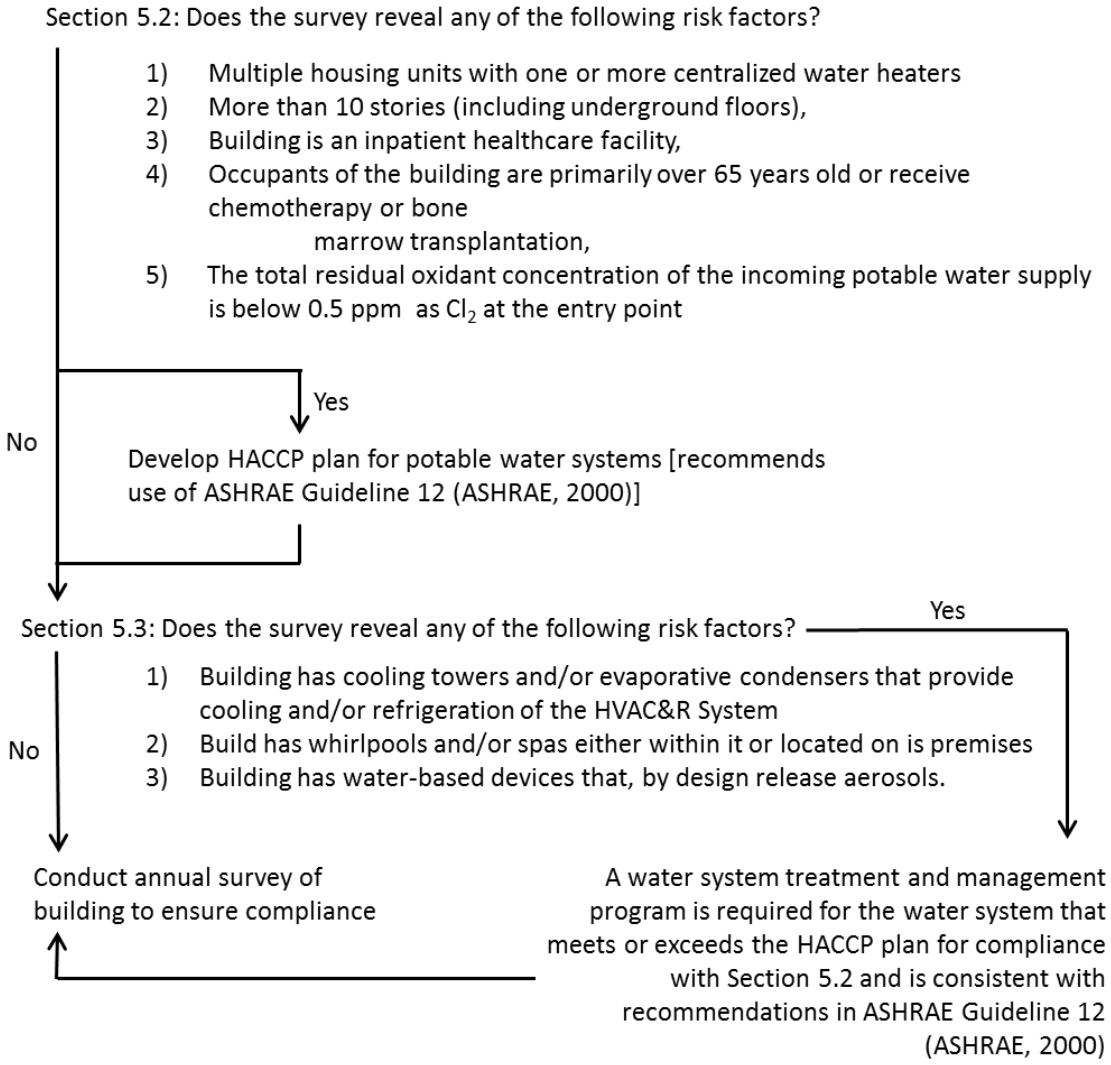


Fig. 4.1 ASHRAE Standard 188 flow diagram of requirements

IN-BUILDING DISINFECTION METHODS

Remediation-Based Techniques. Remedial disinfection techniques are useful for a system that is known or suspected to be contaminated with pathogenic bacteria, sometimes in response to an outbreak of waterborne disease. Two types of remediation include thermal and chlorine shock treatments.

Thermal Shock. Although guidelines vary (Table 4.1), thermal shock imposes water temperatures above 60˚C for 20-30 minutes while flushing taps, or achieving temperatures greater than 80˚C at least momentarily at all points in the plumbing system. It can be logistically challenging to maintain water temperatures throughout the plumbing network in large systems (Muraca et al., 1990). Plastic pipes used in hot water systems such as chlorinated polyvinyl chloride (CPVC) pipes are rated to withstand temperatures up to 82˚C (180˚F) for continuous use (e.g. Harvel, Easton, PA); however, there has been no research on the effects of continuous or repeated heat exposure on physical integrity or leaching propensity from other types of plastic pipes including cross-linked polyethylene (PEX) and high density polyethylene (HDPE). None of the guidelines that recommend thermal shock treatment for *Legionella* remediation warn building owners or operators against the potential for rapid scaling, or the precipitation of calcium carbonate [$CaCO_3$] catalyzed by elevated temperatures.

The risk of scalding consumers must also be avoided as the health risks are very serious (CPSC, 2005; NSKC, 2004). Although this technique can be at least temporarily effective for controlling *Legionella* (Kim et al., 2002; Muraca et al., 1987; Lin et al., 1998), recent work has demonstrated the potential for rapid *Legionella* regrowth following heat treatment, presumably due to necrotrophic consumption of dead cell biomass resulting from thermal shock (Temmerman et al., 2006).

Chlorine Shock. Control of pathogens by implementing a high concentration of free chlorine (hyperchlorination) is another remedial strategy. Target concentrations in *Legionella* guidelines have a wide range (from 2 to 50 ppm, or even higher), and in some cases a sustained level of residual without more than 30% loss is required. In many systems, reactions between the pipes and disinfectant make meeting the residual disinfection requirements difficult (BOCA, 1997; Edwards et al., 2011; International Code Council, 2000; Nguyen et al., 2011), even upon repeated doses of disinfectant. There are also concerns about initiating non-uniform pitting corrosion associated with high chlorine levels (Rushing and Edwards, 2004; Cong and Scully, 2010; Sarver et al., 2011). To combat this effect, pH may be maintained at lower levels (< pH 8) or a corrosion inhibitor may be introduced to reduce copper corrosion, adding further complexity to the method. Repeated shock chlorination can also damage the surface of HDPE pipes, and possibly reduce the overall lifetime of the plumbing system (Whelton and Dietrich, 2008; Whelton et al., 2011).

Continuous Disinfection Practices. After an outbreak has occurred and remediation has been successful, or if a plumbing system is considered to be conducive to pathogen growth under the new ASHRAE standard, continuous in-building disinfection practices may be considered. Factors mediating disinfection efficacy towards target pathogens such as interactions with plumbing are often unclear, especially in relation to application in different water qualities and across different premise plumbing materials. In addition, the U.S. Environmental Protection Agency (U.S. EPA) defines any building serving more than 25 individuals and dosing a disinfectant as a public utility. This definition triggers onerous drinking water standards and associated monitoring requirements. This makes installation of a secondary disinfection system considerably less attractive, and may even encourage undocumented use of building-level disinfection systems. Although some work has been done to define the efficacy of these systems (e.g. Lin et al., 2011), the following sections highlight expectations for the most common disinfection methods and specific types of premise plumbing.

Table 4.1. Summary of Thermal-based Guidelines for Control of Legionellosis

Entity	Remedial Recommendation	Long-term Recommendation	Caution Against Scalding?	Caution Against Pipe Damage in Long-Term Rec.? (e.g. scaling)	Recommend Repeated Thermal Shock?
Florida DoH	Heat hot water heatersto 71-77ºC for 24 hrs, then flush each tap for 5-20 minutes	Set hot water heaters to 60ºC; drain periodically to clean; hot-water recirculation pumps run continuously	Yes	No; allude to systems where thermal shock is not possible	Yes; when systems cannot be set at 60ºC (because of scalding or retrofit with mixing valves not possible)
OSHA	Heat hot water heaters to 70ºC for 24 hrs; flush each tap for 20 minutes	Set hot water heaters to 60ºC with minimum delivery temperature of 50ºC; drain periodically to clean; hot-water recirculation pumps run continuously	Yes	No	No
WHO	No temperature or exposure time recommended for pasteurization; Periodic flushing with waters 50-60ºC	Maintain all water temperatures > 50ºC	Yes	No	No
ASHRAE	Periodically raising hot water heaters to 66ºC followed by flushing	Store water at 60ºC; Maintain minimum return temperature 51ºC; Where not practical maintain all water temperature above 49ºC	Yes	No	Yes; when long-term temperature settings not possible
AWT	Heat hot water heaters>60ºC; preferably 66ºC; flush for up to 30 minutes	Has no long-term recommendations, but states in review that at 50ºC, there is 90% kill with 2 hour exposure	Yes	No	No

Chlorine and Chloramine. While there are over 190 years of experience using chlorine and/or chloramine as disinfectants (U.S. EPA, 2011), it has only recently been recognized that these disinfectants can quickly disappear via reactions within premise plumbing systems, even when they tend to be stable in the main distribution network (Zhang et al., 2009; Zhang and Edwards, 2009; Nguyen et al., 2011; 2012). Dosing these disinfectants within buildings poses additional maintenance and corrosion challenges that exceed the capabilities of many building staff. Unfortunately, there is not a sufficient body of research to assess the effects of the application of chlorine and chloramine at the building-level.

Chlorine is a strong oxidant that can react with and be consumed by many common plumbing materials including lead, PEX, copper, and brass, but not PVC (Sarver et al., 2011; Nguyen et al., 2012; Whelton et al., 2011; Edwards and Dudi, 2004). Certain water chemistries can accelerate or inhibit corrosivity of chlorine towards specific building plumbing materials (Rushing and Edwards, 2004; Sarver et al., 2011; Lytle and Schock, 2008). However, chlorine can also accelerate aging of copper pipes by converting soluble cupric hydroxide [$Cu(OH)_2$] to tenorite [CuO], markedly reducing aqueous copper and chlorine concentrations (Edwards et al, 1996; Edwards et al., 1999; Hidmi and Edwards, 1999; Lagos et al., 2001; Patterson et al., 1991; Schock et al., 1995). In some waters without disinfectant residuals this aging process never occurs, resulting in persistent elevated copper concentrations until at least a trace of chlorine residual is present (Edwards et al., 2000). However, starting new chemical disinfection treatments or changing the type of chemical disinfection can contribute to the release of inorganic contaminants. Changes to the oxidation-reduction potential of the water or other changes to the water chemistry/microbiology that can lead to shifts in pH (e.g. nitrification) can cause the oxidation and destabilization of inorganic contaminants including lead and arsenic (Edwards and Dudi, 2004; Lytle and Schock, 2005).

Many utilities switched to chloramine as a secondary disinfectant in response to the Disinfection Byproduct Rule (Seidel et al., 2005) because chloramine reacts weakly with natural organic matter to form disinfection byproducts such as trihalomethanes (THMs) and tends to be more persistent than chlorine in the main distribution system (Neden et al., 1992). In premise plumbing, with longer stagnation times, greater surface area to volume ratios, more reactive materials, higher levels of microbes (especially nitrifying bacteria), and warmer waters, chloramines can decay rapidly (Nguyen et al., 2012; Zhang et al., 2009; Zhang and Edwards, 2009). Chloramines also react differently with pipe scale, causing high lead leaching in premise plumbing with lead components (Edwards and Dudi, 2004). In addition, there is some evidence that monochloramine preferentially selects for *Mycobacterium* over *Legionella* (Pryor et al., 2004).

Chlorine Dioxide. There is little research that examines the effects of water chemistry and pipe material on chlorine dioxide efficacy, corrosivity or stability at distal taps. At levels below the U.S. EPA Maximum Residual Disinfectant Level (MRDL) of 0.8 mg/L, it has been effective in eliminating *Legionella* in at least some hospital applications (Sidari et al., 2004). One study claimed there was no significant increase in corrosion rates for copper pipes at these levels (Srinivasan et al., 2003); however, other research suggests there is an increased rate of decay at higher temperatures, higher total organic carbon (TOC) concentrations (Zhang et al, 2006 as cited in Zhang et al., 2008), and in the presence of iron and copper corrosion scale (Zhang et al., 2008).

Chlorine dioxide has been reported to be effective for infrequently used branches of the plumbing system over long periods of time, but it does not effectively eliminate amoeba hosts, such as *Acanthamoeba* and *Hartmanella,* in the biofilms of cool (35˚C) water systems (Thomas et al., 2004). One research group reported a chlorine dioxide resistant subpopulation of *L. pneumophila* and *E. coli* in controlled batch experiments (Berg et al., 1988).

Ozone. Efficacy of ozone is not generally affected by temperature or pH changes in bench-scale studies (Domingue et al., 1988), but it reacts more rapidly and less discriminately at higher pHs (>7) (Hoigne and Bader, 1976). Unlike chlorine dioxide, ozone is capable of reducing established biofilms (at 0.5 mg/L) at the point of treatment (Thomas et al., 2004). However, controlled studies on the corrosivity of ozone (and higher oxygen) are lacking.

Copper-Silver Ionization. The recommended dose of copper and silver ions for disinfection (0.2-0.4 mg/L for copper and 0.02-0.08 mg/L silver) depends on water quality, water system design, use, and interactions with existing plumbing materials (Cachafeiro et al., 2007). Copper levels above these recommended levels may be maintained naturally by corrosion and dissolution from existing copper pipes (Edwards and Jacobs, 2000). In addition, the concentrations required to be effective in some waters may conflict with goals of lower copper loading to sewage treatment plants (Cachafeiro et al., 2007; Boulay and Edwards, 2000). It is likely that pH, alkalinity, phosphate and other water constituents affect efficacy of these metals for controlling OPPPs (Rohr et al., 1999; Lin and Vidic, 2006), as has been observed for other premise plumbing microorganisms such as nitrifying bacteria (Zhang et al., 2009). Copper ions can also cause severe deposition corrosion of galvanized or steel plumbing systems (Kentworthy, 1943; Cruse, 1971), and silver ions may attack copper pipes and other metals by the same mechanism (Clark et al., 2011).

Although this approach has been reported to be successful in field studies, basic lab-scale research on its effectiveness is lacking (e.g. Liu et al., 1998). Copper silver ionization is most effective when concentrations can continually be monitored and adjusted (WHO, 2007), requiring special equipment and expertise. Although there is no one optimum concentration of copper and silver ions, there is a synergistic effect when they are used at the upper range of the allowable concentrations (Lin et al., 2002). Studies on the effect of speciation of metal ions dosed as they travel throughout plumbing systems would be of interest (e.g., Stout and Yu, 2003). Toxicity literature suggests that speciation is an important factor and highly dependent on pH (Franklin et al., 2000)

A few studies have generally examined the effect of pH and other water quality parameters on the efficacy of silver and copper ions. Lin et al. (2002) determined that at pH 9, copper ions were not effective in eliminating *Legionella* due the speciation of copper ions in the water compared to pH 7. At pH 7, more free copper ions exist in the water than at pH 9, where solids begin to form that are less bioavailable to the bacteria. They determined that there was no obvious effect of pH on the potency of silver ions. Other literature reports the effects of lowered pH (and thus presence of free copper ions) on drinking water nitrifying bacteria is a strong function of dissolved oxygen, and their detection varies markedly in different buildings of the same distribution system (Zhang et al., 2010). As of February 1, 2013, copper ionization systems are no longer permitted to be marketed or installed in countries under European Union jurisdiction because “no manufacturer

took sufficient action to support the biocidal use of elemental copper during a review period that ended in September 2011" (HSE, 2013).

Ultraviolet Light. Ultraviolet (UV) light requires minimal maintenance and expertise. While efficacy decreases with higher turbidity water and temperature fluctuations alter its efficiency, the main concern is that no disinfection residual is provided (Muraca et al., 1990). Therefore, there is no control over microbes that may proliferate downstream of the treatment, which is an essential aspect of OPPP control. Making matters worse, UV-killed cells in the absence of a disinfectant residual could stimulate necrotrophic growth of *Legionella*, as has been observed after short-term thermal disinfection (Temmerman et al., 2006). Consequently, UV may be most effective at mitigating OPPP growth in combination with other treatment options (WHO, 2007).

Thermal Disinfection. There is a major need for practical research aimed at guiding selection of target temperatures in hot water infrastructure. Maintaining thermal targets below 20˚C in cold water systems (WHO, 2007, AHSRAE, 2000, Florida DOH, n.d.) and above 60˚C in hot water systems is thought to limit pathogen growth (Muraca et al., 1987). In contrast, the U.S. EPA recommends a hot water heater set point of 48˚C for domestic systems for assumed energy savings of cooler water temperature and to reduce scalding danger (U.S. EPA, 2010). The new ASHRAE 188 standard for control of *Legionella* growth recommends maintaining hot water tanks above 60˚C and above 51˚C throughout the entire water network.

THERMAL DISINFECTION: SECONDARY IMPACTS AND UNINTENDED CONSEQUENCES

The ASHRAE recommendation to maintain 51˚C throughout the system may be practically unrealistic, as portions of the system typically remain stagnant long enough for water to cool to room temperature. In an attempt to meet this requirement, buildings may desire to continuously recirculate hot water systems, but these can consume more energy than traditional systems (Brazeau and Edwards, 2013), and water to distal taps may still cool to room temperature during stagnation.

The U.S. EPA recommendation of 48˚C in water heaters may not be aggressive enough to reduce the growth of *Legionella*, which has an optimal range between 32-42˚C (Yee and Wasowsky, 1982) and evidence of growth as high as 51.6˚C (Kusnetsov et al., 1996). In addition, pathogenic *Legionella* have been shown to survive successive thermal (70°C for 30 minutes) and chemical (hydrogen peroxide) treatments, with the overall biofilm mass intact and the microbial community only temporarily affected (Farhat et al., 2012). The presence of bacterial pathogens within an amoeba host can also aid their survival by shielding them from heat treatment (Greub and Raoult, 2004). Moreover, since *L. pneumophila* grow better in temperatures above room temperature (30 – 37 °C range as compared to 24 °C), the recirculation of warm water through the main pipes may result in dead-end lines with low disinfectant residual and with ideal growth temperatures of 25 °C to 45 °C (Buse and Ashbolt, 2011).

Increasing the water temperature in premise plumbing also increases the propensity of certain water chemistries to rapidly precipitate scale, such as calcium carbonate, due to decreasing solubility at higher temperatures. For example, at moderate levels of calcium hardness (80 mg/L

as Ca^{2+}), moderate alkalinity (100 mg/L as $CaCO_3$), and at pH 7, water is undersaturated with respect to $CaCO_3$ at 48°C (Fig. 4.2). If temperature is raised to 60°C or higher in response to ASHRAE 188 Section 5.2 (Fig. 4.1), 11 mg/L of $CaCO_3$ is predicted to precipitate to reach equilibrium.

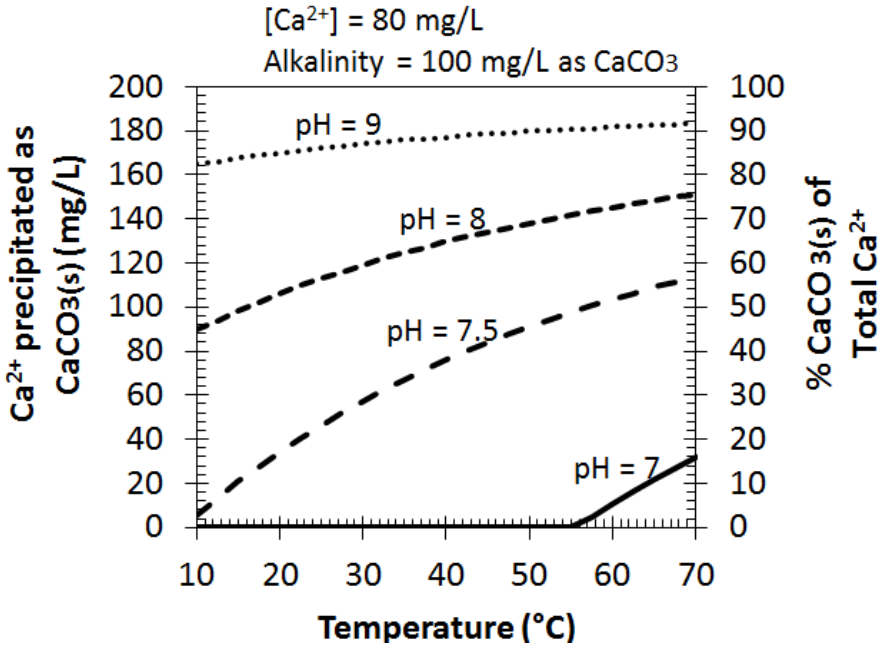


Fig. 4.2. Scaling propensity of waters with 80 mg/L Ca^{2+}, 100 mg/L alkalinity as $CaCO_3$, and a pHs 7, 7.5, 8, and 9 (Created using MINEQL +4.6 assuming $CaCO_3$ was the only solid formed)

The Langelier Saturation Index (LSI), while widely discredited for use in determining the relative corrosivity of water and estimating the leaching potential of lead, zinc, and copper from pipes and other premise plumbing materials (AWWARF, 1996; Edwards et al., 1996; Schock, 1999), is useful in predicting the tendency for certain waters to precipitate $CaCO_3$ scale (Langelier, 1936; Elfil and Hannachi, 2006). The LSI does not predict precipitation rates, which is especially problematic given certain chemicals such as polyphosphate or orthophosphate that are added to some tap waters, which slow or stop precipitation rates (Lin and Singer, 2005). Polyphosphates proved to be two orders of magnitude more effective than orthophosphate as an inhibitor of $CaCO_3$ precipitation (Lin and Singer, 2005; 2006).

Other tests that involve consideration of kinetics have also been used to predict problems with precipitation. van Raalte-Drewes et al. (2004) includes a nucleation index (NI), incorporating the initial amount of time it takes for precipitates to form. Many waters in this study were predicted to have problems with scaling due to high calcium carbonate precipitation potentials (CCPPs) and saturation indices (Sis) that did not scale quickly in practice, as evidenced by low NIs. This contradictory result is possibly due to the effects of certain compounds on the reaction kinetics mentioned earlier. This emphasizes the importance of testing an individual water type before elevated temperatures for disinfection are used.

Regardless of whether particular water chemistry has the tendency to precipitate calcium carbonate, magnesium hydroxides, or magnesium silicates, a building owner must take into account the rate at which the precipitates form. For some waters, even though they are superstaturated and theoretically tend to form scales, the residence times of most premise plumbing hot water systems are too short for the reactions to occur before the water is used. For larger systems, scaling inhibitors can be explored (Fig. 4.3). Although more practical tests that incorporate kinetics to some degree are better than saturation indices, local experiences relative to problems with scaling at high temperatures should be synthesized on a case-by-case basis.

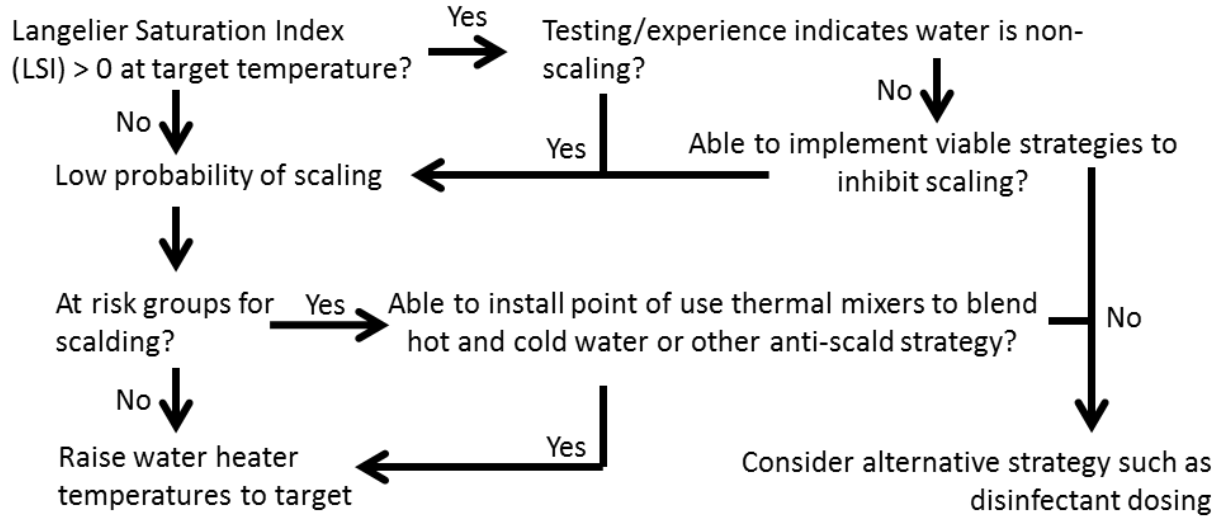


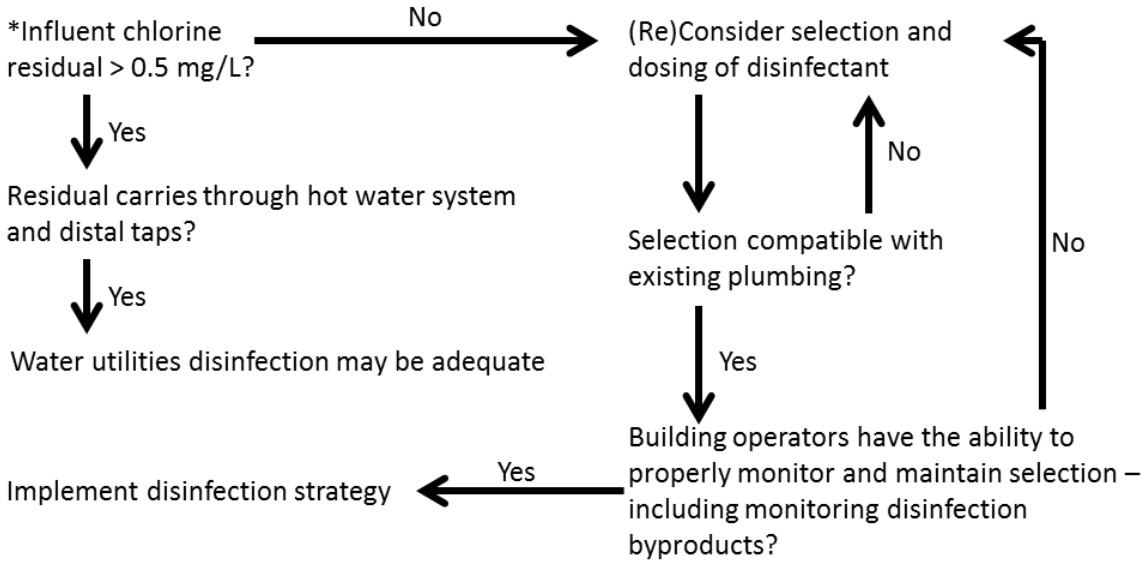
Fig. 4.3. Logical framework/decision tree to determine if thermal treatment (shock or continuous) is likely to damage pipes at 60°C.

Other issues that could arise from increased water temperatures include increased corrosion rates of metallic pipes and higher scalding risk to consumers. Increased water temperature is believed to increase the leaching propensity of lead from plumbing devices (Sarver and Edwards, 2011; U.S. EPA, 2013) and cause markedly higher erosion corrosion rates of copper pipes in hot waters under moderate to continuous flow conditions have been observed (Obrecht and Quill 1960a-d). The degradation of the physical attributes of the plumbing system could necessitate using a design flow rate in some situations (Knutsson et al., 1972).

Building owners and operators must take the propensity for the water to scale and induce corrosion into account before deciding to employ thermal disinfection to control pathogen growth (Fig. 4.3). In some circumstances, it may be possible to dose inhibitors or use water softening as strategies to avoid issues. If the water is subject to scaling, and inhibitors are not a viable option, chemical disinfection methods must be considered (Fig. 4.4). Current guidelines and standards do not provide a formal decision-making process for how and when to select a certain disinfection procedure. Fig. 4.3 and 4.4 provide basic information on how to approach this while maintaining the integrity of the plumbing system and safety of consumers.

Instead of reducing the temperature of the entire hot water system to avoid scalding, thermostatic mixers can be installed at each tap to blend both hot and cold water to reduce the maximum temperature of dispensed water as per Australian code (Spinks et al., 2003). However, automatic

mixing valves in metered and hands-free faucets have been implicated as triggers for OPPP growth (Halabi et al., 2001; Yapicioglu et al., 2011). Similar concerns may emerge with thermal mixing valves installed to reduce scalding potential. In addition, the mixing devices can fail, causing the potential for cross-connections between the hot and cold water lines to develop. There is some work that raises concerns of thermostatic mixing and tempering valves not working properly under all flow conditions observed in premise plumbing (Stephen and Murray, 1993).



**Implementation of 0.5 mg/L residual measurement is ambiguous*

Fig. 4.4. Logical framework/decision tree for implementing a treatment technique other than thermal disinfection.

CONCLUSIONS AND RESEARCH GAPS

There is a shared responsibility amongst stakeholders to prevent colonization of OPPPs in in-building potable water systems. While ASHRAE 188 is a critical step in this direction, requiring the development of a plan for preventing, responding to, and following up on LD outbreaks, new research is necessary to address implementation of the standard. However, practical guidance on the implementation of basic measurements employed by the standard should be provided. For instance, defining where, how, and when to measure the level of disinfection residual in the plumbing system is an important step in determining the overall risk associated with growth in a system. There is also a need for updated and more readily available information on the costs of the design, construction, and operation of the various in-building disinfection techniques. The most thorough work in this area is now outdated. In addition, relatively little is known about interactions between plumbing materials and in-building disinfectants, and these reactions have important implications for maintaining disinfectant residuals, controlling corrosion, and forming harmful disinfection byproducts. Research is needed to determine the background reactions occurring between the disinfectants, water chemistry, and plumbing materials and the effects of these reactions on the efficacy of pathogen inactivation. Many systems will damage their plumbing systems if they raise water temperatures to 60°C due to rapid scaling and increased corrosion. While the Langelier Saturation Index (LSI) provides a useful starting point for considering whether

waters are at risk to scaling, more practical tests may also be of value in predicting the physical risks associated with the implementation of thermal disinfection. Other strategies to maintain safe water temperature at the point of use in a system with elevated hot water heater temperature, such as thermostatic mixing valves, deserves increased scrutiny. Guidance should be provided in the standards and treatment guidelines that outline the required maintenance and monitoring expertise of the operator to facilitate the selection of an effective disinfection regimen.

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REFERENCES

Allegheny County Health Department (ACHD). (1997) *Approaches to Prevention and Control of Legionella Infection in Allegheny County Health Care Facilities*., Pittsburg, PA. URL: http:// www.legionella.org/achd_guideline.pdf (accessed November 20, 2012)

American Society for Heating Refrigeration, and Air-Conditioning Engineers (ASHRAE). (2000) *ASHRAE 12-2000 Guideline: Minimizing the Risk of Legionellosis Associated with Building Water Systems.* Atlanta, GA.

American Society for Heating Refrigeration, and Air-Conditioning Engineers (ASHRAE). (2013) *ASHRAE Standard 188: Prevention of Legionellosis Associated with Building Water Systems.* Atlanta, GA.

American Water Works Association Research Foundation & DVGW-Technologiezentrum Wasser. (1996) Internal Corrosion of Water Distribution Systems, 2nd Edition. American Water Works Association, Denver, CO.

Association of Water Technologies (AWT). (2003) *Legionella 2003: An Update and Statement by the Association of Water Technologies*. Rockville, MD, URL: http://www.legionella.com/images/awtlegionella2003.pdf (Accessed December 2, 2013).

Berg, J. D.; Hoff, J. C.; Roberts, P. V.; Matin, A. (1988) Resistance of Bacterial Subpopulations to Disinfection by Chlorine Dioxide. *J. Am. Water Works Ass.*, **80(9)**, 115-119.

Best, M.; Yu, V.L.; Stout, J.; Goetz, A.; Muder, R.R.; Taylor, F. (1983) Legionellaceae in hospital water-supple: epidemiological link with disease and evaluation of a method for control of nosocomial Legionnaires' disease and Pittsburgh pneumonia. *Lancet. **2**, 307-310.*Boulay, N.; Edwards, M. (2000) Copper in the Urban Water Cycle. *Crit. Rev. Env. Sci Tec.* **30(3)**, 297-326.

Boulay, N., & Edwards, M. (2000). Copper in the urban water cycle. *Critical reviews in Environ. Sci. and Technol.*, **30(3)**, 297-326.

Brazeau, R.H.; Edwards, M.A. (2012) A Review of the Sustainability of Residential Hot Water Heater Infrastructure: Public Health, Environmental Impacts, and Consumer Drivers. *J. Green Build.*, **6(4)**: 77-95.

Brazeau, R.H.; Edwards, M.A. (2013) Water and Energy Savings from On-Demand and Hot Water Recirculating Systems, To be published in the *Journal of Green Building*.

Building Officials and Code Administrators (BOCA). (1997) BOCA National Plumbing Code, 9th ed. BOCA, Country Club Hills, IL.

Buse, H. Y.; Ashbolt, N.J. (2011). Differential growth of Legionella pneumophila strains within a range of amoebae at various temperatures associated with in-premise plumbing. Lett Appl Microbiol **53(2)**: 217-224.

Cachafeiro, S. P.; Naveira, I. M.; García, I. G. (2007) Is copper–silver ionisation safe and effective in controlling *Legionella*?. *J. Hosp. Infect.*, **67(3)**, 209-216.

Centers for Disease Control and Prevention (CDC). (2011) Surveillance for Waterborne Disease Outbreaks Associated with Drinking Water --- United States, 2007--2008. *Morbidity and Mortality Weekly Report (MMWR)*. **60(12)**, 38-68.

Clark, B.N.; A.L. Hernandez; M.A. Edwards. (2011) Deposition Corrosion of Water Distribution System Materials, in *Water Quality Technology Conference*, Phoenix, AZ.

Cong, H.; Scully, J. R. (2010) Effect of chlorine concentration on natural pitting of copper as a function of water chemistry. *J Electrochem. Soc.*, **157(5)**, 200-211.

Consumer Product Safety Commission (CPSC). (2005) Tap water scalds. Document #5098. URL: http://www.cpsc.gov/cpscpub/pubs/5098.html (accessed December 20, 2012).

Cordes, L.G.; Fraser, D.W.; Skaliy, P.; Perlino, C.A.; Elsea, W.R.; Mallison, G.F.; Hayes, P.S. (1980) Legionnaires' Disease Outbreak at an Atlanta, Georgia, Country Club: Evidence for Spread from an Evaporative Condenser. *Am. J. Epidemiol.*, **111(4)**, 425-431.

Cruse, H. (1971) Dissolved-Copper Effect on Iron Pipe. *J. Am Water Works Ass.*, **63(2)**, 79-81.

Domingue, E. L.; Tyndall, R. L.; Mayberry, W. R.; Pancorbo, O. C. (1988) Effects of three oxidizing biocides on *Legionella pneumophila* serogroup 1. *Appl. Environ. Microb.*, **54(3)**, 741-747.

Dondero Jr, T. J.; Rendtorff, R. C.; Mallison, G. F.; Weeks, R. M.; Levy, J. S.; Wong, E. W.; Schaffner, W. (1980). An outbreak of Legionnaires' disease associated with a contaminated air-conditioning cooling tower. *New Engl. J. Med.*, **302(7)**, 365-370.

Edwards, M.; Dudi, A. (2004) Role of chlorine and chloramines in corrosion of lead-bearing plumbing materials. *J. Am. Water Works Assoc.* **96**, 69-81.Edwards, M. (2004) Corrosion Control in Water Distribution Systems. One of the Grand Engineering Challenges for the 21st Century. Edited by Simon Parsons, Richard Stuetz, Bruce Jefferson and Marc Edwards. *Water Sci. Technol.*, **49(2)**, 1-8.

Edwards, M.; Dudi, A. (2004) Role of chlorine and chloramine in corrosion of lead-bearing plumbing materials. *J. Am. Water Works Ass.*, **96(8)**; 69-81.

Edwards, M.; Jacobs, S.; Taylor, R. J. (2000) The blue water phenomenon. *J. Am. Water Works Ass.*, **92(7)**; 72-82.

Edwards, M.; Jacobs,S.; Dodrill, D. (1999) Desktop guidance for mitigating Pb and Cu corrosion by-products. *J. Am. Water Works Ass.*, **91(5)**, 66-77.

Edwards, M.; Schock, M. R.; Meyer, T. E. (1996) Alkalinity, pH, and copper corrosion by-product release. *J. Am. Water Works Ass.*, **88(3)**, 81-94.

Edwards, M.A.; Parks, J.; Griffin, A.; Raetz, M.; Martin, A.; Scardina, P.; Elfland, C. (2011) Lead and Copper Corrosion Control in New Construction. Water Research Foundation, Denver, CO.

Elfil, H.; Hannachi, A. (2006) Reconsidering water scaling tendency assessment. *AIChE Journal*, **52(10)**, 3583-3591.

Farhat, M.; Moletta-Denat, M.; Frère, J.; Onillon, S.; Trouilhé, M. C.; Robine, E. (2012) Effects of Disinfection on *Legionella* spp., Eukarya, and Biofilms in a Hot Water System. *Appl. Environ. Microb.*, **78(19)**, 6850-6858.

Florida Department of Health (Florida DOH).Guidelines for the Surveillance, Investigation, and Control of Legionnaires' Disease in Florida. URL: http://www.doh.state.fl.us/ environment/medicine/foodsurveillance/investigation_docs/Legionella_Guidelines.pdf (accessed December 20, 2012).

Franklin, N. M., Stauber, J. L., Markich, S. J., & Lim, R. P. (2000). pH-dependent toxicity of copper and uranium to a tropical freshwater alga *Chlorella*. *Aquatic toxicology*, **48(2)**, 275-289.

Greub, G.; Raoult, D. (2004) Microorganisms resistant to free-living amoebae. *Clin. Microbiol Rev.*, **17(2)**, 413-433.

Halabi, M.; Wiesholzer-Pittl, M., Schoberl, J.; Mittermayer, H. (2001) Non-touch fittings in hospitals: a possible source of Pseudomonas aeruginosa and Legionella spp. *J. Hosp. Infect.*, **49(2)**, 117-121.

Harvel Webpage. (2013) Product Specifications. CPVC Industrial Pipe: Schedule 40 & 80. Accessed at: http://www.harvel.com/sites/www.harvel.com/files/documents/ Specifications-CPVC_Pipe.pdf

Health and Safety Executive (HSE). *Legionella* and Legionnairs' disease. Accessed at: http://www.hse.gov.uk/legionnaires/index.htm.

Hidmi, L.; Edwards, M.A. (1999) Role of temperature and pH in Cu (OH) 2 solubility. *Environ. Sci. Technol.*, **33(15)**, 2607-2610.

Hoigne, J., & Bader, H. (1976). The role of hydroxyl radical reactions in ozonation processes in aqueous solutions. Water Research, **10(5),** 377-386.

International Code Council, 2000. International Plumbing Code. International Code Council, Falls Church, VA.

Isaacs, W.P.; Stockton, G.R. (1981) Softened Water Energy Savings Study Controlled Experimental Testing Program on Household Water Heaters, New Mexico State University, Las Cruces, NM in association with Water Quality Research Council (WQRC).

Kenworthy, L. (1943) The Problem of Copper and Galvanized Iron in the Same Water System. *J.I. Met.*, **69**, 67-90.

Kim, B. R.; Anderson, J. E.; Mueller, S. A.; Gaines, W. A.; Kendall, A. M. (2002) Literature review—efficacy of various disinfectants against *Legionella* in water systems. *Water Res.*, **36(18)**, 4433-4444.

Knutsson, L.; Mattsson, E.; Ramberg, B.E. (1972). Erosion corrosion in copper water tubing. *British Corrosion Journal*, **7(5)**, 208-211.

Kusnetsov, J. M.; Ottoila, E.; Martikainen, P. J. (1996) Growth, respiration and survival of *Legionella pneumophila* at high temperatures. *J. Appl. Microbiol.*, **81(4)**, 341-347.

Lagos, G. E.; Cuadrado, C. A.; Letelier, M. V. (2001) Aging of copper pipes by drinking water. *J. Am. Water Works Ass.*, **93(11)**, 94-103.

Langelier, W.F. (1936) The analytical control of anticorrosion water treatment. *J. Am. Water Works Ass.*, **28**, 1500-1521.

Lin, Y. P.; Singer, P. C. (2005) Inhibition of calcite crystal growth by polyphosphates. *Water Res.*, **39(19)**, 4835-4843.

Lin, Y. P.; Singer, P. C. (2006) Inhibition of calcite precipitation by orthophosphate: Speciation and thermodynamic considerations. *Geochim. Cosmochim. Ac.*, **70(10)**, 2530- 2539.

Lin, Y. S. E.; Vidic, R. D.; Stout, J. E.; Yu, V. L. (2002) Negative effect of high pH on biocidal efficacy of copper and silver ions in controlling *Legionella pneumophila*. *App. Environ. Microb.*, **68(6)**, 2711-2715.

Lin, Y.; Stout, J.; Yu, V. (2011) Controlling *Legionella* in Hosital Drinking Water: An evidence-Based Review of Disinfection Methods. *Infect. Cont. Hosp. Ep.* **32(2),** 166-173.

Lin, Y.E., Vidic, R.D. (2006). Possible phosphate interference with copper-silver ionization for *Legionella* control. *J. Hosp. Infect.*, **62**, 119-XXX.

Lin, Y-S.E.; Stout, J.E.; Yu, V.L.; Vidic, R.D. (1998) Disinfection of water distribution systems for *Legionella*. *Semin. Respir. Infect.*, **13(2)**, 147–159.

Liu, Z.; Stout, J.E.; Boldin, M.; Rugh, J.; Diven, W.F.; Yu, V.L. (1998). Intermittent use of copper-silver ionization for *Legionella* control in water distribution systems: a potential option in buildings housing individuals at low risk of infection. *Clin Infect. Dis*, **26(1)**, 138-140.

Lytle, D. A.; Schock, M. R. (2008) Pitting corrosion of copper in waters with high pH and low alkalinity. *J. Am. Water Works Ass.*, **100(3)**, 115-129.

Lytle, D.A.; Schock, M.R. (2005) The formation of Pb (IV) oxides in chlorinated water. *J. Am. Water Works Assoc.*, **97**, 102–114.

Muraca, P. W.; Yu, V. L.; Goetz, A. (1990) Disinfection of water distribution systems for *Legionella*: a review of application procedures and methodologies. *Infect. Cont. Hosp. Ep.*, **11(2)**, 79-88.

Muraca, P.; Stout, J. E.; Yu, V. L. (1987) Comparative assessment of chlorine, heat, ozone, and UV light for killing *Legionella pneumophila* within a model plumbing system. *Appl. Enviorn. Microb.*, **53(2)**, 447-453.

National Research Council (NRC). (2006) *Alternatives to premise plumbing*, in: Drinking Water Distribution Systems: Assessing and Reducing Risk, 316-340.

National SAFE KIDS Campaign (NSKC). (2004) Burn injury fact sheet. Washington, DC: National SAFE KIDS Campaign. URL: http://www.safekids.org/our-work/research/fact-sheets/burn-and-scald-prevention-fact-sheet.html (accessed March 18, 2013).

Neden, D. G.; Jones, R. J.; Smith, J. R.; Kirmeyer, G. J., Foust, G. W. (1992) Comparing chlorination and chloramination for controlling bacterial regrowth. *J. Am. Water Works Ass.*, **84(7)**, 80-88.

Nguyen, C. K.; Powers, K. A., Raetz, M. A.; Parks, J. L.; Edwards, M. A. (2011) Rapid free chlorine decay in the presence of $Cu(OH)_2$: Chemistry and practical implications. *Water Res.*, **45(16)**, 5302-5312.

Nguyen, C.; Elfland, C.; Edwards, M. (2012) Impact of advanced water conservation features and new copper pipe on rapid chloramine decay and microbial regrowth. *Water Res.*, **46(3)**, 611-621

Nguyen, T. M. N.; Ilef, D.; Jarraud, S.; Rouil, L.; Campese, C.; Che, D.; Desenclos, J. C. (2006) A community-wide outbreak of legionnaires disease linked to industrial cooling towers—how far can contaminated aerosols spread? *J. Infect. Dis.*, **193(1)**, 102-111.

Obrecht M. F.; Quill L. L. (1960a) How temperature, treatment, and velocity of potable water affect corrosion of copper and its alloys in heat exchanger and piping systems. *Heat. Piping Air Cond*. **January 1960**,165-169.

Obrecht M. F.; Quill L. L. (1960b) How temperature, treatment, and velocity of potable water affect corrosion of copper and its alloys; different softened waters have broad corrosive effects on copper tubing. *Heat. Piping Air Cond*. **July 1960**, 115-122.

Obrecht M. F.; Quill L. L. (1960c) How temperature, treatment, and velocity of potable water after corrosion of copper and its alloys; monitoring system reveals effects of different operating conditions. *Heat. Piping Air Cond*. **April 1960**, 131-137.

Obrecht M. F.; Quill L. L. (1960d) How temperature, treatment, and velocity of potable water affect corrosion of copper and its alloys; tests show effects of water quality at various temperatures, velocities. *Heat. Piping Air Cond*. **May 1960**, 105-113.

Occupational Safety and Health Administration (OSHA). (1999) OSHA Technical manual TED 1-0.15 A. US Dept. of Labor.

Patterson, J. W.; Boice, R. E.,; Marani, D. (1991) Alkaline precipitation and aging of copper from dilute cupric nitrate solution. *Enviorn. Sci. Technol.*, **25(10)**, 1780-1787.

Paul, D.D.; Gadkari, V.V.; Evers, D.P.; Goshe, M.E.; Thornton, D.A. (2010) Final Report: Study on Benefits of Removal of Water Hardness (Calcium and Magnesium Ions) from a Water Supply. Battelle Memorial Institute, Columbus, OH. URL: http://www.flawater treatment.com/pdfs/Battelle_Final_Report.pdf (accessed January 10, 2013).

Pruden, A.; Edwards, M.; Falkinham III, J. (2012) Research Needs for Opportunistic Pathogens in Premise Plumbing: Experimental Methodology, Microbial Ecology and Epidemiology. Water Research Foundation Project 4379, Denver, CO.

Pryor, M., Springthorpe, S., Riffard, S., Brooks, T., Huo, Y., Davis, G., & Sattar, S. A. (2004). Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. Water Science & Technology, **50(1)**, 83-90.

Rohr, U., Senger, M., Selenka, F., Turley, R., & Wilhelm, M. (1999). Four years of experience with silver-copper ionization for control of Legionella in a German university hospital hot water plumbing system. *Clin. Infect. Dis.*, **29(6)**, 1507-1511.

Rushing, J. C.; Edwards, M. (2004) Effect of aluminium solids and chlorine on cold water pitting of copper. *Corros. Sci.*, **46(12)**, 3069-3088.

Sarver, E.; Dodson, K.; Scardina, P.; Lattyak-Slabaugh, R.; Edwards, M.; Nguyen, C. (2011) Copper Pitting in Chlorinated, High pH Potable Waters. *J. Am. Water Works Assoc*, **103(3),** 86-98.

Schock, M. R. (1990). Internal corrosion and deposition control. In: American Water Works Association (ed.), Water Quality and Treatment: a handbook of community water supplies. McGraw-Hill, New York, p. 1–17.

Schock, M. R.; Lytle, D. A.; Clement, J. A. (1995) Effect of pH, DIC, orthophosphate and sulfate on drinking water cuprosolvency. National Risk Management Research Lab., Cincinnati, OH (United States).

Seidel, C. J.; McGuire, M. J.; Summers, R. S.; Via, S. (2005) Have utilities switched to chloramines?. *J. Am. Water Works Ass.*, **97(10)**, 87-97.

Sidari, F. P. I.; Stout, J. E.; Van Briesen, J. M.; Bowman, A. M.;Grubb, D., Neuner, A.; Yu, V. L. (2004) Keeping *Legionella* Out of Water Systems. *J. Am. Water Works Ass.*, **96(1)**, 111-119.

Spinks, A. T.; Coombes, P.; Dunstan, R. H.; Kuczera, G. (2003) Water quality treatment processes in domestic rainwater harvesting systems. In 28th International Hydrology and Water Resources Symposium: About Water; Symposium Proceedings (p. 2). Institution of Engineers, Australia.

Srinivasan, A.; Bova, G.; Ross, T.; Mackie, K.; Paquette, N.; Merz, W.; Perl, T. M. (2003) A 17-month evaluation of a chlorine dioxide water treatment system to control *Legionella* species in a hospital water supply. *Infect. Cont. Hosp. Ep.* , **24(8)**, 575-579.

Stephen, F. R., & Murray, J. P. (1993). Prevention of hot tap water burns-a comparative study of three types of automatic mixing valve. Burns, **19(1),** 56-62.

Stout, J. E.; Muder, R. R.; Mietzner, S.; Wagener, M. M.; Perri, M. B.; DeRoos, K.; Yu, V. L (2007) Role of environmental surveillance in determining the risk of hospital-acquired legionellosis: a national surveillance study with clinical correlations. *Infect. Cont. Hosp. Ep.*, **28(7)**, 818-824.

Stout, J. E.; Yu, V. L. (2003) Experiences of the first 16 hospitals using copper-silver ionization for *Legionella* control: implications for the evaluation of other disinfection modalities. *Infect. Cont. Hosp. Ep.*, **24(8)**, 563-568.

Temmerman, R.; Vervaeren, H.; Noseda, B.; Boon, N.; Verstraete, W. (2006) Necrotrophic growth of *Legionella pneumophila*. *Appl. Envrion. Microb.*, **72(6)**, 4323-4328.

Thomas, V.; Bouchez, T.; Nicolas, V.; Robert, S.; Loret, J. F.; Levi, Y. (2004) Amoebae in domestic water systems: resistance to disinfection treatments and implication in *Legionella* persistence. *J. Appl. Microbiol.*, **97(5)**, 950-963.

United States Environmental Protection Agency (U.S. EPA). (2010) Energy Efficiency. URL: http://www.epa.gov/p2/pubs/energy.htm (accessed May 29, 2013).

United States Environmental Protection Agency (U.S. EPA). (2011) Information about Chloramine in Drinking Water. URL: http://www.epa.gov/ogwdw/disinfection /chloramine/pdfs/chloramine2.pdf (accessed December 20, 2012).

United States Environmental Protection Agency (U.S. EPA). (2006) Inorganic Contaminant Accumulation in Potable Water Distribution Systems. URL: http://www.epa.gov/ogwdw /disinfection/tcr/pdfs/issuepaper_tcr_inorganiccontaminantaccumulation.pdf (accessed October 7, 2013).

United States Environmental Protection Agency (U.S. EPA). (2013) Lead in Drinking Water. URL: http://water.epa.gov/drink/info/lead/ (accessed May 7,2013).

van Raalte-Drewes, M.J.C.; Brink, H.; Feij, L.A.C.; Slaats, P.G.G.; van Soest, E.A.M; Vaal, P.P.R.; Veenendaal, G. (2004), Scaling Propensity of Water: New Predictive Parameters. KIWA Report. International Water Association.

Whelton, A. J.; Dietrich, A. M.; Gallagher, D. L. (2011) Impact of chlorinated water exposure on contaminant transport and surface and bulk properties of high-density polyethylene and cross-linked polyethylene potable water pipes. *J. Environ. Eng.-ASCE*, **137(7)**, 559-568.

Whelton, A.J.; A.M. Dietrich. (2008) Critical Considerations for the Accelerated Ageing of High Density Polyethylene Potable Water Materials. *Polm. Degrad. Stabil.*, **94(7)**, 1163-1175.

World Health Organization (WHO). (2007) *Legionella* and the prevention of Legionellosis. WHO Press. Geneva, Switzerland.

Yapicioglu, H.; Gokmen, T. G.; Yildizdas, D.; Koksal, F.; Ozlu, F.; Kale-Cekinmez, E.; Candevir, A. (2011) Pseudomonas aeruginosa infections due to electronic faucets in a neonatal intensive care unit. *J. Paediatr. Child H.*, **48(5)**, 430-437.

Yee, R. B.; Wadowsky, R. M. (1982) Multiplication of *Legionella pneumophila* in unsterilized tap water. *Appl. Envion. Microb.*, **43(6)**, 1330-1334.

Zhang, Y.; Edwards, M. (2009) Accelerated chloramine decay and microbial growth by nitrification in premise plumbing. *J. Am. Water Works Ass.*, **101(11)**, 51-62.

Zhang, Y.; Griffin, A.; Edwards, M. (2010). Effect of nitrification on corrosion of galvanized iron, copper, and concrete. *J. Am. Water Works Ass*, **102(4)**, 83-93.

Zhang, Y.; Love, N.; Edwards, M. (2009) Nitrification in drinking water systems. *Crit. Rev. Envion. Sci. Technol.* **39 (3)**, 153-208.

Zhang, Z.; Stout, J. E.; Yu, V. L.; Vidic, R. (2008) Effect of pipe corrosion scales on chlorine dioxide consumption in drinking water distribution systems. *Water Res.*, **42(1)**, 129-13.

CHAPTER 5. WATER HEATER TEMPERATURE SET POINT AND WATER USE PATTERNS INFLUENCE *LEGIONELLA PNEUMOPHILA* AND ASSOCIATED MICROORGANISMS AT THE TAP

William J. Rhoads, Pan Ji, Amy Pruden, Marc A. Edwards

ABSTRACT

BACKGROUND: Lowering water heater temperature set points and using less drinking water are common approaches to conserving water and energy; yet, there are discrepancies in past literature regarding the effects of water heater temperature and water use patterns on the occurrence of opportunistic pathogens, and in particular *Legionella pneumophila*. Our objective was to conduct a controlled, replicated pilot-scale investigation to address this knowledge gap using continuously recirculating water heaters to examine five water heater set points (39-58° C) under three water use conditions. We hypothesized that *L. pneumophila* levels at the tap depend on the collective influence water heater temperature, flow frequency, and the resident plumbing microbiome.

RESULTS: We confirmed temperature setting to be a critical factor in suppressing *L. pneumophila* growth both in continuously recirculating hot water lines and at distal taps. For example, at 51° C, planktonic *L. pneumophila* in recirculating lines was reduced by a factor of 28.7 compared to 39° C and was prevented from re-colonizing biofilm. However, *L. pneumophila* still persisted up to 58° C, with evidence it was growing under the conditions of this study. Further, exposure to 51° C water in a low use tap appeared to optimally select for *L. pneumophila* (e.g., 125 times greater numbers than in high use taps). We subsequently explored relationships among *L. pneumophila* and other ecologically-relevant microbes, noting that elevated temperature did not have a general disinfecting effect in terms of total bacterial numbers. We documented the relationship between *L. pneumophila* and *Legionella* spp., and noted several instances of correlations with *Vermamoeba vermiformis*, and generally found that there is a dynamic relationship with this amoeba host over the range of temperatures and water use frequencies examined.

CONCLUSION: Our study provides a new window of understanding into the microbial ecology of potable hot water systems and helps to resolve past discrepancies in the literature regarding the influence of water temperature and stagnation on *L. pneumophila*, which is the cause of a growing number of outbreaks. This work is especially timely, given society's movement towards "green" buildings and the need to reconcile innovations in building design with public health.

KEY WORDS**:** *Legionella pneumophila*, hot water, stagnation, water use, temperature

BACKGROUND

The growth of opportunistic pathogens (OPs) in building plumbing systems is an increasing public health threat with no clear solutions (Pruden et al., 2012; Bartrand et al., 2014; Falkinham, 2015; Bedard et al., 2015). In particular, the warm, stagnant conditions in building plumbing create ideal conditions for regrowth of a number of OPs and their free-living amoeba hosts (Fig. C1). *Legionella* spp., including *L. pneumophila*, are model organisms for understanding the interplay between building plumbing design and operation and OP proliferation. *Legionella* are now recognized as the most common agents of waterborne disease outbreak, resulting in an estimated 8,000 to 18,000 hospitalizations (which are likely underreported) due to the severe pneumonia that characterizes Legionnaire's disease (Yoder et al., 2008; Brunkard et al., 2011). With community-acquired infections representing 96% (n=31) of reported drinking water-associated Legionnaries' disease outbreaks from 2007-2010, the majority of drinking water-associated Legionnaire's cases result from exposure to aerosols from drinking water systems in the built environment. A fundamental feature of *Legionella* and other OPs is that they can grow and thrive as part of complex microbial communities inhabiting building plumbing supplied by "clean" drinking water and therefore do not necessarily respond to traditional approaches for pathogen control geared towards fecal organisms (Berry and Raskin, 2006; Camper et al., 1985). At the same time, the characteristic conditions in building plumbing (e.g., warm temperature, high surface area, and long residence time) make it difficult to maintain an effective chlorine residual usually depended upon to kill pathogens (Falkinham, 2015, Chiao et al., 2014; Feazel et al., 2009). Thus, new strategies are needed for building plumbing design and operation that are informed by how they influence *Legionella* and its microbial ecology.

Prior field studies provide some clues about key factors that trigger *Legionella* colonization and amplification in building plumbing (Straus et al., 1996; Alary and Joly, 1991; Borella et al., 2004). *Legionella* are notorious for growing in hot water systems and while the optimal temperatures for inhibiting their growth have been well characterized in laboratory culture, they are not necessarily applicable to field conditions where *Legionella* colonize biofilm and may be protected within an amoeba host (Storey et al., 2004). Further, water heater set points do not directly translate into the temperature experienced at the tap, where it can quickly cool to room temperature.

Stagnation has also received a great deal of attention as a major risk factor for *Legionella* amplification and is interrelated with temperature setting (Ciesielski et al., 1984; Harper, 1988; OSHA, 2015; ASHRAE, 2000; Health and Safety Executive, 2015; Muraca et al., 1987; Stout et al., 1987; ASHRAE, 2015; Liu et al., 2006). For example, an advantage of recirculating systems is that they maintain water temperature in the recirculating pipes closer to the water heater temperature, which will ideally kill *Legionella* and prevent further colonization. Although the majority of guidance criteria advise against stagnation (OSHA, 2015; ASHRAE, 2000; Health and Safety Executive, 2015; ASHRAE, 2015), prior reports are inconsistent and indicate that it sometimes stimulates (Ciesielski et al., 1984) and sometimes deters (Liu et al., 2006; Moore et al., 2006; Mathys et al., 2008) *Legionella* growth. In the absence of disinfectant (thermal or chemical), stagnation may limit the delivery of new nutrients to distal taps, reducing the potential for regrowth (Liu et al., 2006; Ohl et al., 2004). However, nutrient gradients have not been examined in an integrated fashion considerate of how plumbing temperature and water use conditions together might ultimately impact nutrient availability. Finally, given that *Legionella* and many other OPs

are intimately dependent on host free-living amoebae for their replication in oligotrophic drinking water systems, better understanding this ecological relationship as influenced by temperature and stagnation is critical (Storey et al., 2004; Thomas and Ashbolt, 2010; Lau and Ashbolt, 2009).

A major limitation of field studies is the inherent complexity encountered in actual building systems, which make it difficult to pinpoint precise factors that trigger *Legionella* proliferation. Therefore, our objective was to conduct a controlled, replicated laboratory investigation examining the interrelationship of water heater temperature set point and distal tap use frequency on *Legionella* occurrence. Identical experimental and control hot water plumbing systems were constructed in which continuously recirculating pipe loops delivered water to distal taps subject to high, medium, and low water use frequency (Fig. 5.1). Both systems were initially acclimated for five months at 39° C to establish a baseline with stable microbial communities before incrementally increasing the water heater temperature of the experimental system to 42° C, 48° C, 51° C, and 58° C, while the control system was maintained at 39° C for the duration of the 15 month experiment. Genetic markers of *Legionella* spp. (23S rRNA gene), *L. pneumophila* (macrophage infectivity potentiator (*mip*) gene), *Vermamoeba vermiformis* (18S rRNA gene; an important ecological host for *Legionella*), and total bacteria (16S rRNA gene) were tracked by quantitative polymerase chain reaction (q-PCR) to measure regrowth in the recirculating lines relative to the influent water and in distal taps relative to the recirculating lines (Fig. 5.1). Given the current trend towards "green" buildings that are intended to conserve both water (i.e., to decrease water use frequency, which increases corresponding stagnation) and energy (i.e., lower temperature settings), the present moment is critical for untangling the complexities that trigger *Legionella* growth and identifying practical solutions for their control that can be considered in building system design.

RESULTS AND DISCUSSION

Both *Legionella* spp. and *L. pneumophila* naturally colonized both the experimental and control building plumbing systems and established a comparable baseline, which provided the unique opportunity to systematically examine the effect of changes in building plumbing operation and microbial response under replicated and controlled conditions. Our overarching hypothesis was that *L. pneumophila* levels at the tap depend on the interrelationship between water heater temperature set point and use frequency and their collective influence on the microbiome. Table 5.1 breaks this hypothesis down more specifically, summarizing four representative conditions (I-IV) under which increased use frequency would be expected to increase, decrease, or have no effect on *L. pneumophila* levels. Across this study, we conducted testing with little to no disinfectant residual, as can occur in building plumbing, especially under water conservation scenarios and at the end of water main networks (Nguyen et al., 2012; Elfland et al., 2010; EPA, 2002; Vasconcelos and Boulos, 1996). If disinfectant can be effectively delivered and maintained above about 0.5 mg/L as Cl_2 (Condition IV), it is generally believed that *L. pneumophila* will effectively be controlled (ASHRAE, 2000; 2015). In the following sections, we first discuss physicochemical trends in temperature and chlorine, and subsequently examine occurrence of *L. pneumophila* and other ecologically-relevant microbes relative to these trends and in the context of the specific hypotheses presented in Table 2.1. Table 2.2 provides an overview of the calculations we employed in this study to compare the distribution of *L. pneumophila* between the experimental and control rigs, and across various system compartments.

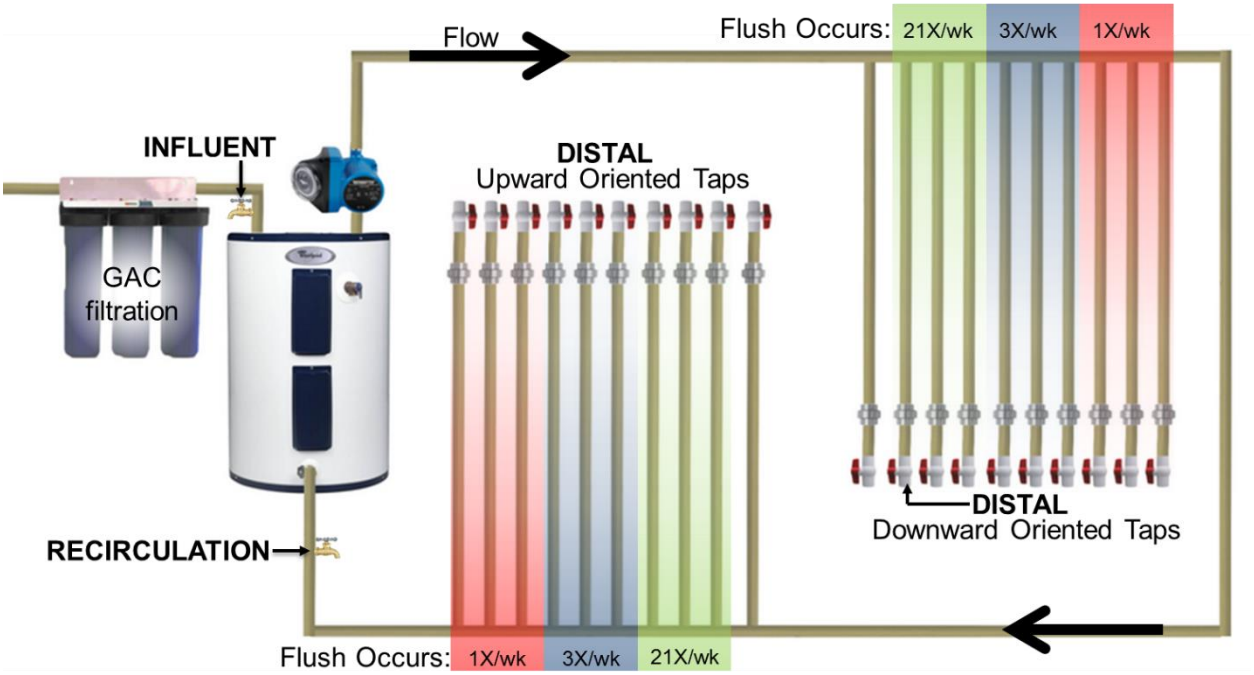

Fig. 5.1. Overview of experimental design of replicated building plumbing systems

Two identical systems were constructed to examine the effect of water heater temperature setting and water use frequency on Legionella proliferation. One remained at 39° C (control system) while the other was incrementally increased to 58° C (experimental system) over 15 months. Influent water was flushed through three granular activated carbon whole-house filters (Sample port: Inf), a recirculating pump continuously pumped water around the return loop back to the water heater creating a completely mixed reservoir (Sample port: Recirc), six replicate distal taps (three upward + three downward) were flushed at 3.8 L/min (1 gallon/min) 21x/week, 3x/week, and 1x/week for a total of 36 pipes (Sample ports: at end of distal pipes).

Table 5.1. Hypothesized effects of increased water use frequency under various hot water system operating conditions on L. pneumophila in distal taps.

Condition[1]	Dominant Impact	Hypothesized Result	Experiment herein
I. No disinfectant and low water heater set point (T < 48° C)	Growth due to increased delivery of nutrients to distal taps at ideal growth T	Greatest total numbers produced in distal taps with time, but lower concentrations due to more frequent use	Control System, over time (T = 39° C)
II. No disinfectant and moderate water heater set point (T = 48-51°C)	Low use condition provides optimal ecological selection by transient sub-lethal T events	Lower numbers produced in distal taps and lower concentrations at higher use	Exp. 2 (T = 51°C)
III. No disinfectant and high water heater set point (T >55° C)	Regrowth limited only to distal taps during stagnation events	Lower concentrations	Exp. 3 (T = 58° C)
IV. Stable and high disinfectant	Disinfection effect dominates	Lower number and concentrations	Not tested in this work

[1]See Fig. 5.2 for hypothetical temperature effects from the literature

Table 5.2. Calculations for determining *L. pneumophila* distribution across various system compartments and effects of operating conditions

Analysis	Calculation	Practical Meaning/Interpretation
Temperature effect on total planktonic loads (Table 4)	$\frac{(\sum[Lp]_{Distal} \times \forall_{Distal} + [Lp]_{Recirc} \times \forall_{Recirc})_{Cont}}{(\sum[Lp]_{Distal} \times \forall_{Distal} + [Lp]_{Recirc} \times \forall_{Recirc})_{Experir}}$	Factor by which planktonic *L. pneumophila* increase in the total system if the temperature is not elevated to the experimental setting
Temperature effect on recirculating line and tank loads (Table 4)	$\frac{(\sum[Lp]_{Recirc} \times \forall_{Recirc})_{Control}}{(\sum[Lp]_{Recirc} \times \forall_{Recirc})_{Experimental}}$	Factor by which planktonic *L. pneumophila* increase in the recirculating portions of the system if the temperature is not elevated to the experimental setting
Temperature effect on distal tap loads (Table 3)	$\frac{(\sum[Lp]_{Distal} \times \forall_{Distal})_{Control}}{(\sum[Lp]_{Distal} \times \forall_{Distal})_{Experimental}}$	Factor by which planktonic *L. pneumophila* increase at the tap if the temperature is not elevated to the experimental setting
Regrowth in recirculating lines (Fig. 3)	$[Lp]_{\mathrm{Recirc}} - [Lp]_{\mathrm{Inf}}$	Regrowth in the recirculating lines relative to the influent
Concentration (Fig. 4a)	$[Lp]$	*L. pneumophila* concentration
Factor Increase (Fig. 4b)	$\frac{[Lp]_{Distal}}{[Lp]_{Recirc}}$	Regrowth in the distal taps relative to the recirculating line
Weekly yield (Fig. 4c)	$[Lp]_{Distal} \times \forall_{Distal} \times Use$	Total amount of *L. pneumophila* delivered at the tap per week

Notes:

1. [Lp] – *L. pneumophila* concentration in gene copies/mL;
2. Distal – "in Distal Tap";
3. Recirc – "in Recirculating line and tank";
4. ∀ - "Volume";
5. Inf = "influent"
6. Use = use per week (21, 3, or 1)

Physicochemical Trends. *Distal Pipe Temperatures.* We documented a clear disconnect between water heater set point and temperatures observed at the distal taps. Water at the distal taps cooled to room temperature (26.1° C ± 0.2° C) within 25 minutes of each water use event, regardless of water heater set point temperature (Fig. 5.2). In general, water in the distal taps never exceeded the temperature × time requirements to achieve 99% disinfection of *Legionella.* Stagnation temperature differed by only ~1° C (Fig. C.2) and *Legionella* spp. and *L. pneumophila* levels were not significantly different in upward versus downward oriented pipes (paired t-test, n=177, p-value = 0.48 and 0.31, respectively), so these data were pooled for subsequent analysis, resulting in six replicates for each water use frequency.

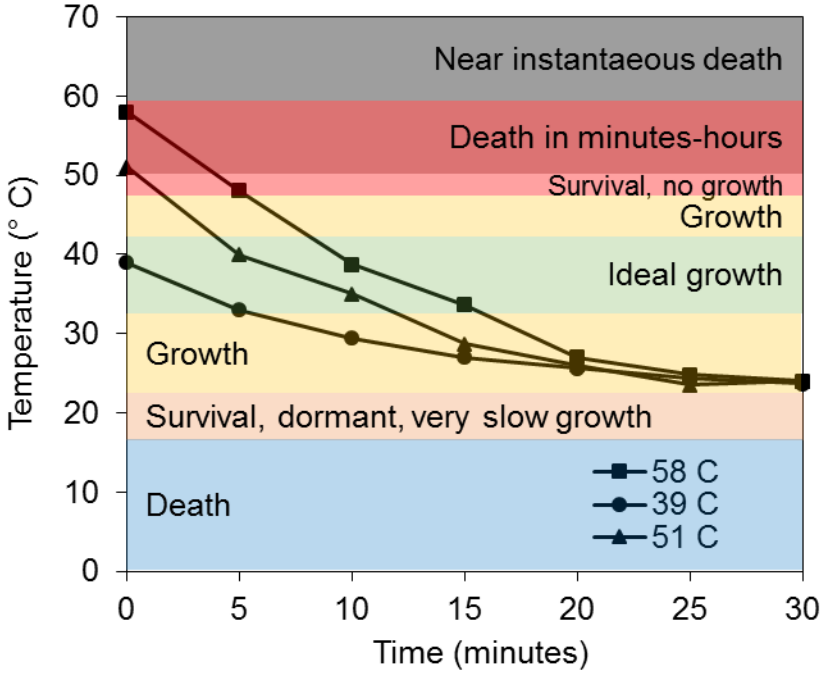

Fig. 5.2. Water temperatures (and reported effects on *Legionella*) at distal taps with stagnation time

Targeted water temperatures were not maintained in pipes for sufficient durations after each use to effectively disinfect *Legionella*. Shaded temperatures regions labelled on the plot represent the time required to achieve 90% inactivation of *Legionella.* [Time to 90% death and growth temperature ranges based on references (Ohno et al., 2008; Dennis et al., 1984; Schulze-Röbbecke et al., 1987; Darelid et al., 2002; Söderberg et al., 2004; Wadowsky and Yee, 1981; Wadowsky et al., 1985)]

*Total Chlorine***.** Chloramine was removed from influent Blacksburg, VA drinking water using three granular activated carbon filters (Pentek, Upper Saddle River, NJ). Average total chlorine concentrations in the influent water samples were always less than 0.10 mg/L as Cl_2 and remained near the detection limit (0.02 mg/L as Cl_2) in the water heaters throughout the experiment (Fig. S3). Therefore we achieved the goal of eliminating disinfectant from the system, which we hypothesize would have over-ridden the effects of temperature and water use that are the focus of this study (Table 2.1).

General Trends in *L. pneumophila* Occurrence and Effect of Water Heater Temperature. *L. pneumophila* were found to naturally colonize the systems at comparable levels following the 5 month baseline conditioning at 39° C (Table 5.3), which facilitated subsequent comparisons throughout the study. Further, elevated levels of *L. pneumophila* in the recirculating lines relative to the influent across all samplings confirmed that at least some portion of the *L. pneumophila* detected was actively regrowing in the building plumbing, and not just passing through from the influent water (Fig. 2.3, 1.7-3.5 logs higher in the recirculating lines; Kruskal-Wallis Test, p-value=0.002-0.035, except the control system baseline sampling, p-value=0.11 and the experimental system at 51° C, p-value=0.080). Unless otherwise stated, we focus our discussion here on behavior of planktonic *L. pneumophila*, which is ultimately what consumers will be exposed to in buildings, and later describe what was observed with respect to other target microbes and in the biofilm.

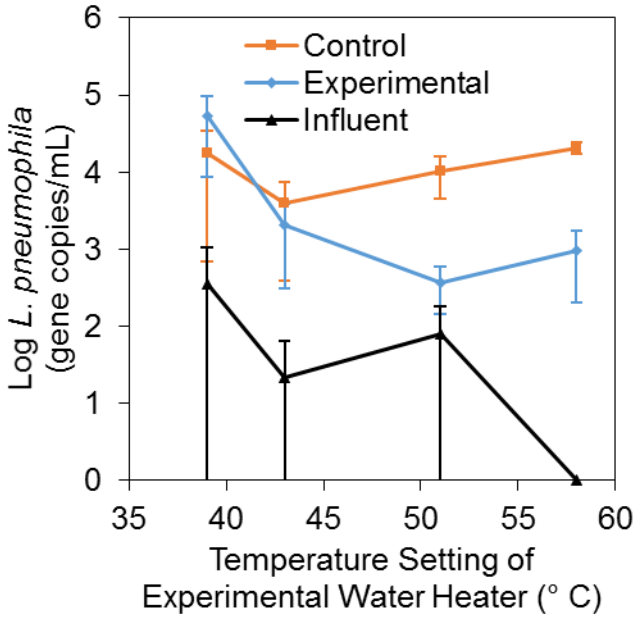

Fig. 5.3. *L. pneumophila* concentrations in the recirculating lines compared to the influent
L. pneumophila concentration in the recirculating lines compared to the influent. X axis reports the temperature setting for the experimental water heater, with the corresponding values for the control and influent plotted for the same time point. The control system remained at 39° C throughout the experiment. Error bars indicate 95% confidence intervals on biological replicate samples (n=2-6).

Generally, it was found that *L. pneumophila* decreased as the water heater setting increased, as was apparent in comparing levels in the control versus experimental recirculating lines (Fig. 2.3). More detailed comparisons were made by normalizing the levels of *L. pneumophila* gene copies in the control to the experimental system as an indicator of how much higher they would be without the elevated temperature intervention (Table 3). When the experimental system was set to 51° C, *L. pneumophila* were 28.7 times lower in the recirculating portion of the experimental system than the control system (Kruskal-Wallis Test, p-value = 0.019, n=12), but the benefits of increased temperature were not observed at the distal taps until the highest experimental temperature setting, where *L. pneumophila* was 43.6 times lower in distal taps in the experimental system set to 58°C than in the control system set to 39° C (Kruskal-Wallis Test, p-value=0.0005, n=18) (Table 5.3). The overall trend illustrated that the elevated water heater temperature settings were more immediately effective in the recirculating lines, which are continuously exposed to the

Table 5.3. Average total number of planktonic *L. pneumophila* gene copies in each reservoir during each sampling (for each sampling, n=18 for distal taps; n=2-6 for tank+recirc).

System	Reservoir	Baseline (5 months)	Exp. 1 (8 months)	Exp. 2 (13 months)	Exp.3 (15 months)
Control (Always 39° C)	Distal Taps (water)	5.01E+07	3.02E+07	1.02E+08	2.17E+08
	Tank+Recirc (water)	1.30E+09	2.94E+08	7.55E+08	1.55E+09
Experimental	Distal Taps (water)	5.60E+07	5.92E+06	2.44E+07	4.98E+06
	Tank+Recirc (water)	3.94E+09	1.55E+08	2.63E+07	7.12E+07
Control normalized to Experimental:		39 °C /39 °C	39 °C/42 °C	39 °C/51 °C	39 °C/58 °C
Total system *L. pneumophila* genes (water)		0.30	2.0	16.9	23.2
Tank+Recirc *L. pneumophila* genes (water)		0.33	1.9	28.7	21.8
Distal tap *L. pneumophila* genes (water)		0.89	5.1	4.2	43.6

hot water, whereas higher temperature settings were needed to best control *L. pneumophila* at the tap, where the water stagnates and quickly cools.

***L. pneumophila* in the Control System and Effect of Use Frequency (Condition I).** Examination of the control system provided the opportunity to directly evaluate the effect of water use frequency, as described in Condition I (Table 5.1). Interestingly, we observed that there was initially little difference in the concentration of *L. pneumophila* (gene copies/mL) as water use frequency changed (Fig. 5.4A; Kruskal-Wallis Test, p-value=0.31-0.52). However, this initial assessment can be deceiving as the actual yield of *L. pneumophila* at the tap (gene copies per week) typically increased by about one log from low use to high use, because the concentrations are multiplied by the number of times per week each tap was used (Table 5.2; Fig. 5.4C). This trend was also true for the experimental system when operated at the baseline condition before the temperature was elevated. We hypothesize that this phenomenon is due to increased delivery of nutrients under higher use frequency conditions, which broadly stimulates the microbial community at the tap. If true, this would suggest that increasing water use frequency alone will not necessarily fix a *Legionella* problem associated with stagnant conditions and could partially explain discrepancies in the effects of stagnation in prior reports (Health and Safety Executive, 2015; Liu et al., 2006; Moore et al., 2006; Mathys et al., 2008).

Comparing the distal taps to the recirculating lines is another approach to evaluate the effect of use frequency and stagnation (Fig. 5.4B). The *L. pneumophila* regrowth factor (defined in Table 5.2) under Condition I tended to strengthen with time, indicating that *L. pneumophila* could become more concentrated under the stagnant conditions at distal taps relative to the recirculating line as a system ages. Specifically, the *L. pneumophila* growth factor was less than 1 for all three water use conditions at the time of the baseline sampling, but increased to 5.5 and 3.2 in the low and medium use frequencies, respectively, by 15 months (Fig. 5.4B).

Monitoring the control system with time was also essential for this study in order to be certain that the trends observed in the experimental condition were a result of the temperature elevation and not necessarily natural succession of the microbial populations. Notably, *L. pneumophila* levels generally increased with time at the tap of the control system over the 15 month study (Table 5.3, by a factor of 4.3; Kruskal-Wallis Test, p-value<0.0001, n=16-18 per sampling event), especially in the low use condition (Fig. 5.4A). By the end of the study, *L. pneumophila* was 6.3 times higher (1.1×10^5 gene copies/mL) in the low use relative to high use distal taps (a factor of 6.3) (Kruskal-Wallis Test, p-value=0.004), suggesting that differences induced by water use frequency became more pronounced as the microbial ecology of the systems matured. In contrast, *L. pneumophila* levels were relatively stable with time in the recirculating portions of the system (Table 5.3; Fig. 5.3, Kruskal-Wallis Test, p-value = 0.22-0.40; n=6 per sampling event). Consistent with the nutrient delivery hypothesis, this suggests that a stable microbial ecology may take longer to establish at the tap, where flow is intermittent, than in a continuously flowing system. A random survey of 452 household hot water systems also suggests that it may take time for *Legionella* to colonize new pipes, where it was found that homes with new plumbing systems (<2 years old) had no *Legionella* spp. positive samples while 14% of older homes were colonized (Mathys et al., 2008).

A *L. pneumophila* concentration (log gene copies/mL)

Control System (39° C)	Water Use Freqency Low	Medium	High	Experimental System	Water Use Freqency Low	Medium	High
5 months	4.2	4.2	4.1	Baseline (39° C)	4.3	4.3	4.2
8 months	4.2	3.4	3.9	Exp.1 (42° C)	3.5	3.1	3.0
13 months	4.3	4.7	4.3	Exp. 2 (51° C)	4.3	2.3	2.2
15 months	5.1	4.8	4.3	Exp. 3 (58° C)	3.4	3.2	2.7

B *L. pneumophila* regrowth factor (distal taps/recirculating lines)

Control System (39° C)	Water Use Freqency Low	Medium	High	Experimental System	Water Use Freqency Low	Medium	High
5 months	0.8	0.9	0.7	Baseline (39° C)	0.3	0.4	0.3
8 months	4.2	0.7	2.1	Exp.1 (42° C)	1.5	0.5	0.5
13 months	1.9	5.6	1.9	Exp. 2 (51° C)	68.2	0.7	0.6
15 months	5.5	3.2	1.0	Exp. 3 (58° C)	2.7	1.6	0.5

C Total *L. pneumophila* yield per week (log gene copies)

Control System (39° C)	Water Use Freqency Low	Medium	High	Experimental System	Water Use Freqency Low	Medium	High
5 months	6.9	7.4	8.1	Baseline (39° C)	7.0	7.5	8.2
8 months	6.9	6.6	7.9	Exp.1 (42° C)	6.2	6.2	7.0
13 months	7.0	7.9	8.3	Exp. 2 (51° C)	7.0	5.5	6.3
15 months	7.8	8.0	8.4	Exp. 3 (58° C)	6.1	6.4	6.7

Fig. 5.4. Heat map of *L. pneumophila* occurrence at the distal taps

Heat maps of *L. pneumophila* comparing A) concentration in bulk water at each distal tap (log gene copies/mL), B) distal taps normalized to the recirculating lines (regrowth factor), and C) total yield of *L. pneumophila* per week at the tap (log gene copies). Colors are on a continuous scales from green (low) to red (high). Table 5.3 provides a detailed description of each calculation.

***L. pneumophila* in the Experimental System at Moderate Temperature (51° C) (Condition II).** A major finding of this study may best be described as an ecological "sweet spot" that occurred when the water heater was set at 51° C and the water use frequency was low. In this specific condition, enrichment of *L. pneumophila* at the tap relative to the recirculating line was striking (68.2 times higher; Fig. 5.4B). Interestingly, *L. pneumophila* concentrations decreased at the tap as expected in the medium and high use scenarios relative to both low use and the recirculating lines as the temperature was elevated to 51° C (Fig. 5.4A, B), suggesting a unique phenomenon when a moderate water heater temperature is combined with low water use frequency. Besides being enriched relative to the recirculating line, *L. pneumophila* under the 51° C/low use condition were also uncharacteristically high in concentration (Fig. 5.4A), equivalent to that of the control system maintained at optimal growth temperature (Kruskal-Wallis Test, p-value=1.0), and was the only case where low use distal taps yielded greater total *L. pneumophila* than high use distal taps (Fig. 5.4C, by a factor of 5, Kruskal-Wallis Test, p-value=0.044). We hypothesize that a brief exposure to a sub-optimal disinfection temperature (i.e., Fig. 5.2) combined with sufficient stagnation time for recovery and re-growth can lead to selection of *L. pneumophila* at the tap. Others have also noted evidence that brief exposures to elevated temperatures could have unintended negative consequences by decreasing competition or enhancing nutrient availability

via necrotrophic growth (Allegra et al., 2011; Temmerman et al., 2006), and rapid recolonization after thermal disinfection has been observed in the field (Steinert et al., 1998). Importantly, new guidelines on effective control of *Legionella* in building systems suggest maintaining at least 51° C in all portions of the hot water system (ASHRAE, 2000; 2015). It is apparent from these results that it will be difficult (if not impossible) to maintain set point temperatures throughout distal portions of the system (Fig. 5.2; Fig. C.2), and may inadvertently increase *Legionella* risk under certain circumstances. The 51° C "sweet spot" warrants further investigation.

***L. pneumophila* in the Experimental System at High Temperature (58° C) (Condition III).** While elevating the water heater temperature to 58° C effectively eliminated the selective effect of the 51° C/low use condition, the advantages were not striking in terms of *L. pneumophila* concentrations (Fig. 5.4A) or yields (Fig. 5.4C) in medium or high use distal taps relative to 42° C or 51° C. Nevertheless, the advantages of elevated water heater temperature were clear when comparing the experimental to the control system (Fig. 5.4; 40-50 times reduction in total weekly yield at 58° C vs 39° C), suggesting that the gradual *L. pneumophila* colonization of both systems with time may have muted the benefits of the elevated temperature. Further, *L. pneumophila* tended to be positively selected at the tap in the control system (Fig. 5.4B, ratios generally >1.0) and negatively selected at the tap in the experimental system at 58° C/high use frequency (Fig. 5.4B, ratios <1.0). This suggests that, if applied properly, elevated temperature can have a lasting effect for *L. pneumophila* control at the tap. Interestingly, the enhanced nutrient delivery hypothesis appeared to hold true as the temperature was elevated in the experimental system, with increased total yields of *L. pneumophila* as water use frequency increased (Fig. 5.4C). However, increased water use decreased *L. pneumophila* concentrations by a factor of 5.0 relative to lower use in the experimental system at 58° C (Fig. 5.4A).

Microbial Ecological Relationships of *L. pneumophila* in Hot Water Plumbing. *Trends in Biofilm-associated L. pneumophila.* We repeatedly swabbed the same area (65 cm^2) to collect biofilm at the end of each experimental period, providing a measurement of *L. pneumophila* that re-colonized pipe surfaces at each temperature setting (Fig. 5.5). *L. pneumophila* in influent pipe biofilms were consistently below the detection limit, except for the 11 month sampling date (Fig. 5.5A, during a period of elevated influent water temperature of 22-23° C versus 11-13° C for subsequent sampling events), further lending confidence that *L. pneumophila* gene copies observed in the plumbing systems were representative of regrowth and not an artifact of the influent. Interestingly, *L. pneumophila* levels were consistently below detection in the recirculating pipe biofilm of the experimental system when the water heater setting was ≥ 48° C, while they consistently increased with time in the control rig set to 39° C (Fig. 5.5A). Thus, it appears that *L. pneumophila* was not adept at re-colonizing biofilms at moderate-high water heater temperature set points, though it cannot be certain how it behaved in intact portions of the biofilm not subject to re-sampling.

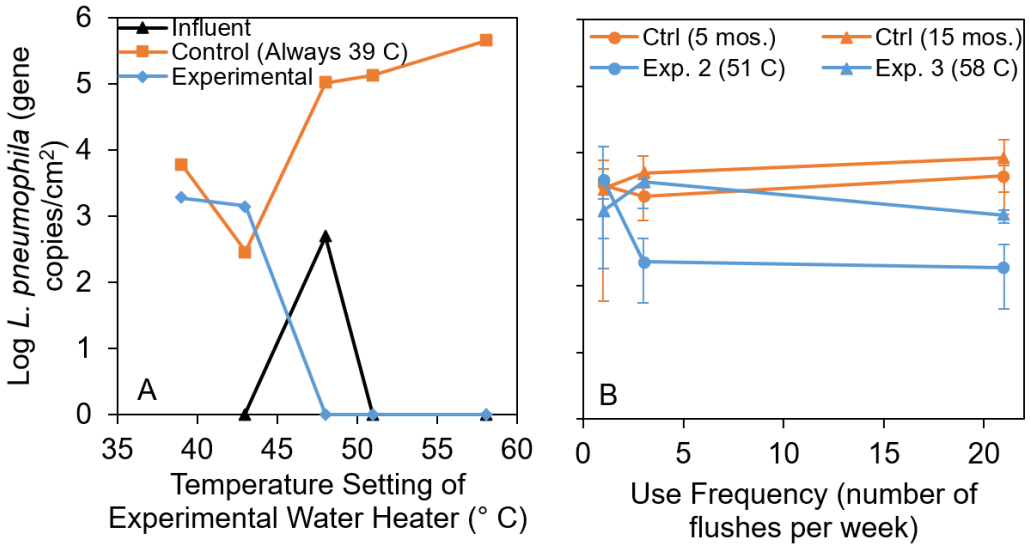

Fig. 5.5. Biofilm-associated *L. pneumophila* concentrations

A) *L. pneumophila* concentrations in recirculating lines as a function of water heater temperature setting. The x-axis reports the temperature setting for the experimental water heater, with the corresponding values for the control and influent plotted for the same time point. The control system remained at 39° C throughout the experiment. No error bars were calculated due to the biofilm sampling approach used. B) *L. pneumophila* concentrations at the distal taps as a function of flush frequency. Error bars indicate 95% confidence intervals on biological replicate samples (n=6). Note that biofilms were subject to repeated sampling of the same area, thus the numbers represent re-growth between sampling events.

Water use frequency also appeared to affect regrowth of biofilm-associated *L. pneumophila*. For example, in the control system biofilm, *L. pneumophila* increased with increased use frequency, with 55 times more *L. pneumophila* in the continuously recirculating line than the most frequently used distal taps by the end of the study (Fig. 5.5A vs 5.5B). This is consistent with the nutrient delivery hypothesis (Liu et al., 2006). However, where there was a trend in the experimental system, it was the opposite, with 19.2 times less biofilm-associated *L. pneumophila* in high use distal taps than low use taps when the heater was set at 51 ° C (Fig. 5.5B, Kruskal-Wallis Test, p-value=0.037, n=12). Notably, this was also the ecological "sweet spot" condition noted above, suggesting that the brief exposure to sub-optimal disinfection temperature followed by long stagnation selected for *L. pneumophila* in the biofilm as well as the bulk water. Although water use frequency can be subordinate to other factors, such as temperature and corresponding microbial ecological responses, analyzing water use conditions in conjunction with water temperature helps reconcile discrepancies in prior reports of effects of stagnation on *L. pneumophila* (Ciesielski et al., 1984; Liu et al., 2006; Mathys et al., 2008).

Relationships among L. pneumophila and Other Ecologically-Relevant Microorganisms. Relationships were explored among total bacteria, *Legionella* spp., and *V. vermiformis* to gain insight into how *L. pneumophila* behaved in the context of the broader plumbing microbiome (Fig. 5.6 and 5.7). Remarkably, elevated temperatures did not have a significant effect on levels of total bacteria in the recirculating lines or at the tap (Kruskal-Wallis Test, p-value=0.27, n=58). While it was expected that the disinfecting properties of the hotter water would reduce total microbial populations, our results suggest that instead the elevated temperature merely shifted the

microbiome composition, which can be seen by reductions in the other specific targets in the experimental relative to the control system (Fig. 5.6 and 5.7).

Of particular interest was the relationship between *Legionella* and *V. vermiformis*, which is among free-living amoebae thought to act as obligate hosts for *Legionella* replication in drinking water systems and thus could be an important player in pathogen control (Thomas and Ashbolt, 2010; Wang et al., 2012; Rowbotham, 1980). Here we found that *Legionella* spp. and *L. pneumophila* were correlated with *V. vermiformis* under certain circumstances. During the baseline sampling, when the microbial community was still developing, there were no correlations between *V. vermiformis* and *Legionella* spp. or *L. pneumophila* (Spearman Rank Correlations, Rho=-0.19-.47, p-value=0.15-0.47). However, later in the experiment (13 months), significant correlations developed in the distal pipes in the mature experimental system set to 51° C (Spearman Rank Correlation, Rho=0.52-0.68, p-value=0.002-0.031). This suggests *V. vermiformis* may have played a role in the much higher levels of *L. pneumophila* observed in the water- and biofilm-associated *L. pneumophila* as thermal stresses reached the "sweet spot" in the experimental system at 51° C (Fig. 5.4B and 5.4C). While correlations did occasionally exist in the recirculating line samples, seven of eight correlations of *V. vermiformis* compared to *Legionell*a spp. and *L. pneumophila* during the last two sampling periods were inconsistent and insignificant (Spearman Rank Correlation, Rho=0.02-0.70, p-value=0.19-0.95). Lack of a consistent correlation suggests a dynamic relationship between *V. vermiformis* and *Legionell*a, which is intuitive given their predator-prey relationship.

Relationship between Legionella spp. and L. pneumophila. The genus *Legionella* contains other pathogens, besides *L. pneumophila*, as well as non-pathogenic members. Thus, there is interest in how *L. pneumophila* behaves in hot water systems relative to *Legionella* spp. *L. pneumophila* and *Legionella* spp. were strongly correlated across all water samples (R^2=0.70, n=484) and across all distal tap water samples (R^2=0.75, n=357), but in most other cases correlations were weak (e.g., R^2=0.57 in water samples of recirculating lines, n=90) or non-existent. This indicates that there are situations under which *L. pneumophila* trends with other *Legionella* spp., and other cases where it does not. In particular, we observe an apparent decrease in the ratio of *L. pneumophila* to *Legionella* spp. with elevated temperature. For instance, when the temperature of the experimental system was increased to 48° C or 58° C (but not 51° C possibly due to the unique selective condition), the ratio of *L. pneumophila* to *Legionella* spp. was significantly lower in the experimental than in the control system (Paired t-Test, p-value<0.0001, n=36-48). While temperature may truly be the dominating factor influencing the type of *Legionella* that prevail, other selectors have been noted in the literature, such as other microorganisms (e.g., *B. subtilis*) inhibiting *L. pneumophila* growth within amoeba or lysing cells (Temmerman et al., 2006; Guerrieri et al., 2008; Wang et al., 2013).

Survival of *L. pneumophila* at Elevated Temperatures. Importantly, this study demonstrated that, even at the highest temperature of 58° C, *L. pneumophila* was not eliminated from the hot water plumbing and continued to persist at levels greater than the influent (Fig. 5.3). We did not expect this result given that it is thought that *L. pneumophila* is unable to replicate above 50° C (Söderberg et al., 2004; Wadowsky and Yee, 1981; Wadowsky et al., 1985; Kuchta et al., 1993; Schulze-Röbbecke et al., 1992), though it has been observed to survive short periods of time at 55-70° C and long periods (on the order of months) as free organism in hot springs water (Söderberg

et al., 2004; Wadowsky and Yee, 1981; Wadowsky et al., 1985; Sheehan et al., 2005; Ohno et al., 2003; Dennis et al., 1984; Schulze-Röbbecke et al., 1987; Darelid et al., 2002). Nonetheless, our work is strongly suggestive that *L. pneumophila* growth does occur in this temperature range under representative plumbing conditions. Given that 99.97% (3-logs) of planktonic *L. pneumophila* would theoretically be washed out of both systems each week, regrowth is the most likely

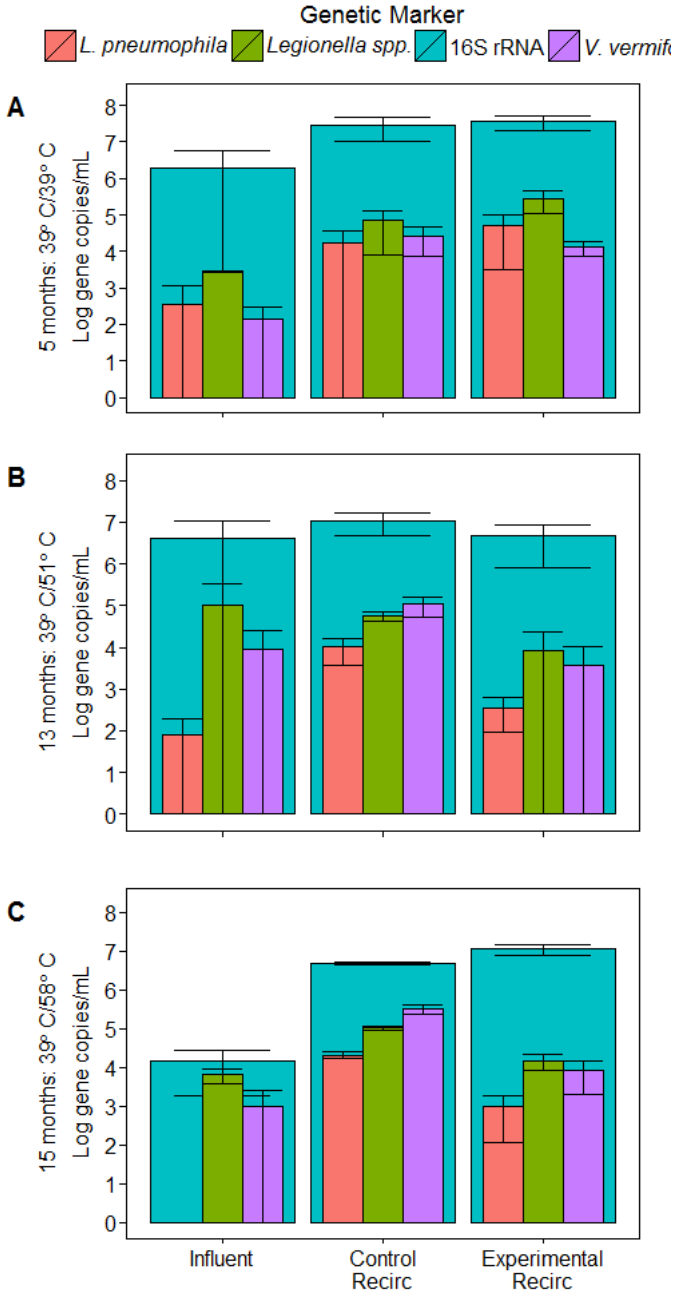

Fig. 5.6. Relative levels of *L. pneumophila* and ecologically-relevant microbes in the influent and recirculating line

Log *L. pneumophila*, *Legionella* spp., and *V. vermiformis* nested within total bacteria concentrations (gene copies/mL) in the influent and recirculating lines for A) the baseline sampling (both systems set to 39° C at 5 months), B) Exp. 2 (control system set to 39° C, experimental system set to 51° C at 13 months), and C) Exp. 3 (control system set to 39° C, experimental system set to 58° C at 15 months).

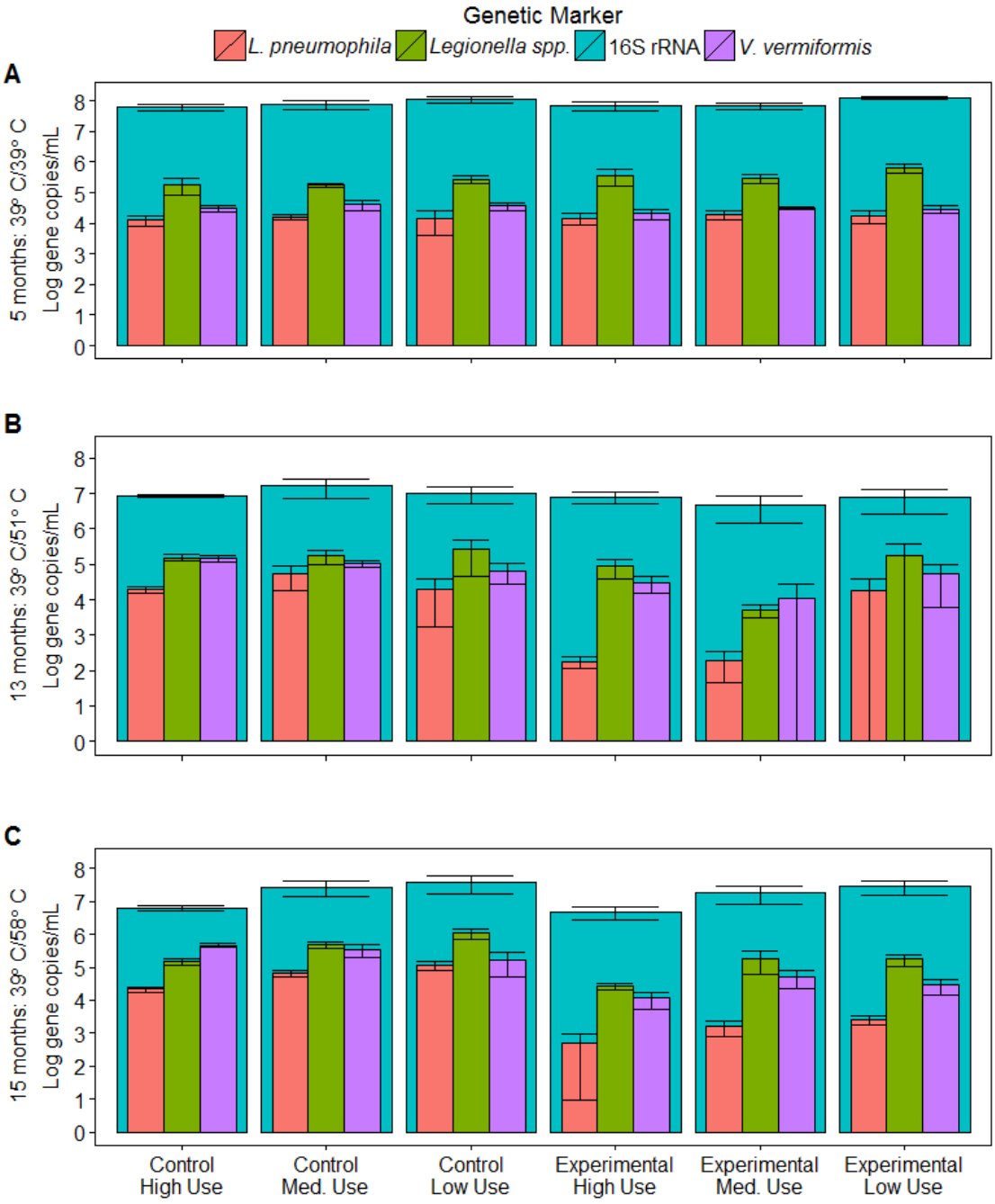

Fig. 5.7. Relative levels of *L. pneumophila* and ecologically-relevant microbes in the distal taps
Log *L. pneumophila*, *Legionella* spp., and *V. vermiformis* nested within total bacteria concentrations (gene copies/mL) in the distal taps for each water use frequency for A) the baseline sampling (both systems set to 39° C at 5 months), B) Exp. 2 (control system set to 39° C, experimental system set to 51° C at 13 months), and C) Exp. 3 (control system set to 39° C, experimental system set to 58° C at 15 months).

explanation for the persistence observed at elevated temperature. Even though biofilm-associated *L. pneumophila* was shown to not be able to re-colonize the swabbed areas at higher temperatures, it is possible that *L. pneumophila* persisted in and was released from the vast majority of the biofilm not disturbed by sampling, perhaps within amoebae hosts. Notably, high levels of planktonic *V. vermiformis* were detected at 58° C (Fig. 2.6, average of 8.4×10^3 gene copies/mL), which could extend the range at which *L. pneumophila* grows (Kuchta et al., 1993; Storey et al., 2004).

CONCLUSION

Here we examined the effect of water heater temperature setting and water use frequency, which are two critical factors for energy and water conservation, on *L. pneumophila* as a representative OP resident to the building plumbing microbiome. This controlled, replicated, pilot-scale approach aided in resolving complexities encountered in prior field studies and addressing discrepancies with respect to effects of temperature and stagnation reported in the literature. Overall, it was found that elevated temperature was a critical factor in suppressing *L. pneumophila* growth both in continuously recirculating hot water lines and at the tap, where water quickly cools to room temperature following heat shock. Nonetheless, naturally occurring *L. pneumophila* persisted up to 58° C, with strong evidence for growth within this pilot-scale plumbing system, relative to prior understanding that it does not grow above 50° C under simplified laboratory conditions. Further, it was found that temperature and water use frequency can have interactive effects; for example, optimal *L. pneumophila* selection at the tap was observed when the water use frequency was low following a heat shock at 51° C. At the same time, while higher use frequency can dilute *L. pneumophila* and result in lower concentrations at the tap, it still tended to result in higher overall yields, given that concentration is multiplied by higher use frequency. We hypothesize that this is a result of increased water use frequency replenishing nutrients required for *L. pneumophila* re-growth in combination with temperature. Overall, this study takes a step towards untangling the complexity of the factors shaping the microbial ecology of hot water plumbing and lays the groundwork for an integrated approach for opportunistic pathogen control.

MATERIALS AND METHODS

Experimental Setup and Operation. Two identical household hot water systems with 71.9 L (19 gallon) electric water heaters and continuously recirculating pipe loops were constructed with nominal ¾" chlorinated polyvinyl chloride (CPVC; Charlotte Pipe, Charlotte, NC) pipe (Fig. 2.1). Each system tested two pipe orientations (upward/downward) with three water use patterns in triplicate, including low use (1 flush/week), medium use (3 flushes/week), and high use (21 flushes/week) for a total of 36 distal taps (2 systems × 2 orientations × 3 use patterns × triplicate = 36). Each distal tap pipe was 1.7 m (5.5 ft) for a total distal tap volume of 0.43 L (0.11 gallons) and internal surface area of 0.87 m^2 (0.94 ft^2). Each recirculating line was a total of 7.6 m (25 ft). The water heaters and recirculating lines were completely mixed, resulting in a combined volume of 73.9 L (19.5 gallons) and surface area of 1.46 m^2 (15.7 ft^2). Each flush was conducted for 28 seconds at 3.8 L/min (1 gallon/min). Influent water consisted of well-flushed (10 minutes at 11.3 L/min), granular activated carbon (GAC) filtered Blacksburg, VA tap water. Both systems were initially acclimated for 5 months at 39° C. Afterwards, the experimental system water heater temperature was increased approximately by 5° C increments while the control system remained at 39° C. During periods of stagnation, distal pipes cooled to room temperature.

Water Quality Analysis. Disinfectant residual, total ammonia, temperature, pH, dissolved oxygen (DO), total organic carbon (TOC), and total and dissolved cations were generally characterized at each temperature setting beginning one week after each temperature adjustment. Chloramine and total ammonia were measured according to Standard Method 4500-Cl25 and 5310-NH3 using a DR2700 or DR5000 spectrophotometer (HACH, Loveland, CO). pH and temperature was measured using a pH 110 meter with automatic temperature correction (Oakton Research, Vernon Hills, IL). DO was monitored using a Thermo Scientific Orion 3-star meter. TOC was measured by persulfate-ultraviolet detection using a Sievers Model 5300C with an autosampler according to Standard Method 5310 C. Cations were measured by inductively coupled plasma mass spectrometry after acidification with 2% nitric acid (v/v) and >24 hours holding time.

Microbiological Sample Collection and DNA Extraction

After a minimum of two-months acclimation period at each experimental condition, approximately 0.5 L of first-flush water was collected directly from the influent, recirculating lines, and each distal tap at the end of regular stagnation periods for each use condition and filtered through sterile 0.22 µm pore-size mixed cellulose ester filters (Millipore, Billerica, MA). Filters were fragmented and subject to DNA extraction. For biofilm sampling, 65 cm^2 of influent, recirculating line, and ends of the distal tap pipes accessible by threaded union connections were swabbed using sterile cotton-tip applicators (Fisherbrand, Fisher Scientific, UK). DNA was extracted directly from the fragmented filters and cotton swabs using a FastDNA Spin Kit (MP Biomedicals, Solon, OH) according to the manufacturer protocol. Field, trip, and equipment negative controls consisting of pre-sterilized water in identical sampling bottles were included each time samples were collected.

Quantitative Polymerase Chain Reaction

Gene markers for *Legionella* spp., *L. pneumophila*, and *V. vermiformis*, along with bacterial 16S rRNA genes, were enumerated by quantitative polymerase chain reaction (q-PCR) assays using previously established methods (Wang et al., 2012). In brief, all q-PCR assays were performed in 10 µL reaction mixtures containing SsoFast Probes or Evagreen Supermix (Bio-Rad, Hercules, CA), 250 or 400 nM primer, 93.75 nM probe (Taqman assay only) with 1 µL of DNA template. DNA extracts (diluted 1:10 to minimize inhibition), a negative control, 10-fold serial dilutions of standards, and a positive spike into sample DNA matrix were included in triplicate wells with each q-PCR run. The quantification limit (QL) for all q-PCR assays ranged from 10 to 1,000 gene copies/reaction and was implemented as appropriate for each run. Samples yielding threshold cycles ≥ QL in at least two q-PCR triplicate wells were considered quantifiable. Sample with only one triplicate above the QL threshold cycle, or samples otherwise below the QL were re-analyzed undiluted to increase the QL of the assays. On each re-run plate, standard DNA template was spiked into the experimental DNA matrix to confirm amplification reactions were not inhibited in undiluted samples. If inhibited, the sample was marked as below the QL. All values are reported as log(gene copies/mL + 1).

Statistical Analyses. All error bars on figures and ±calculations are 95% confidence intervals, calculated based on the normal cumulative distribution function, degrees of freedom, and standard error. For graphing and statistical purposes, any positive detection below q-PCR QL was entered as half of the quantification limit. All data exploration was conducted in Microsoft Excel 2013 or JMP Pro 11. Spearman's rank coefficient and associated significance tests were conducted in JMP Pro 11 to detect and quantify relationships between gene markers (using "Multivariate Methods").

Other statistical tests were performed using RStudio with R version 3.2.0. Student's t-test ("t.test()") and multiple comparison Kruskal-Wallis tests with a Holm p-value adjustment for multiple comparisons were conducted to compare sample means (initially with "kruskal.test()," then using package and function "dunn.test()" for multiple comparisons). Significance was determined at p=0.05.

Competing Interests. The authors declare that they have no competing interests.

Authors' contributions. WJR designed, built, and maintained the experiment, collected (and prepared) samples for analysis, performed chemical/physical analyses, analyzed data, constructed all tables and figures, preformed all statistical analyses, and drafted the manuscript. PJ performed the q-PCR assays and provided feedback on the manuscript. AP and MAE designed the experiment, assisted in data analysis approach, and revised the manuscript. All authors read and approved the final manuscript.

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REFERENCES

Alary M, Joly JR. Risk factors for contamination of domestic hot water systems by legionellae. Applied and Environmental Microbiology. 1991;57(8):2360-2367.

Allegra S, Grattard F, Girardot F, Riffard S, Pozzetto B, Berthelot P. Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella* spp. in hospital water systems by using a flow cytometric assay. Applied and environmental microbiology. 2011;77(4):1268-1275.

AMSI/ASHRAE Standard 188-2015. Legionellosis: Risk Management for Building Water Systems. Atlanta, GA.

Anand C. M, Skinner A. R, Malic A, Kurtz J. B. Interaction of *L. pneumophila* and a free living amoeba (*Acanthamoeba palestinensis*). Journal of hygiene. 1983;91(02):167-178.

ASHRAE Guideline 12-2000. Minimizing the Risk of Legionellosis Associated with Building Water Systems.

Bartrand TA, Causey JJ, Clancy, J. L. Naegleria fowleri: An emerging drinking water pathogen. JAWWA. 2014;106:10.

Bédard E, Fey S, Charron D, Lalancette C, Cantin P, Dolcé P, Prévost M, et al. Temperature diagnostic to identify high risk areas and optimize *Legionella pneumophila* surveillance in hot water distribution systems. Water research. 2015;71:244-256.

Berry D, Xi C, Raskin L. Microbial ecology of drinking water distribution systems. Current opinion in biotechnology. 2006;17(3):297-302.

Borella P, Montagna M. T, Romano-Spica V, Stampi S, Stancanelli G, Triassi M, D'alcalà G.R, et al. *Legionella* infection risk from domestic hot water. Emerging infectious diseases. 2004;10(3):457.

Brown A. D. Some general properties of a psychrophilic pseudomonad: the effects of temperature on some of these properties and the utilization of glucose by this organism and Pseudomonas aeruginosa. Journal of general microbiology. 1957;17(3):640-648.

Brunkard JM, Ailes E, Robers VA, Hill V, Hilborn ED, Craun GF, Rajasingham A, Kahler A, Garrison L, Hicks L, Carpenter J, Wade TJ, Bearch MJ, Yoder JS. Surveillance for Waterborne Disease and Outbreaks Associated with Drinking Water and Water not Intended for Drinking --- United States, 2007—2008. MMWR Surveillance Summaries. 2011;60 (SS12):38-68.

Buse H. Y, Ashbolt N. J. Differential growth of *Legionella pneumophila* strains within a range of amoebae at various temperatures associated with in-premise plumbing. Letters in applied microbiology. 2011;53(2):217-224.

Camper AK, LeChevallier MW, Broadaway SC, McFeters GA. Growth and persistence of pathogens on granular activated carbon filters. Applied and Environmental Microbiology. 1985;50(6):1378-1378.

Chiao TH, Clancy TM, Pinto A, Xi C, Raskin, L. Differential resistance of drinking water bacterial populations to monochloramine disinfection. Environmental science & technology. 2014;48(7):4038-4047.

Ciesielski C. A, Blaser M. J, Wang W. L. Role of stagnation and obstruction of water flow in isolation of *Legionella pneumophila* from hospital plumbing. Applied and environmental microbiology. 1984;48(5):984-987.

Darelid J, Löfgren S, Malmvall B. E. Control of nosocomial Legionnaires' disease by keeping the circulating hot water temperature above 55 C: experience from a 10-year surveillance programme in a district general hospital. Journal of Hospital Infection. 2002;50(3):213-219.

Dennis P. J. L, Wright A. E, Rutter D. A, Death J. E, Jones B. P. C. *Legionella pneumophila* in aerosols from shower baths. Journal of hygiene. 1984;93(02):349-353.

Elfland C, Scardina P, Edwards MA. Lead-contaminated water from brass plumbing devices in new buildings. JAWWA. 2010;102(11):66.

Environemental Protection Agency (EPA). Effects of Water Age on Distribution System Water Quality. Office of Water. Office of Ground Water and Drinking Water. 2002. http://water.epa.gov/lawsregs/rulesregs/sdwa/tcr/upload/2007_05_18_disinfection_tcr_whitepaper_tcr_waterdistribution.pdf. Accessed 12 Sept 2015.

Falkinham JO. Common Features of Opportunistic Premise Plumbing Pathogens. International journal of environmental research and public health, 2015;12:(5):4533-4545.

Feazel LM, Baumgartner LK, Peterson KL, Frank DN, Harris JK, Pace NR. Opportunistic pathogens enriched in showerhead biofilms. Proceedings of the National Academy of Sciences. 2009;106(38):16393-16399.

Fliermans C. B, Cherry W. B, Orrison L. H, Smith S. J, Tison D. L, Pope D. H. Ecological distribution of *Legionella pneumophila*. Applied and Environmental Microbiology. 1981;41(1):9-16.

Guerrieri E, Bondi M, Sabia C, de Niederhäusern S, Borella P, Messi P. Effect of bacterial interference on biofilm development by *Legionella pneumophila*. Current microbiology. 2008;57(6):532-536.

Harper D. Legionnaires' disease outbreaks—the engineering implications. Journal of Hospital Infection. 1988;11:201-208.

Health and Safety Executive. *Legionella* and Legionnaires' disease. http://www.hse.gov.uk/legionnaires/ Accessed 12 Sept 2015.

Kuchta J. M, Navratil J. S, Shepherd M. E, Wadowsky R. M, Dowling J. N, Yee R. B. Impact of chlorine and heat on the survival of *Hartmannella vermiformis* and subsequent growth of *Legionella pneumophila*. Applied and environmental microbiology. 1993;59(12):4096-4100.

Lau H.Y, Ashbolt N.J. The role of biofilms and protozoa in *Legionella* pathogenesis: Implications for drinking water. J. Appl. Microbiol. 2009;107:368-378

Liu Z, Lin Y. E, Stout J. E, Hwang C. C, Vidic R. D, Yu V. L. Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. Journal of applied microbiology. 2006;101(2):437-442.

Mathys W, Stanke J, Harmuth M, Junge-Mathys E. Occurrence of *Legionella* in hot water systems of single-family residences in suburbs of two German cities with special reference to solar and district heating. International journal of hygiene and environmental health. 2008;211(1):179-185.

Moore M. R, Pryor M, Fields B, Lucas C, Phelan M, Besser R. E. Introduction of monochloramine into a municipal water system: impact on colonization of buildings by *Legionella* spp. Applied and environmental microbiology. 2006;*72*(1):378-383.

Muraca P, Stout J. E, Yu V. L. Comparative assessment of chlorine, heat, ozone, and UV light for killing *Legionella pneumophila* within a model plumbing system. Applied and Environmental Microbiology. 1987;53(2):447-453.

Nagington J, Smith D. J. Pontiac fever and amoebae. The Lancet. 1980;316(8206):1241.

Nguyen C, Elfland C, Edwards MA. Impact of advanced water conservation features and new copper pipe on rapid chloramine decay and microbial regrowth. Water research. 2012;46(3):611-621.

Ohl L, Horn H, Hempel D. Behaviour of biofilm systems under varying hydrodynamic conditions. Water Science & Technology. 2004;49(11-12):345-351.

Ohno A, Kato N, Yamada K, Yamaguchi K. Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. Applied and environmental microbiology. 2003;69(5):2540-2547.

OSHA technical manual. Section III: Chapter 7. Legionnaires' Disease. https://www.osha.gov/dts/osta/otm/otm_iii/otm_iii_7.html. Accessed 12 Sept 2015.

Pruden A, Edwards MA, Falkinham, III JO. Research Needs for Opportunistic Pathogens in Premise Plumbing: Experimental Methodology, Microbial Ecology and Epidemiology. Water Research Foundation Project 4379. 2012. http://www.waterrf.org/PublicReportLibrary/4379.pdf. Accessed 12 Sept 2015.

Rowbotham T. J. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. Journal of clinical pathology. 1980;33(12):1179-1183.

Schulze-Röbbecke R, Buchholtz K. A. T. R. I. N. Heat susceptibility of aquatic mycobacteria. Applied and environmental microbiology. 1992;58(6):1869-1873.

Schulze-Röbbecke R, Rödder M, Exner M. Multiplication and killing temperatures of naturally occurring legionellas. Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene. 1987;184(6):495-500.

Sheehan K. B, Henson J. M, Ferris M. J. *Legionella* species diversity in an acidic biofilm community in Yellowstone National Park. Applied and Environmental Microbiology. 2005;71(1):507-511.

Söderberg M. A, Rossier O, Cianciotto N. P. The type II protein secretion system of *Legionella pneumophila* promotes growth at low temperatures. Journal of bacteriology. 2004;186(12):3712-3720.

Steinert M, Ockert G, Lück C, Hacker J. Regrowth of *Legionella pneumophila* in a heat-disinfected plumbing system. Zentralblatt für Bakteriologie. 1998;288(3):331-342.

Storey M. V, Ashbolt N. J, Stenström T. A. Biofilms, thermophilic amoebae and *Legionella pneumophila*- A quantitative risk assessment for distributed water. Water Science & Technology. 2004;50(1):77-82.

Stout J. E, Victor L. Y, Muraca P. Legionnaires' disease acquired within the homes of two patients: link to the home water supply. *JAMA*. 1987;*257*(9):1215-1217.

Straus WL, Plouffe JF, File TM, Lipman HB, Hackman BH, Salstrom SJ, Breiman RF, et al. Risk factors for domestic acquisition of Legionnaires disease. Archives of Internal Medicine. 1996;156(15):1685-1692.

Temmerman R, Vervaeren H, Noseda B, Boon N, & Verstraete W. Necrotrophic growth of *Legionella pneumophila*. Applied and environmental microbiology. 2006;72(6):4323-4328.

Thomas J. M, Ashbolt N. J. Do free-living amoebae in treated drinking water systems present an emerging health risk? Environmental science & technology. 2010;45(3):860-869.

Van der Kooij D, Oranje J. P, Hijnen W. A. Growth of *Pseudomonas aeruginosa* in tap water in relation to utilization of substrates at concentrations of a few micrograms per liter. Applied and Environmental Microbiology. 1982;44(5):1086-1095.

Vasconcelos J. J, Boulos P. F. (1996). Characterization and modeling of chlorine decay in distribution systems. American Water Works Association. http://www.waterrf.org/ExecutiveSummaryLibrary/90705_815_profile.pdf. Accessed 13 Sept 2015.

Wadowsky R. M, Wolford R, McNamara A. M, Yee R. B. Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. Applied and environmental microbiology. 1985;49(5):1197-1205.

Wadowsky R. M, Yee R. B. Glycine-containing selective medium for isolation of Legionellaceae from environmental specimens. Applied and Environmental Microbiology. 1981;42(5):768-772.

Wallace Jr R. J, Brown B. A, Griffith D. E. Nosocomial outbreaks/pseudo outbreaks caused by nontuberculous mycobacteria. Annual Reviews in Microbiology. 1998;52(1):453-490.

Wang H, Edwards M, Falkinham J. O, Pruden A. Molecular survey of the occurrence of *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa*, and amoeba hosts in two chloraminated drinking water distribution systems. Applied and environmental microbiology. 2012;78(17):6285-6294.

Wang H, Edwards M. A, Falkinham III J. O, Pruden A. Probiotic approach to pathogen control in premise plumbing systems? A review. Environmental science & technology. 2013;47(18):10117-10128.

Yoder J, Roberts V, Craun GF, Hill V, Hicks L, Alexander NT, Radke V, Calderon RL, Hlavsa MC, Beach MJ, Roy SL. Surveillance for Waterborne Disease and Outbreaks Associated

with Drinking Water and Water not Intended for Drinking --- United States, 2005—2006. MMWR Surveillance Summaries. 2008;57 (SS09):39-62.

APPENDIX C - SUPPLEMENTARY INFORMATION FOR CHAPTER 5.

Table C.1. P-values for Kruskal-Wallis multiple comparisons with Holm p-value adjustment for planktonic *L. pneumophila* concentrations (gene copies/mL) in the influent and recirculating lines (See Fig. 2.3, n=2 for Influent baseline; otherwise n=6 for each system)

Comparison	Baseline (39° C v 39° C)	Exp. 1 (39° C v 42° C)	Exp. 2 (39° C v 51° C)	Exp. 3 (39° C v 58° C)
Influent vs Control	0.11	0.0019	0.0051	0.0002
Influent vs Experimental	0.016	0.0074	0.080	0.035
Control vs Experimental	0.084	0.29	0.019	0.033

Table C.2. P-values for Kruskal-Wallis multiple comparisons with Holm p-value adjustment for planktonic *L. pneumophila* concentrations (gene copies/mL) in distal taps as a function of flow frequency (See Fig. 2.4A)

Comparison	Control System, Baseline (5 mos, n=16)	Control System, Sample 4 (15 mos, n=18)	Experimental System, 39° C (5 mos, n=18)	Experimental System, 42° C (8 mos, n=18)	Experimental System, 48° C (11 mos, n=18)	Experimental System, 51° C (13 mos, n=18)	Experimental System, 58° C (15 mos, n=18)
High vs Medium	0.31	0.027	0.50	0.41	0.010	0.43	0.058
High vs Low	0.34	0.0004	0.52	0.088	0.18	0.0003	0.0015
Medium vs Low	0.43	0.080	0.37	0.094	0.088	0.042	0.080

Table C.3. P-values for Kruskal-Wallis with Holm p-value adjustment for multiple comparisons for total weekly planktonic *L. pneumophila* yield (gene copies) in distal taps as a function of flow frequency (See Fig. 2.4C)

Comparison	Control System (all samplings, n=90)	Experimental System, 39° C (5 mos, n=18)	Experimental System, 42° C (8 mos, n=18)	Experimental System, 48° C (11 mos, n=18)	Experimental System, 51° C (13 mos, n=18)	Experimental System, 58° C (15 mos, n=18)
High vs Medium	<0.0001	0.052	0.099	0.052	0.033	0.31
High vs Low	<0.0001	0.0001	0.084	0.0001	0.44	0.42
Low vs Medium	0.012	0.026	0.46	0.026	0.044	0.29

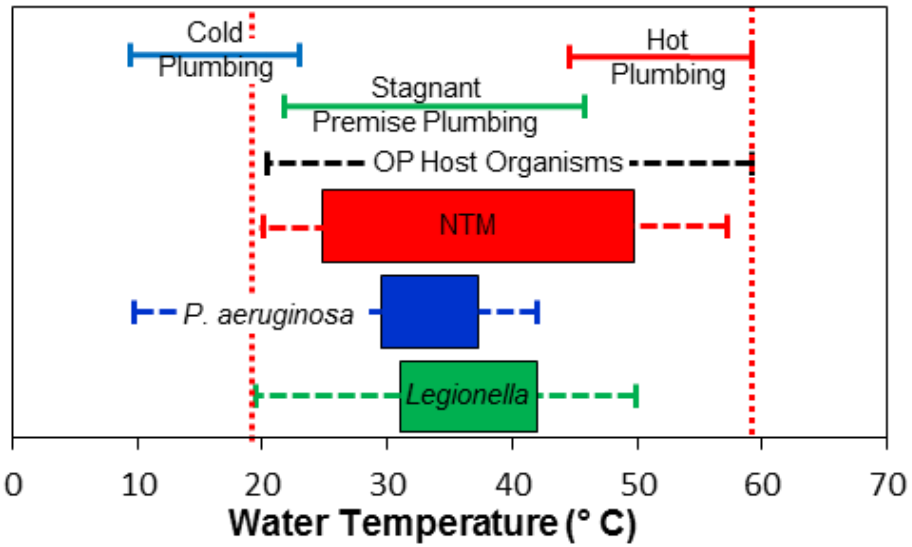
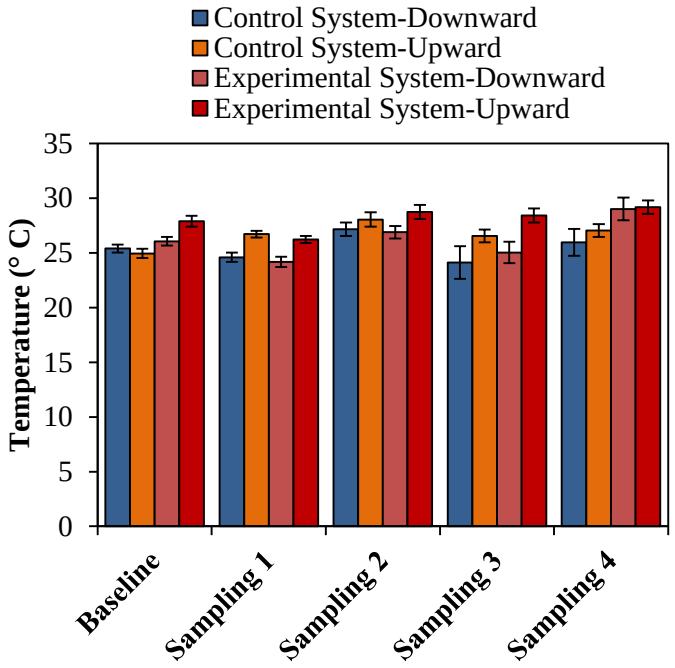


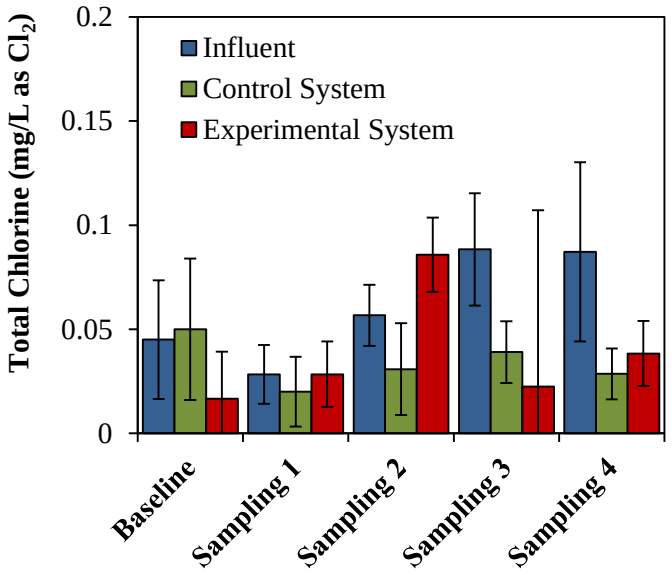
Fig. C.1. Opportunistic pathogen growth and survival temperatures relative to premise plumbing water temperatures
Solid boxes indicate ideal growth temperatures associated with reference opportunistic pathogens (non-tuberculous mycobacteria (NTM), *Pseudomonas aeruginosa*, and *Legionella*) and amoeba host organisms (e.g., *Vermamoeba* and *Acanthamoeba*) of important to premise plumbing ecology, dashed lines indicate the temperature ranges in which growth and/or survival of each organism has been documented, and the solid lines indicate water temperatures commonly encountered in cold and hot water supplies as well as stagnant premise plumbing (Ohno et al., 2003; Dennis et al., 1984; Schulze-Röbbecke et al., 1987; Darelid et al., 2002; Söderberg et al., 2004; Wadowsky and Yee, 1981; Wadowsky et al., 1985; Kuchta et al., 1993; Schulze-Röbbecke et al., 1992; Wallace et al., 1998; Anand et al., 1983; Buse and Ashbolt, 2011; Nagington et al., 1980; Brown et al., 1957; Van der Kooij et al., 1982)



Sampling Period	Months of Operation	Exp. Heater T Setting
Baseline	5	39° C
Sampling 1	8	42° C
Sampling 2	11	48° C
Sampling 3	13	51° C
Sampling 4	15	58° C

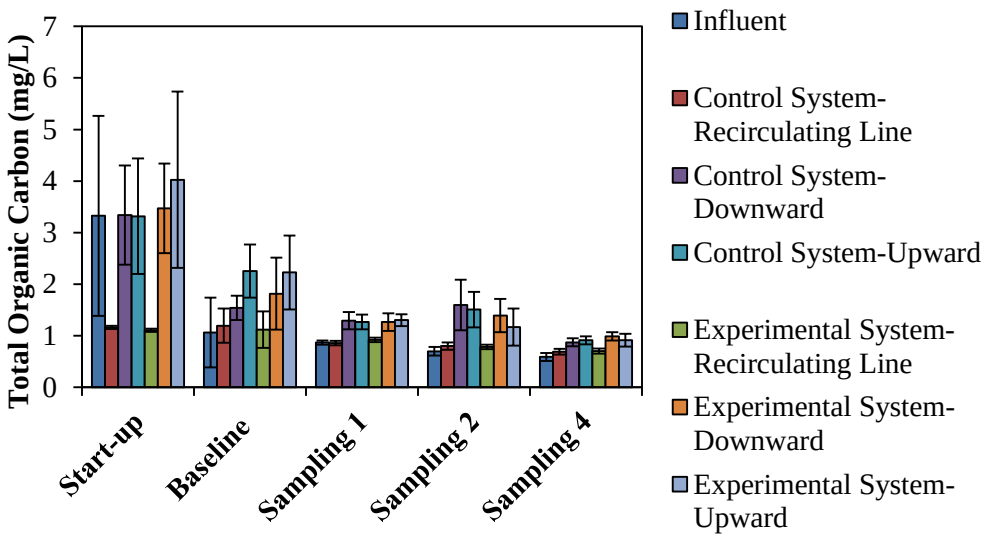
Fig. C.2. Temperature measurements in the control and experimental system in upward and downward oriented pipes for all five experimental conditions; error bars represent 95% confidence intervals on the triplicate pipes.

Upward oriented pipes were significantly warmer than downward oriented pipes (paired t-test, n=318, p-value<0.0001); however, the difference was small (on average 1.1° C). Generally speaking, water in the distal taps never exceeded the temperature and time requirements to achieve 99% disinfection of *Legionella*, and, as a result, here were also no significant differences in *L. pneumophila* or *Legionella* spp. genetic marker concentrations between upward and downward pipes (paired t-test, n=177, p-value = 0.31-0.48).



Sampling Period	Months of Operation	Exp. Heater T Setting
Baseline	5	39° C
Sampling 1	8	42° C
Sampling 2	11	48° C
Sampling 3	13	51° C
Sampling 4	15	58° C

Fig. C.3. Total chlorine concentrations in the influent and both recirculating lines throughout the study (detection limit = 0.02 mg/L as Cl_2); error bars represent 95% confidence intervals on repeat sampling of the recirculating line at the beginning and end of stagnation periods for the distal taps (n=9-12 for each system at each sampling period).



Sampling Period	Months of Operation	Exp. Heater T Setting
Baseline	5	39° C
Sampling 1	8	42° C
Sampling 2	11	48° C
Sampling 3	13	51° C
Sampling 4	15	58° C

Fig. C.4. Total organic carbon concentrations in the control and experimental system distal taps during each sampling period; error bars represent 95% confidence intervals on two samplings of the triplicate pipes and repeat independent samples of the recirculating lines.

Organic carbon initially leached from the chlorinated polyvinyl chloride (C-PVC) pipes at about 3 mg/L during the first two weeks of startup operation. During the experimental phase, TOC increased marginally in the recirculating lines by an average of 0.07-0.08 mg/L compared to the influent. TOC initially increased in distal taps by 0.50-0.52 mg/L during the baseline testing, and over time TOC leaching was reduced to 0.17-0.21 mg/L during the last sampling period.

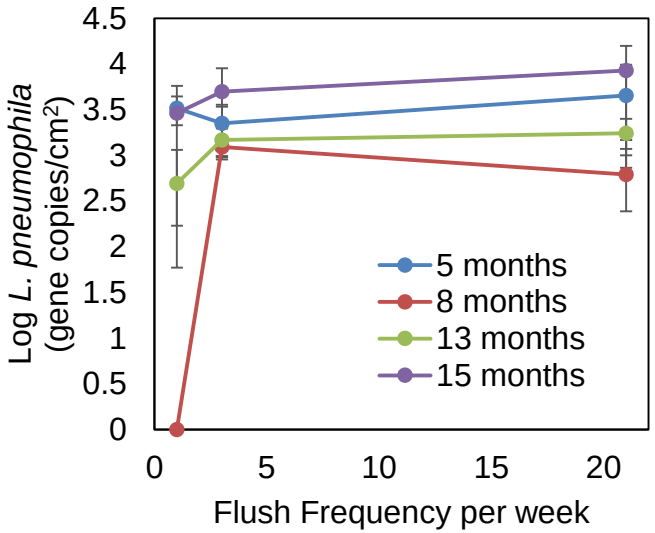


Fig. C.5. Biofilm *L. pneumophila* concentration as a function of flush frequency per week in the control system

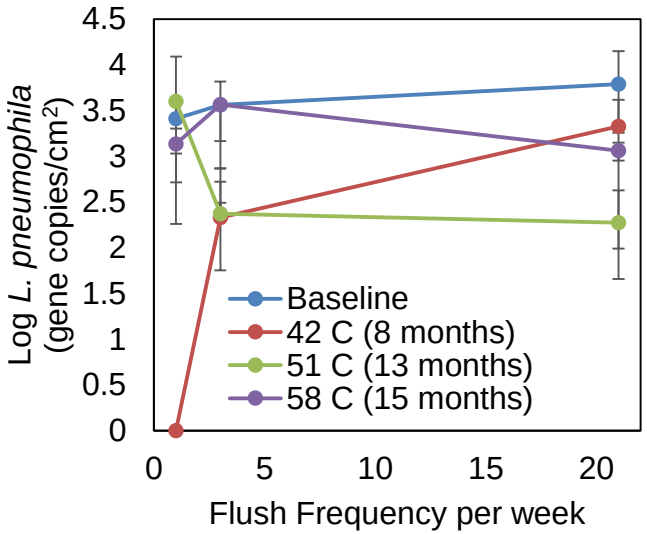
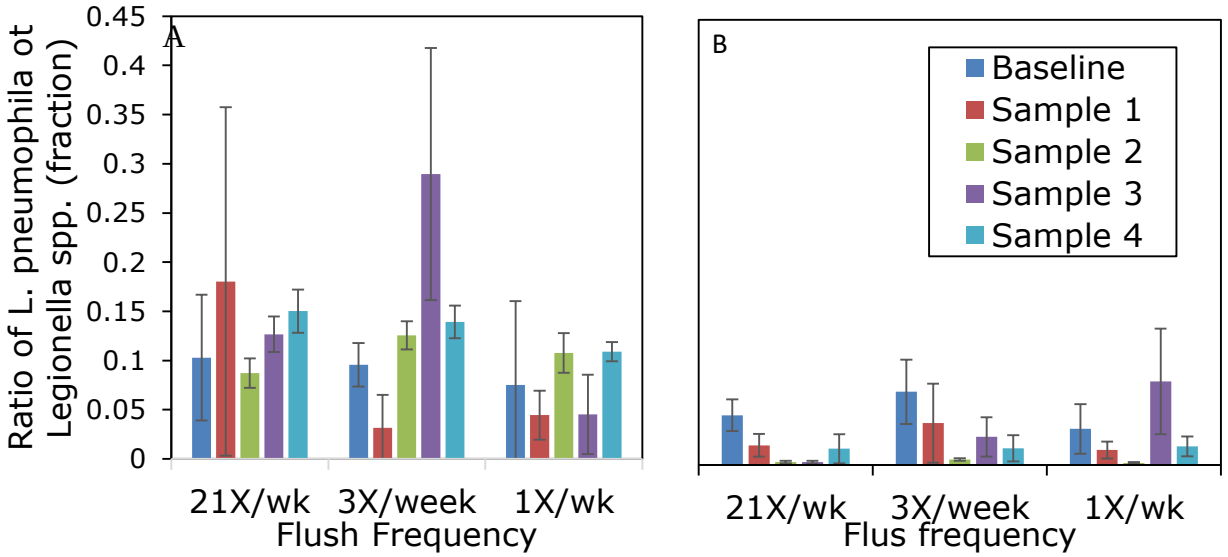


Fig. C.6. Biofilm *L. pneumophila* concentration as a function of flush frequency per week in the experimental system



Sampling Period	Months of Operation	Exp. Heater T Setting
Baseline	5	39° C
Sampling 1	8	42° C
Sampling 2	11	48° C
Sampling 3	13	51° C
Sampling 4	15	58° C

Fig. C.8. Ratio of *L. pneumophila* to *Legionella* spp. in the distal tap pipes for A) the control system and B) the experimental system for each flush frequency across all samples; Error bars represent 95% confidence intervals on the six biological replicates for each condition.

APPENDIX D - CORRELATION ANALYSES FOR TRENDS IN GENE MARKER LEVELS FROM CHAPTER 5.

Table D1. Least-squares regression for gene correlations

Non-transformed data R-squared						
	Comparison Being Made:					
Sample Subset:	Lspp-16S	Lp-Lspp	Lp-Vv	Lp-16S	Vv-16S	Lspp-Vv
ALL DATA (n=433 per gene)	0.26	0.45	0.38	0.03	0.16	0.20
Biofilm-ALL (n=191 per gene)	0.20	0.39	0.54	0.10	0.16	0.20
Water-ALL (n=242 per gene)	0.40	**0.70**	0.26	0.08	-0.25	0.12
Biofilm Recirc (N=10 per gene)	Too few samples for R-squared					
Water Recirc (n=57 per gene)	**0.66**	0.57	0.02	0.26	-0.30	0.04
Biofilm Distal (n=180)	0.37	0.15	0.10	0.21	0.41	0.03
Water Distal (n=177 per gene)	0.34	**0.75**	0.32	0.05	-0.28	0.12
Note:	Red indicates well-correlated gene pairs					

Table D2. Spearman Rank Correlation analysis for all water samples

1. DISTAL TAP – ALL DATA

Comparison			Rho	p-value
Lp	**vs**	**Lspp**	**0.72**	**<0.0001**
Lp	**vs**	**16S**	**0.24**	**0.001**
Lspp	**vs**	**16S**	**0.41**	**<0.0001**
Lp	**vs**	**Vv**	**0.67**	**<0.0001**
Lspp	**vs**	**Vv**	**0.37**	**<0.0001**
Vv	vs	16S	0.202	0.0933
Notes:	n=177 for Lp, Lspp, and 16S and n=105 for Vv (didn't analyze middle temperatures)			

2. DISTAL TAPS – SPLIT BY SYSTEM

System	Temp	Comparison			Rho	p-value
Control	39	**Lp**	**vs**	**Lspp**	**0.763**	**<0.0001**
		Lp	**vs**	**Vv**	**0.513**	**<0.0001**
		Vv	**vs**	**16S**	**-0.59**	**<0.0001**
		Lspp	vs	16S	0.1061	0.328
		Lp	vs	16S	-0.143	0.185
		Lspp	vs	Vv	0.1861	0.187
Note:	Did not do for Exp. b/c across time, temperature was not constant.					

3. DISTAL TAPS – SPLIT BY SAMPLE

Sample	Duration	Comparison			Rho	p-value
Baseline	5 months	**Lspp**	**vs**	**16S**	**0.638**	**<0.0001**
		Lp	**vs**	**Vv**	**0.495**	**0**
		Lp	vs	Lspp	0.1256	0.479
		Lp	vs	16S	0.1982	0.261
		Lspp	vs	Vv	-0.003	0.986
		Vv	vs	16S	0.2822	0.106
Notes:	n=34 for baseline					
	Did not do for other sample dates b/c baseline was the only one where both systems were at 39C					

4. DISTAL TAPS – SPLIT BY SAMPLE DATE AND SYSTEM

Sample	Duration	System	Temp.	Comparison			Rho	p-value
Baseline	5 months	Control	39	**Lspp**	**vs**	**16S**	**0.64**	**0.008**
				Lp	vs	Lspp	-0.353	0.18
				Lp	vs	16S	-0.162	0.5495
				Lp	vs	Vv	0.377	0.1506
				Lspp	vs	Vv	-0.194	0.4713
				Vv	vs	16S	0.291	0.2739
		Exp	39	**Lspp**	**vs**	**16S**	**0.71**	**9E-04**
				Lp	vs	Lspp	0.127	0.6157
				Lp	vs	16S	0.348	0.1573
				Lp	vs	Vv	0.367	0.1626
				Lspp	vs	Vv	0.342	0.1653
				Vv	vs	16S	0.294	0.2361
Sample 1	8 months	Control	39	Lp	vs	Lspp	0.348	0.1573
				Lp	vs	16S	-0.092	0.717
				Lspp	vs	16S	-0.061	0.8103
		Experime	42	Lp	vs	Lspp	0.443	0.0658
				Lp	vs	16S	-0.03	0.8997
				Lspp	vs	16S	0.207	0.4089
Note:	Vv was not analyzed for Sample 1 and Sample 2							

Table D.2 CONT'D

Sample 2	11 month:	Control	39	**Lp**	**vs**	**Lspp**	**0.86**	**<0.0001**
				Lspp	**vs**	**16S**	**0.51**	**0.031**
				Lp	vs	16S	0.459	0.0552
		Experime	48	**Lp**	**vs**	**Lspp**	**0.47**	**0.048**
				Lspp	**vs**	**16S**	**0.67**	**0.002**
				Lp	vs	16S	0.397	0.1025
Note:	Vv was not analyzed for Sample 1 and Sample 2							
Sample 3	13 month	Control	39	**Lp**	**vs**	**Lspp**	**0.85**	**<0.0001**
				Lp	**vs**	**16S**	**0.65**	**0.005**
				Lspp	**vs**	**16S**	**0.57**	**0.016**
				Lp	vs	Vv	0.422	0.0808
				Lspp	vs	Vv	0.445	0.0644
				Vv	vs	16S	0.049	0.8515
		Experime	51	**Lp**	**vs**	**Lspp**	**0.59**	**0.013**
				Lp	**vs**	**16S**	**0.68**	**0.003**
				Lspp	**vs**	**16S**	**0.8**	**1E-04**
				Lp	**vs**	**Vv**	**0.52**	**0.031**
				Lspp	**vs**	**Vv**	**0.68**	**0.003**
				Vv	**vs**	**16S**	**0.56**	**0.02**
Sample 4	15 month	Control	39	**Lp**	**vs**	**Lspp**	**0.99**	**<0.0001**
				Lp	**vs**	**16S**	**0.85**	**<0.0001**
				Lspp	**vs**	**16S**	**0.8**	**<0.0001**
				Lp	**vs**	**Vv**	**-0.62**	**0.006**
				Lspp	**vs**	**Vv**	**-0.62**	**0.006**
				Vv	**vs**	**16S**	**-0.7**	**0.001**
		Exp	58	**Lp**	**vs**	**Lspp**	**0.77**	**2E-04**
				Lp	**vs**	**16S**	**0.7**	**0.001**
				Lspp	**vs**	**16S**	**0.54**	**0.02**
				Vv	**vs**	**16S**	**0.58**	**0.012**
				Lp	vs	Vv	0.323	0.1911
				Lspp	vs	Vv	0.577	0.095
Notes:	n=16-18 for each system in each sampling							

Table D.2 – CONT'D

5. DISTAL TAPS – SPLIT BY SAMPLE DATE, SYSTEM, AND FLUSH									
NOTE:	*ONLY SIGNIFICANT CORRELATIONS ARE SHOWN FOR THE BELOW*								
Sample	Duration	System	Temp	Flush	Comparison			Rho	p-value
Baseline	5 months	Control	39	High	**Lspp**	**vs**	**16S**	**0.821**	**0.042**
				Med	**Vv**	**vs**	**16S**	**0.9**	**0.037**
		Exp	39	High	**Lp**	**vs**	**16S**	**0.829**	**0.042**
					Lspp	**vs**	**16S**	**0.943**	**0.005**
				Med	**Vv**	**vs**	**16S**	**0.943**	**0.005**
Sample 1	8 months	Control	39	Med	**Lspp**	**vs**	**16S**	**0.886**	**0.019**
				Low	**Lp**	**vs**	**16S**	**1**	**<0.0001**
Sample 2	11 month:	Control	39	High	**Lp**	**vs**	**16S**	**0.886**	**0.019**
				Med	**Lp**	**vs**	**Lspp**	**0.943**	**0.005**
				Low	**Lspp**	**vs**	**16S**	**0.943**	**0.005**
		Exp	48	High	**Lp**	**vs**	**Lspp**	**0.829**	**0.042**
				Low	**Lp**	**vs**	**16S**	**0.943**	**0.005**
Sample 3	13 month	Control	39	High	**Vv**	**vs**	**Lspp**	**0.943**	**0.005**
				Med	**Lp**	**vs**	**Lspp**	**0.943**	**0.005**
					Lp	**vs**	**16S**	**0.943**	**0.005**
					Lspp	**vs**	**16S**	**0.829**	**0.042**
				Low	**Lp**	**vs**	**Lspp**	**0.886**	**0.019**
					Lp	**vs**	**16S**	**0.9**	**0.037**
					Vv	**vs**	**Lspp**	**0.829**	**0.047**
		Exp	51	High	**Lspp**	**vs**	**16S**	**0.943**	**0.005**
				Med	**Lp**	**vs**	**16S**	**0.886**	**0.019**
					Lspp	**vs**	**16S**	**0.943**	**0.005**
				Low	**Lp**	**vs**	**Lspp**	**0.9**	**0.037**
					Lp	**vs**	**16S**	**0.9**	**0.037**
					Lspp	**vs**	**16S**	**1**	**<0.0001**
Sample 4	15 month	Control	39	High	**Lp**	**vs**	**Lspp**	**0.829**	**0.042**
				Med	**Lp**	**vs**	**Lspp**	**0.886**	**0.019**
				Low	**Lp**	**vs**	**Lspp**	**1**	**<0.0001**
					Lp	**vs**	**16S**	**0.943**	**0.005**
					Lspp	**vs**	**16S**	**0.943**	**0.005**
					Lp	**vs**	**Vv**	**-0.94**	**0.005**
					Lspp	**vs**	**Vv**	**-0.94**	**0.005**
					Vv	**vs**	**16S**	**-1**	**<0.0001**
		Exp	58	High	**Lp**	**vs**	**Lspp**	**0.886**	**0.018**
					Lspp	**vs**	**16S**	**0.829**	**0.042**
				Med	**Lp**	**vs**	**16S**	**1**	**<0.0001**
Notes:	n=5-6 for each flush condition in each system during each sampling.								

Table D.3. Spearman Rank Coefficient Analysis for Recirculating Line Biofilm samples

1. RECIRCULATING LINES – ALL DATA

Comparison			Rho	p-value
Lp	**vs**	**Lspp**	**0.793**	**<0.0001**
Lp	**vs**	**Vv**	**0.503**	**0.003**
Lspp	**vs**	**Vv**	**0.604**	**2E-04**
Lspp	**vs**	**16S**	**0.301**	**0.05**
Lp	vs	16S	0.2149	0.1664
Vv	vs	16S	-0.071	0.706
Note:	n=45 for Lp, Lspp, and 16S and n=33 for Vv			

2. RECIRCULATING LINES – SPLIT BY SYSTEM

System	Temp	Comparison			Rho	p-value
Control	39	**Lp**	**vs**	**Lspp**	**0.493**	**0.02**
		Lspp	**vs**	**Vv**	**0.585**	**0.017**
		Vv	**vs**	**16S**	**-0.61**	**0.012**
		Lspp	vs	16S	-0.219	0.3286
		Lp	vs	16S	-0.368	0.0924
		Vv	vs	Lp	0.3176	0.2306
Note:	Did not do for Exp. b/c across time, temperature was not constant.					

3. RECIRCULATING LINES – SPLIT BY SAMPLE

Sample	Duration	Comparison			Rho	p-value
Baseline	5 months	**Lspp**	**vs**	**16S**	**0.762**	**0.004**
		Lp	**vs**	**Lspp**	**0.657**	**0.02**
		Vv	**vs**	**16S**	**0.629**	**0.028**
		Lp	vs	16S	0.2238	0.4845
		Lspp	vs	Vv	0.1259	0.6967
		Lp	vs	Vv	-0.252	0.4299
Notes:	n=32 for baseline					
	Did not do for other sample dates b/c baseline was the only one where both systems were at 39C					

4. RECIRCULATING LINES – SPLIT BY SAMPLE AND SYSTEM

Sample	Duration	System	Tempert	Comparison			Rho	p-value
Baseline	5 months	Control	39	**Vv**	**vs**	**16S**	**0.886**	**0.019**
				Lspp	**vs**	**16S**	**0.771**	**0.072**
				Lp	vs	16S	-0.143	0.7872
				Lp	vs	Lspp	0.4286	0.3965
				Lspp	vs	Vv	0.4857	0.3287
				Lp	vs	Vv	-0.486	0.3287
		Exp	39	**Lspp**	**vs**	**16S**	**0.886**	**0.019**
				Lp	vs	16S	0.6571	0.1562
				Lp	vs	Lspp	0.7714	0.0724
				Vv	vs	16S	0.7143	0.1108
				Lspp	vs	Vv	0.6	0.208
				Lp	vs	Vv	0.4857	0.3287
Sample 1	8 months	Control	39	Lp	vs	Lspp	-0.143	0.7872
				Lp	vs	16S	-0.086	0.8712
				Lspp	vs	16S	-0.086	0.8712
		Exp	39	Lp	vs	Lspp	0.3143	0.5441
				Lp	vs	16S	-0.543	0.2657
				Lspp	vs	16S	0.5271	0.6228
Note:	Vv was not analyzed for Sample 1 and Sample 2							

Table D.3 – CONT'D

Sample 2	11 months							
Note:	Recirculating line samples for this sampling were lost due to laboratory error							
Sample 3	13 month	Control	39	Lspp	vs	16S	0.5	0.391
				Lp	vs	16S	0.5	0.391
				Lp	vs	Lspp	0	1
				Vv	vs	16S	0.3	0.6238
				Lspp	vs	Vv	0.4	0.5046
				Lp	vs	Vv	0.7	0.1881
		Experime	51	Lspp	vs	16S	-0.5	0.6667
				Lp	vs	16S	0.5	0.6667
				Lp	vs	Lspp	0.1	0.8729
				Vv	vs	16S	0.5	0.6667
				Lspp	vs	Vv	0.8721	0.0539
				Lp	vs	Vv	0.1026	0.8696
Sample 4	15 month	Control	39	Lp	vs	Lspp	0.9	0.0374
				Lspp	vs	Vv	0.9	0.0374
				Vv	vs	16S	0.9	0.0374
				Lspp	vs	16S	0.7	0.1881
				Lp	vs	16S	0.6	0.2848
				Lp	vs	Vv	0.7	0.1881
		Exp	58	Lp	vs	Lspp	0.9429	0.0048
				Lspp	vs	Vv	0.1429	0.7872
				Vv	vs	16S	0.2	0.704
				Lspp	vs	16S	0.7714	0.0724
				Lp	vs	16S	0.7134	0.1108
				Lp	vs	Vv	0.0286	0.9572
Note:	n=2-6 for each sampling period for each system							

Table D.4. Spearman Rank Correlation Analysis for all Biofilm Samples

1. DISTAL TAP - ALL DATA								
Comparison			Rho	p-value				
Lp	**vs**	**Lspp**	**0.15**	**0.0416**				
Lp	**vs**	**Vv**	**0.36**	**0.0001**				
Lp	**vs**	**16S**	**0.33**	**<0.0001**				
Lspp	**vs**	**16S**	**0.53**	**<0.0001**				
Vv	**vs**	**16S**	**0.42**	**<0.0001**				
Lspp	vs	Vv	0.11	0.2592				
Notes:	n=180 for Lp, Lspp, and 16S and n=108 for Vv (didn't analyze middle temperatures)							

2. DISTAL TAPS - SPLIT BY SYSTEM						
System	Temp	Comparison			Rho	p-value
Contorl	39	**Lp**	**vs**	**16S**	**0.04**	**0.0002**
		Lspp	**vs**	**16S**	**0.52**	**<0.0001**
		Vv	**vs**	**16S**	**0.49**	**0.0012**
		Lp	vs	Lspp	-0.08	0.4732
		Lspp	vs	Vv	0.104	0.4533
		Lp	vs	Vv	0.263	0.0547
Note:	Did not do for Exp. b/c across time, temperature was not constant.					

4. DISTAL TAPS - SPLIT BY SAMPLE DATE AND SYSTEM								
Sample	Duration	System	Temp	Comparison			Rho	p-value
Baseline	5 months	Control	39	Lspp	vs	16S	0.3705	0.1302
				Lp	vs	16S	0.0402	0.874
				Lp	vs	Lspp	0.3127	0.2065
				Vv	vs	16S	0.3168	0.2002
				Lspp	vs	Vv	0.2281	0.3627
				Lp	vs	Vv	-0.0857	0.7354
		Exp	39	**Lspp**	**vs**	**16S**	**0.5934**	**0.0094**
				Lp	vs	16S	0.4303	0.0746
				Lp	vs	Lspp	0.451	0.0603
				Vv	vs	16S	0.0774	0.76
				Lspp	vs	Vv	-0.1828	0.4679
				Lp	vs	Vv	-0.3604	0.1418
Sample 1	8 months	Control	39	Lspp	vs	16S	0.5039	0.033
				Lp	vs	16S	-0.0844	0.7393
				Lp	vs	Lspp	0.0737	0.7714
		Experiment	42	Lspp	vs	16S	-0.0815	0.7478
				Lp	vs	16S	-0.1118	0.6691
				Lp	vs	Lspp	0.1663	0.5234
Note:	Vv was not analyzed for Sample 1 and Sample 2							

Table D.4 CONT'D

Sample :	11 months	Control	39	**Lspp**	**vs**	**16S**	**0.5916**	**0.0097**
				Lp	vs	16S	0.3777	0.1223
				Lp	vs	Lspp	0.1541	0.5416
		Exp	48	Lspp	vs	16S	0.3519	0.1521
				Lp	**vs**	**16S**	**-0.514**	**0.0291**
				Lp	vs	Lspp	-0.1663	0.5096
Note:	Vv was not analyzed for Sample 1 and Sample 2							
Sample :	13 months	Control	39	**Lp**	**vs**	**Vv**	**0.5231**	**0.0256**
				Lspp	**vs**	**16S**	**0.5784**	**0.0126**
				Vv	**vs**	**16S**	**0.6484**	**0.0036**
				Lp	vs	16S	0.2106	0.4016
				Lp	vs	Lspp	-0.277	0.2658
				Lspp	vs	Vv	0.3356	0.1734
		Experiment	51	Lspp	vs	16S	0.3239	0.1898
				Lp	vs	16S	-0.3656	0.1357
				Lp	vs	Lspp	0.0884	0.7272
				Vv	vs	16S	0.0733	0.7724
				Lspp	vs	Vv	0.2149	0.3919
				Lp	vs	Vv	0.1938	0.4409
Sample :	15 months	Control	39	**Lp**	**vs**	**16S**	**0.6726**	**0.0022**
				Lp	**vs**	**Vv**	**0.4891**	**0.0394**
				Vv	**vs**	**16S**	**0.8638**	**<0.001**
				Lspp	vs	16S	-0.1229	0.6272
				Lp	vs	Lspp	-0.3193	0.1966
				Lspp	vs	Vv	-0.1683	0.5044
		Exp	58	**Lp**	**vs**	**16S**	**0.682**	**0.0018**
				Lspp	**vs**	**Vv**	**-0.49**	**0.0389**
				Lspp		16S	0.0444	0.8612
				Lp		Lspp	-0.009	0.9715
				Vv		16S	0.162	0.5207
				Lp		Vv	0.3286	0.1831
Notes:	n=18 for each system during each							
5. DISTAL TAPS - SPLIT BY SAMPLE DATE, SYSTEM, AND FLUSH								
NOTE: ONLY SIGNIFICANT CORRELATIONS ARE SHOWN FOR THE BELOW								
Sample	Duration	System	Temp	Flush	Comparison		Rho	p-value
Baseline	5 months	Control	39	**Low**	**Lspp**	**16S**	**0.8286**	**0.0416**
					Lspp	**Vv**	**0.9429**	**0.0048**
		Exp	39	**High**	**Lp**	**Vv**	**-0.812**	**0.0499**
				Med	**Lspp**	**Vv**	**-0.943**	**0.0048**
				Low	**Lp**	**16S**	**0.9429**	**0.0048**
Sample :	8 months	Control	39	**High**	**Lspp**	**16S**	**0.8857**	**0.0188**
		Exp	42	**High**	**Lspp**	**16S**	**-0.886**	**0.0119**

Table D.4. CONT'D

Sample :	11 months							
Sample :	13 months	Control	39	**High**	**Vv**	**16S**	**0.8266**	**0.0416**
				Med	**Lspp**	**Vv**	**0.8857**	**0.0188**
				Low	**Lp**	**Lspp**	**-0.845**	**0.0341**
		Exp	51	**High**	**Lspp**	**16S**	**0.8857**	**0.0188**
Sample :	15 months	Control	39	**High**	**Lp**	**Lspp**	**-0.812**	**0.0499**
					Vv	**16S**	**0.9429**	**0.0048**
				Med	**Lp**	**Lspp**	**0.8286**	**0.0188**
					Vv	**16S**	**0.8857**	**0.0416**
				Low	**Lp**	**16S**	**0.8117**	**0.0499**
					Vv	**16S**	**0.8857**	**0.0188**
		Exp	58	**High**	**Lp**	**16S**	**0.8452**	**0.0341**
					Lp	**Lspp**	**-0.845**	**0.0341**
				Low	**Lp**	**16S**	**-0.941**	**0.0051**
Note:	n=6 for each water use in each system during each sampling							

Table D5. Spearman Rank Correlation Analysis for Recirculating Line Biofilm Samples

1. RECIRCULATING LINES - ALL DATA

Comparison			Rho	p-value
Lp	**vs**	**Lspp**	**0.7547**	**0.0116**
Lp	**vs**	**Vv**	**0.7412**	**0.0051**
Lspp	vs	16S	-0.1905	0.6514
Lp	vs	16S	-0.6347	0.0909
Vv	vs	16S	-0.5218	0.2883
Lspp	vs	Vv	0.6957	0.1248
Note:	n=10 for each gene			

2. RECIRCULATING LINES - SPLIT BY SYSTEM

System	Tempera	Comparison			Rho	p-value
Control	39	Lspp	vs	16S	0.8	0.2
		Lp	vs	16S	0.4	0.6
		Lp	vs	Lspp	0.8	0.1041
		Vv	vs	16S	0.5	0.6667
		Lspp	vs	Vv	0.5	0.6667
		Lp	vs	Vv	0.5	0.6667
Note: n=5 for each gene						

3. RECIRCULATING LINES - SPLIT BY SAMPLE AND SYSTEM

Sample	Duration	System	Tempert	Comparison		Rho	p-value
Did not do b/c n=1 for each sample/system combination							

CHAPTER 6. CONVECTIVE MIXING IN DISTAL PIPES EXACERBATES *L. PNEUMOPHILA* GROWTH IN HOT WATER PLUMBING

William J. Rhoads, Amy Pruden, and Marc A. Edwards

ABSTRACT

Legionella pneumophila is known to proliferate in hot water plumbing systems, but little is known about the specific physicochemical factors that contribute to its regrowth. Here, *L. pneumophila* trends were examined in controlled, replicated pilot-scale hot water systems with continuous recirculation lines subject to two water heater settings (40°C and 58°C) and three distal tap water use frequencies (high, medium, and low) with two pipe configurations (oriented upward to promote convective mixing with the recirculating line and downward to prevent it). Water heater temperature setting determined where *L. pneumophila* regrowth occurred in each system, with an increase of up to 4.4 log gene copies/mL in the 40°C system tank and recirculating line relative to influent water compared to only 2.5 log gene copies/mL regrowth in the 58°C system. Distal pipes without convective mixing cooled to room temperature (23-24°C) during periods of no water use, but pipes with convective mixing equilibrated to 30.5°C in the 40°C system and 38.8°C in the 58°C system. Corresponding with known temperature effects on *L. pneumophila* and enhanced delivery of nutrients, distal pipes with convective mixing had on average 0.2 log more gene copies/mL in the 40°C system and 0.8 log more gene copies/mL in the 58°C system. Importantly, this work demonstrated the potential for thermal control strategies to be undermined by distal taps in general, and convective mixing in particular.

KEY WORDS: *Legionella pneumophila*; premise plumbing; hot water; design; installation; mixing; convection

INTRODUCTION

Legionella pneumophila is the most frequently reported causal agent of waterborne disease outbreaks in developed countries, causing a respiratory infection known as Legionnaires' disease (a severe and life-threatening pneumonia) or Pontiac Fever (a milder flu-like illness that is usually self-limiting) (Brunkard et al., 2011; Yoder et al., 2008). Inhalation of *Legionella*-entrained aerosols is the primary exposure pathway for infection, rather than ingestion, which is the infection route for traditional fecal pathogens that are the primary basis of water regulations (Casini et al., 2008; Cowen et al., 2006; Bollin et al., 1985). *L. pneumophila* serogroup 1 is estimated to have caused 84% of the 6,868 reported cases of legionellosis in the United States between 2009-2010 and is thought to be the most common strain associated with disease (Brunkard et al., 2011; Yoder et al., 2008; Hlavsa et al., 2014; Hilborn et al., 2013). Other species and serogroups can also be pathogenic, but are not as well documented (Fields et al., 2002; Fang et al., 1989).

Controlling *L. pneumophila* is a major challenge in building plumbing systems. In a recent survey, nearly half of 68 cold drinking water taps were positive for *L. pneumophila* genetic markers (272 total samples, from 25 states across the U.S.) (Donohue et al., 2014). In particular, hot water systems are a key reservoir for *Legionella* and source of disease, due to ideal growth temperatures along with dissipation of chemical disinfectant residuals (Pruden et al., 2013; Alary and Joly, 1991; Wadowsky et al., 192; Cordes et al., 1981; Dondero et al., 1980). The temperature dependence of *L. pneumophila* survival in hot water has been well-characterized (Wadowsky et al., 1982; Konishi et al., 2006; Sheehan et al., 2005; Ohno et al., 2003; Darelid et al., 2002; Schulze-Röbbecke et al., 1987; Dennis et al., 1981). Although some strains of *L. pneumophila* have been observed to survive brief periods exposed >70° C water (Allegra et al., 2008; 2011),maintaining an elevated water heater setting (>55°C) is a widely accepted strategy for minimizing regrowth of *Legionella* (Alary and Joly, 1991; Darelid et al., 2002; Kool et al., 1991). However, it is important to recognize that actual water temperatures at the tap can cool considerably relative to the water heater set points (Rhoads et al., 2015), and maintaining desired temperatures throughout the entire plumbing system is challenging (Bedard et al., 2015). For example, distal taps experience a form of thermal shock every time hot water flows and return to ambient temperature during stagnation periods. Such conditions can actually stimulate the proliferation of *Legionella*, rather than inhibit it (Rhoads et al., 2015; Mouchtouri et al., 2007; Borella et al., 2005; 2004; Leoni et al., 2004).

Water heater set points, along with plumbing configuration and water use patterns, may also have interactive effects on *L. pneumophila* proliferation. Here, we hypothesize that convective mixing induced by upward-oriented pipes and increased flow frequency at high use taps can influence the flux of nutrients to taps and the microbes that colonize there, including *Legionella*. Recent work demonstrated that AOC control strategies can also be undermined by the generation of organic carbon by nitrifiers, hydrogen oxidation, and other autotrophic processes (LeChevallier et al., 2001; Martin, 2012; Zhang et al., 2009; Morton et al., 2005; Camper, 2004; Haddix et al., 2004; Grady et al., 1999), and that AOC is not the master variable controlling *L. pneumophila* regrowth in a typical plumbing system (Williams et al., 2015).

Systematic, controlled studies of water heater set point on *Legionella* proliferation in plumbing are lacking, but a few available studies suggest there may be interactive effects with plumbing configuration. Culture-based surveys found that having electric water heaters and vertically

oriented hot water tanks are additional risk factors for *Legionella* colonization (Alary and Joly, 1991; Vickers et al., 1987). Some water heaters thermally stratify with warm water on the bottom of the tank and hot water at the top, creating zones of ideal *L. pneumophila* growth temperature (Brazeau and Edwards, 2013; Gopalakrishnan et al., 2009). With respect to pipe orientation and convective mixing specifically, thermal gradients in otherwise stagnant water have been documented to exacerbate pipe corrosion under cold water pipe conditions between 5-25° C (Rushing and Edwards, 2004), but have never been studied with respect to possible impacts on microbial communities. In hot water systems, the potential for convective mixing is high given the stark temperature differential between hot water in tanks and recirculating lines (up to 60° C) and ambient room temperature influencing stagnant pipes (20° C). Water in downward oriented distal pipes (e.g., pipes from a recirculating hot water loop located in the ceiling running downwards to distal taps) will not convectively mix (Rushing and Edwards, 2004). However, we hypothesize that horizontal and upward oriented distal pipes will convectively mix with hot water in recirculating loops, warming the otherwise stagnant water in the distal lines and enhancing flux of nutrients and corresponding regrowth of microorganisms (Table 6.1) (Rushing and Edwards, 2004; Agathokleous , 2004).

Herein we report the first controlled, large-scale, replicated laboratory examination of interrelationships between water heater temperature set point, convective mixing within distal plumbing, and distal tap use frequency on *L. pneumophila* occurrence and regrowth. Identical "Warm Temperature Set Point" and "Hot Temperature Set Point" hot water plumbing systems were constructed in which continuously recirculating pipe loops delivered water to two sets of distal taps subject to high, medium, and low water use frequency. One set of pipes was oriented to promote convective mixing with the recirculating loop while the other set was oriented to prevent convective mixing (Fig. 6.1). The Warm Temperature system was set to 40° C and the Hot Temperature system to 58° C. Genetic markers of *Legionella* spp. (23S rRNA gene), *L. pneumophila* (macrophage infectivity potentiator (*mip*) gene), *Vermamoeba vermiformis* (18S rRNA gene; an important ecological host for *Legionella*), and total bacteria (16S rRNA gene) were tracked by quantitative polymerase chain reaction (q-PCR) to measure regrowth in the recirculating lines and distal taps.

RESULTS AND DISCUSSION

After describing the effects of convective mixing on temperature profiles in distal taps, its effect on other physicochemical trends including chlorine, ammonia, and total organic carbon (TOC) are presented. Thereafter, regrowth and occurrence of *Legionella* (*L. pneumophila* and *Legionella* spp.) and other ecologically relevant microorganisms is examined as a function of water use patterns and convective mixing (Table 6.1). The discussion is focused on behavior of target organisms measured in water flushed from the system, as this is the source of consumer exposure, but biofilm results are included as well. An overview of the calculations used in this study to compare the distribution of *L. pneumophila* between the Hot and Warm Temperature systems, and across their various niches, are provided in the supplementary materials (Table E1).

Convective Mixing in Distal Taps. The two experimental systems were aged 15 months without significant convective mixing of water in distal taps as previously reported elsewhere (Rhoads et al., 2015). After the initial 15 month period, convective mixing was initiated in one set of distal

taps by orienting the pipe 30° off vertical, significantly altering the temperature profile of water in the pipes (Agathokleous, 2004). During periods of stagnation, distal pipes without convective mixing cooled to room temperature while distal pipes with convective mixing equilibrated to warmer temperatures. The measured pipe surface temperature profiles along the length of distal taps when water was not being used confirmed convective mixing in upward oriented pipes, maintaining higher temperature throughout the pipes (Fig. 6.2A). There was no evidence of convective mixing in downward oriented pipes, as pipes cooled to room temperature at distances less than 30 cm from the recirculation line (Fig. 6.2A). Notably, the last 1 meter of distal pipes with convective mixing were 5-12° C warmer in the Hot Temperature system than pipes that did not convectively mix when water was not being used. Convective mixing also altered temperature profiles within the distal taps immediately following water use. Water temperatures in otherwise stagnant distal pipes without convective mixing cooled to ambient room temperature (23-24° C) within 30 minutes after use in both systems, but equilibrated in distal pipes that did convectively mix to 30.5° C in the Warm system and to 38.8° C in the Hot system (Fig. 6.2B). Convective mixing was stronger in the Hot Temperature system because it had a larger temperature differential between the water heater set point and ambient room temperature. In general, the entire volume of the Warm Temperature system water heater and recirculating line were consistently maintained at 40.6° C, near the upper limit of the ideal growth range for *L. pneumophila*, while water in the distal taps was in the ideal growth range only up to 3% of the time due to cooling during stagnation (Table 2). In contrast, the Hot water heater and recirculating line were maintained in the ideal growth range 0% of the time, while the distal taps were in the ideal growth range at least 95% of the time due to convective mixing (Table 6.2). The entire length of pipe would like equilibrate at higher temperatures if more conductive pipe materials (e.g., copper) or insulation had been used, which would have different implications for bacterial growth.

Other Physicochemical Trends. *Total Chlorine.* Influent chloramine disinfectant residual was reduced by three granular activated carbon (GAC) filters in series. Average total chlorine concentration in the influent water was always less than 0.10 mg Cl_2/L; thus, achieving the goal of isolating the effects of convective mixing, temperature setting, and water use pattern, which are the focus of this study.

Ammonia (NH_3) Oxidation and Total Organic Carbon (TOC) Production. In the Warm Temperature system, 100% (0.56 mg/L NH_3-N) of the influent ammonia was oxidized in the tank and recirculating line reservoir between distal pipe flushes, indicating the presence of active nitrifying microorganisms (Zhang et al., 2009; Grady et al., 1999). In the Hot Temperature system, only 52% of the total influent ammonia was oxidized between flushes in the tank and recirculating lines, likely because nitrifying bacteria reduce in activity above 45-55° C (Neufeld et al., 1980; Sawyer and Bradney, 1946; Gibbs, 1920). In fact, at these high temperatures, it is possible that nitrification was not occurring in the main tank and recirculating line, but mainly in the distal taps where temperature was cooler and 100% of the ammonia delivered by convective mixing was oxidized in medium and low use distal taps (Fig. 6.3A). In that case the ammonia drop in the main tank could be due to convective exchange with the distal taps. (Fig. 6.3A). Assuming this is the case, we estimated that the convective mixing exchange rate (net convectively driven flow of water from the recirculation line to the distal tap during stagnation) would need to be about 0.14 L/hr to

Table 6.1. Hypothesized effects of convective mixing on *Legionella* spp. and *L. pneumophila* in stagnant distal taps under Warm (40° C) and Hot (58° C) operating conditions.

Experimental Condition	Water in tank and recirculating line	Impact of convective mixing in distal taps	Net effect of mixing on regrowth in distal taps
I. No disinfectant and warm water heater set point (T = 40° C).	Active microbial community with high levels of *L. pneumophila* in tank and recirculating line due to continuous flow of nutrients at ideal growth T	Very weak convective mixing due to low ΔT delivers some nutrients and slightly increases temperature	Marginally more regrowth in taps subject to convective mixing, but dwarfed by regrowth in tank and recirculating line
II. No disinfectant and hot water heater set point (T = 58° C)	Thermophilic microbial community with low levels of *L. pneumophila* due to consistent inhibitory T	Stronger mixing due to greater ΔT delivers nutrients and maintains temperatures closer to ideal growth range	High concentrations in low use convectively mixed taps due to warm temperatures, continuous nutrient delivery, and sufficient regrowth time

T = Temperature

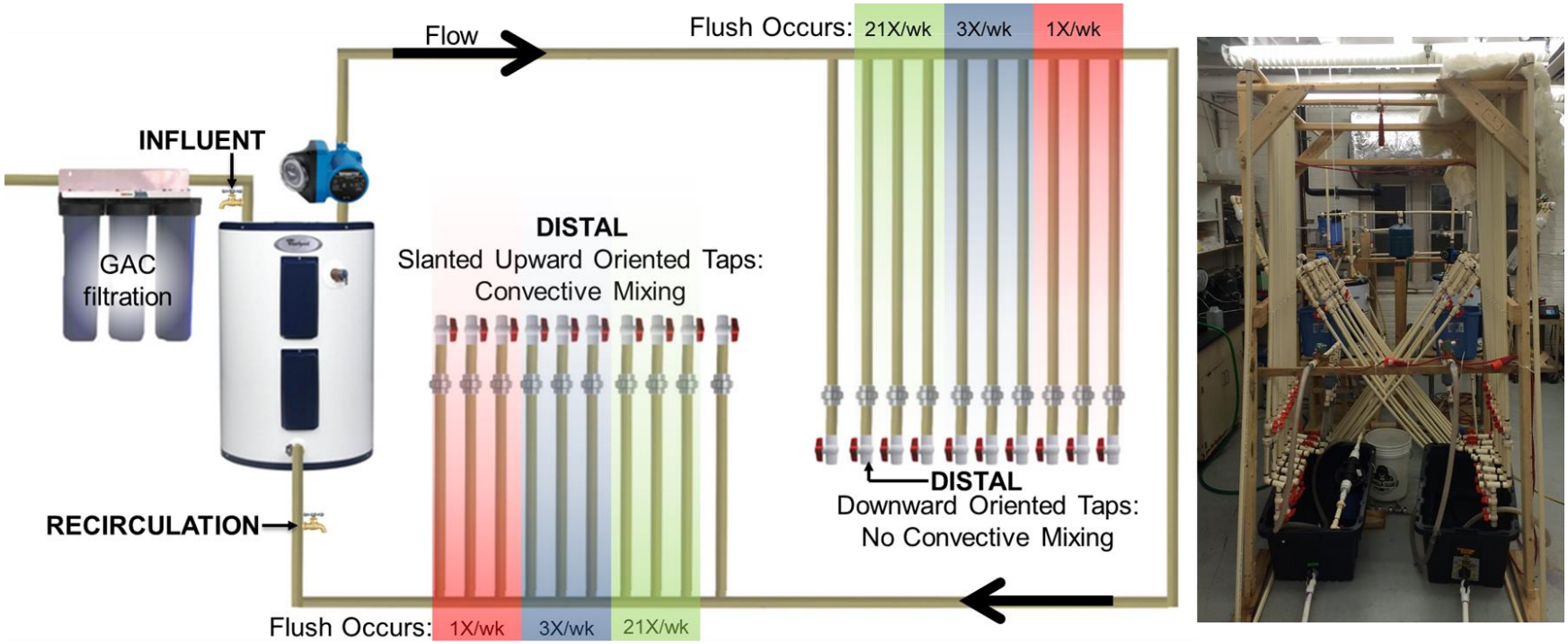


Fig. 6.1. Overview of experimental design of replicated building plumbing systems.

Convective mixing was induced in upward oriented pipes by slanting each pipe 30° from vertical, whereas downward oriented pipes provide a control without convective mixing. One plumbing system tank and recirculating line was maintained at 40° C (Warm Temperature) while the other was maintained at 58° C (Hot Temperature) over 4 months. Influent water was flushed through three granular activated carbon whole-house filters (Sample port: Influent), a recirculating pump continuously pumped water around the return loop back to the water heater creating a completely mixed reservoir (Sample port: Recirculation), six replicate distal taps (three upward + three downward) were flushed at 3.8 L/min (1 gallon/min) 21x/week, 3x/week, and 1x/week for a total of 36 pipes (Sample ports: at end of distal pipes).

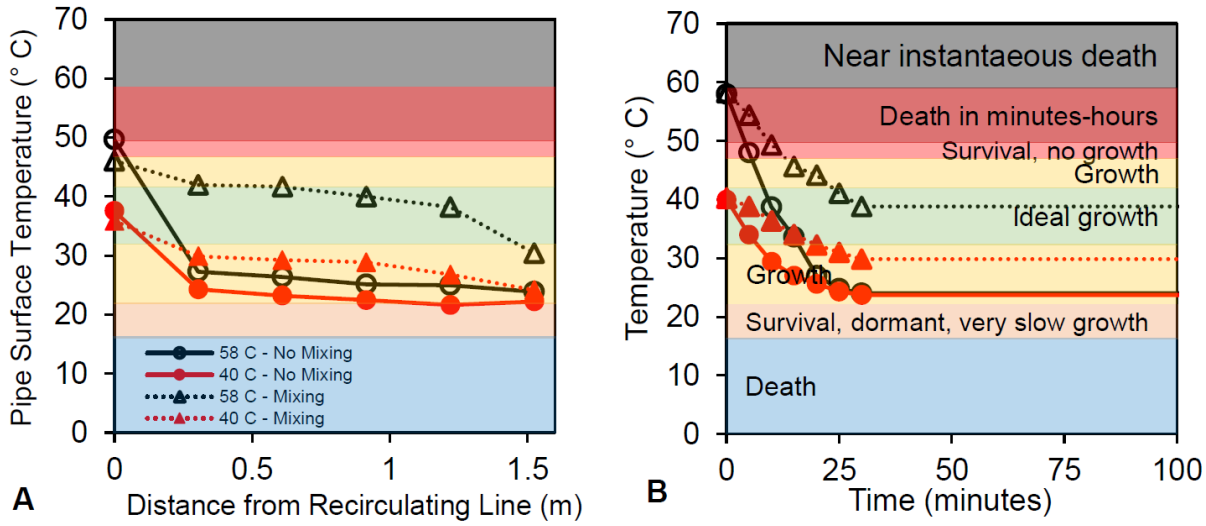


Fig. 6.2. Temperature profiles in the Warm and Hot Temperature systems.
Temperature profiles with and without convective mixing A) along the length of distal pipes during periods of no water use (pipe surface temperature measured with infrared emissivity gun, each point represents average of n=9) and B) within pipes as a function of time immediately after flushing (each data point represents temperature of a ~100 mL first flush sample collected from individual distal taps at the time indicated after all distal pipes were flushed with water from the recirculating line); shaded regions on the plots illustrate temperature dependent ranges of *Legionella* growth (Ohno et al., 2008; Dennis et al., 1984; Schulze-Röbbecke et al., 1987; Darelid et al., 2002; Söderberg et al., 2004; Wadowsky and Yee, 1981; Wadowsky et al., 1985).

Table 6.2. Percent of overall time the tank/recirculation lines or distal taps were maintained at temperatures suitable or ideal for *Legionella* growth; ranges represent differences resulting from different water use conditions.

System	System Volume	Ideal Growth (32-42°C)	Growth (22-32°C)	Limited Growth (>50°C)
Warm Temperature Set Point	Tank+Recirc Line	100	0	0
	Mixing Distal Pipes	0.2-3.1	96.9-99.9	0
	Non-Mixing Distal Pipe	0.05-1.0	99.0-99.9	0
Hot Temperature Set Point	Tank+Recirc Line	0	0	100
	Mixing Distal Pipe	94.8-99.8	0	0.1-2.1
	Non-Mixing Distal Pipe	0.05-1.0	96.9-99.9	0.04-0.8

achieve the measured steady state ammonia concentration of 0.27± 0.07 mg/L NH_3-N observed in the tank (Appendix F). A potassium tracer, dosed into the experimental tank and monitored at the distal taps over time by taking very small (~20 mL) aliquot samples, resulted in an estimated water exchange rate of 0.12 L/hr, which is very close to our estimate.

Total organic carbon (TOC) increased by a factor of 2.7 (from 0.54 mg/L in the filtered influent water to 1.47 mg/L in the tank and recirculating line) in the Warm Temperature system, presumably due to autotrophic growth. In addition to nitrifying bacteria using residual ammonia from chloramines, hydrogen oxidizing bacteria may also have contributed to the increase, by using hydrogen created from corrosion of the water heater sacrificial anode rod (Brazeau and Edwards, 2013). There was relatively little further increase of TOC in the high use distal taps, but in the medium and low use taps TOC decreased by 0.26-0.39 mg/L relative to the recirculating line, presumably due to microbial uptake for growth and respiration. Convective mixing in upward oriented pipes did not have a significant effect on the TOC, with only 0.09-0.13 mg/L more TOC than pipes that did not mix (Fig. 6.3B; Paired t-Test, p-value = 0.47, n=18). In contrast, TOC increased by only a factor of 1.8 (from 0.54 mg/L to 0.96 mg/L) in the Hot Temperature tank and recirculating line, presumably due to lower levels of autotrophic growth as illustrated by the lower levels of ammonia removal noted previously. The 1.1-1.3 mg/L increase in TOC observed in the high use pipes relative to the recirculating line may reflect the high activity of autotrophs in these taps, and the smaller increases in TOC in medium and low use pipes could indicate greater uptake by heterotrophic bacteria. Pipes with convective mixing generally had more TOC than pipes without convective mixing (0.07-0.2 mg TOC/L), except the Hot Temperature system high use taps (which had 0.16 mg TOC/L less) (Paired t-Test, p-value = 0.70, n=18). In general, the trends observed in the data were qualitatively consistent with our hypothesis (Table 1), even though they were not generally statistically significant. Given that our system had ample nutrients (nitrogen and organic carbon), convective mixing could be playing only a small role in regrowth potential in these systems, but be increasingly important in systems with lower nutrients, as mixing in those cases might deliver a higher fraction of total nutrients relative to that supplied during flushing.

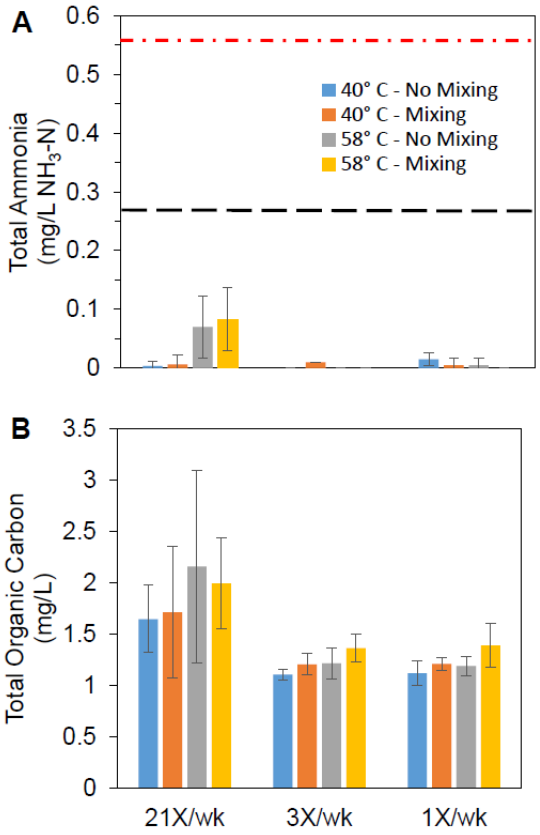


Fig. 6.3. Ammonia and total organic carbon trends in tank and distal taps

A) Average ammonia (NH_3-N) in distal taps. Influent water had 0.56 mg/L NH_3-N, the Hot Temperature system had 0.27 mg/L NH_3-N at the end stagnation periods, and Warm Temperature system had 0.0 mg/L NH_3-N at the end stagnation periods. B) Average total organic carbon (TOC) concentration after regular stagnation period between flushes (i.e., immediately before flushing occurs). NH_3 and TOC concentrations are indicated in the 40° C system tank and recirculating line (solid black line; note ammonia in the 40° C tank and recirculating line is 0 mg/L), in the 58° C system tank and recirculating line (dashed blackline), and in the influent water (dashed-dot red line). Error bars represent 95% confidence intervals (n=2-6).

General Trends in Bulk Water Legionella. *L. pneumophila* levels in the Warm Temperature system recirculating line and distal taps remained stable after fifteen months of aging and over the four month timeframe of this experiment (Fig. 6.4; Fig. E1). In our prior study that took place during the 6-15 month window of operation, baseline levels of *L. pneumophila* steadily increased in the recirculating line from 5.0×10^3 to 1.6×10^4 copies/mL and in the medium and low use distal taps from 1.6×10^4 to 1.3×10^5 copies/mL (Rhoads et al., 2015). In the present study, *L. pneumophila* was below the quantification limit in the cold, filtered influent water while the recirculating line of the Warm system remained at 2.51×10^4-6.31×10^4 gene copies/mL. There was no change from these values in the high use distal pipes and only a 0.1 log decrease to a 0.6 log increase in gene copies/mL in low and medium use distal pipes (Fig. 4c). In other words, the vast majority of the *L. pneumophila* growth occurred within the tank and recirculating line of the Warm Temperature system (Fig. 4a). The highest total yield of *L. pneumophila* was from the highest use taps (Fig. 4b), given that concentration is multiplied by water use coefficient due to the increased frequency of use (Table E1) (Rhoads et al., 2015). In the Warm Temperature system, the high concentrations of

L. pneumophila that regrew in the tank and recirculating lines were delivered to the distal taps with each use. Under such conditions, orientation of the distal taps and use frequency had little effect on levels of *L. pneumophila* at each tap.

Consistent with our hypothesis, the elevated temperature setting in the Hot Temperature system produced much lower populations of *L. pneumophila* in the tank and recirculating line, but the benefits of the elevated temperature setting did not fully translate to the distal pipes (Table E2 and E3) (Rhoads et al., 2015). In general, there was a 1.6-3.5 log decrease in total yield of *L. pneumophila* in the Hot Temperature system compared to corresponding pipe conditions in the Warm Temperature system (Fig. 4b). However, convective mixing in distal pipes in this work reduced the effectiveness of the elevated temperature. For instance, in low use taps without convective mixing, the elevated temperature in the Hot Temperature system decreased *L. pneumophila* levels by an average of 2.3 logs compared to the Warm Temperature system, but in low use pipes with mixing, the elevated temperatures only reduced *L. pneumophila* levels by an average of 1.6 logs (Fig. 4a; Mann-Whitney U-Test, $p = 0.020$, $n_{mixing} = n_{no\ mixing} = 6$). Similar trends were observed with medium and high use taps (Fig. 4a). In addition, the only case where low use taps had a greater total *L. pneumophila* yield than high use taps occurred in the Hot Temperature system pipes with convective mixing, with 1.2 log more total weekly yield of *L. pneumophila* than high use taps (Fig. 6.4b, 4 month sampling). Clearly, in the Hot Temperature system, orientation of the distal taps and frequency of use were major factors in determining the levels of *L. pneumophila* at each tap.

Legionella spp. occurrence was also of interest, given that there are more than 50 species and 70 serogroups of *Legionella*, and that many besides *L. pneumophila* are also pathogenic. Interestingly, *Legionella* spp. was not as highly impacted by the elevated temperature settings in the Hot Temperature system as *L. pneumophila* (Fig. 6.4b; Fig. E.2). In general, total yield of *Legionella* spp. was reduced up to only 1.5 logs in the Hot versus Warm Temperature system, compared to the 3.5 log decrease in *L. pneumophila* under some conditions (Fig. E.2b). However, in the Hot Temperature system, low use pipes had substantially more *Legionella* spp. regrowth (increasing to 4.7-5.1 log gene copies/mL from 3.6 log gene copies/mL in the recirculating line) than in medium or high use pipes (increasing to only 3.4-4.7 log gene copies/mL from 3.6 log gene copies/mL in the recirculating line). Similar to our previous work (Rhoads et al., 2015), the elevated operating temperature of the Hot Temperature system significantly decreased the ratio of *L. pneumophila* compared to *Legionella* spp., from 0.42 to 0.05 (Paired t-Test, p-value<0.0001, n=36). Thus, higher temperatures in this experiment appeared to select other *Legionella* spp. relative to *L. pneumophila.*

(a) Log average *L. pneumophila* concentration (gene copies/mL)

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Time	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Tank & Recirc		4.8		Tank & Recirc		2.5	
	Distal (No Mixing)	4.9	4.7	4.8	Distal (No Mixing)	2.4	BQL	1.7
	Distal (w/ Mixing)	5.0	5.3	4.8	Distal (w/ Mixing)	3.2	3.7	3.0
4 months	Tank & Recirc		4.4		Tank & Recirc		0.0	
	Distal (No Mixing)	4.9	4.5	4.5	Distal (No Mixing)	2.8	2.7	2.0
	Distal (w/ Mixing)	5.0	4.9	4.6	Distal (w/ Mixing)	3.5	2.9	BQL

(b) Log total average *L. pneumophila* weekly yield (gene copies)

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Time	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Distal (No Mixing)	7.6	7.9	8.8	Distal (No Mixing)	5.1	4.4	5.7
	Distal (w/ Mixing)	7.7	8.5	8.8	Distal (w/ Mixing)	5.9	6.9*	7.0
4 months	Distal (No Mixing)	7.6	7.7	8.5	Distal (No Mixing)	5.5	5.9	6.0
	Distal (w/ Mixing)	7.7	8.1	8.6	Distal (w/ Mixing)	6.2	6.0	4.9*

(c) Log average change in *L. pneumophila* in distal taps relative to recirculating lines (gene copies/mL)

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Time	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Distal (No Mixing)	0.1	-0.1	0.0	Distal (No Mixing)	-0.1	-1.3	-0.8
	Distal (w/ Mixing)	0.2	0.5	0.0	Distal (w/ Mixing)	0.6	1.2*	0.4
4 months	Distal (No Mixing)	0.5	0.1	0.1	Distal (No Mixing)	2.8	2.7	2.0
	Distal (w/ Mixing)	0.6	0.5	0.2	Distal (w/ Mixing)	3.5	2.9	0.9*

Fig. 6.4. Heat map of bulk water *L. pneumophila*.

Heat map of bulk water *L. pneumophila* compares (**a**) concentration in the tank & recirculating lines and each set distal taps (log gene copies/mL), (**b**) total yield of *L. pneumophila* per week at the tap (log gene copies), and (**c**) log change in concentration in distal taps with respect to the recirculating lines (regrowth factor). Colors are on a continuous scales from green (low) to red (high). Table S1 provides a detailed description of each calculation. "*" indicates calculations done with estimated values that were below the quantification limit. BQL indicates "below quantification limit."

Impact of Convective Mixing on Bulk Water *Legionella*. A direct comparison of *Legionella* spp. and *L. pneumophila* in pipes with mixing to pipes without mixing yields 12 comparisons (3 use frequencies × 2 temperature settings × 2 samplings=12).

Convective mixing increased *L. pneumophila* regrowth in both the Hot and Warm Temperature systems in 11 of 12 conditions (4 of the 11 were statistically higher based on 95% confidence intervals; Fig. 6.5). In the one condition where pipes without convective mixing had higher *L. pneumophila* concentrations than pipes with mixing, there was one outlier in the triplicate pipes without mixing that may have skewed the results (Fig. E1). The sharpest increases due to convective mixing (3 of the 4 significant conditions) occurred in the low and medium use pipes, consistent with the hypothesis that the convective mixing gradients help supply ideal growth temperatures and nutrients in the Hot Temperature system. In fact, the low use distal taps with convective mixing in the Hot Temperature system had only 0.9-1.6 log fewer *L. pneumophila* than the recirculating line of the Warm Temperature system. This is expected since this small volume of Hot Temperature low use distal tap water was effectively mixed during stagnation and held at ideal temperature, much as is the case in the Warm Temperature tank and recirculating line (Fig. 6.4A).

Legionella spp. occurrence was not as strongly impacted by convective mixing gradients as *L. pneumophila* (Fig. 6.5). *Legionella* spp. concentrations were elevated in pipes with mixing compared to pipes without mixing in 8 of 12 conditions (5 of those 8 were statistically higher based on 95% confidence intervals). However, the conditions with significant increases were distributed between all three use frequencies and both temperature settings, producing no clear trend. Convective mixing also tended to decrease the proportion of *L. pneumophila* relative to all *Legionella* spp. in the Warm Temperature system distal taps (by 0.14-0.26) and increase it in the Hot Experimental system distal taps (by 0.013-0.15) (Fig. E.5). In these same conditions (Warm and Hot Temperature system distal taps with convective mixing), *L. pneumophila* and *Legionella* spp. were significantly, albeit weakly, correlated (Spearman's Rank ρ=0.37-0.57, p-value=0.005-0.013). In the Warm Temperature system, convective mixing supplied the distal taps with an influx of diverse and active microorganisms, potentially reducing the competitiveness of *L. pneumophila* and providing nutrients for all *Legionella* spp. to regrow. Hence, we observed weak correlations between *L. pneumophila* and *Legionella* spp. In the Hot Temperature system, convective mixing continuously inoculated distal taps with a less active microbial community with respect to *Legionella* growth compared to the Warm Temperature system, along with unused nutrients from the recirculating line, and were maintained at ideal growth temperatures. This apparently resulted in regrowth of all *Legionella* spp., but favored *L. pneumophila.* Thus, *Legionella* spp. and *L. pneumophila* were better correlated in the Hot Water system and the relative ratio of *L. pneumophila* slightly increased compared to pipes with no convective mixing. However, both systems initially had high levels of total bacteria genetic markers in the recirculating lines and tank (7.2-7.7 log gene copies/mL), suggesting a shift in the microbial community instead of inhibiting regrowth altogether. Convective mixing also increased total bacteria in distal pipes relative to pipes without mixing (with 0.1-0.7 log more 16S rRNA gene copies/mL). Overall, this study suggests that there is variable response across *Legionella* spp. to water stressors experienced in hot water plumbing. While many species share the ability to infect hosts, and even share specific virulence genes, pathogenesis is differentiated among *Legionella* spp., which may extend to survival and growth mechanisms within plumbing systems as witnessed here (Pereira et al., 2011; Joshi et al.,

Condition	Water Heater Set to 40° Low	Medium	High	Water Heater Set to 58° C Low	Medium	High
***Legionella* spp.**						
2 months	**0.55**	**0.79**	0.30	0.07	**0.89**	**0.61**
4 months	0.02	**0.45**	0.20	0.37	-0.23*	0.29
L. pneumophila						
2 months	0.11	**0.60**	0.02	**0.76**	**2.51**	1.27
4 months	0.12	0.37	0.06	**0.73**	0.17	-1.08*

Fig. 6.5. Log difference in bulk water *Legionella* spp. and *L. pneumophila* concentration in pipes with thermal mixing compared to pipes without thermal mixing.

Numbers near zero indicate no change between pipes with and without thermal mixing; numbers near to 1 or -1 indicate a 10X increase or decrease in concentration in pipes with thermal mixing compared to pipes without thermal mixing. Positive numbers indicate that pipes with thermal mixing have greater concentrations than pipes with no thermal mixing. Bold numbers are statistically significant (Kruskal-Wallis Test, $p<0.05$). * indicates calculations carried out with estimated values that were below the quantification limit.

1999; Ratcliff et al., 1997; Wadowsky et al., 1991; Fields et al., 1990; Cervero-Aragó et al., 2015; Storey et al., 2004).

Trends in Biofilm *Legionella*. To provide a measurement of biofilm re-colonization, the same area (65 cm^2) was swabbed in each reservoir during each experimental period. At the end of our previous study (Rhoads et al., 2015), there were 4.4×10^5 gene copies/cm^2 in the system maintained at approximately 40° C. In the Warm Temperature system, the recirculating lines had the greatest *L. pneumophila* biofilm densities (up to 1.3×10^4 gene copies/cm^2), again consistent with continuous recirculation and delivery of nutrients to biofilms at ideal temperature (Fig. 6.6). In the distal taps at cooler temperatures, *L. pneumophila* did not recolonize the biofilm to the same extent after the previous sampling, having 0.4-2.5 log fewer *L. pneumophila* gene copies/cm^2 than the recirculating line (Fig. 6.6; Fig. E3). High use distal taps tended to have slightly higher densities of *L. pneumophila* recolonize the biofilm after each sampling compared to less frequently used taps (0.1-0.5 log more gene copies/cm^2 in 3 of 4 conditions), and pipes with convective mixing tended to have higher densities recolonize the biofilm than pipes without mixing (0.70-1.27 log more gene copies/cm^2); however, neither trend had statistically significant differences. Similar trends occurred with respect to *Legionella* spp. (Fig. E4). These findings support the hypothesis that the majority of regrowth occurred in the recirculating line of the Warm Temperature system, where all the ammonia was consumed and the majority of the TOC was produced.

L. pneumophila appeared unable to recolonize the biofilm after each resampling in the Hot Temperature system. The one condition where recolonization was observed (2.5 log gene copies/cm^2 in the 2 month sampling of the high use pipes with mixing) had significant regrowth in only one of three triplicate pipes (Fig. E3B). More regrowth would be expected if the entire distal pipe was maintained at ideal growth temperatures instead of the gradient we observed (Fig. 6.2A), as might be the case if pipe insulation or heat-conducing pipe (e.g., copper) had been used.

Thus, the repeated swabbing of a small portion of the system surface provides insights into the ability of the system to re-establish a biofilm rapidly. More regrowth was expected considering that the bulk water *L. pneumophila* results were consistent with the hypotheses in Table 6.1. However, these results do indicate that the use of elevated temperatures reduce the likelihood of *L. pneumophila* colonization if temperatures are maintained at hot enough levels to inhibit regrowth, which is difficult to achieve and maintain in complex plumbing networks (Kool et al., 1999; Bedard et al., 2015).

Ecological relationships. *Legionella* proliferation in drinking water systems is widely thought to be facilitated by growth within a host amoeba cell, with a broad range of host organisms having been described. Here, we focus on biofilm-associated *V. vermiformis* because it is among the most frequently detected host organisms in drinking water (Scheikl et al., 2014; Coşkun et al., 2013; Ovrutsky et al., 2013), was found to be the most prevalent amoeba and was weakly correlated to *Legionella* spp. in a prior investigation of Blacksburg, VA tap water (Wang et al., 2012), and amoeba primarily graze on organisms inhabiting biofilms. In the Warm Temperature system tank and recirculating lines, high levels of *V. vermiformis* were detected (average 1.1×10^4 gene copies/cm^2) relative to the influent (3.2×10^3 gene copies/cm^2), as were high levels of *Legionella* spp. (2.36×10^3 gene copies/cm^2), *L. pneumophila* (8.2×10^3 gene copies/cm^2), TOC production (0.93 mg/L increase), and ammonia oxidation (0.54 mg/L consumed). Thus, under these ideal conditions, all indicators of regrowth were in agreement. In the distal taps, which received high levels of all bacteria and maintained favorable regrowth conditions, *V. vermiformis* strongly correlated with *Legionella* spp. and *L. pneumophila* (Fig. 6.7A and B; R^2=0.78-0.79). However, in the Hot Temperature system, there were no *V. vermiformis* detected in the recirculating line and tank, and no correlations were found in the distal taps with *Legionella* spp. or *L. pneumophila* (Fig. 6.7A and B; R^2=0.001-0.008). In addition, there were 1.2 log fewer *V. vermiformis* gene copies/cm^2 in the distal taps of the Hot Temperature system than the Warm Temperature system on average (paired t-test, p-value=0.0011, n=72). In the Warm Temperature system, the strong correlations of *Legionella* and *V. vermiformis* are consistent with the expectation of the pathogen-host relationship. However, in the Hot Temperature system, the elevated temperatures may have caused *V. vermiformis* to encyst, resulting in decreased phagocytosis of *Legionella* cells and decreased recovery of the amoebae genetic material in general. Interestingly, low use distal taps in the Hot Temperature system had higher concentrations of *V. vermiformis* than high or medium use taps (681 vs 92 gene copies/mL, Mann-Whitney U-test, p-value=0.023). This suggests the week long stagnation in the low use distal taps was sufficient for the amoebae to recover from the encysted phase that may have been induced by the elevated temperature in the Hot Temperature system. Finally, convective mixing in the Warm Temperatures system was associated with an increase in the number of detected *V. vermiformis* detected (paired t-test, p-value=0.025, n=36) and improved correlations with *Legionella* spp. (R^2=0.71 without mixing vs 0.99 with mixing), but not with *L. pneumophila* (R^2=0.76 without mixing vs 0.80 with mixing). No such trends were found in the bulk water (Fig. E7).

Log average *L. pneumophila* concentration (gene copies/cm^2)

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Condition	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Recirculating Line		3.9		Recirculating Line		0.0	
	Distal (No Mixing)	2.2	2.2	2.4	Distal (No Mixing)	0.0	0.0	0.0
	Distal (w/ Mixing)	3.0	3.1	3.5	Distal (w/ Mixing)	0.0	BQL	2.5
4 months	Recirculating Line		4.1		Recirculating Line		BQL	
	Distal (No Mixing)	1.9	BQL	BQL	Distal (No Mixing)	BQL	0.0	0.0
	Distal (w/ Mixing)	2.6	2.7	2.7	Distal (w/ Mixing)	0.0	BQL	0.0

Fig. 6.6. Heat map of *L. pneumophila* comparing concentration in the tank & recirculating line biofilm to each set distal tap biofilm (log gene copies/cm^2).

Colors are on a continuous scales from green (low) to red (high). BQL indicates values that were detectable, but below the quantification limit. ND indicates there were no genes detected.

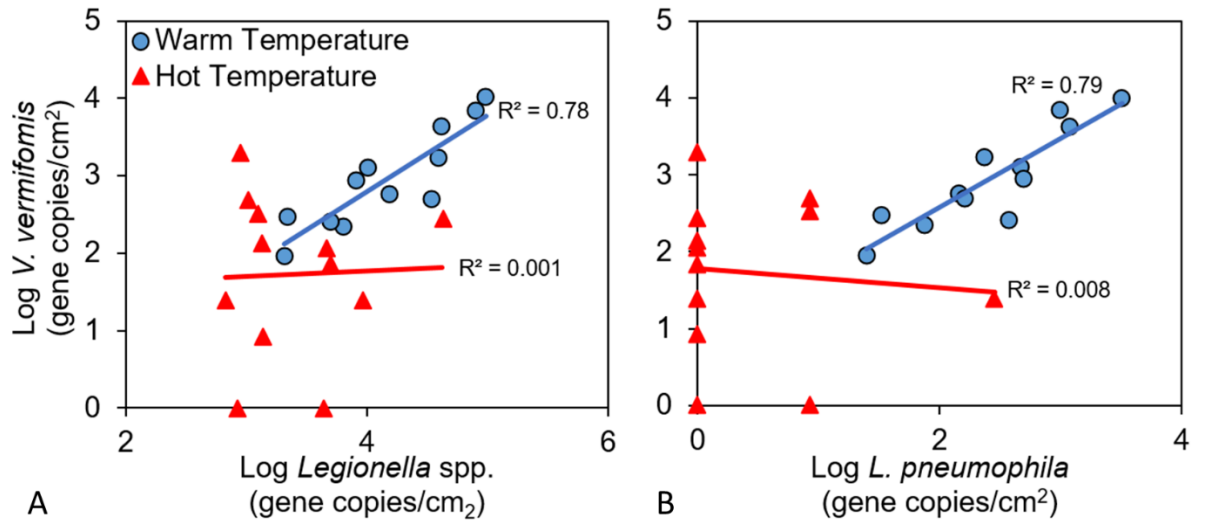


Fig. 6.7. Correlation of *V. vermiformis* with A) *Legionella* spp. and B) *L. pneumophila* in distal tap biofilms.

Limitations of Experimental Conditions. Despite the consistent impact of convective mixing in distal pipes that occurred in this study, which facilitated additional growth of *Legionella* spp. and *L. pneumophila* relative to distal pipes without convective mixing, it is important to note that under other operating conditions the opposite effect is expected to occur. For instance, in later experiments using the same experimental systems, we introduced a 0.42 ± 0.05 mg/L (as total Cl_2) chloramine residual to both water heaters. After a typical stagnation period, chloramine concentrations in the recirculating lines were 0.28 ± 0.08 mg/L. In low use downward oriented taps with no convective mixing, the residual decayed to 0.06 ± 0.02 mg/L while pipes with convective mixing maintained 0.13 ± 0.02 mg/L of chloramine delivered from the recirculating line. Under this scenario, convective mixing would be expected to decrease the ability for *Legionella* and *L. pneumophila* to regrow relative to pipes without convective mixing due to the increased presence of chloramine (Want et al., 2012; Moore et al., 2006; Pryor et al., 2004). Similarly, factors that impact the convective water exchange rate between the recirculating line and distal taps (i.e., pipe diameter, insulation, conductive copper vs. non-conductive plastics) may also create different trends than results observed in this study.

In addition, there are inherent limitations to the methodology applied in this study. While qPCR provides quantitative insights into the presence and increase in gene targets, it does not insure only live cells are quantified. Culturing data could have provided insight into the species and strains of *Legionella* growing at elevated temperatures that grow on isolation media; however, the viable but non-cultivable state of *Legionella* makes definitive conclusions over the dominant species difficult.

EXPERIMENTAL SECTION

Experimental Setup and Operation. Details of the experimental system design were previously published in a study examining the effect of water heater set points without convective mixing (Rhoads et al., 2015). Briefly, two identical household hot water systems were constructed with

71.9 L (19 gallon) electric water heaters and continuously recirculating pipe loops (Fig. 6.1). For this work, the "Warm Temperature Set Point" system water heater was set to 40° C and the "Hot Temperature Set Point" system water heater was set to 58° C to represent worst and best management practices with respect to *Legionella* control. Both systems were acclimated for 15 months with no convective mixing prior to commencing this study (month 15 in our previous work is month 0 for this study) (Rhoads et al., 2015). For this study, convective mixing was induced in half the distal pipes by slanting upward oriented pipes 30° from vertical. Sampling occurred at 2 months and 4 months after inducing convective mixing to compare the two pipe orientations (with and without convective mixing) with three water use patterns in triplicate, including low use (1 flush/week), medium use (3 flushes/week), and high use (21 flushes/week) for a total of 36 distal taps (2 systems × 2 orientations × 3 use patterns × triplicate = 36) to simulate the range of use frequencies encountered in homes. Each distal pipe was 1.5 m long with a 0.2 m section accessible by a union for repeated biofilm swab, had a volume of approximately 430 mL, and each flush described above was 28 seconds long at 3.8 L/min (1 gallon/min). Influent water consisted of well-flushed (10 minutes at 11.3 L/min), GAC filtered Blacksburg, VA tap water. During periods of no use, distal pipes were allowed to equilibrate with the ambient temperature. All pipes were uninsulated.

Sample collection and microbiological analyses. At the end of each two-month period, approximately 0.5 L of first-flush water was collected directly from the influent, recirculating lines, and each distal tap at the end of regular stagnation periods for each use condition and filtered through sterile 0.22 µm pore-size mixed cellulose ester filters (Millipore, Billerica, MA). Exact volume collected for each sample was measured by weight after filtration. For biofilm sampling, 65 cm^2 of influent, recirculating line, and ends of the distal tap pipes accessible by threaded union connections were swabbed using sterile cotton-tip applicators (Fisherbrand, Fisher Scientific, UK). DNA was extracted directly from fragmented filters and cotton swabs using a FastDNA Spin Kit (MP Biomedicals, Solon, OH) according to the manufacturer protocol. Field, trip, and equipment negative controls consisting of pre-sterilized (autoclaved) nanopure water in identical sampling bottles were included each time samples were collected.

Gene markers for *Legionella* spp. (23S rRNA), *L. pneumophila* (macrophage infectivity potentiator (*mip*) gene), *V. vermiformis* (18S rRNA), and total bacteria (16S rRNA gene), were enumerated by q-PCR assays using previously established methods (Wang et al., 2012). In brief, all q-PCR assays were performed in 10 µL reaction mixtures containing SsoFast Probes or Evagreen Supermix (Bio-Rad, Hercules, CA), 250 or 400 nM primer, 93.75 nM probe (Taqman assay only) with 1 µL of DNA template. For each set of samples, serial dilutions (ranging from 1:1 to 1:100) with positive template spikes were used to identify the optimum dilution to minimize q-PCR inhibition (1:10) on a subset of samples. A negative control, 10-fold serial dilutions of standards, and a positive spike into sample DNA matrix were included in triplicate wells with each q-PCR run. The quantification limit (QL) for all q-PCR assays ranged from 10 to 100 gene copies/reaction, except 16S rRNA which ranged from 200-1,000 gene copies/reaction. The QL was applied to samples based on standard curves obtained on each thermocycler run. Samples yielding threshold cycles ≥ QL in at least two q-PCR triplicate wells were considered quantifiable. Samples with only one triplicate above the QL threshold cycle, or samples otherwise below the QL were re-analyzed undiluted to increase the QL of the assays. On each re-run plate, standard DNA template was spiked into the experimental DNA matrix to confirm amplification reactions

were not inhibited in undiluted samples. If inhibited, the sample was marked as below the QL. All values are reported as log(gene copies/mL + 1).

Disinfectant residual, total ammonia, temperature, pH, dissolved oxygen (DO), total organic carbon (TOC), and total and dissolved cations were generally characterized in the first week of each 2-month operation period. Once trends were established, disinfectant, ammonia, temperature, and pH were monitored to confirm parameters were not significantly changing. Chloramine and total ammonia were measured according to Standard Method 4500-Cl25 and 5310-NH3 using a DR2700 or DR5000 spectrophotometer (HACH, Loveland, CO). pH and temperature were measured using a pH 110 meter with automatic temperature correction (Oakton Research, Vernon Hills, IL). Pipe surface temperature was measured using an infrared emissivity temperature gun (Kintrex Infrared Thermometer IRT0421). Pipe surface profiles were quantified by measuring the pipe surface temperature at 0.3 m (1 ft) intervals at the end of regular stagnation period. Dissolved Oxygen was monitored using a Thermo Scientific Orion 3-star meter. Total organic carbon was measured by persulfate-ultraviolet detection using a Sievers Model 5300C with an autosampler according to Standard Method 5310 C. Cations were measured by inductively coupled plasma mass spectrometry after acidification with 2% nitric acid (v/v) and >24 hours holding time.

Statistical Analyses. All error bars on figures and error margin calculations are 95% confidence intervals, calculated based on the normal cumulative distribution function, degrees of freedom, and standard error. For graphing and statistical purposes, any positive detection below the q-PCR QL was entered as half of the lowest observed QL. All data analysis was conducted in Microsoft Excel 2013, JMP Pro 11, or RStudio using R version 3.2.0. Spearman's rank coefficient and associated significance tests were conducted in JMP Pro 11 to detect and quantify relationships between gene markers (using "Multivariate Methods"). Other statistical tests were performed in RStudio. Student's t-test ("t.test()") and Kruskal-Wallis tests with a Holm p-value adjustment for multiple comparisons were conducted to compare sample means (initially with "kruskal.test(()," then using package and function "dunn.test()" for multiple comparisons). Significance was determined at p=0.05.

CONCLUSIONS

Here we examined the interactive effects of convective mixing induced by plumbing configuration along with water heater temperature setting and water use frequency on the growth of *Legionella* spp. and *L. pneumophila* in recirculating lines and otherwise stagnant distal pipes. This large pilot-scale experiment supports the conventional wisdom that maintaining elevated water temperatures at all points in a hot water system is a critical engineering control for inhibiting regrowth of *Legionella,* especially *L. pneumophila*. This work demonstrated that convective mixing currents in hot water systems can maintain ideal growth temperatures and continuously supply nutrients to otherwise stagnant distal taps. As a result, convective mixing has the potential to undermine thermal control strategies. In these experiments, the benefits of maintaining water above 55° C in the water heater and recirculating line were reduced in the distal taps, and convective mixing facilitated additional *L. pneumophila* regrowth. Thus, holistic approaches are needed to control *L. pneumophila* regrowth with attention to subtle design considerations that can inadvertently impact the extent of regrowth. Other factors expected to alter convective mixing exchange between

reservoirs in hot water plumbing (such as pipe diameter, use of insulation, and use of conductive metallic pipe) is expected to affect regrowth at distal taps.

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Author Contributions. W.R., A.P., and M.E. conceived and designed the experiments; W.R. performed the experiments, analyzed the data, and led the preparation of the manuscript; A.P. and M.E. contributed reagents/materials/analysis tools and assisted with writing the manuscript.

Conflicts of Interest. The authors declare no conflict of interest.

REFERENCES

Agathokleous, R.A. Optimization of Thermosiphon Solar Water Heating systems working in a Mediterranean Environment of Cyprus. Archimedes Solar Energy Laboratory, Cyprus University of Technology. **2004**. Available online: http://www.poemconference.org/poem2013/uploads/presentations/08-10-2013/Special_Session-Current_research_at_Archimedes_solar_energy_laboratory/Optimization_of_Thermosiphon_Solar_Water_Heaters_working_in_a_Mediterranean_Environment.pdf (Accessed April 30, 2014).

Alary M.; Joly J.R. Risk factors for contamination of domestic hot water systems by legionellae. *Appl. Environ. Microbiol*. **1991**, 57(8), 2360-2367.

Allegra, S.; Berger, F.; Berthelot, P.; Grattard, F.; Pozzetto, B.; Riffard. S. Use of flow cytometry to monitor *Legionella* viability. *Appl. Environ. Microbiol.* **2008**, 74(24), 7813-7816.

Allegra, S.; Grattard, F.; Girardot, F.; Riffard, S.; Pozzetto, B.; Berthelot, P. Longitudinal evaluation of the efficacy of heat treatment procedures against *Legionella* spp. in hospital water systems by using a flow cytometric assay. *Appl. Environ. Microbiol*. **2011**, 77(4), 1268-1275.

Bédard E, Fey S, Charron D, Lalancette C, Cantin P, Dolcé P, Prévost M. Temperature diagnostic to identify high risk areas and optimize *Legionella pneumophila* surveillance in hot water distribution systems. *Water Res*. **2015**, 71, 244-256.

Bollin, G.E.; Plouffe, J.F.; Para, M.F.; Hackman, B. Aerosols containing *Legionella pneumophila* generated by shower heads and hot-water faucets. *Appl. Env. Microbiol*. **1985**, 50(5), 1128-1131.

Borella, P.; Montagna, M. T.; Stampi, S.; Stancanelli, G.; Romano-Spica, V.; Triassi, M.; Boccia, S. *Legionella* contamination in hot water of Italian hotels. *Appl. Environ. Microbiol.* 2005, 71(10), 5805-5813.

Borella, P.; Montagna, M.T.; Romano-Spica, V.; Stampi, S.; Stancanelli, G.; Triassi, M.; D'alcalà, G.R. *Legionella* infection risk from domestic hot water. Emerging infectious diseases. **2004**, 10(3), 457.

Brazeau, R.H.; Edwards, M.A. Role of Hot Water System Design on Factors Influential to Pathogen Regrowth: Temperature, Chlorine Residual, Hydrogen Evolution, and Sediment. *Environmental engineering science*. **2013**, 30(10), 617-627.

Brunkard J.M.; Ailes E.; Robers V.A.; Hill V.; Hilborn E.D.; Craun G.F.; Rajasingham A.; Kahler A.; Garrison L.; Hicks L.; Carpenter J.; Wade T.J.; Bearch M.J.; Yoder J.S. Surveillance for Waterborne Disease and Outbreaks Associated with Drinking Water and Water not Intended for Drinking --- United States, 2007—2008. *Morb.Mor. Wkly. Rep.* **2011**, 60(SS12), 38-68.

Camper, A.K. Involvement of humic substances in regrowth. International Journal of Food Microbiology, **2004**, 92(3), 355-364.

Casini, B.; Valentini, P.; Baggiani, A.; Torracca, F.; Frateschi, S.; Nelli, L.C.; Privitera, G. Molecular epidemiology of *Legionella pneumophila* serogroup 1 isolates following long-term chlorine dioxide treatment in a university hospital water system. *J. Hosp. Infect.* **2008**, 69(2), 141-147.

Cervero-Aragó, S.; Rodríguez-Martínez, S.; Puertas-Bennasar, A.; Araujo, R.M. Effect of Common Drinking Water Disinfectants, Chlorine and Heat, on Free *Legionella* and Amoebae-Associated *Legionella*. *PloS one*, **2015**,*10*(8).

Cordes, L.G.; Wiesenthal, A.M.; Gorman, G.W.; Phair, J.P.; Sommers, H.M.; Brown, A.; Yu, V.L. Isolation of *Legionella pneumophila* from hospital shower heads. *Annals of internal medicine.* **1981**, 94(2), 195-197.

Coşkun K.A.; Özçelik S.; Tutar L.; Elald N.; Tutar, Y. Isolation and identification of free-living amoebae from tap water in Sivas, Turkey. *BioMed research international*. **2013**, doi: 10.1155/2013/675145.

Cowen, K.A.; Ollison, W.M. Continuous monitoring of particle emissions during showering. Journal of the Air & Waste Management Association. **2006**, 56(12), 1662-1668.

Darelid, J.; Löfgren, S.; Malmvall, B.E. Control of nosocomial Legionnaires' disease by keeping the circulating hot water temperature above 55 C: experience from a 10-year surveillance programme in a district general hospital. *J. Hosp. Infect.* **2002**, 50(3), 213-219.

Dennis, P.J.L; Wright, A.E; Rutter, D. A.; Death, J.E,; Jones, B.P.C. *Legionella pneumophila* in aerosols from shower baths. Journal of hygiene. **1984**, 93(02), 349-353.

Dondero Jr, T.J.; Rendtorff, R.C.; Mallison, G.F.; Weeks, R.M.; Levy, J.S.; Wong, E.W.; Schaffner, W. An outbreak of Legionnaires' disease associated with a contaminated air-conditioning cooling tower. *New England Journal of Medicine*. **1980**, 302(7), 365-370.

Donohue, M.J.; O'Connell, K.; Vesper, S.J.; Mistry, J.H.; King, D.; Kostich, M.; Pfaller, S. Widespread molecular detection of Legionella pneumophila serogroup 1 in cold water taps across the United States. *Environ. Sci. & Technol.* 2014, 48(6), 3145-3152.

Fang, G.D.; Yu, V.L.; Vickers, R.M. Disease due to the Legionellaceae (other than *Legionella pneumophila*): historical, microbiological, clinical, and epidemiological review. *Medicine*, **1989**, *68*(2), 116.

Fields, B.S.; Barbaree, J.M.; Sanden, G.N.; Morrill, W.E. Virulence of a *Legionella anisa* strain associated with Pontiac fever: an evaluation using protozoan, cell culture, and guinea pig models. *Infection and immunity*. **1990**, 58(9), 3139-3142.

Fields, B.S.; Benson, R.F.; Besser, R.E. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clinical microbiology reviews*, 2002, 15(3), 506-526.

Gibbs, W.M. The isolation and study of nitrifying bacteria. *Soil Science*. **1920**, 8, 427.

Gopalakrishnan, N.; Murthy, S.S. Mixed convective flow and thermal stratification in hot water storage tanks during discharging mode. *Applied Solar Energy*. **2009**, 45(4), 254-261.

Grady, C.P.L.; Daigger, G.T.; Lim, H.C. Biological wastewater treatment, 2nd ed. Marcel Dekker, Inc: Basel, New York, USA, **1999**.

Haddix, P.L.; Shaw, N.J.; LeChevallier, M.W. Characterization of Bioluminescent Derivatives of Assimilable Organic Carbon Test Bacteria. *Appl. Environ. Microbiol.* **2004**, 70(2), 850-854.

Hilborn, E. D.; Wade, T. J.; Hicks, L.; Garrison, L.; Carpenter, J.; Adam, E.; Mull, B.; Yoder, J.; Roberts, V.; Gargano, J. W. Surveillance for Waterborne Disease Outbreaks Associated with Drinking Water and Other Nonrecreational Water - United States, 2009-2010. *Morb.Mor. Wkly. Rep.* **2013**, 62(35), 714-720.

Hlavsa, M. C.; Roberts, V. A.; Kahler, A. M.; Hilborn, E. D.; Wade, T. J.; Backer, L. C.; Yoder, J. S. Recreational water-associated disease outbreaks - United States, 2009-2010. *Morb. Mor. Wkly. Rep*. **2014**, 63(1), 6-10.

Joshi, A.D.; Swanson, M.S. Comparative Analysis of *Legionella pneumophila* and *Legionella* micdadeiVirulence Traits. *Infection and immunity*. **1999**, 67(8), 4134-4142.

Konishi, T.; Yamashiro, T.; Koide, M.; Nishizono, A. Influence of temperature on growth of *Legionella pneumophila* biofilm determined by precise temperature gradient incubator. *Journal of bioscience and bioengineering*. **2006**, 101(6), 478-484.

Kool, J.L.; Bergmire-Sweat, D.; Butler, J.C.; Brown, E.W.; Peabody, D.J.; Massi, D.S.; Fields, B.S. Hospital characteristics associated with colonization of water systems by *Legionella* and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals. *Infection Control*. **1999**, 20(12), 798-805.

LeChevallier, M.W.; Schulz, W.; Lee, R. G. Bacterial nutrients in drinking water. *Appl. Environ. Microbiol.* **2001**, 57(3), 857-862.

Leoni, E.; Legnani, P.P. Comparison of selective procedures for isolation and enumeration of Legionella species from hot water systems. *J. Appl. Microbiol*. **2001**, 90(1), 27-33.

Martin, A. K. Organic carbon generation mechanisms in main and premise distribution systems. Virginia Tech Masters Thesis, **2012.** Available online: http://scholar.lib.vt.edu/theses/available/etd-08192012-202007/unrestricted/Martin_AK_T_2012.pdf (accessed Jan. 15, 2016).

Moore, M. R.; Pryor, M.; Fields, B.; Lucas, C.; Phelan, M.; Besser, R. E. Introduction of monochloramine into a municipal water system: impact on colonization of buildings by *Legionella* spp. *Appl. Environ. Microbiol.* **2006**, *72*(1), 378-383.

Morton, S.C.; Zhang, Y.; Edwards, M.A. Implications of nutrient release from iron metal for microbial regrowth in water distribution systems. *Water Res*. **2005**, 39(13), 2883-2892.

Mouchtouri, V.; Velonakis, E.; Hadjichristodoulou, C. Thermal disinfection of hotels, hospitals, and athletic venues hot water distribution systems contaminated by *Legionella* species. American journal of infection control, 2007, 35(9), 623-627.

Neufeld, R.D.; Hill, A.J.; Greenfield, J. Factors influencing biological nitrification of steel industry wastewaters. *AlChe Symposium Series*. **1980**, 77(209), 151–158.

Ohno, A.; Kato, N.; Yamada, K.; Yamaguchi, K. Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water. *Appl. Environ. Microbiol*. **2003**, 69(5), 2540-2547.

Ovrutsky A.R.; Chan E.D.; Kartalija M.; Bai X.; Jackson M.; Gibbs S.; Fakinham J.O. 3rd, Iseman M.D.; Reynolds P.R.; McDonnell G.; Thomas V. Cooccurrence of free-living amoebae and

nontuberculous *Mycobacteria* in hospital water networks, and preferential growth of *Mycobacterium avium* in *Acanthamoeba lenticulata*. *Appl. Environ. Microbiol.* **2013**, 79(10), 3185-3192.

Pereira, M.S.; Morgantetti, G.F.; Massis, L.M.; Horta, C.V.; Hori, J.I.; Zamboni, D.S. Activation of NLRC4 by flagellated bacteria triggers caspase-1–dependent and–independent responses to restrict Legionella pneumophila replication in macrophages and in vivo. *The Journal of Immunology*. **2011**, 187(12), 6447-6455.

Pruden, A.; Edwards, M.A.; Falkinham, J.O., III. Research Needs for Opportunistic Pathogens in Premise Plumbing: Methodology, Microbial Ecology, and Epidemiology. Water Research Foundation Project 4379 Final Report; Water Research Foundation: Denver, CO, USA, **2013**.

Pryor, M.; Springthorpe, S.; Riffard, S.; Brooks, T.; Huo, Y.; Davis, G.; Sattar, S.A. Investigation of opportunistic pathogens in municipal drinking water under different supply and treatment regimes. *Water Sci. & Technol.* **2004**, 50(1), 83-90.

Ratcliff, R.M.; Donnellan, S.C.; Lanser, J.A.; Manning, P.A.; Heuzenroeder, M.W. Interspecies sequence differences in the Mip protein from the genus *Legionella*: implications for function and evolutionary relatedness. *Molecular microbiology*. **1997**, 25(6), 1149-1158.

Rhoads, W.J.; Ji, P.; Pruden, A.; Edwards, M.A. Water heater temperature set point and water use patterns influence *Legionella pneumophila* and associated microorganisms at the tap. *Microbiome*. **2015**, 3(1), Available Online: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4666224/pdf/40168_2015_Article_134.pdf (accessed Jan. 15, 2016).

Rushing, J.C.; Edwards, M. Effect of aluminium solids and chlorine on cold water pitting of copper. *Corrosion science*. **2004**, 46(12), 3069-3088.

Sawyer, C.N.; Bradney, L. Modernization of the BOD test for determining the efficiency of sewage treatment processes. *Science of Sewage Works*. **1946**, 18, 1113.

Scheikl U.; Sommer R.; Kirschner A.; Rameder A.; Schrammel B.; Zweimüller I.; Wesner W.; Hinker M.; Walochnik J. Free-living amoebae (FLA) co-occurring with legionellae in industrial waters. *European journal of protistology*. **2014**;50(4):422

Schulze-Röbbecke R., Rödder M., Exner M. Multiplication and killing temperatures of naturally occurring legionellas. *Zentralblatt fur Bakteriologie, Mikrobiologie und Hygiene*. **1987**, 184(6), 495-500.

Sheehan, K. B.; Henson, J. M.; Ferris, M. J. *Legionella* species diversity in an acidic biofilm community in Yellowstone National Park. *Appl. Environ. Microbiol*. **2005**, 71(1), 507-511.

Storey, M.V.; Ashbolt, N.J.; Stenström, T.A. Biofilms, thermophilic amoebae and *Legionella pneumophila*- A quantitative risk assessment for distributed water. *Water Science & Technology*, **2004** *50*(1), 77-82.

Vickers, R.M.; Yu, V.L.; Hanna, S.S.; Muraca, P.; Diven, W.; Carmen, N.; Taylor, F.B. Determinants of *Legionella pneumophila* Contamination of Water Distribution Systems 15-Hospital Prospective Study. *Infect. Cont.* **1987**, 8(09), 357-363.

Wadowsky, R.M.; Wilson, T.M.; Kapp, N.J.; West, A.J.; Kuchta, J.M.; Dowling, J.N.; Yee, R.B. Multiplication of *Legionella* spp. in tap water containing *Hartmannella vermiformis*. *Applied and environmental microbiology*. **1991**, 57(7), 1950-1955.

Wadowsky, R.M.; Yee, R.B.; Mezmar, L.; Wing, E.J.; Dowling, J.N. Hot water systems as sources of Legionella pneumophila in hospital and nonhospital plumbing fixtures. *Appl. Environ. Microbiol*. 1982, 43(5), 1104-1110.

Wang, H.; Edwards, M.A.; Falkinham, J. O. 3rd; Pruden, A. Molecular survey of the occurrence of *Legionella* spp, *Mycobacterium* spp., *Pseudomonas aeruginosa*, and amoeba hosts in two chloraminated drinking water distribution systems. *Appl. Environ. Microbiol*. **2012**, 78(17), 6285-6294.

Williams, K.; Pruden, A.; Falkinham, J.O.; Edwards, M.A. Relationship between organic carbon and opportunistic pathogens in simulated glass water heaters. *Pathogens*, **2015**, 4(2), 355-372.

Yoder J.; Roberts V.; Craun G.F.; Hill V.; Hicks L.; Alexander N.T.; Radke V.; Calderon R.L.; Hlavsa M.C.; Beach M.J.; Roy S.L. Surveillance for Waterborne Disease and Outbreaks Associated with Drinking Water and Water not Intended for Drinking --- United States, 2005—2006. *Morb.Mor. Wkly. Rep*. **2008**, 57(SS09), 39-62.

Zhang, Y.; Love, N.; Edwards, M. Nitrification in drinking water systems. *Critical Reviews in Environmental Science and Technology*. **2009**, 39(3), 153-208.

APPENDIX E - SUPPLEMENTAL INFORMATION FOR CHAPTER 6.

Table E1. Example calculations for determining distribution of genetic markers across various system compartments and effects of operating conditions

Analysis	Calculation	Practical Meaning/Interpretation
Concentration (Fig. 4A)	$log_{10}([Lp])$	*L. pneumophila* concentration
Weekly yield (Fig. 4B)	$log_{10}([Lp]_{Distal} \times \forall_{Distal} \times Use)$	Total amount of *L. pneumophila* delivered at the tap per week
Change in Distal (Fig. 4C)	$log_{10}\left(\frac{[Lp]_{Distal}}{[Lp]_{Recirc}}\right)$	Regrowth in the distal taps relative to the recirculating line
Convective Mixing Impact (Fig. 5)	$log_{10}\left(\frac{[Lp]_{Mixing}}{[Lp]_{No\ Mixing}}\right)$	Increase in L. pneumophila in pipes due to convective mixing gradients

Notes:

1. [Lp] – *L. pneumophila* concentration in gene copies/mL;
2. Distal – "in Distal Tap";
3. Recirc – "in Recirculating line and tank";
4. ∀ - "Volume"
5. Mixing – Pipes with convective mixing
6. No Mixing – Pipes without convective mixing
7. Use = Use per week (21, 3, or 1)

Table E2. Average total number of bulk water *Legionella* spp. gene copies in each reservoir during each sampling (for each sampling, n=18 for Distal Taps; n=2 for Tank & Recirc).

System	Reservoir	2 months	4 months
Water Heater Set to 40° C	Distal Taps	1.62E+09	2.15E+09
	Tank & Recirc	6.53E+09	1.25E+10
Water Heater Set to 58° C	Distal Taps	3.74E+08	3.35E+08
	Tank & Recirc	2.93E+08	2.99E+08
Log increase in 39°C System normalized to 58° C System:		40 °C - 58 °C	40 °C - 58 °C
Distal tap *Legionella* spp. genes		0.6	0.8
Tank+Recirc *Legionella* spp. genes		1.3	1.6
Total *Legionella* spp. genes		1.1	1.4

Table E3. Average total number of bulk water *L. pneumophila* gene copies in each reservoir during each sampling (for each sampling, n=18 for Distal Taps; n=2 for Tank & Recirc).

System	Reservoir	2 months	4 months
Water Heater Set to 40° C	Distal Taps	6.83E+08	2.26E+08
	Tank & Recirc	4.67E+09	1.77E+09
Water Heater Set to 58° C	Distal Taps	1.02E+07	1.66E+06
	Tank & Recirc	2.50E+07	0.00E+00
Log increase in 40°C System normalized to 58° C System:		40 °C - 58 °C	40 °C - 58 °C
Distal tap *L. pneumophila* genes		1.8	2.1
Tank & Recirc *L. pneumophila* genes		2.3	9.2
Total system *L. pneumophila* genes		2.2	3.1

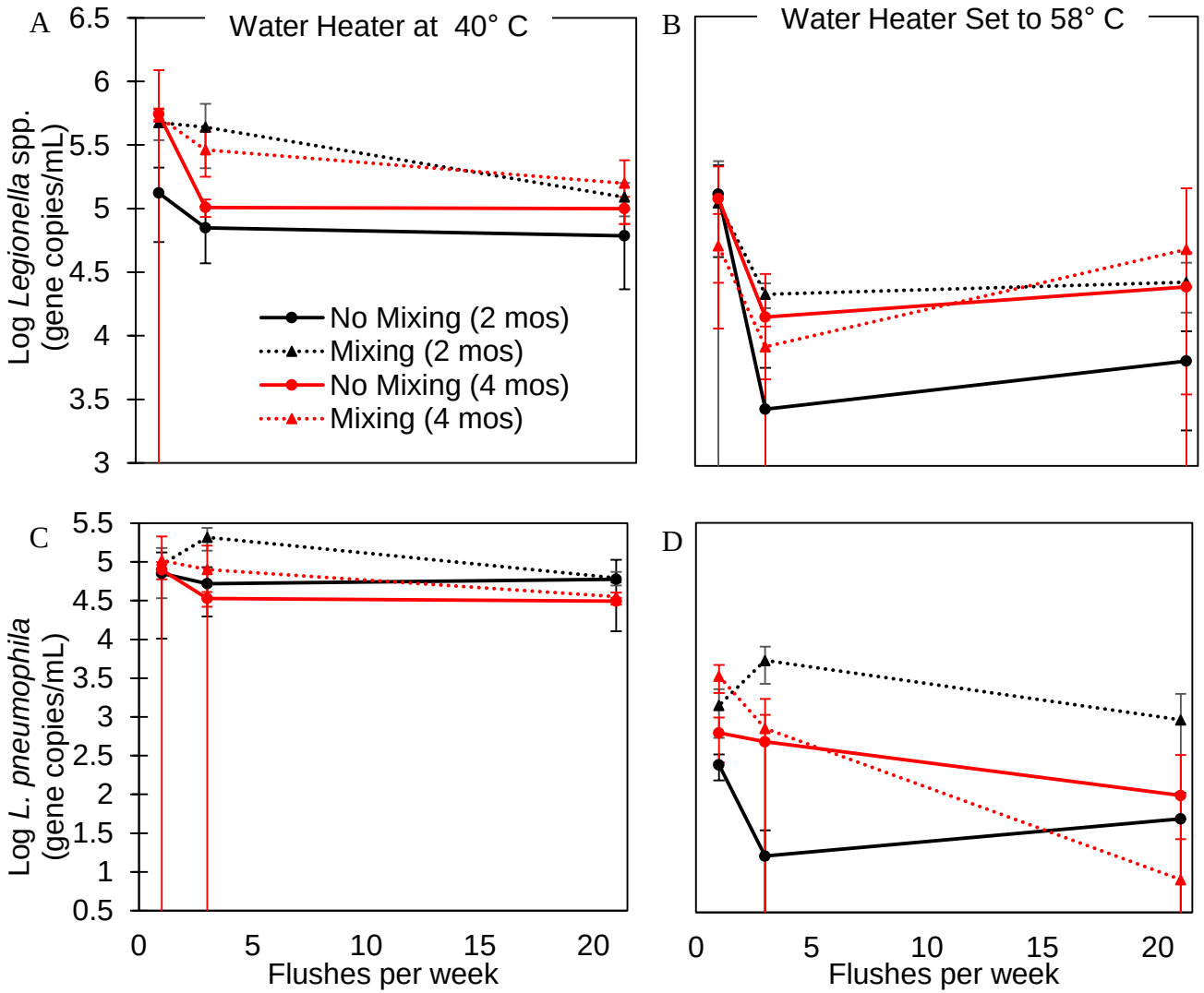

Fig. E1. *Legionella* spp. and *L. pneumophila* concentrations in distal tap water when water heater is set to A) and C) 40° C and B) and D) 58° C

(a) Average Log *Legionella* spp. concentration (gene copies/mL)

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Time	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Tank & Recirc	4.9			Tank & Recirc	3.6		
	Distal (No Mixing)	5.1	4.8	4.8	Distal (No Mixing)	5.1	3.4	3.8
	Distal (w/ Mixing)	5.7	5.6	5.1	Distal (w/ Mixing)	5.0	4.3	4.4
4 months	Tank & Recirc	5.2			Tank & Recirc	3.6		
	Distal (No Mixing)	5.7	5.0	5.0	Distal (No Mixing)	5.1	4.2	4.4
	Distal (w/ Mixing)	5.7	5.5	5.2	Distal (w/ Mixing)	4.7	3.9	4.7

(b) Average Log total *Legionella* spp. weekly yield from distal taps (gene copies)

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Time	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Distal (No Mixing)	7.8	8.0	8.8	Distal (No Mixing)	7.8	6.6	7.8
	Distal (w/ Mixing)	8.4	8.8	9.1	Distal (w/ Mixing)	7.7	7.5	8.5
4 months	Distal (No Mixing)	8.4	8.2	9.0	Distal (No Mixing)	7.8	7.3	8.4
	Distal (w/ Mixing)	8.4	8.6	9.2	Distal (w/ Mixing)	7.4	7.1	8.7

(c) Average Log change in *Legionella* spp. in distal taps relative to recirculating lines (gene copies/mL)

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Time	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Distal (No Mixing)	0.2	-0.1	-0.2	Distal (No Mixing)	1.5	-0.2	0.2
	Distal (w/ Mixing)	0.7	0.7	0.1	Distal (w/ Mixing)	1.4	0.7	0.8
4 months	Distal (No Mixing)	0.5	-0.2	-0.2	Distal (No Mixing)	1.5	0.6	0.8
	Distal (w/ Mixing)	0.5	0.2	0.0	Distal (w/ Mixing)	1.1	0.3	1.1

Fig. E2. Heat map of planktonic *Legionella* spp.

Comparing (**a**) concentration in the tank & recirculating lines and each set distal taps (average log gene copies/mL), (**b**) total yield of *L. pneumophila* per week at the tap (average log gene copies), and (**c**) average log change in concentration in distal taps with respect to the recirculating lines (regrowth factor). Colors are on a continuous scales from green (low) to red (high).

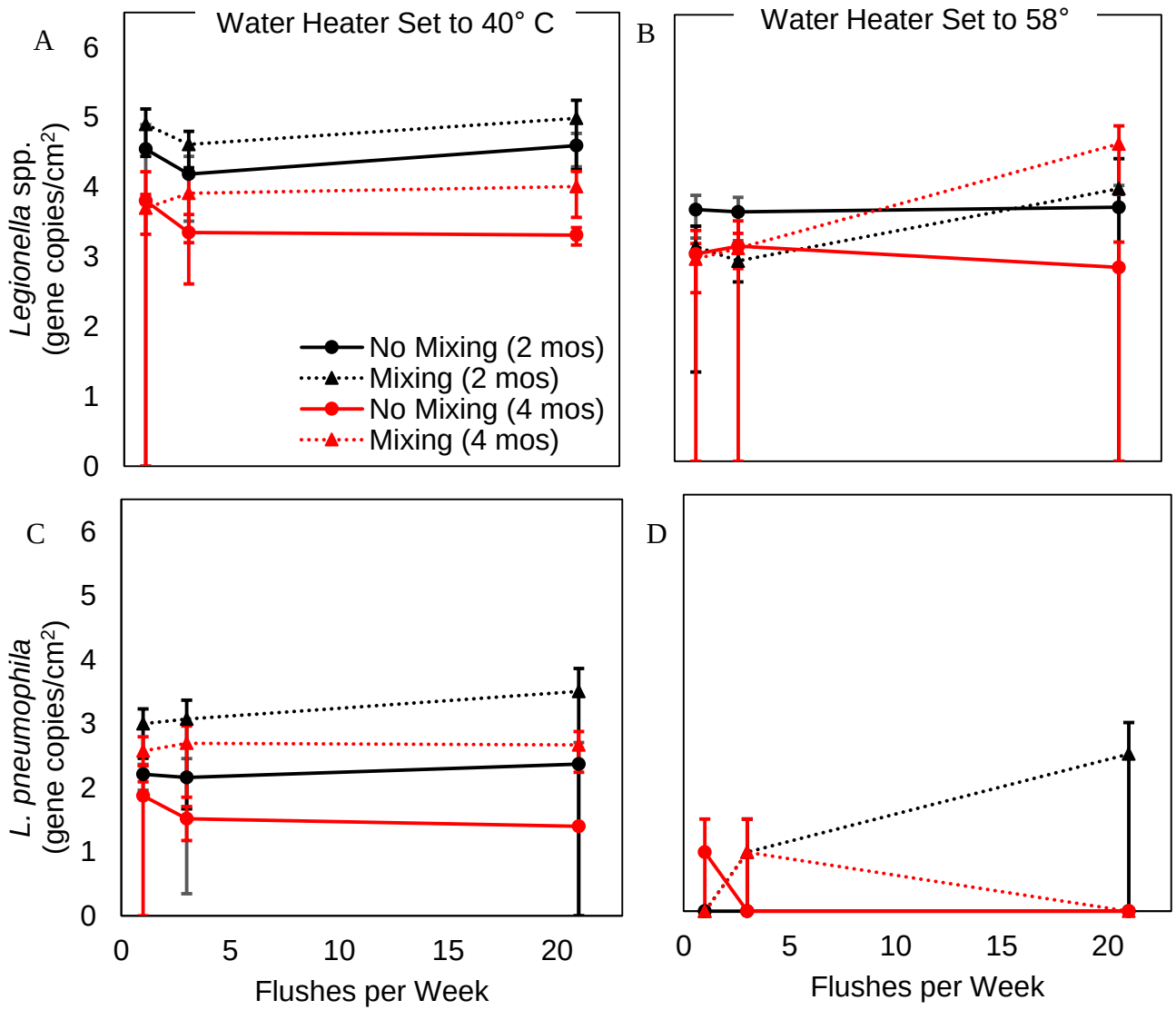

Fig. E3. Average log *Legionella* spp. and *L. pneumophila* concentrations in distal tap biofilms when water heater is set to A) and C) 40° C and B) and D) 58° C

	Condition I: Water Heater Set to 40° C				Condition II: Water Heater Set to 58° C			
Condition	Orientation	Low	Medium	High	Orientation	Low	Medium	High
2 months	Recirculating Line	5.4			Recirculating Line	3.7		
	Distal (No Mixing)	4.5	4.2	4.6	Distal (No Mixing)	3.7	3.6	3.7
	Distal (w/ Mixing)	4.9	4.6	5.0	Distal (w/ Mixing)	3.1	2.9	4.0
4 months	Recirculating Line	4.6			Recirculating Line	1.4		
	Distal (No Mixing)	3.8	3.3	3.3	Distal (No Mixing)	3.0	3.1	2.8
	Distal (w/ Mixing)	3.7	3.9	4.0	Distal (w/ Mixing)	2.9	3.1	4.6

Fig. E4. Heat map of *Legionella* spp.

Comparing concentration in the tank & recirculating line biofilm to each set distal tap biofilm (average log gene copies/cm^2). Colors are on a continuous scales from green (low) to red (high). BQL indicates values that were detectable, but below the quantification limit.

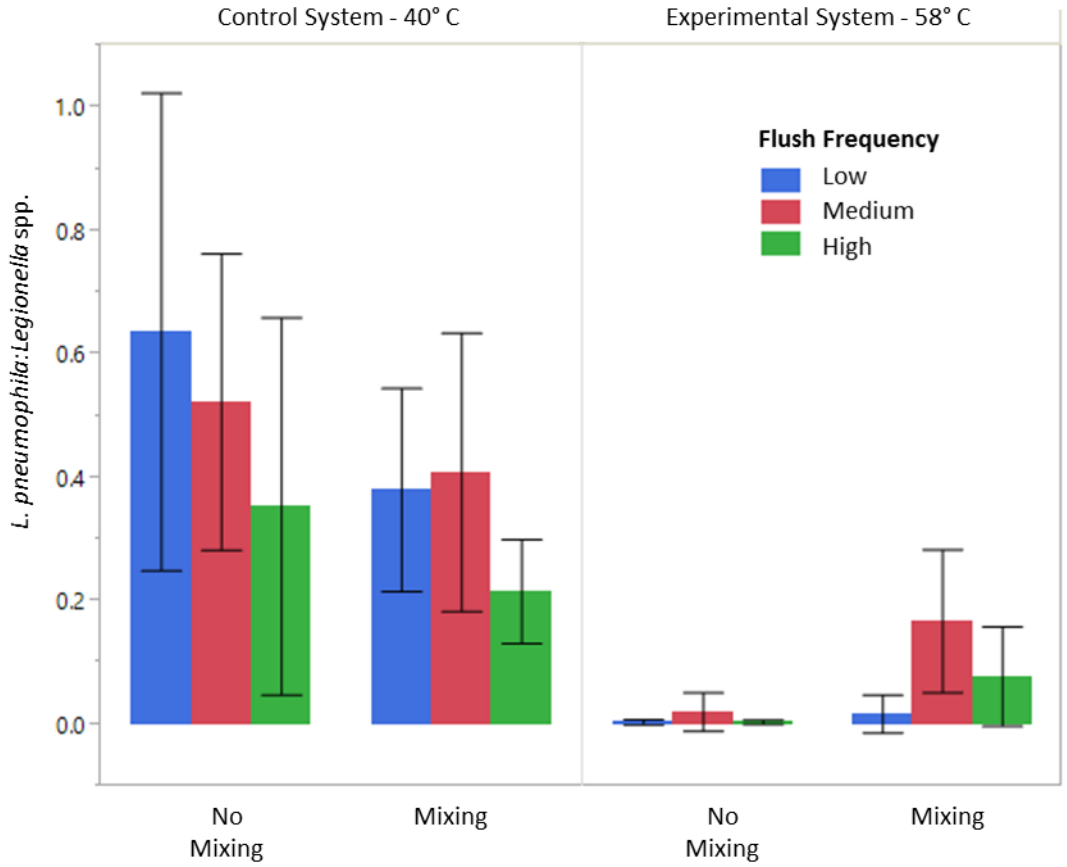

Fig. E5. Relationship between the *L. pneumophila* to *Legionella* spp. ratio by system, convective mixing gradient, and use frequency.

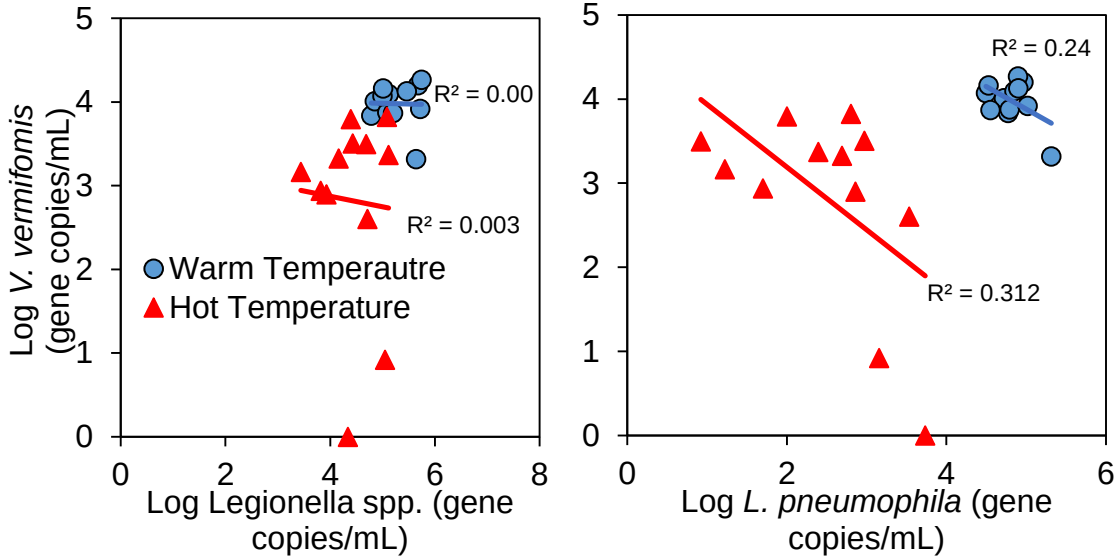

Fig. E6. Correlation of *V. vermiformis* with A) *Legionella* spp. and B) *L. pneumophila* in distal tap bulk water.

APPENDIX F -CORRELATION ANALYSES AND SUPPORTING EXCEL WORKBOOK

Table F.1 Overall Spearman Rank Coefficients within Distal Taps

Bolded values indicate insignificant correlations.

OVERALL	n=81					
			Spearman Rho	P-value	Pairwise R-sq	P-value
Lspp	to	16S	0.6212	<0.0001	0.3343	0.0023
Lp	to	16S	0.457	<0.0001	0.1868	0.0949
Lp	to	Lspp	0.7588	<0.0001	0.7523	<0.0001
Vv	to	16S	**0.1209**	**0.2823**	**-0.0271**	**0.81**
Vv	to	Lspp	0.6269	<0.0001	0.4704	<0.0001
Vv	to	Lp	0.5784	<0.0001	0.3974	0.0002

Table F.2 Spearman Rank Coefficients within Distal Taps without Convective Mixing

		n=36			WITHOUT MIXING, CONTROL SYSTEM				n=18
			Spearman Rho	P-value				Spearman Rho	P-value
Lspp	to	16S	0.7166	<0.0001	**Lspp**	**to**	**16S**	**0.451**	**0.0603**
Lp	to	16S	0.5932	<0.0001	Lp	to	16S	0.7606	0.0002
Lp	to	Lspp	0.6714	<0.0001	**Lp**	**to**	**Lspp**	**0.4613**	**0.054**
Vv	to	16S	0.4268	0.0094	Vv	to	16S	0.4902	0.0389
Vv	to	Lspp	0.6656	<0.0001	Vv	to	Lspp	0.581	0.0115
Vv	to	Lp	0.7203	<0.0001	Vv	to	Lp	**0.3911**	**0.1085**
					WITHOUT MIXING, EXPERIMENTAL SYSTEM				n=18
								Spearman Rho	P-value
					Lspp	to	16S	0.6656	0.0026
					Lp	to	16S	0.706	0.0011
					Lp	to	Lspp	0.3619	0.0049
					Vv	**to**	**16S**	**0.1105**	**0.6625**
					Vv	**to**	**Lspp**	**0.4491**	**0.0615**
					Vv	**to**	**Lp**	**0.2102**	**0.4024**

Table F3 Spearman Rank Coefficients within Distal Taps with Convective Mixing

		n=36			WITH MIXING, CONTROL SYSTEM				n=18
			Spearman Rho	P-value				Spearman Rho	P-value
Lspp	to	16S	0.477	0.0033	Lspp	to	16S	0.8246	<0.0001
Lp	to	16S	0.3824	0.0214	Lp	to	16S	0.6244	0.0056
Lp	to	Lspp	0.8075	<0.0001	Lp	to	Lspp	0.5728	0.013
Vv	to	16S	-0.1228	0.4754	**Vv**	**to**	**16S**	**0.1393**	**0.5814**
Vv	to	Lspp	0.6291	<0.0001	**Vv**	**to**	**Lspp**	**0.1125**	**0.6568**
Vv	to	Lp	0.5141	0.0013	**Vv**	**to**	**Lp**	**-0.2322**	**0.3538**
					WITH MIXING, EXPERIMENTAL SYSTEM				n=18
								Spearman Rho	P-value
					Lspp	to	16S	0.6615	0.0028
					Lp	to	16S	0.6532	0.0033
					Lp	**to**	**Lspp**	**0.3102**	**0.2103**
					Vv	to	16S	-0.5865	0.0105
					Vv	**to**	**Lspp**	**0.0211**	**0.9937**
					Vv	to	Lp	-0.5657	0.0144

Supporting Excel Workbook on Thermal Mixing Model:

1. Estimate the convective mixing rate in distal lines:

Protocol for a potassium tracer model in the recirculating system:
Using the experimental rig, inject 1.46 g K+ into tank in concentrated solution (dissolve into ~10 mL). Turn the recirculating pump on to fully mix the tank and recirculating line (<5 minutes). Monitor mixing between the distal tap and the tank+recirc line reservoir via small samples (~50 mL) collected directly from ends distal taps with time. Assuming that each upward tap mixes at a similar rate, each subsequent distal tap sampled provides information across time as to the amount of mixing that has occurred (i.e., sample distal taps at specified time intervals).

Rate based on fraction of steady tracer concentration measured in the recirc line that was achieved after 70 minutes of operation.

All [K+] reported are "above background" levels (i.e., subtracting out [K+] of Blacksburg tap water.

Recirculating line [K+] = 10.39 mg/L @ 70 minutes mixing
Distal tap [K+] = 3.54 mg/L @ 70 minutes
Fraction mixed = 3.45 mg/L / 10.39 mg/L = 0.341

If 34.1% of the 0.42 L distal tap is mixed in 70 minutes, estimated convective mixing rate is:
0.341 × 0.42 L / (70/60 hours) = 0.12 L/hr

2. Apply convective mixing rate estimation to simplified mass balance on ammonia concentration to compare estimated vs measured steady state ammonia concentration in the recirculating line.

This iterative model is based on hourly increments of NH_3 delivered and consumed in each distal tap. An iterative model was used for simplicity as well as to account for the unequal time steps between regular water changes. High use pipes were changed at 12AM, 8AM, and 4PM 7 days a week. Medium use pipes were changed at 12PM on M,W,F. Low use pipes were changed at 12PM every Tues. Therefore, at the end of each hour:

EQ1: Total NH_3 in $System_{t+1}$= Total NH_3 in $Tank+Recirc_t$ - NH_3 delivered to distal taps due to convective mixing in 1 hour + NH_3 introduced to system via influent during regular water changes

Assumptions:

Recirculating line and tank are well mixed

All ammonia delivered to distal lines via convective mixing is consumed

Total NH_3 in $Tank+Recirc_t$ = Concentration NH_3 in Recirc × Total Volume of system

and

NH_3 delivered to distal taps due to convective mixing = Concentration NH_3 in Recirc × $Q_{convective\ mixing}$ × number of distal taps with convective mixing * 1 hour

When a water change was conducted (which occurred 29 times per week on a set schedule = 3X/day*7days + 3X/week + 1X/week for all water use frequencies):

NH_3 introduced to system via influent during regular water changes = NH_3 concentration of influent * Total volume of water change

In an hour where a water change did not occur:

NH_3 introduced to system via influent during regular water changes = 0

Constants assumed are:

Total Volume of system = 81 L

$Q_{convective\ mixing}$ = 0.123 L/hr (calculated based on tracer model)

NH_3 concentration of influent = 0.56 mg/L (average, measured over 4 month experiment)

Total volume of water change= 11.4 L

We also assume 100% of NH_3 delivered is consumed, since measured NH_3 levels at the tap were near the detection limit throughout the study.

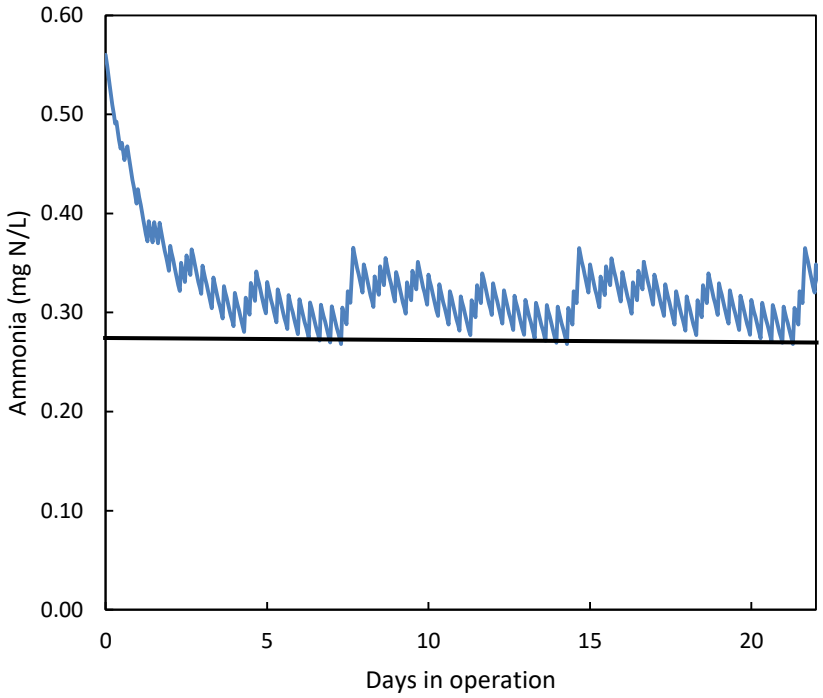

Fig. F1. Convective mixing model results

The convective mixing model estimates that the average steady state ammonia concentration should be 0.31 mg/L NH_3-N, assuming that the tank and recirculating line are mixing, influent ammonia concentration is constant, all ammonia delivered to distal taps is consumed, and all convective mixing flow rates in all upward pipes are identical. This estimate agrees with the actual average steady state ammonia concentration of 0.27 mg/L NH_3-N (±0.07 mg/L NH_3-N). This indicates that the estimated convective mixing rate of 0.12 L/hr (based on mixing studies) is indeed a fair estimate of the actual exchange rate.

CHAPTER 7. INTERACTIVE EFFECTS OF CORROSION, COPPER, AND CHLORAMINES ON LEGIONELLA AND MYCOBACTERIA IN HOT WATER PLUMBING

William J. Rhoads, Amy Pruden, Marc A. Edwards

ABSTRACT

Complexities associated with drinking water plumbing systems can result in undesirable interactions among plumbing components, undermining engineering controls for opportunistic pathogens (OPs). In this study, we examine the effects of plumbing system materials and two commonly applied disinfectants, copper and chloramines, on water chemistry and the growth of *Legionella* and *Mycobacteria* across a transect of bench- and pilot-scale hot water experiments carried out with the same municipal water supply. We discovered that copper released from corrosion of plumbing materials can initiate evolution of >1,100 times more hydrogen (H_2) from water heater sacrificial anode rods than does presence of copper dosed as soluble cupric ions. H_2 is a favorable electron donor for autotrophs and causes fixation of organic carbon that could serve as a nutrient for OPs. Dosed cupric ions acted as a disinfectant in stratified stagnant pipes, inhibiting culturable *Legionella* and biofilm formation, but promoted *Legionella* growth in pipes subject to convective mixing. This difference was presumably due to continuous delivery of nutrients to biofilm on the pipes under convective mixing conditions. Chloramines eliminated culturable *Legionella* and prevented *L. pneumophila* from re-colonizing biofilms, but *M. avium* gene numbers increased by 0.14-0.75 logs in the bulk water and were unaffected in the biofilm. This study provides practical confirmation of past discrepancies in the literature regarding the variable effects of copper on *Legionella* growth, and confirms prior reports of trade-offs between *Legionella* and *Mycobacteria* if chloramines are applied as secondary disinfectant residual.

Key words: *Legionella*, *Mycobacteria*, copper, chloramines, corrosion, hydrogen

INTRODUCTION

Opportunistic pathogens (OPs) *Legionella pneumophila* and Nontuberculous *Mycobacteria* (NTM) are prominent causes of drinking water-associated disease in several developed countries (Brunkard et al., 2011; Yoder et al., 2007; CDC, 2008; Falkinham et al., 2008; Marras et al., 2007; Kuchta et al., 1985; Cooper and Hanlon, 2010). Control of OPs is challenging because, unlike traditional pathogens, they are native members of drinking water microbial communities. In addition, they can be resistant to disinfectant residuals (Kuchta et al., 1985; Cooper and Hanlon, 2010) and other biocides, such as copper ions (Rohr et al., 1999; Thomas et al., 2004), and participate as part of a complex microbial ecological web (Rowbotham, 1980). For instance, *L. pneumophila* reproduction in drinking water is dependent on infection of free-living amoebae hosts (Declerck et al., 2009; Kuiper et al., 2004), within which they can proliferate, resist thermal and chemical disinfection, and become more infectious (Cargill et al., 1992; Surman et al., 2002; Donlan et al., 2005; Barker et al., 1992; Storey et al., 2004; Lau and Ashbolt, 2009; Thomas and Ashbolt, 2009).

There are many factors that impact colonization and proliferation of OPs in building plumbing systems (Alary and Joly, 1991). Maintaining a monochloramine secondary disinfectant residual is one popular engineering control to reduce Legionnaires' disease incidence (Kool et al., 1999; Victor and Stout, 2000; Donlan et al., 2002; Fields et al., 2002; Moore et al., 2006). It has been suggested that 90% of cases could be prevented by using monochloramine as a secondary disinfectant residual (Kool et al., 1999), but chloramines sometimes have other drawbacks (Edwards et al., 2005; Zhang et al., 2008; Zhang et al., 2009; Nguyen et al., 2012; Elfland et al., 2010; Rhoads et al., 2014; Rhoads et al., 2015). While introduction of chloramines reduced the culturability and levels of *Legionella* in several field investigations, in a few cases it was associated with increased levels of *Mycobacteria* (Moore et al., 2006; Flannery et al., 2006; Marchesi et al., 2011; Wang et al., 2015). However, no well-controlled large-scale laboratory studies have confirmed this finding under realistic plumbing operational conditions.

Disinfectant residuals are most effective if they can be maintained throughout building systems, but this is challenging in buildings with high water age or reactive plumbing. In two green buildings with high water age, disinfectant residual decayed up to 144 times faster at taps than the residual in the same municipal water delivered to the buildings (Nguyen et al., 2012; Rhoads et al., 2016). While the factors governing disinfectant residual stability within buildings are not well-characterized, certain domestic water plumbing materials, such as copper, along with growth of microbes, such as nitrifiers, can contribute to accelerated residual decay (Zhang et al., 2009; Nguyen et al., 2012; Lytle and Liggett, 2016).

Another potential strategy to control OP growth is to select plumbing materials that can inhibit colonization. Copper ions released from copper-bearing plumbing (i.e., copper pipes or in-line brass devices), or added to water as a disinfectant, have the potential to inhibit *Legionella* regrowth (Leoni et al., 2005; Marrie, 1994; Miuetzner et al., 1997; Rogers et al., 1994; van der Kooij et al., 2005; Zeybek and Cotuk, 2002; Buse and Ashbolt, 2012). For example, in one building, copper levels above 50 μg/L had significantly lower culturable *Legionella* spp. positivity (Borella et al., 2004; 2005). In another study, copper coupons were found to inhibit regrowth of culturable *Legionella*, but the effect was not sustained after two years (van der Kooij et al., 2005). As pipes

age, rates of copper ion release decreases, reducing their potential to inhibit *Legionella* regrowth. Higher pH or corrosion inhibitors can also diminish the disinfectant properties of copper pipe by limiting copper ion release (van der Kooij et al., 2005; Demirjian et al., 2015; Triantafyllidou et al., 2016; Buse et al., 2014; Lin et al., 2002). In other studies, the presence of copper pipe was believed to facilitate *Legionella* growth (Buse et al., 2014; Mathys et al., 2008; Stout et al., 2003). A possible explanation is that copper catalyzes decay of chloramines which then releases ammonia nutrients (Zhang et al., 2008), but this would not explain the findings of Mathys et al. (2008), where occurrence of *Legionella* was associated with copper pipes in hot water recirculating systems in a water system without a disinfectant residual. It is also possible that certain amoebae hosts that are supportive of *Legionella* growth are sometimes enriched, or selected for, by the presence of copper. Table 1 summarizes several hypotheses that could explain why copper appears to have variable impacts on *Legionella* growth.

Table 7.1. Hypothesized impacts of copper on *Legionella* growth

Condition	Supporting References
Copper sometimes inhibits *Legionella* growth	
a) Copper directly toxic to *Legionella*	Lin et al., 1998
b) Copper toxic to microorganisms that facilitate *Legionella* growth (e.g., nitrifiers that fix organic carbon, host amoeba)	Hypothesized here
Copper sometimes has no impact of *Legionella* growth	
a) Copper is not bioavailable (e.g., due to precipitation at pH>6)	Lin et al., 2002
b) Copper levels are insufficient (i.e., below threshold response range)	Lin et al., 1998
c) *Legionella* developed resistance to copper	
d) Amoebae protect *Legionella* from copper	
Copper sometimes stimulates the growth of *Legionella*	
a) Copper acts a micronutrient for microorganisms that facilitate *Legionella* growth	Zhang et al., 2009
b) Amoebae community shifted by copper to support *Legionella*	Buse et al., 2014
c) Copper (Cu^{1+}) shifts anode corrosion pathway towards H_2 evolution, which stimulates autotrophic carbon fixation and (indirectly) heterotrophic growth.	Morton et al., 2005; Brazeau et al., 2012
d) Copper catalyzes decay of disinfectant residual, increasing regrowth potential	Nguyen et al., 2012
e) Copper decreases microbial diversity	Buse et al., 2014

Copper-enhanced production of hydrogen (H_2) from water heater sacrificial anode rods (which are designed to corrode instead of the iron tank) is an unexplored phenomenon that could also indirectly stimulate *Legionella*. Copper can catalyze evolution of H_2 from water heater anode rods via deposition corrosion, especially in recirculating systems (Brazeau and Edwards, 2011; Morton et al., 2005; Martin, 2012). The evolved H_2 may stimulate autotrophic hydrogen-oxidizing bacteria, which could subsequently fix organic carbon and feed a downstream ecological food web, potentially supporting OPs and associated host organisms. While, to our knowledge, no prior

report has explored the role of H_2 as a stimulant of OPs growth in plumbing systems, analogous deleterious consequences of stimulating autotrophic organisms in drinking water are well-established in the case of nitrifying bacteria. Specifically, nitrifying bacteria oxidize ammonia, fix organic carbon, and catalyze chloramines disinfectant residual decay (Zhang et al., 2008; 2009).

The purpose of this study was to evaluate interactive effects of copper and chloramines for controlling *Legionella* and *Mycobacteria* under realistic hot water plumbing conditions where there is potential for H_2-evolution via water heater anode rod corrosion. Effects of copper in various forms (i.e., released naturally from pipe vs dosed as $CuSO_4$), chloramines, and hydrogen-evolution were systematically examined across a transect of controlled pilot-scale hot water pipe rigs and complementary batch reactors, receiving the same municipal water supply, to provide fundamental insight into factors limiting or stimulating OP growth.

EXPERIMENTAL

Three experimental apparatuses were analyzed in this study.

Copper Pipe Rigs. Brazeau and Edwards examined physicochemical water quality trends in common residential hot water system designs, including a standard hot water storage tank with no hot water recirculation (STANDARD) and with recirculation (RECIRC), each with an aluminum anode rod (Fig. G.1; Brazeau and Edwards, 2011; 2012). For this study, we have re-examined a subset of H_2 evolution data when both water heaters were set to 49 °C, and 25% of the tank (4.75 gallons) was flushed from distal taps twice daily for comparison to new data gathered in the present study. All pipes were ¾" new (at the time study was initiated) copper pipe.

CPVC Rigs. This apparatus had two hot water storage tanks with chlorinated polyvinyl chloride (CPVC) recirculation lines and simulated distal taps modified from previous studies (Fig. G.2; Rhoads et al., 2015; 2016). Briefly, two identical hot water recirculation systems, originally constructed in Oct. 2012, were set to 39 °C to simulate worst-case scenarios for OP growth. Two distal tap orientations were tested: downward oriented pipes became thermally stratified during periods of no use (i.e., pipes with no convective mixing) while upward oriented pipes convectively mixed with the recirculating line (i.e., pipes with convective mixing). Previous work demonstrated that pipes with convective mixing facilitated nutrient transport, as well as re-colonization of *L. pneumophila* after each sampling relative to pipe with no mixing (Rhoads et al., 2016). Distal taps simulated high (21 times/week), medium (3 times/week), and low (1 times/week) use frequencies in triplicate at 3.8 L/min (1 gallon/min) for 28 seconds (15% tank turn over per flush; 6 pipes per flush (i.e., 2 orientations × 3 for triplicate=6 pipes) × 3 use frequencies × 2 systems = 36 distal taps total). Use of copper-bearing materials was limited to one brass solenoid valve, union, and check valve in each system.

For Phase 1, influent water was filtered through three granular activated carbon (GAC – Pentek 10" Big Blue Model) filters in series to remove chloramines residual. Starting 2/2/2015, the experimental system was dosed with a 4 g Cu^{2+}/L ($CuSO_4$) stock solution (pre-filtered through a 0.45 µm pore-size polytetrafluoroethylene (PTFE) filter and maintained at pH 4) directly to the influent during each water change to continuously achieve a target concentration of 0.3 mg Cu^{2+}/L in influent water using a chemical dosing pump (Model BL10, Hanna Instruments, Melrose, MA).

The control system received no copper. After copper dosing was initiated, the systems were operated for 22 days to allow sufficient time for all chemical and microbial reactions to acclimate to the operating conditions. Water chemistry trends were then established through routine monitoring, with bulk water and biofilm microbiological data collected on 4/3/2015 and 5/27/2015 (days 60 and 114) to determine the impact of copper on the growth of OPs. See Appendix 3 for additional water use and sampling details.

During Phase 2, 1.5 mg/L chloramines disinfectant residual (as total Cl_2) was dosed into both systems by blending ~50% GAC-filtered water and ~50% Blacksburg, VA municipal water, beginning on 5/28/2015 (day 115). Copper was still dosed into the experimental system, while the control system continued to receive no copper. These conditions were acclimated for 20 days before water chemistry trends were again characterized, followed by microbiological sampling on 7/2/2015 and 8/19/2015 (days 150 and 198).

Simulated Glass Water Heaters (SGWHs). SGWHs, comprised of 500 mL French square glass bottles, were operated in triplicate and received influent water identical to the GAC-filtered CPVC rig described above. Each set of SGWHs received no, low (4.25 nM H_2), or high (40,600 nM H_2) hydrogen additions; 0.5 mg Cu^{2+}/L as soluble copper sulfate ($CuSO_4$); 0.5 mg/L chloramines as total Cl_2; or combinations of all three (hydrogen, copper, chloramines) conditions (11 conditions total; Fig. G.3). Chloramines were dosed from a 100 mg/L free chlorine stock solution into GAC-filtered CPVC rig influent water. Background free ammonia (~0.5 mg/L) was used to generate combined monochloramine in situ. Copper was dosed from a 4 g/L Cu^{2+} (as $CuSO_4$) stock solution in the same manner as it was to the CPVC rig. Hydrogen was dosed from a 100 ppm analytical standard in nitrogen (Scott Gas, Plumsteadville, PA) for the lower H_2 doses, and from generated pure H_2 for higher H_2 doses.[61] At the onset of the SGWH experiments, protocols were established to ensure hydrogen dosed was not lost to the environment during water changes (Table G.3). Low hydrogen doses were representative of concentrations found in the CPVC rigs while the high dose represented an order of magnitude higher H_2 than was detected by Brazeau et al.[58] Hydrogen was dosed directly to the headspace of inverted SGWHs to achieve target aqueous hydrogen concentrations estimated by Henry's Law (Appendix H). Water changes were executed with an 80% water volume dump-and-fill protocol 3X/week. SGWHs were incubated at 37 °C, agitated on a shaker table at 150 rpm, and acclimated for 37 days before characterizing chemistry trends over a period of three weeks, followed by biological sampling on day 55.

Physical and Chemical Analyses. General physical and chemical water quality parameters were well characterized through repeat measurement at the beginning of each experimental phase. Chloramines and ammonia were measured using Standard Method 4500-Cl2 and 5310-NH3, respectively, using a DR2700 or DR5000 spectrophotometer (HACH, Loveland, CO). Headspace hydrogen gas (H_2) was measured using a headspace gas chromatograph with a reduced gas and flame ionization detector (Amtek Trace Analytical RGA-5) and Henry's Law to back calculate aqueous concentrations (Appendix H). Total organic carbon (TOC) was measured by persulfate-ultraviolet detection (Sievers Model 5300C) according to Standard Method 5310 C. Galvanic current was measured using a digital amp meter by electrically separating the anode and cathode for measurement and reconnecting them with copper wire.

Microbiological Analyses. *Legionella* cultivation media was prepared according to International Organization for Standardization Draft International Standard 11731 (Appendix I). Quantitative polymerase chain reaction assays (qPCR) for *Legionella* spp. (23S rRNA), *L. pneumophila* (macrophage infectivity potentiator) and *M. avium* (16S rRNA) were carried out using previously published methods (Appendix 3; Rhoads et al., 2015; 2016; Wang et al., 2012). Total cell counts were conducted using a fluorescent nucleic acid stain (SYBRs Green I) with quantitative flow cytometry (BDAccuri C6) using previously developed methods (Appendix J; Hammes et al., 2008; SLMB, 2012).

Statistics. Where applicable, paired t-tests were applied to log-transformed qPCR data to establish statistical differences among conditions. For non-normally distributed data, non-parametric paired Wilcoxon rank-sum tests were performed. R version 3.2.0 was used for t-tests, Wilcoxon rank sum tests, and pairwise Wilcoxon rank sum test, as appropriate. Significance was determined at p=0.05.

RESULTS AND DISCUSSION

After establishing the effects of copper ions and system design on corrosion of anode rods and potential for OP growth, chemical and microbiological impacts of copper and chloramines dosing are presented. *L. pneumophila* is the most commonly reported disease-associated species (Fields, et al., 2002; CDC, 2016; Kozak et al., 2009), and thus were the primary focus of this study. Culture-based methods were used to evaluate trends related to viable *L. pneumophila*; while qPCR served as a composite measure of live, dead, and unculturable cells, in order to further evaluate culture-based trends and provide more consistent quantification and lower detection limits. *M. avium* was monitored solely by qPCR because there are currently no standardized culture-based techniques and existing methods are extremely tedious and lacking in quantitative capability (Brooks et al., 1984; Griffith et al., 2007).

Effect of Various Cu Forms, Recirculation, and System Design. System design impacted the anode rod corrosion rate and amount of H_2 evolved. The anode rod in the system with copper pipes and recirculation corroded 1.6-1.8 times faster than any other condition (Table 7.2). The system with copper pipe and recirculation also yielded 4.1 times more H_2 compared to the standard design system with copper pipe and produced 1129-1533 times more H_2 than either the system with no copper or dosed with cupric ions. However, the anode rod in system with copper pipe corroded 60% faster (measured by direct weight loss) than the system dose with cupric ions, but evolved 1,129 times more H_2 (Table 7.2). This leaves a factor of 719 times more H_2 evolved in the system with copper pipe that is not explained by anode rod corrosion rate (Table 7.2; See Appendix K for calculation). These results indicate that copper released from copper pipe (and not that dosed as cupric ions), together with recirculation has a combined effect in dramatically increase H_2 release from water heater anode rods. Orthophosphate present in Blacksburg tap water could have played a role in decreasing solubility of available copper (at ~0.9 mg/L as PO_4); however, this would have been a uniform factor across all experiments. We hypothesize that Cu^{1+} released from copper pipes changed the nature of the anode rod corrosion to favor the H_2 evolution (instead of O_2 consumption) pathway in a distinct manner relative to Cu^{2+}, as dosed to the CPVC rig. Thermodynamically and kinetically, Cu^{1+} would preferentially plate onto anodes versus Cu^{2+} (Bard and Faulkner, 2001; Kanani, 2003). In addition, work by Clark et al. demonstrated Cu^{1+} plating onto galvanized iron pipes preferentially occurred under conditions with recirculation or

agitation (Clark et al., 2015). To our knowledge, H_2 levels in potable water as a result of corrosion has not been previously reported for comparison. However, copper released from copper pipe correlated with galvanized weight loss up to 0.3 ppm copper (Kentworthy, 1943) while copper dosed (as cupric sulfate or Cu^{2+}) did not have obvious impacts up to 2 ppm copper in some studies (Fox et al., 1986; Hu et al., 2012).

We conducted two follow-up head-to-head bench-scale experiments to confirm the phenomena observed under the different experimental design conditions of the Brazeau and CPVC rigs. The bench-scale experiments confirmed that the copper released from copper pipe increased corrosion of aluminum sacrificial anode rods (via direct weight loss measurements; Fig. G.4) and resulted in much higher H_2 levels (through headspace analysis; Fig. G.5) relative to conditions dosed with similar levels of cupric ions from $CuSO_4$. At the pH of these experiments (~7.2), the soluble fraction of copper from dosed $CuSO_4$ would likely exist as Cu^{2+} and the insoluble fraction as $Cu(OH)_2$ (Schock et al., 1996). However, because corrosion of copper pipes proceeds through a Cu^{1+} intermediate, which would be absent in the rigs dosed with $CuSO_4$. Cu^{1+} may be present as a small fraction of total copper released from copper pipes. This provides a possible explanation for the drastic differences in H_2 detected in the pilot-scale systems (Table 7.2). The excess H_2 could be used by hydrogen-oxidizing bacteria to fix organic carbon, in an oligotrophic potable water system where thresholds >10 µg/L assimilable organic carbon have been suggested to support OP regrowth (van der Wiele and van der Kooij 2013). Therefore, deposition corrosion and associated H_2 evolution, could provide a mechanistic explanation for how copper systems can sometimes support higher levels of bacteria, *Legionella*, and/or amoeba hosts, especially in waters that are carbon limited.

Chemical Trends in CPVC Rigs. Background copper in GAC-filtered influent water was consistently below 10 µg/L, and remained low in the system with no cupric ion addition (Table 7.3). Copper was dosed to the experimental system at a target of 0.3 mg Cu^{2+}/L, comparable to that measured in the Brazeau and Edwards (2012) copper pipe rig with recirculation (Table 7.2). During the first phase, without chloramines, and in the second phase, with chloramines, average total copper concentrations were 0.22 mg/L and 0.31 mg/L, respectively (Table 7.3). In the system dosed with cupric ions, aluminum levels increased by a factor of only 1.5-2.0, galvanic corrosion current measured between the iron water heater and aluminum anode increased by a factor of 1.6-1.8, and no additional H_2 was detected. This further supports the hypothesis that cupric ions are not as effective at stimulating H_2 evolution as copper pipe.

Before chloramines were introduced (Phase 1), influent ammonia was 0.52 mg/L NH_3-N. After a typical 8-hour stagnation, an average of 89% of the ammonia was consumed in the system without copper dosing (Table 7.3), indicating presence of active nitrifying microorganisms (Zhang et al., 2008; 2009). In the system with cupric ions, an average of 65% of the influent ammonia was consumed, consistent with reported copper inhibition of nitrifying bacteria growth (Zhang et al., 2009; Skinner et al., 1961; Loveless and Painter, 1968). Inhibitory levels of copper vary greatly among nitrifying bacteria, but a strong inhibitory effect has been reported above 0.90 mg/L (Zhang et al., 2009). The relatively small amount of inhibition observed suggests that the copper was not high enough in concentration or was not fully bioavailable, the latter scenario supported by the fact that only 22% of the copper dosed was soluble after it had recirculated in the tank, despite being dosed as soluble copper (Table 7.2). When chloramines were present in the second phase of

Table 7.2. Corrosion indicators associated with copper (either dosed or from copper pipes) in standard and recirculating hot water systems fed with local tap water

Copper Source in System	System Type[c]	Copper Total (ppb)	Copper % Soluble	Aluminum Total (ppb)	Aluminum % Soluble	Anode Rod Weight Loss (% · year^{-1})	Aqueous H_2 (nM)[d]
No Copper [a]	Recirculating	11 ± 3	40%	856 ± 189	14%	8.8	1.4 ± 0.39
Dosed Cu^{2+} [a]	Recirculating	223 ± 46	22%	1,439 ± 202	17%	9.6	1.9 ± 0.22
Copper pipe[b]	Recirculating	310	18%	2,680	11%	15.1	2,146
Copper pipe[b]	Standard	24	50%	126	63%	8.2	527

[a] - This work, using CPVC Rigs in Fig. S2, water heater set point = 40° C, n = 13 for metals, n = 1 for weight loss, n = 3 for H_2, error represents 95% confidence intervals

[b] - Brazeau and Edwards (2013) Copper Rigs, see Fig. S3 for design, water heater set point = 49° C, n not reported, metal and H_2 values represent averages over approximately 5 month portions of 19 month experiment; n = 1 for weight loss

[c] - Standard indicates typical design with no hot water return; Recirculating indicates continuous water recirculation, See Fig. S2 and S3

[d] - Headspace analysis. See calculation example in SI

± indicates 95% confidence intervals calculated using a t-distribution multiplied based on the number of samples collected

Table 7.3. Water quality characteristics of GAC-filtered influent water (See Fig. S3 – INFLUENT sample port) and water from each experimental system tank and recirculating reservoir (See Fig. S3 – RECIRCULATION sample port)

(n as follows: Phase 1 temperature – 21; Phase 2 temperature – 19; Phase 1 metals – 13; Phase 2 metals – 6; Phase 1 galvanic current – 25; Phase 2 galvanic current – 19; Phase 1 and 2 H_2 analysis – 3; Phase 1 and 2 chloramine – 10; Phase 1 ammonia – 12; Phase 2 ammonia – 11)

Time	System	Temp (°C)	Cu (ppb)	Al (ppb)	Fe (ppb)	I_{galv}[a] (mA)	H_2 evolved (nM)	Chloramine (mg/L) After Flush	Chloramine (mg/L) Before Flush[c]	Ammonia (mg/L NH_3-N)
Phase 1 2/2/2015-5/27/2015	Influent	15.7±1.7	5±3	14±3	ND	NA	0.52	0.04		0.52 ± 0.06
	No addition	40.0±0.6	11±3	924±293	0.8±0.6	2.82±0.19	1.4±0.39	ND	ND	0.06 ± 0.04[d]
	Cu^{2+}	40.0±0.7	223±46	1414±252	3.9±4	4.40±0.25	1.9±0.22	ND	ND	0.18 ± 0.02 [d]
Phase 2 5/28/2015-8/19/2015	Influent	23.3±1.3	4±2	21±4	8.6±0.5	NA	NA[b]	1.76 ± 0.18		0.50 ± 0.04
	NH_2Cl	38.8±0.7	7±2	748±126	16.7±7	2.37±.14	NA[b]	0.41 ± 0.07	0.31 ± 0.14	0.31 ± 0.02 [d]
	$NH_2Cl + Cu^{2+}$	38.7±0.9	313±25	1493±286	22.6±9	4.35±0.37	NA[b]	0.44 ± 0.08	0.35 ± 0.10	0.35 ± 0.03 [d]

[a] – indicates galvanic corrosion current measured between the iron water heater tank and aluminum anode rod, [b] – No reliable data because of equipment malfunction, [c] – "Before" flush indicates chloramine levels 8 hours after last flush; "After" indicates immediately after a flush occurs, [d] – measured at the end of regular 8-hour stagnation period, NA – no data available, ND – non-detect, ± indicates 95% confidence intervals calculated using a t-distribution multiplied based on the number of samples collected

experiments, only 30-35% of influent ammonia was consumed, regardless of whether or not cupric ions were dosed, suggesting that any inhibitory effects of cupric ions on ammonia oxidizing bacteria were relatively minor compared to the effects of chloramines.

When chloramines were introduced, both the systems with and without cupric ions exerted a small, yet consistent, instantaneous chlorine demand of 0.08-0.09 mg/L as total Cl_2. The residual in both systems was subsequently stable during stagnation and only decreased by 0.09-0.10 mg/L total Cl_2 on average during a typical 8-hour stagnation period. Distal taps were exposed to chloramines from the tank and recirculating line while being flushed and subsequently decayed during stagnation. In pipes with convective mixing between the distal and recirculating lines, chloramines were higher than in pipes with no convective mixing (on average, 0.14-0.25 mg/L more total Cl_2), which were characterized by stagnation and stratification (Fig. G.6). This suggests that the more consistent disinfectant residual maintained by pipes with mixing may more effectively control *Legionella*, relative to pipes with no mixing. However, as discussed in the following sections, observations in this study were not consistent with this expectation.

Baseline OPs in CPVC Rig: No copper or Chloramines Added. Culturable *Legionella* levels were high in the recirculating line (479 CFU/mL; Table 7.4A) of the control rig with no cupric or chloramines during Phase 1. In medium and high pipes with no mixing, culturable levels decreased relative to the recirculating line (on average, 241-267 CFU/mL in high and medium use pipes; Table 4A). No culturable *Legionella* was detected in low use pipes without mixing, likely due to decreased nutrient delivery with low flushing frequency (Rhoads et al., 2016). In distal pipes with convective mixing, the highest concentrations were detected in the low use pipes (572 CFU/mL; Table 4A), possibly due to the continuous supply of nutrients with infrequent turbulent flow events that slough off biofilm.

The majority of *L. pneumophila* gene copy numbers occurred in the recirculating lines and tank reservoir, consistent with our previous work (Table 7.4B; Rhoads et al., 2015; 2016). *L. pneumophila* was consistently detected at high levels in recirculating water ($10^{4.1}$ gene copies/mL) and biofilm ($10^{5.1}$ gene copies/cm^2) relative to influent water (below quantification level for water and $10^{3.6}$ gene copies/cm^2 for biofilm; Table 4B; Table G.1.A and B). A slight increase in gene numbers was observed in low use distal taps, especially in pipes with convective mixing ($10^{4.9}$-$10^{5.0}$ gene copies/mL; Table 4B; Table S1.B), but not in high use taps. *L. pneumophila* was more readily able to reestablish in biofilm in distal pipes with convective mixing or in the recirculating line with continuous recirculation ($10^{4.1}$-$10^{5.1}$ gene copies/cm^2; Table 4C) relative to pipes with no mixing.

Table 7.4. Average of A) *Legionella* culture counts, B) Log *L. pneumophila* gene copy numbers in bulk water samples, and C) Log *L. pneumophila* gene copy numbers in biofilm swabs in the CPVC rig with and without soluble cupric ions dosed (as cupric sulfate) during Phase 1 without chloramines and Phase 2 with chloramines.

± indicates 95% confidence intervals of colony forming units or log-transformed gene copy data, Distal tap n=3 for culture and Phase 1 biofilm data and n=6 bulk water and Phase 2 biofilm, recirculating line n=1-3.

Target	Condition	Orientation	Phase 1 (2/2/2015-5/27/2015) No Chloramine Added			Phase 2 (5/28/2015-8/19/2015) Chloramine Added		
			Low Use	Medium Use	High Use	Low Use	Medium Use	High Use
A) *Legionella* spp. colony forming units/mL	System with No Copper Added	Recirc. Line		479			ND	
		Taps with no mixing	ND	267+388	241±22	ND	ND	ND
		Taps with mixing	572±118	195+214	401±165	ND	ND	ND
	System with Copper Added	Recirc. Line		58			ND	
		Taps with no mixing	ND	35±78	ND	ND	BQL	ND
		Taps with mixing	1066±485	1013±576	1080±220	ND	BQL	ND
B) Log *L. pneumophila gene copies/mL*	System with No Copper Added	Recirc. Line		4.1±0.2			2.5±1.2	
		Taps with no mixing	4.32±0.39	4.21±0.33	4.15±0.22	3.18±0.87	2.83±0.66	2.67±0.80
		Taps with mixing	4.99±0.07	4.60±0.2	4.40±0.05	3.82±0.87	3.02±0.74	3.14±0.82
	System with Copper Added	Recirc. Line		4.2±0.2			2.9±0.1	
		Taps with no mixing	4.57±0.35	4.50±0.25	4.27±0.12	3.41±0.72	3.14±0.56	3.28±0.60
		Taps with mixing	5.02±0.14	4.57±0.21	4.44±0.18	3.89±0.91	3.49±0.39	3.45±0.63
C) Log *L. pneumophila* gene copies/cm^2	System with No Copper Added	Recirc. Line		5.1			4.90	
		Taps with no mixing	1.76±1.95	BQL	BQL	BQL	BQL	BQL
		Taps with mixing	4.06±0.04	4.05±0.10	4.27±0.18	1.8±0.9	BQL	BQL
	System with Copper Added	Recirc. Line		4.8			4.25	
		Taps with no mixing	BQL	BQL	BQL	BQL	BQL	BQL
		Taps with mixing	4.54±0.47	4.67±0.66	5.12±0.04	3.1±1.0	2.6±1.5	3.1±1.0

Table 7.5. A) *M. avium* gene copy numbers in bulk water samples and B) *M. avium* gene copy numbers in biofilm swabs in the CPVC rig with and without soluble cupric ions dosed (as cupric sulfate) during Phase 1 without chloramines and Phase 2 with chloramines.

± indicates 95% confidence intervals of colony forming units or log-transformed gene copy data, Distal tap n=3 Phase 1 biofilm data and n=6 bulk water and Phase 2 biofilm, recirculating line n=1-3.

Target	Condition	Orientation	Phase 1 (2/2/2015-5/27/2015) No Chloramine Added			Phase 2 (5/28/2015-8/19/2015) Chloramine Added		
			Low Use	Medium Use	High Use	Low Use	Medium Use	High Use
A) Log *M. avium* gene copies/mL	System with No Copper Added	Recirc. Line		3.03			3.49±1.30	
		Taps with no mixing	3.19±0.14	2.78±0.13	2.95±0.16	3.93±0.15	3.54±0.11	3.09±0.52
		Taps with mixing	4.17±0.11	4.05±0.11	4.04±0.19	4.60±0.33	4.80±0.45	4.48±0.31
	System with Copper Added	Recirc. Line		2.78			2.79±0.09	
		Taps with no mixing	2.27±0.96	2.71±0.13	2.65±0.15	3.12±0.62	2.54±0.74	2.25±0.67
		Taps with mixing	3.30±0.67	3.61±0.58	2.78±0.39	2.98±0.20	3.44±0.42	3.22±0.56
B) Log *M. avium* gene copies/cm^2	System with No Copper Added	Recirc. Line		4.58			5.36	
		Taps with no mixing	3.79±0.33	3.54±0.34	4.06±1.23	2.21±1.11	1.71±0.64	1.76±0.75
		Taps with mixing	3.9±0.3	4.5±0.9	3.9±0.1	3.76±0.29	4.60±0.82	4.42±0.60
	System with Copper Added	Recirc. Line		4.29			4.94	
		Taps with no mixing	2.92±1.69	3.08±1.84	3.72±0.64	BQL	1.86±1.22	BQL
		Taps with mixing	4.01±0.37	3.15±1.91	4.35±0.11	2.80±1.03	3.53±1.03	4.39±0.39

There were high levels of *M. avium* gene copies in recirculating line water ($10^{3.0}$ gene copies/mL; Table 7.5A) relative to influent water (below quantification), but comparable levels in the recirculating line biofilm relative to the biofilm on the influent cold water pipe (~$10^{4.6}$ gene copies/cm$^{2;}$; Table 7.5B). In the distal taps, bulk water-associated *M. avium* consistently increased in pipes with convective mixing ($10^{4.0}$-$10^{4.2}$ gene copies/mL), but not in pipes without convective mixing (Table 7.5A). Biofilm-associated *M. avium* reestablished in distal tap biofilms to nearly the same extent as the recirculating line ($10^{2.8}$-$10^{4.2}$ gene copies/cm^2; Table 7.5B).

Impact of Cupric Ions on OPs in CPVC Rig without Chloramines. The addition of soluble cupric ions alone markedly decreased the culturability of *Legionella* in the recirculating lines by nearly an order of magnitude (58 CFU/mL versus 479 CFU/mL; Table 7.4A). Inhibitory impacts of cupric ions were observed as expected in distal taps without convective mixing, in which only one of three medium use pipes had detectable *Legionella* (at 107 CFU/mL). However, copper did not appear to have an inhibitory effect in pipes with convective mixing, where very high levels of culturable *Legionella* were observed (1,013-1,080 CFU/mL; Table 7.4A).

Although there were not significant impacts of cupric ions on bulk water *L. pneumophila* gene numbers (p-value=0.076, paired t-test on log transformed data, n=70; Table 7.4B), likely due to detecting both live and dead cells, biofilm-associated *L. pneumophila* gene numbers agreed with culture data (Table 7.5A and C). Biofilm-associated *L. pneumophila* gene numbers were not consistently detected in pipes with no mixing relative to the recirculating line, and were significantly higher in pipes with mixing and cupric ions relative to without cupric ions (Table 7.5C; Wilcox Rank Sum, p-value=0.014, $n_{cupric}=n_{nocupric}=9$).

H_2-derived organic carbon was not likely the source of the additional growth in this case, since there was only a relatively small amount of H_2 evolved from corrosion of the anode rod (Table 7.3). However, since there was active nitrification, additional organic carbon could have been fixed by in the pipes with convective mixing, but this would have applied to both the system with and without cupric. It is likely that biofilm-associated *L. pneumophila* was protected from the disinfection by cupric ions within amoeba hosts (Buse et al., 2014; Buse and Ashbolt, 2014), and could have used cupric-killed microorganisms as a substrate, similar to what has been observed for *Legionella* growth using heat-killed microorganisms as a substrate (Table 7.1; Temmerman et al., 2006). In this experiment, convective mixing provided an ecological niche, supplying nutrients to cupric-resistant *Legionella* biofilms where *Legionella* thrived. In the pipes without convective mixing, no additional nutrients were supplied, and the *Legionella* populations suffered (Table 1.7). The abrupt change in flow conditions from the very slow convective mixing during periods of no use to 1 gpm could have caused the *Legionella* rich biofilm to slough off into the distal water sample, resulting in the high colony forming unit culture concentrations (Table 7.4A).

The cupric ions reduced bulk water *M. avium* gene numbers by an average of 0.64 logs (p-value<0.001, paired t-test, n=36; Table 5A), but did not have a significant impact on biofilm-associated *M. avium* gene numbers after two months (p-value=0.12, paired t-test, n=36; Table 7.5B). Nontuberculous *mycobacteria* are reportedly resistant to disinfection by copper (and silver) ions (Kusnetsov et al., 2001; Lin et al., 1998; Norton et al., 2004), thus, cupric ions may have only acted to induce a stress response for maintaining biofilm populations, as has been identified for oxidative stress of *M. avium* (Geier et al., 2008).

Impact of Chloramines on OPs in CPVC Rig without Copper. No culturable *Legionella* was quantifiable in any condition after the introduction of chloramines (Table 7.4A). The efficacy of chloramines were also observed via qPCR. For instance, bulk water-associated *L. pneumophila* decreased significantly in all conditions (p-value<0.0001, paired t-test, n=33; Table 7.4B) and chloramines became more effective with time (p-value<0.0001, Wilcoxon test, $n_{\text{first sampling}}$=16, $n_{\text{second sampling}}$=18; Table G.1.A), likely due to decreased levels in the biofilm over time (Table G.1.C). In distal pipes without convective mixing, since biofilm-associated *L. pneumophila* was already at or below quantification in 6/9 pipes, and non-detect in 2/9 pipes, chloramines appeared to have no additional impact on biofilm-associated *L. pneumophila* gene numbers in these pipes. In pipes with convective mixing, chloramines prevented *L. pneumophila* from re-establishing on swabbed surfaces in all distal taps (on average, by 3.04 logs, p-value=0.00042, Wilcoxon test, $n_{\text{no chloramines}}$=$n_{\text{chloramines}}$=9; Table 7.4C).

Consistent with prior field work (Moore et al., 2006; Flannery et al., 2006; Marchesi et al., 2011; Wang et al., 2012), bulk water-associated *M. avium* increased in all distal pipes when chloramines were introduced (Wilcoxon Rank Sum, p-value=0.014, $n_{\text{no chloramines}}$=$n_{\text{chloramines}}$=18; Table 7.5A). Similarly, *M. avium* biofilms in pipes with convective mixing were not significantly impacted by chloramines (p-value=0.21, Wilcoxon test, $n_{\text{no chloramines}}$=$n_{\text{chloramines}}$=9; Table 7.5B), despite more uniform exposure to chloramines due to mixing (Fig. G.6). However, biofilm-associated *M. avium* were not able to reestablish in the area swabbed after the introduction of chloramines in pipes without convective mixing (p-value<0.0001, Wilcoxon test, $n_{\text{no chloramines}}$=$n_{\text{chloramines}}$=9; Table 7.5B). These data suggest that the underlying conditions of individual pipes (i.e., ideal growth conditions with continuous delivery of nutrients in pipes with mixing versus stratified pipes with no mixing) help to determine response of *M. avium*. Prior studies reporting an increase in nontuberculous mycobacteria with the introduction of chloramines have not been able to provide potential explanations for this phenomenon due to the lack of specific knowledge (use, flow, temperature profiles, etc.) about individual samples. To our knowledge, this is the first controlled pilot-scale demonstration that chloramines have obvious deleterious effects on the presence of *Legionella*, but can have a varied impact on *Mycobacteria* depending on configuration and phase (bulk water vs biofilm).

Combined Cupric Ions and Chloramines on OPs in CPVC Rig. Due to the efficacy of chloramines alone, it was difficult to identify synergistic effects on bulk water-associated *L. pneumophila* due to cupric ions and chloramines together. Consistent with the addition of cupric ions without chloramines, *L. pneumophila* gene copies increased slightly in the bulk water with copper and chloramines relative to chloramine alone (Table 7.4). However, changes were not consistently significant and culture and biofilm data were consistently at or below detection, impeding further interpretation (Table 7.4A and C). Bulk water *M. avium* levels did not change in distal taps with cupric ions and chloramines relative to cupric ions alone (Wilcox Rank Sum, p-value=, n_{Cu}=$n_{Cu+NH2Cl}$=; Table 7.5A). Similarly, biofilm *M. avium* levels did not change in with cupric ions and chloramine relative to chloramines alone (Wilcox Rank Sum, p-value=, n_{NH2Cl}=$n_{Cu+NH2Cl}$=; Table 7.5B). Therefore, there were no apparent synergistic effects of copper and chloramines on *M. avium.*

Confirmation Tests with Simulated Glass Water Heaters (SGWHs). Because no additional H_2 was detected in the CPVC Rig with added cupric ions, SGWHs were used to directly test the hypothesis that elevated levels of H_2 could support additional microbial growth, including *Legionella*. Hydrogen consumption corresponded with an increase in TOC and total cell counts (via flow cytometry) in SGWHs with a high dose of H_2 relative to the control, but not in those with low H_2 (Fig. 7.1A, B). However, there was no significant corresponding impact on *Legionella* growth (Fig. 7.1C). Instead, higher H_2 additions were associated with a slight decrease in average *Legionella* spp. and *L. pneumophila* gene copy numbers and corresponding culture counts were variable, with unquantifiable CFUs in the low H_2 dose reactors and similar levels of colonies in the control and high H_2 dose reactors.

The potential impact of excess hydrogen on *Legionella* growth may have been overshadowed in these SGWHs by the presence of ammonia and ammonia oxidizing bacteria, which also fix organic carbon, even though all reactors had identical influent ammonia. There were high levels of ammonia oxidation in all conditions, with 87% of the influent ammonia oxidized over a typical 2-day stagnation period in conditions without chloramines and 69% oxidized in the conditions with chloramines. Total chlorine levels in all SGWHs decayed over ~24 hours, leaving the subsequent ~24 hours with no or very low levels of chloramines before the next water change (Fig. G.7). To partially account for nitrification as a confounding factor, short-term follow-up SGWHs were run in an identical manner with break-point chlorinated influent water (to remove ammonia prior to experimentation). While reactors with H_2 and ammonia fixed 1.5 times more TOC than reactors with H_2 alone (Fig. G.8; Wilcox Rank Sum, p-value=0.0011, $n_{H2}=n_{H2+NH3}=24$), reactors with H_2 alone still fixed 3.5 times more TOC than the influent levels (Fig. G.8; Wilcox Rank Sum, p-value=0.0031, $n_{H2}=24$, $n_{influent}=7$) and 2.0 times more than the control reactors with no H_2 or NH_3 (Fig. G.8; Wilcox Rank Sum, p-value=0.0027, $n_{H2}=n_{control}=24$). More investigation is needed to determine the combined effects of autotrophic H_2 and NH_3 carbon fixation on *Legionella*, as *Legionella* were not consistently established in these follow up reactors.

In the present study, cupric ions and chloramines had significant deleterious effects on average *Legionella* spp. and *L. pneumophila* gene copy numbers in the SGWHs relative to the control (Fig. 7.1C). However, the largest impact was on culturable *Legionella*, which was 77 times lower in the reactors with cupric ions and was not detectable with chloramines and generally agree with trends in the recirculating lines of the CPVC rig (Table 4). Contrary trends were observed for *M. avium* (Fig. 7.1D). Although not significant due to variability in the low levels detected, *M. avium* trended towards increasing by 0.29-1.58 log gene copies/mL in SGWHs with copper and chloramines. The highest concentrations of *M. avium* were observed when chloramines were present, either alone ($10^{4.9}$ gene copies/mL; Fig. 7.1D) or in combination with copper and hydrogen ($10^{4.3}$-$10^{4.6}$ gene copies/mL; data not shown). These general trends provide a key line of evidence consistent with prior field observations that there is sometimes an inherent trade-off between *Legionella* control using chloramines and the regrowth of *Mycobacteria* (Moore et al., 2006; Flannery et al., 2006; Marchesi et al., 2011; Wang et al., 2012).

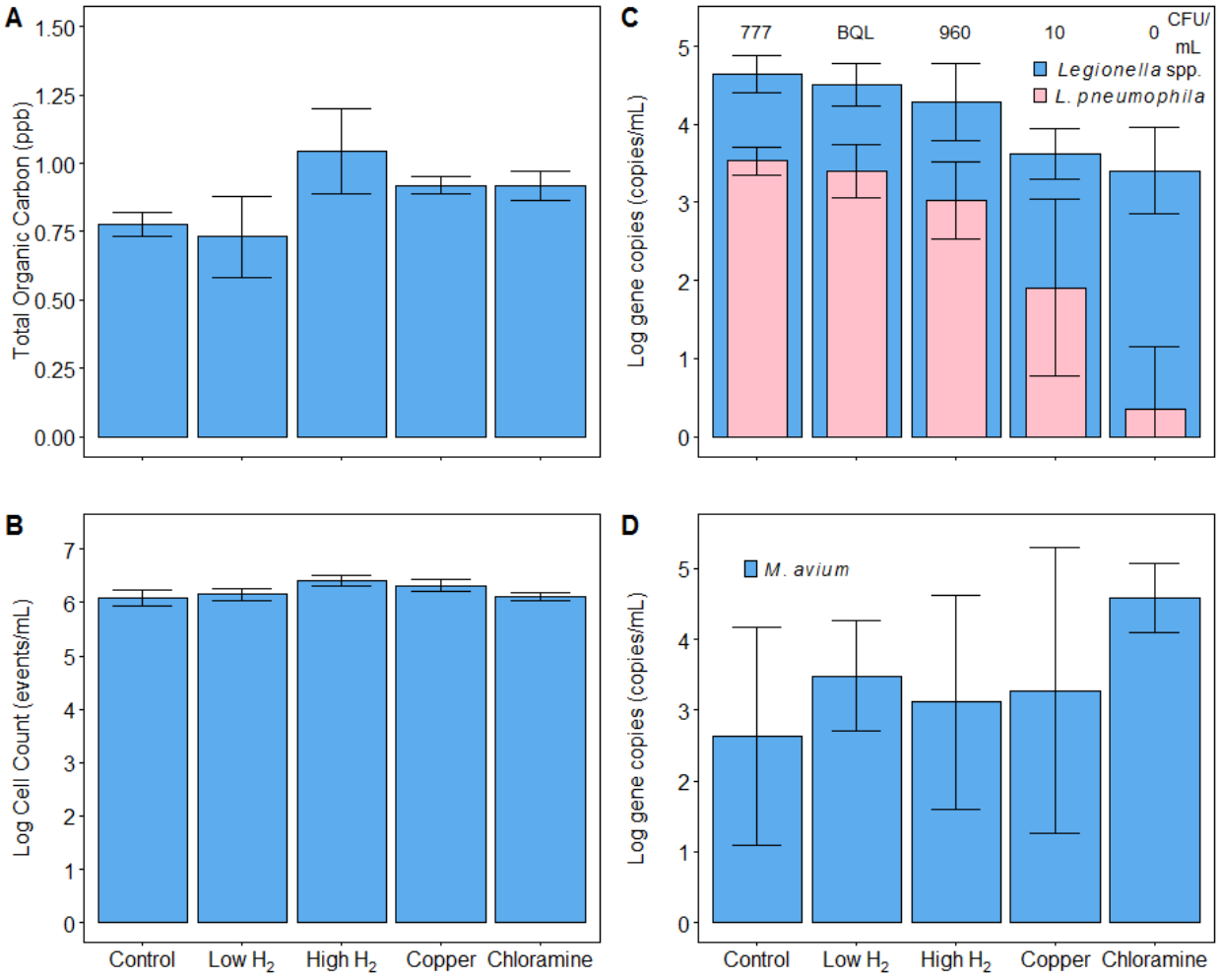

Fig. 7.1. Comparison of simulated glass water heater (SGWH) conditions operated at 37 °C Control – GAC-filtered water only, Low H_2 – hydrogen dosed at 4.25 nM H_2, High H_2 – hydrogen dosed at 40.6 µM H_2, Copper – $CuSO_4$ dosed at 0.5 mg/L total copper, and Chloramine – NH_2Cl dosed at 0.5 mg/L total Cl_2. A) Average total organic carbon concentrations (n=12), B) Log average total cell counts (n=12), C) *Legionella* spp. and *L. pneumophila* gene copy numbers, with colony forming units denoted above each bar (n=3), and D) *M. avium* gene copy concentrations (n=3). Note: BQL – Below quantification limit.

The hypothesized stimulation of OPs growth by elevated H_2 production was not consistently observed. It is possible that this water already had sufficient background nutrients and levels of nitrifying bacteria, in which case the hydrogen-oxidizing bacteria did not play a significant role in amoeba or OP lifecycles because growth was not carbon limited. There was evidence of strong nitrification in all SGWH reactors and nitrifying organisms, which also fix organic carbon (0.2 mg/L per 1 mg/L NH_3-N oxidized; Grady et al., 1999) and are native to the pilot-scale rig influent water as well. Further research on the role of autotrophic organisms in either directly or indirectly stimulating OP growth, is of interest for future research, given that anode rods are so frequently used in modern plumbing systems.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI: to be issued, and includes all extra referenced materials.

AUTHOR INFORMATION

Corresponding Author
* Email: wrhoads@vt.edu; Phone: (417) 437-2550

Notes:
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REFERENCES

Alary, M.; Joly, J. R. Risk factors for contamination of domestic hot water systems by legionellae. *Appl. Environ. Microbiol.* **1991**, *57* (8), 2360-2367.

American Public Health Association (APHA), American Water Works Association (AWWA), and Water Environment Federation (WEF). Standard Methods for the Examination of Water and Wastewater, 19th, ed.; Washington, D.C. 1995.

Bard, A. J.; Faulkner, L. R. *Electrochemical Methods: Principles and Applications*, 2nd Ed., John Wiley & Sons, New York, NY 2001; pp. 386-428.

Barker, J.; Brown, M. R.; Collier, P. J.; Farrell, I.; Gilbert, P. Relationship between *Legionella pneumophila* and *Acanthamoeba polyphaga*: physiological status and susceptibility to chemical inactivation. *Appl. Environ. Microbiol.* **1992**, *58* (8), 2420-2425.

Borella, P.; Montagna, M. T.; Romano-Spica, V.; Stampi, S.; Stancanelli, G.; Triassi, M.; Neglia, R.; Marchesi, I.; Fantuzzi, G.; Tato, D.; Napoli, C.; Quaranta, G.; Laurenti, P.; Leoni, E.; de Lucas, G.; Ossi, C.; Moro, M.; D'Alcalà, G. R. *Legionella* infection risk from domestic hot water. *Emerg. Infect. Dis.* **2004**, *10* (3), 457-64.

Borella, P.; Montagna, M. T.; Stampi, S.; Stancanelli, G.; Romano-Spica, V.; Triassi, M.; Marchesi, I.; Bargellini, A.; Tato, D.; Napoli, C.; Zanetti, F.; Leoni, E.; Moro, M.; Scaltriti, S.; D'Alcalà, G. R.; Santarpi, R.; Boccia, S. *Legionella* contamination in hot water of Italian hotels. *Appl. Environ. Microbiol.* **2005**, *71* (10), 5805-5813.

Brazeau, R. H.; Edwards, M. A. A review of the sustainability of residential hot water infrastructure: Public health, environmental impacts, and consumer drivers. *J. Green Build.* **2011**, *6* (4), 77-95.

Brazeau, R. H.; Edwards, M. A. Role of hot water system design on factors influential to pathogen regrowth: temperature, chlorine residual, hydrogen evolution, and sediment. *Environ. Eng. Sci.* **2013**, *30* (10), 617-627.

Brunkard J. M.; Ailes E.; Robers V. A.; Hill V.; Hilborn E. D.; Craun G. F.; Rajasingham A.; Kahler A.; Garrison L.; Hicks L.; Carpenter J.; Wade T. J.; Bearch M .J.; Yoder J. S. Surveillance for Waterborne Disease and Outbreaks Associated with Drinking Water and Water not Intended for Drinking --- United States, 2007—2008. *Morb.Mor. Wkly. Rep.* **2011**, *60* (SS12), 38-68.

Buse, H. Y.; Ashbolt, N. J. Counting *Legionella* cells within single amoeba host cells. *Appl. Environ. Microbiol.* **2012**, *78* (6), 2070-2072.

Buse, H. Y.; Lu, J.; Struewing, I. T.; Ashbolt, N. J. Preferential colonization and release of *Legionella pneumophila* from mature drinking water biofilms grown on copper versus unplasticized polyvinylchloride coupons. *Int. J. Hyg. Envir. Health*. **2014**, *217* (2), 219-225.

Cargill, K. L.; Pyle, B. H.; Sauer, R. L.; McFeters, G. A. Effects of culture conditions and biofilm formation on the iodine susceptibility of *Legionella pneumophila*. *Can. J. Microbiol*. **1992**, *38* (5), 423-429.

Centers for Disease Control (CDC). Primary Amebic Meningoencephalitis --- Arizona, Florida, and Texas, 2007. *Morb. Mor. Wkly. Rep.* **2008**, 58(SS21); 573-577.

Cooper, I. R.; Hanlon, G. W. Resistance of *Legionella pneumophila* serotype 1 biofilms to chlorine-based disinfection. *J. Hosp. Infect.* **2010**, *74* (2), 152-159.

Declerck, P.; Behets, J.; Margineanu, A.; van Hoef, V.; De Keersmaecker, B.; Ollevier, F. Replication of *Legionella pneumophila* in biofilms of water distribution pipes. *Microbiol. Res.* **2009**, *164* (6), 593-603.

Demirjian, A.; Lucas, C. E.; Garrison, L. E.; Kozak-Muiznieks, N. A.; States, S.; Brown, E. W.; Wortham, J. M.; Beaudoin, A.; Casey, M. L.; Marriott, C.; Ludwig, A. M.; Sonel, A. F.; Muder, R. R.; Hicks, L. A. The Importance of Clinical Surveillance in Detecting Legionnaires' Disease Outbreaks: A Large Outbreak in a Hospital With a *Legionella* Disinfection System—Pennsylvania, 2011–2012. *Clin. Infect. Dis.* **2015**, *60* (11), 1596-1602.

Donlan, R. M.; Forster, T.; Murga, R.; Brown, E.; Lucas, C.; Carpenter, J.; Fields, B. *Legionella pneumophila* associated with the protozoan *Hartmannella vermiformis* in a model multi-species biofilm has reduced susceptibility to disinfectants. *Biofouling* **2005**, *21* (1), 1-7.

Donlan, R.; Murga, R.; Carpenter, J.; Brown, E.; Besser, R.; Fields, B. Monochloramine disinfection of biofilm-associated *Legionella pneumophila* in a potable water model system. In *Legionella*; Marre, R.; Abu Kwaik, Y.; Bartlett, C.; Cianciotto, N. P.; Fields, B.; Frosch, M.; Hacker, J.; Luck, P. C., Eds.; ASM Press: Washington, D. C. 2002, 406-410.

Edwards, M.; Marshall, B.; Zhang, Y.; Lee, Y. J. Unintended consequences of chloramine hit home. *Proceedings of the Water Environment Federation* **2005**, 240-256.

Elfland, C.; Scardina, P.; Edwards, M. Lead-contaminated water from brass plumbing devices in new buildings. *J. Am. Water Works Ass.* **2010**, *102* (11), 66.

Falkinham III, J. O.; Iseman, M. D.; de Haas, P.; van Soolingen, D. *Mycobacterium avium* in a shower linked to pulmonary disease. *J. Water Health* **2008**, *6* (2), 209- 213.

Fields, B. S.; Benson, R. F.; Besser, R. E. *Legionella* and Legionnaires' disease: 25 years of investigation. *Clin. Microbiol Rev.* **2002**, *15* (3), 506-526.

Flannery, B.; Gelling, L. B.; Vugia, D. J.; Weintraub, J. M.; Salerno, J. J.; Conroy, M. J.; Stevens, V. A.; Rose, C. E.; Moore, M. R.; Fields, B. S.; Besser, R. E. Reducing *Legionella* colonization in water systems with monochloramine. *Emerg. Infect. Dis*. **2006**, *12* (4), 588-96.

Hammes, F.; Berney, M.; Wang, Y.; Vital, M.; Köster, O.; Egli, T. Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. *Water Res.* **2008**, *42* (1), 269-277.

Heffelfinger, J. D.; Kool, J. L.; Fridkin, S.; Fraser, V. J.; Hageman, J.; Carpenter, J.; Whitney, C. G. Risk of hospital-acquired Legionnaires' disease in cities using monochloramine versus other water disinfectants. *Infect. Cont. Hosp. Ep.* **2003**, *24* (08), 569-574.

Kanani, N., Ed. *Electroplating and electroless plating of copper & its alloys*; ASM International: Materials Park, OH, 2003.

Kool, J. L.; Carpenter, J. C.; Fields, B. S. Effect of monochloramine disinfection of municipal drinking water on risk of nosocomial Legionnaires' disease. *Lancet* **1999**, *353* (9149), 272-277.

Kuchta, J. M.; McGlaughlin, J. E.; Overmeyer, J. H.; Wadowsky, R. M.; McNamara, A. M.; Wolford, R. S.; Yee, R. B. Enhanced chlorine resistance of tap water-adapted *Legionella pneumophila* as compared with agar medium-passaged strains. *Appl. Environ. Microbiol.* **1985**, *50* (1), 21-26.

Kuiper, M. W.; Wullings, B. A.; Akkermans, A. D.; Beumer, R. R.; Van Der Kooij, D. Intracellular proliferation of *Legionella pneumophila* in *Hartmannella vermiformis* in aquatic biofilms grown on plasticized polyvinyl chloride. *Appl. Environ. Microbiol.* **2004**, *70* (11), 6826-6833.

Lau, H. Y.; Ashbolt, N. J. The role of biofilms and protozoa in *Legionella* pathogenesis: implications for drinking water. *J. Appl. Microbiol.* **2009**, *107* (2), 368-378.

Leoni, E.; De Luca, G.; Legnani, P. P.; Sacchetti, R.; Stampi, S.; Zanetti, F. *Legionella* waterline colonization: detection of Legionella species in domestic, hotel and hospital hot water systems. *J. Appl. Microbiol.* **2005**, *98* (2), 373-379.

Lin, Y-S.E.; Vidic, R. D.; Stout, J. E.; Victor, L. Y. Negative effect of high pH on biocidal efficacy of copper and silver ions in controlling *Legionella pneumophila*. *Appl. Environ. Microbiol.* **2002**, *68* (6), 2711-2715.

Lytle, D. A.; Liggett, J. Impact of water quality on chlorine demand of corroding copper. *Water Res.* 2016 *92*, 11-21.

Marchesi, I.; Marchegiano, P.; Bargellini, A.; Cencetti, S.; Frezza, G.; Miselli, M.; Borella, P. Effectiveness of different methods to control legionella in the water supply: ten-year experience in an Italian university hospital. *J. Hosp. Infect.* **2011**, *77* (1), 47-51.

Marras, T. K.; Chedore, P.; Ying, A. M.; Jamieson, F. Isolation prevalence of pulmonary non-tuberculous mycobacteria in Ontario, 1997 - 2003. *Thorax* **2007**, *62* (8), 661-666.

Marrie, T. J. Community-acquired pneumonia. *Clin. Infect. Dis.* **1994**, *18* (4), 501-513.

Martin, A. K. Organic carbon generation mechanisms in main and premise distribution systems. Master's Thesis, Virginia Tech, Blacksburg, VA, 2012.

Mathys, W.; Stanke, J.; Harmuth, M.; Junge-Mathys, E. Occurrence of *Legionella* in hot water systems of single-family residences in suburbs of two German cities with special reference to solar and district heating. *Int. J. Hyg. Envir. Health*. **2008**, *211* (1), 179-185.

Miuetzner, S.; Schwille, R. C.; Farley, A.; Wald, E. R.; Ge, J. H.; States, S. J.; Libert, T.; Wadowsky, R. M. Efficacy of thermal treatment and copper-silver ionization for controlling Legionella pneumophila in high-volume hot water plumbing systems in hospitals. *Am. J. Infect. Cont.* **1997**, *25* (6), 452-457.

Moore, M. R.; Pryor, M.; Fields, B.; Lucas, C.; Phelan, M.; Besser, R. E. Introduction of monochloramine into a municipal water system: impact on colonization of buildings by *Legionella* spp. *Appl. Environ. Microbiol.* **2006**, *72* (1), 378-383.

Morton, S. C.; Zhang, Y.; Edwards, M. A. Implications of nutrient release from iron metal for microbial regrowth in water distribution systems. *Water Res.* **2005**, *39* (13), 2883-2892.

Nguyen, C.; Elfland, C.; Edwards, M. Impact of advanced water conservation features and new copper pipe on rapid chloramine decay and microbial regrowth. *Water Res.* **2012**, *46* (3), 611-621.

Proctor, C. R. Effect of Various Water Chemistry Factors on *Legionella* Proliferation and the Premise Plumbing Microbiome Composition. Master's Thesis, Virginia Tech, Blacksburg, VA, 2014.

Rhoads, W. J.; Chambers, B.; Pearce, A.; Edwards, M. A. Green Building Design: Water Quality Considerations. Water Research Foundation: Denver, CO 2015; http://www.waterrf.org/PublicReportLibrary/4383a.pdf

Rhoads, W. J.; Ji, P.; Pruden, A.; Edwards, M. A. Water heater temperature set point and water use patterns influence *Legionella pneumophila* and associated microorganisms at the tap. *Microbiome* **2015**, *3* (1), 1.

Rhoads, W. J.; Pruden, A.; Edwards, M. A. Anticipating challenges with in-building disinfection for control of opportunistic pathogens. *Water Environ. Res.* **2014**, *86* (6), 540-549.

Rhoads, W. J.; Pruden, A.; Edwards, M. A. Convective mixing in distal pipes exacerbates *Legionella pneumophila* growth in hot water plumbing. *Pathogens*, 2016, *5*(1), 29.

Rhoads, W. J.; Pruden, A.; Edwards, M. A. Survey of green building water systems reveals elevated water age and water quality concerns. *Environ. Sci.: Water Res. Technol.* **2016**, *2*(1), 164-173.

Rogers, J.; Dowsett, A. B.; Dennis, P. J.; Lee, J. V.; Keevil, C. W. Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. *Appl. Environ. Microbiol.* **1994**, *60* (5), 1585-1592.

Rohr, U.; Senger, M.; Selenka, F.; Turley, R.; Wilhelm, M. Four years of experience with silver-copper ionization for control of *Legionella* in a German university hospital hot water plumbing system. *Clin. Infect. Dis.* **1999**, *29* (6), 1507-1511.

Rowbotham, T. J. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *J. Clin. Pathol.* **1980**, *33* (12), 1179-1183.

Schweizerisches Lebensmittelbuch (SLMB). Research Method 366.1: Determining the total cell count and ratios of high and low nucleic acid content cells in freshwater using flow cytometry; Switzerland 2012; http://www.isme-microbes.org/sites/isme-microbes.org/files/2012_ SLMB_366%201%20ME%20EN%20Final.pdf

Storey, M. V.; Ashbolt, N. J.; Stenström, T. A. Biofilms, thermophilic amoebae and *Legionella pneumophila*: A quantitative risk assessment for distributed water. *Water Sci. Technol.* 2004, *50* (1), 77-82.

Stout, J. E.; Rihs, J. D.; Yu, V. L. *Legionella*. In *Manual of clinical microbiology*, 8th, ed; Murray, P. R.; Baron, E. J.; Jorgenson, J. H.; Pfaller, M. A.; Yolken, R. H., Eds.; American Society for Microbiology: Washington, D. C. 2003; pp. 809-23.

Surman, S.; Morton, G.; Keevil, B.; Fitzgeorge, R.; Skinner, A. (2002). *Legionella pneumophila* proliferation is not dependent on intracellular replication. In *Legionella*; Marre, R.; Abu Kwaik, Y.; Bartlett, C.; Cianciotto, N. P.; Fields, B.; Frosch, M.; Hacker, J.; Luck, P. C., Eds.; ASM Press: Washington, D. C. 2002**,** 86-89.

Thomas, J. M.; Ashbolt, N. J. Do free-living amoebae in treated drinking water systems present an emerging health risk? *Environ. Sci. Technol.* **2010**, *45* (3), 860-869.

Thomas, V.; Bouchez, T.; Nicolas, V.; Robert, S.; Loret, J. F; Levi, Y. Amoebae in domestic water systems: resistance to disinfection treatments and implication in *Legionella* persistence. *J. Appl. Microbiol.* **2004**, *97* (5), 950-963.

Triantafyllidou, S.; Lytle, D.; Muhlen, C.; Swertfeger, J. Copper-silver ionization at a US hospital: Interaction of treated drinking water with plumbing materials, aesthetics and other considerations. *Water Res.* **2016**, 102 (1), 1-10.

Van der Kooij, D.; Veenendaal, H. R.; Scheffer, W. J. Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. *Water Res.* **2005**, *39* (13), 2789-2798.

Victor, L. Y.; Stout, J. E. Hospital characteristics associated with colonization of water systems by *Legionella* and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals. *Infect. Cont. Hosp. Ep.* **2000**, *21* (07), 434-435.

Wang, H.; Edwards, M.; Falkinham, J. O.; Pruden, A. Molecular survey of the occurrence of *Legionella* spp.; *Mycobacterium* spp.; *Pseudomonas aeruginosa*, and amoeba hosts in two chloraminated drinking water distribution systems. *Appl. Environ. Microbiol.* **2012**, *78* (17), 6285-6294.

Wang, H.; Masters, S.; Falkinham III, J. O.; Edwards, M. A.; Pruden, A. Distribution system water quality affects responses of opportunistic pathogen gene markers in household water heaters. *Environ. Sci. Technol.* **2015**, *49* (14), 8416-8424.

Yoder J. S.; Roberts V.; Craun G. F.; Hill V.; Hicks L.; Alexander N. T.; Radke V.; Calderon R. L.; Hlavsa M. C.; Beach M. J.; Roy S. L. Surveillance for Waterborne Disease and Outbreaks Associated with Drinking Water and Water not Intended for Drinking --- United States, 2005—2006. *Morb. Mor. Wkly. Rep.* **2008**, *57* (SS09), 39-62.

Zeybek, Z.; Cotuk, A. Relationship between colonization of building water systems by *Legionella pneumophila* and environmental factors. In *Legionella*; Marre, R.; Abu Kwaik, Y.; Bartlett, C.; Cianciotto, N. P.; Fields, B.; Frosch, M.; Hacker, J.; Luck, P. C., Eds.; ASM Press: Washington, D. C. 2002**,** 305-308.

Zhang, Y.; Griffin, A.; Edwards, M. Nitrification in premise plumbing: role of phosphate, pH and pipe corrosion. *Environ. Sci. Technol.* **2008**, *42* (12), 4280-4284.

Zhang, Y.; Love, N.; Edwards, M. Nitrification in drinking water systems. *Crit. Rev. Environ. Sci. Technol.* **2009**, *39* (3), 153-208.

APPENDIX G - SUPPORTING INFORMATION FOR CHAPTER 7.

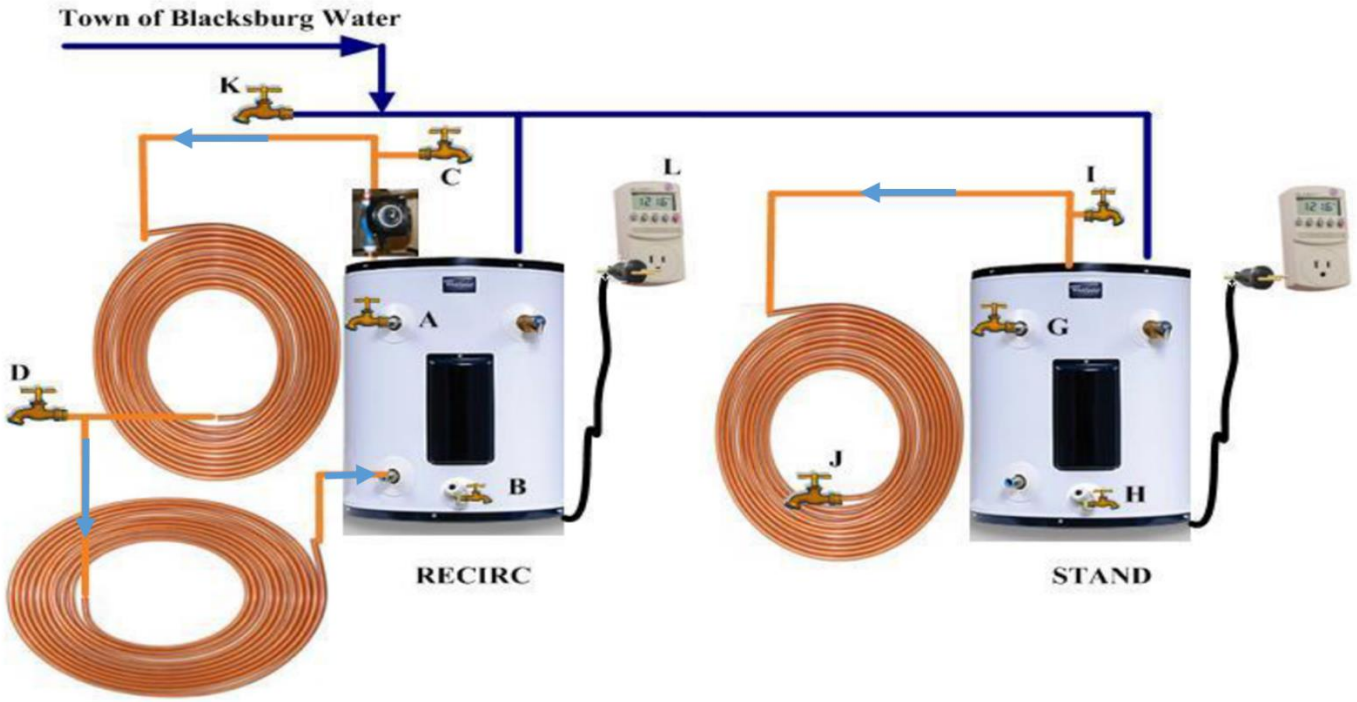


Fig. G.1. Copper Rigs - recirculating and standard system design from Brazeau and Edwards (2012), data from which is compared to data gathered in the present study.

The recirculating system ("RECIRC") continuously recirculated water from the tank through 9.1 m (30 ft) of copper pipe to distal sample port "D," and then back through an additional 9.1 m (30 ft) of copper pipe to the tank. The standard system ("STAND") had no hot water return. Instead, water flowed through 9.1 m (30 ft) of copper pipe when used to sample port "J," otherwise water was stagnant. Water use was characterized by 25% tank turnover (4.75 gallons) twice daily using electronic solenoid timers, flushed from the tap D or J for the recirculating or standard system, respectively. For the portion of data re-analyzed, water heater set point was 49° C, which was chosen to represent EPA recommendations for energy savings (Table 1). There was no granular activated carbon filtration on these rigs.

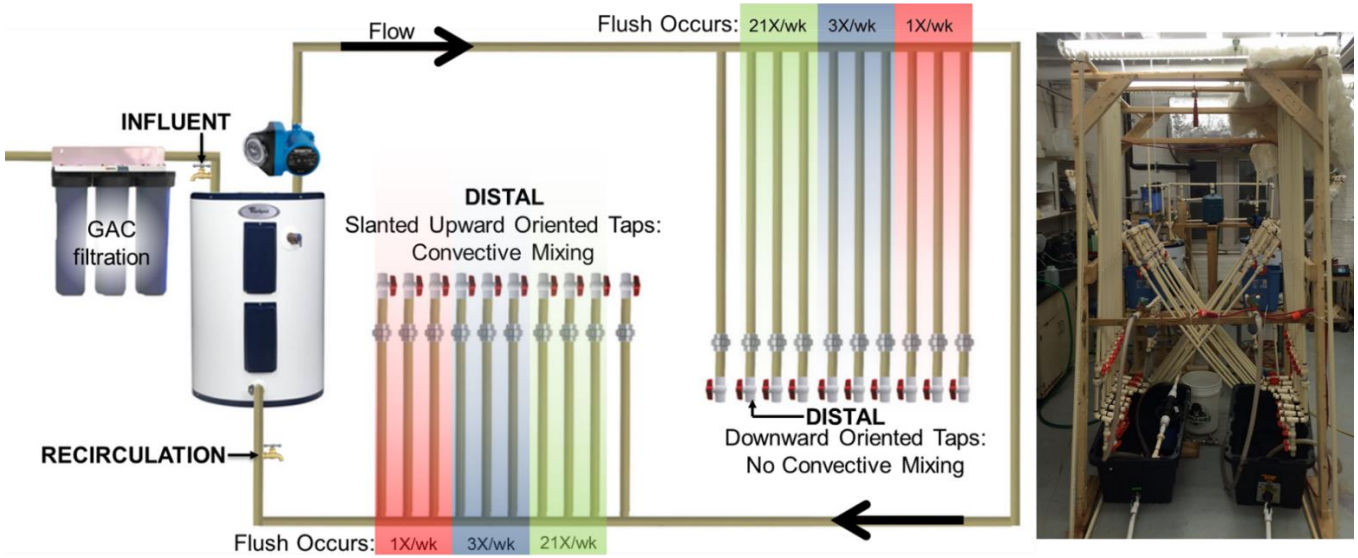


Fig. G.2. CPVC Rigs - Experimental apparatus used to generate new data for this study

Summary of prior experiments using this experimental apparatus:

1. **Rhoads et al., 2015:** "Two identical household hot water systems with 71.9-L (19 gallons) electric water heaters and continuously recirculating pipe loops were constructed with nominal ¾-in. chlorinated polyvinyl chloride (CPVC; Charlotte Pipe, Charlotte, NC) pipe (Fig. 1). Each system tested two pipe orientations (upward/downward) with three water use patterns in triplicate, including low use (1 flush/week), medium use (3 flushes/week), and high use (21 flushes/week) for a total of 36 distal taps (2 systems × 2 orientations × 3 use patterns × triplicate = 36). Each distal tap pipe was 1.7 m (5.5 ft) for a total distal tap volume of 0.43 L (0.11 gallons) and internal surface area of 0.87 m^2 (0.94 ft^2). Each recirculating line was a total of 7.6 m (25 ft). The water heaters and recirculating lines were completely mixed, resulting in a combined volume of 73.9 L (19.5 gallons) and surface area of 1.46 m^2 (15.7 ft^2). Each flush was conducted for 28 s at 3.8 L/min (1 gallon/min). Influent water consisted of well-flushed (10 min at 11.3 L/min), granular activated carbon (GAC)-filtered Blacksburg, VA, tap water. Both systems were initially acclimated for 5 months at 39 °C. Afterwards, the experimental system water heater temperature was increased approximately by 5 °C increments while the control system remained at 39 °C. During periods of stagnation, distal pipes cooled to room temperature."

2. **Rhoads et al., 2016:** “For this work, the “Warm Temperature Set Point” system water heater was set to 40 °C and the “Hot Temperature Set Point” system water heater was set to 58 °C to represent worst and best management practices with respect to *Legionella* control. Both systems were acclimated for 15 months with no convective mixing prior to commencing this study (month 15 in our previous work is month 0 for this study) [Rhoads et al., 2015]. For this study, convective mixing was induced in half the distal pipes by slanting upward oriented pipes 30” from vertical. Sampling occurred at 2 months and 4 months after inducing convective mixing to compare the two pipe orientations (with and without convective mixing) with three water use patterns in triplicate, including low use (1 flush/week), medium use (3 flushes/week), and high use (21 flushes/week) for a total of 36 distal taps (2 systems ˆ 2 orientations ˆ 3 use patterns ˆ triplicate = 36) to simulate the range of use frequencies encountered in homes. Each distal pipe was 1.5 m long with a 0.2 m section accessible by a union for repeated biofilm swab, had a volume of approximately 430 mL, and each flush described above was 28 seconds long at 3.8 L/min (1 gallon/min). Influent water consisted of well-flushed (10 min at 11.3 L/min), GAC filtered Blacksburg, VA tap water. During periods of no use, distal pipes were allowed to equilibrate with the ambient temperature.”
3. **Immediately Prior to this work**: Both water heater temperatures were increased to 60 °C for 30 minutes, following *Legionella* heat shock recommendations in an attempt to return both systems to a similar baseline. Afterwards, both systems were set to 40 °C. Physical (temperature, flow rate, flow frequencies), chemical (amount of ammonia oxidized), and biological (*Legionella* spp. and *L. pneumophila* gene numbers) parameters were not different 1-month after the heat shock occurred (data not shown).

Summary of the experiments that were completed for this work:
For the first phase of work, influent water (Sample Port: INFLUENT) was granular activated carbon (GAC) filtered Blacksburg, VA tap water. Three GAC filters removed >95% of municipal chloramine disinfectant residual. Convective mixing was induced in slanted upward oriented taps by slanting each pipe 30° from vertical, whereas downward oriented taps provide a control without convective mixing in two identical systems. One plumbing system tank and recirculating line was dosed with soluble copper II (as $CuSO^4$) while the other system acted as a control with no additional copper. A recirculating pump continuously pumped water around the return loop back to the water heater creating a completely mixed reservoir (Sample port: Recirculation), six replicate distal taps (three upward + three downward) were flushed for 28 seconds at 3.8 L/min (1 gallon/min) 21x/week, 3x/week, and 1x/week for a total of 36 pipes (Sample ports: at end of distal pipes).For the second phase of work, municipal water was blended with GAC filtered water to achieve a chloramine residual of approximately 1.5 mg/L as Cl_2. Other conditions remained identical to the first phase.

Operation

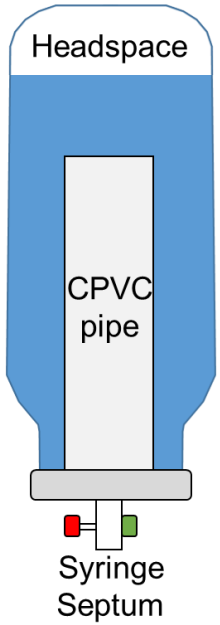
-Incubated inverted to maintain H_2 dosed

-Incubated at 40° C to mimic CPVC rig conditions

-Agitated at 150 rpm

-80% volume change with each water change

-11 conditions total using influent water from CPVC rig

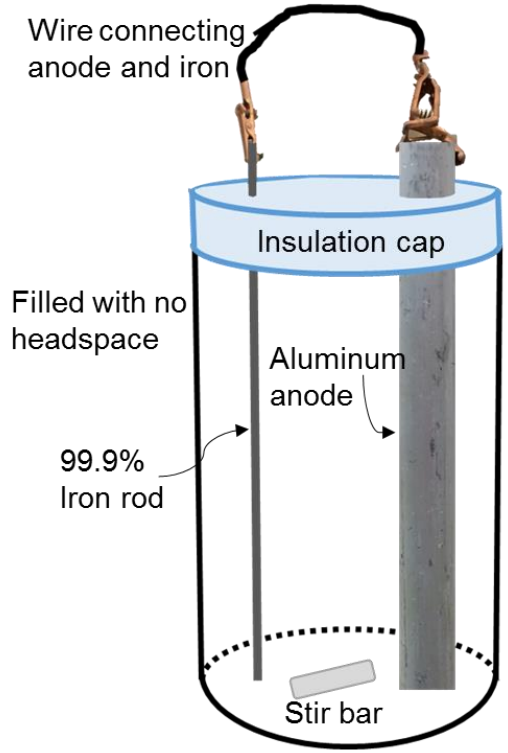


Condition	Description
Control	GAC-filtered tap water (identical to CPVC rigs)
Low H_2	Control influent + 4.25 nM H_2
High H_2	Control influent + 40,600 nM H_2
Cu	Control influent + 0.5 mg/L Cu^{2+} (dosed as $CuSO_4$)
Low H_2 & Cu	Low H_2 condition + 0.5 mg/L Cu^{2+}
High H_2 & Cu	High H_2 condition + 0.5 mg/L Cu^{2+}
Cl	Control influent + 0.5 mg/L chloramine (as total Cl_2)
Low H_2 & Cl	Low H_2 condition + 0.5 mg/L Cl_2
High H_2 & Ci	High H_2 condition + 0.5 mg/L Cl_2
Low Combo	Low H_2 condition + 0.5 mg/L Cu^{2+} + 0.5 mg/L Cl_2
High Combo	High H_2 condition + 0.5 mg/L Cu^{2+} + 0.5 mg/L Cl_2

Fig. G.3. Simulated glass water heater experimental design overview. H_2 was dosed to reactors in orientation shown to minimize loss and incubated inverted, as shown. To sample the reactors, reactors were turned right-side-up. Headspace was sampled directly using the septum port. Water was then transferred to sample containers, as necessary, for analysis by removing cap and pouring water directly from reactors.

Glass reactors were 500 mL French square bottle (Wheaton) with polytetrafluoroethyene (PTFE) caps. Prior to experimentation, all bottles and caps were acid-washed, rinsed in reagent grade nanopure water, and glass bottles were baked in a muffle furnace at 550 °C to combust residual organics. Pipe extracted from the CPVC rigs was epoxied (Devcon 5 minute epoxy) to the interior of the cap to provide a growth surface, a syringe septum sampling port was installed on the exterior of the cap to facilitate headspace hydrogen dosing and analysis, and SGWHs were incubated inverted.

To generate pure H_2, 1 g solid zinc shavings reacted with 100 mL of 12 N hydrochloric acid in deionized water. Gas was collected into a headspace analysis bag with a syringe septum for dosing H_2.



Condition	Aluminum Anode Weight Loss over 2 months (% loss)
Base water: GAC filtered Rhoads rig influent water	0.08
Base water + 0.5 mg/L cupric ions dosed as soluble $CuSO_4$	0.06
Base water + 0.41 mg/L Cu from copper pipe corrosion†	0.11

† Copper from copper pipe corrosion was created by taking the base water and recirculating it for 24 hours through new copper pipes. 0.41 mg/L Cu is the average over a 2 month period.

Fig. G.4. Weight loss experiment.

A segment of an aluminum anode rod and a 99% pure iron wire were placed into water through foam building insulation to simulate the iron and aluminum in a household water heater. They were then submersed into a 1 L glass container and filled with A) GAC-filtered "base" water identical to the pilot-scale CPVC rig influent water), B) base water dosed with 0.5 mg/L cupric ions as soluble $CuSO_4$, or C) base water that had been recirculated through 5 ft of new copper pipe for 24 hours (copper concentrations produced as a result of copper pipe corrosion were measured using a HACH DR 5700, using method 8506 CuVer 1 Powder Pillows Bicinchoninate). An 80% dump and fill protocol was used for water changes 3X/week. These reactors were stirred at 150 RPM to simulate the turbulence associated with recirculating systems and incubated a 37° C. Anode rod weight loss was measured after 2 months of operation.

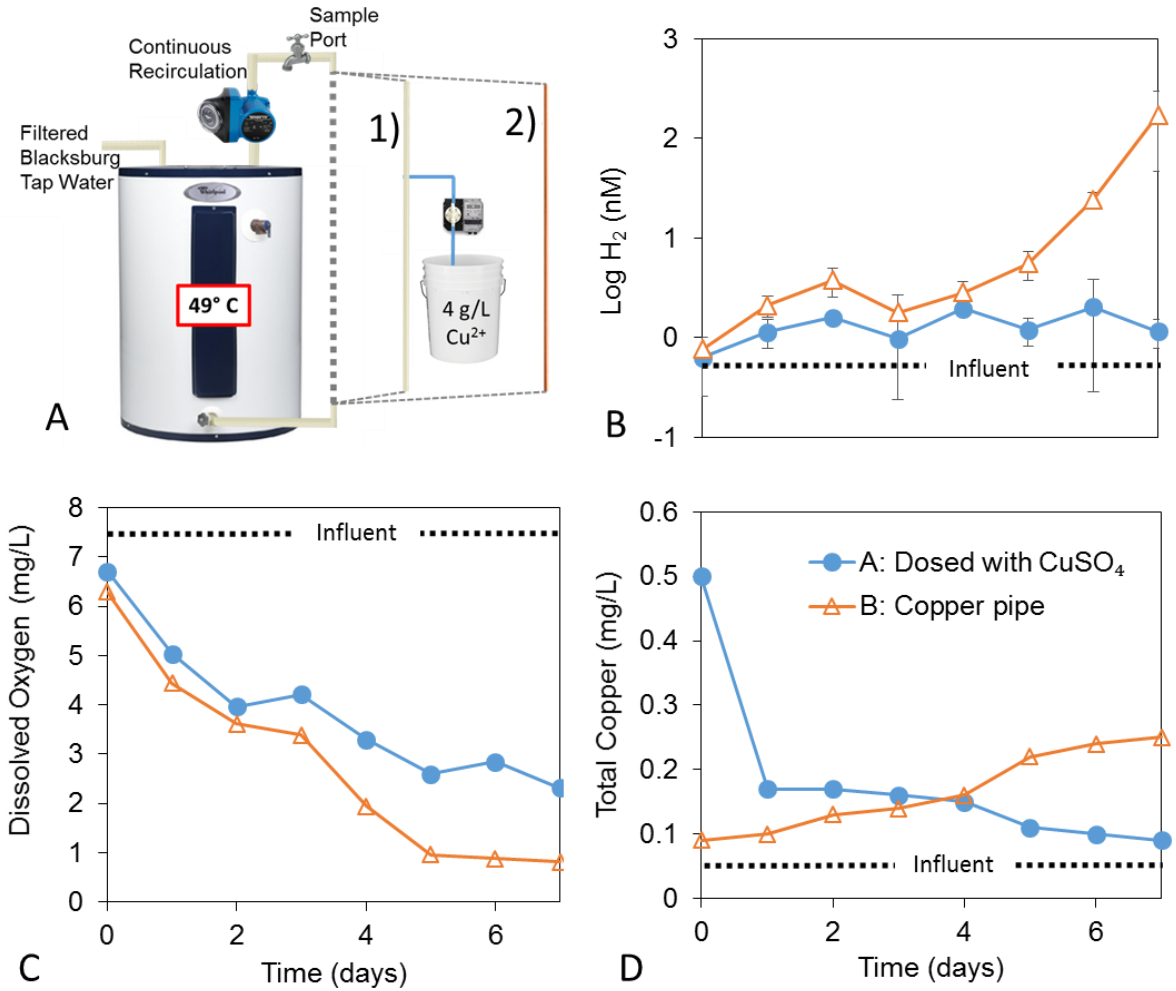


Fig G.5. Impact of copper (as copper sulfate versus from copper pipe corrosion) on biochemical properties of water. A) Experimental apparatus, water heater set to 49° C with continuous recirculation line dosed with either 1) soluble Cu^{2+} (0.5 mg/L as $CuSO_4$) on day zero or 2) copper naturally produced from 1.5 m (5 ft) new copper pipes inserted into the recirculating line over the seven day experimental period, B) aqueous hydrogen concentrations measured over time; C) dissolved oxygen concentration over time, and D) copper concentrations over time.

Discussion of Fig G.5. For this experiment, an extra heater (i.e., not those used in the CPVC Rigs) was outfitted with a recirculating line and a new anode rod was used for each simulation with dosed Cu^{2+} or copper pipe, respectively. For the system dosed with Cu^{2+}, the recirculating line was constructed with CPVC pipes and Cu^{2+} was dosed from a stock solution in the same manner as the CPVC rig. For the system with copper pipes, 5 ft of the recirculating line was replaced with copper pipe to generate copper ions from the pipe. There was no other source of copper in the tank and recirculating line.
The soluble copper dosed as $CuSO_4$ quickly precipitates within the system, so total concentration of copper in the bulk water decreased over time (Fig S6 D – blue circles). Copper gradually increased in the system with copper pipe as the new copper pipes corroded (Fig S6 D – orange triangles). There was no water use during the experiment, so dissolved oxygen naturally decreased during the week long experiment (Fig S6 C). However, the rate of consumption increased as copper concentration increased in the system with copper pipe (Fig S6 D – orange triangles). The increase in copper and decrease in DO in the system with copper pipe coincided with increased aqueous hydrogen. Our hypothesis is that either 1) after an initial incubation period, the copper from the copper pipes plated onto the anode rod and increased the corrosion of the anode rod via the H_2 evolution corrosion pathway, or 2) as the DO decreased in the system, the H2 evolution corrosion pathway of the anode rod began to dominate in the presence of copper from the copper pipes, but not from $CuSO_4$. According to previous work, the form of copper most likely to deposit onto the aluminum anode rod is Cu^{1+} (Clark and Edwards, 2015). At the pH observed (~7.3), any soluble copper dosed as $CuSO_4$ would likely be Cu^{2+} and the insoluble fraction would be $Cu(OH)_2$. Copper from the copper pipe would largely be Cu^{2+}, but there would also be trace amounts of Cu^{1+}. This Cu^{1+}, therefore, could have been reacting with the aluminum anode rod and causing the rapid evolution of H_2.
One concern regarding these data are that the coincidental drop in dissolved oxygen near day 3-4 of the test from >3 mg/L to <1 mg/L could have been the driver of the switch to the hydrogen evolution corrosion pathway. It is possible that the combination of lower DO with copper from the copper pipe led to the H_2 generation, and that H_2 generation would be absent at higher DO levels that are typical of hot water systems in regular use. However, it is our hypothesis that a microgalvanic cell develops where the copper deposits onto the aluminum, and that this site quickly becomes devoid of O_2. This would drive the H_2 evolution pathway, regardless of O_2 in the bulk water of the heater. Mechanistic follow-up work is warranted.

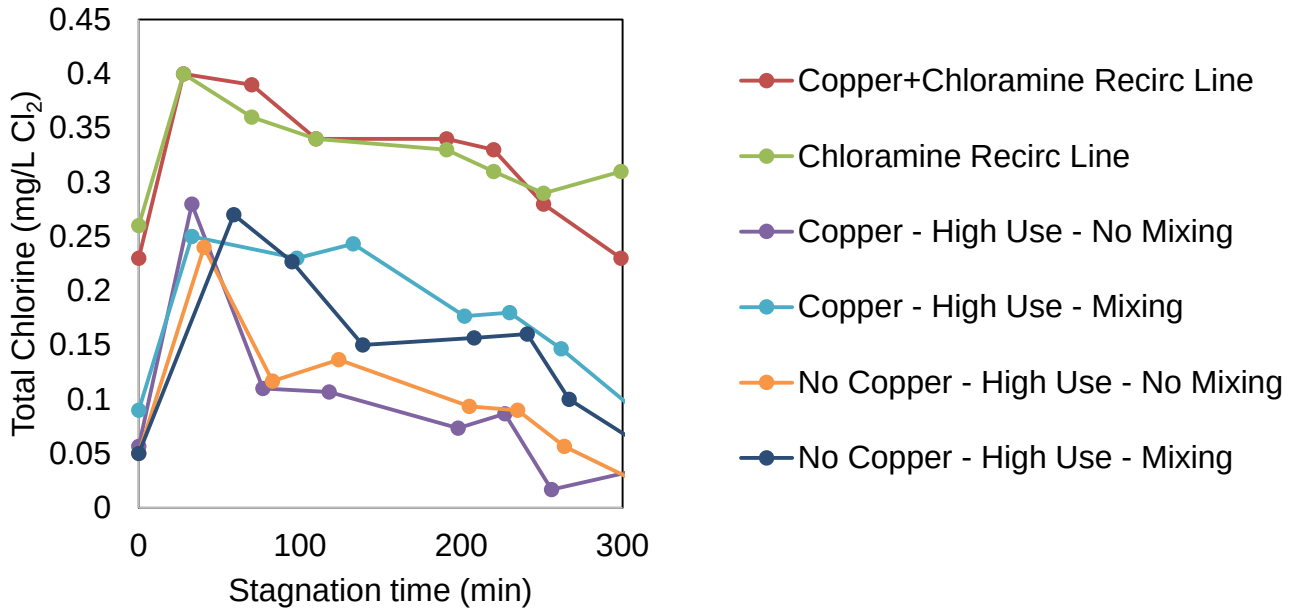


Fig. G.6. Example plot of chloramine concentration as a function of time after a typical 8-hour stagnation; data collected at time = 0 minutes was just before a regular flush of high use pipes (flush 21X/week). Distal tap data is average of triplicate pipes.

Both recirculating lines increased to 0.4 mg/L immediately after the water change occurred, and subsequently decayed at a similar rate (orange and grey lines). Similarly, chloramine concentrations increased in all distal pipes; however, the downward pipes that had no convective mixing decayed faster than the pipes with convective mixing. This is possible due to chloramine being delivered from the recirculating line to the distal taps via mixing.

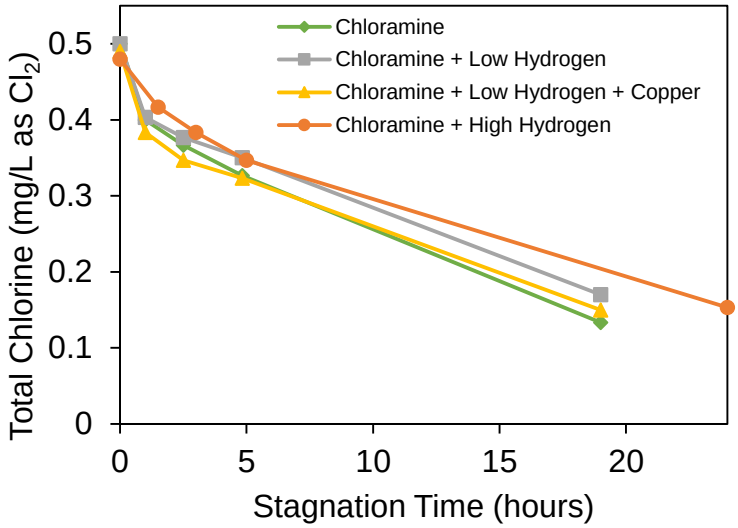


Fig. G.7. Typical simulated glass water heater (SGWH) chloramine decay. All conditions had comparable chloramine decay rates.

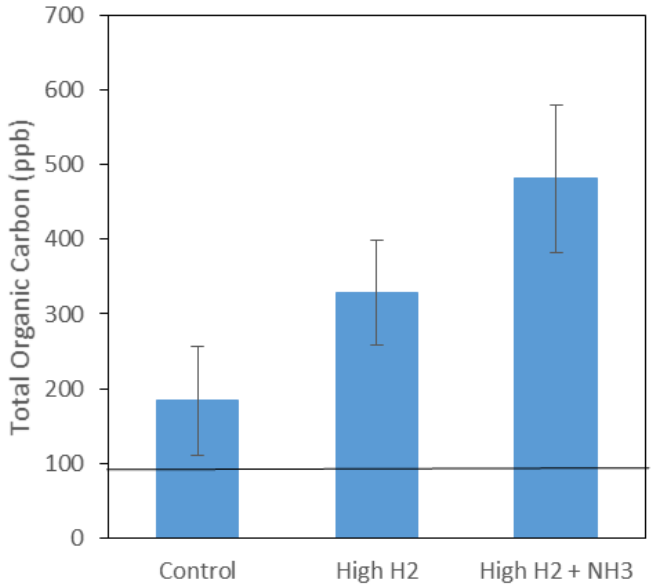


Fig. G.8. Total organic carbon in effluent of follow-up SGWHs using identical procedure to the SGWHs presented in Fig. 1 in the manuscript (and detailed in Fig. S3 above), except influent was breakpoint chlorinated to remove residual ammonia. Ammonia was then added back into these SGWHs at 0.5 ppm NH_3-N. All reactors were seeded with the same influent water, which possessed ammonia oxidizing bacteria.

Table G.1. Average of log A) *Legionella* spp. gene copy numbers in the bulk water, B) *L. pneumophila* gene copy numbers in the bulk water, C) *L. pneumophila* gene copy numbers in the biofilm of the CPVC rig for both sampling dates (Table 4 depicts averages and confidence intervals of combined data sets)

			Phase 1 (2/2/2015-5/27/2015) No Chloramine Added			Phase 2 (5/28/2015-8/19/2015) Chloramine Added		
Target	Condition	Orientation	Low Use	Medium Use	High Use	Low Use	Medium Use	High Use
A. Log Legionella spp. concentration (gene copies/mL)	System with No Copper Added	Recirc Line		4.6			4.0	
		No Mixing	4.6	4.9	4.6	4.7	4.0	3.9
		Mixing	5.2	5.0	4.8	4.9	4.0	4.4
		Recirc Line		4.8			3.4	
		No Mixing	4.9	4.5	4.5	4.3	3.9	3.3
		Mixing	5.5	4.6	4.7	4.3	3.6	3.8
	System with Copper Added	Recirc Line		4.4			4.1	
		No Mixing	5.1	5.0	4.4	4.4	3.9	4.7
		Mixing	5.2	5.0	4.8	4.9	3.9	4.4
		Recirc Line		4.6			3.4	
		No Mixing	4.9	4.8	4.8	3.7	3.7	4.0
		Mixing	5.3	4.7	4.7	4.1	4.1	3.9
B. Log L. pneumophila concentration (gene copies/mL)	System with No Copper Added	Recirc Line		4.1			3.8	
		No Mixing	4.4	4.6	4.3	4.1	3.5	3.5
		Mixing	4.9	4.8	4.5	4.7	3.9	4.0
		Recirc Line		4.2			2.2	
		No Mixing	4.5	4.0	4.0	2.3	2.2	1.9
		Mixing	5.0	4.4	4.4	3.0	2.4	2.3
	System with Copper Added	Recirc Line		4.0			3.8	
		No Mixing	4.8	4.8	4.2	4.1	3.7	3.9
		Mixing	4.9	4.7	4.5	4.8	3.9	4.1
		Recirc Line		4.2			2.6	
		No Mixing	4.3	4.3	4.3	2.7	2.6	2.7
		Mixing	5.1	4.5	4.4	3.0	3.1	2.8
C. Log L. pneumophila concentration (gene copies/cm^2)	System with No Copper Added	Recirc Line		NA			5.1	
		No Mixing	NA	NA	NA	ND	ND	BQL
		Mixing	NA	NA	NA	4.1	3.7	3.4
		Recirc Line		5.1			4.6	
		No Mixing	3.4	BQL	BQL	BQL	BQL	BQL
		Mixing	4.1	4.1	4.3	BQL	ND	BQL
	System with Copper Added	Recirc Line		NA			4.3	
		No Mixing	NA	NA	NA	BQL	BQL	BQL
		Mixing	NA	NA	NA	3.7	3.8	3.7
		Recirc Line		5.1			3.8	
		No Mixing	BQL	BQL	BQL	BQL	ND	ND
		Mixing	4.7	4.9	5.1	1.9	BQL	1.9

Table G2. Average of log A) *M. avium* gene copy numbers in the bulk water, B) *M. avium* gene copy numbers in the biofilm of the CPVC rig for both sampling dates (Table 5 depicts averages and confidence intervals of combined data sets)

Tartget	Condition	Orientation	Phase 1 (2/2/2015-5/27/2015) No Chloramine Added			Phase 2 (5/28/2015-8/19/2015) Chloramine Added		
			Low Use	Medium Use	High Use	Low Use	Medium Use	High Use
A. Log M. avium spp. concentration (gene copies/mL)	System with No Copper Added	Recirc Line		NA			3.7	
		No Mixing	NA	NA	NA	3.9	3.5	3.5
		Mixing	NA	NA	NA	4.8	4.8	4.7
		Recirc Line	3.0				3.6	
		No Mixing	3.2	2.8	3.0	4.0	3.6	2.7
		Mixing	4.2	4.1	4.0	4.4	4.8	4.3
	System with Copper Added	Recirc Line		NA			3.7	
		No Mixing	NA	NA	NA	3.7	2.8	3.6
		Mixing	NA	NA	NA	3.0	3.8	3.8
		Recirc Line	2.8			2.5		
		No Mixing	2.3	2.7	2.7	2.5	2.3	2.3
		Mixing	3.3	3.6	2.8	3.0	3.1	2.7
B. Log M. avium concentration (gene copies/cm^2)	System with No Copper Added	Recirc Line		NA			5.3	
		No Mixing	NA	NA	NA	BQL	BQL	BQL
		Mixing	NA	NA	NA	3.8	5.0	4.5
		Recirc Line	4.6				5.4	
		No Mixing	3.8	3.5	4.1	3.0	2.0	2.1
		Mixing	3.9	4.2	3.9	3.7	4.3	4.3
	System with Copper Added	Recirc Line		NA			5.0	
		No Mixing	NA	NA	NA	BQL	2.1	ND
		Mixing	NA	NA	NA	3.1	4.3	4.7
		Recirc Line		4.3			4.8	
		No Mixing	2.9	3.1	3.7	BQL	1.6	BQL
		Mixing	4.0	3.8	4.4	2.5	2.7	4.1

Table G3. Confirmation of hydrogen concentrations dosed to reactors at start-up
To ensure that hydrogen could be accurately dosed to reactors without appreciable losses (in stock, in syringe used to dose, during transfer, etc), known hydrogen volumes were dosed to reactors containing DI water. Hydrogen was dosed directly to inverted reactors, then recovered immediately. Target concentration in the headspace of the reactors was then calculated based on simple mixing ($M_1V_1=M_2V_2$), and measured concentration samples were collected immediately after dosing to confirm the target concentration was achieved.

Volume of 100 ppm H_2 standard dosed to headspace (mL)	**Target Concentration in headspace (ppbv)**	**Measured Concentration in headspace (ppbv) (n=6)**
0 (i.e., background)	0	Range: 159-592
5	2,340	1701 ± 493
10	4,680	4633 ± 224
15	7,020	6923 ± 619

APPENDIX H – H_2 EXAMPLE CALCULATION

Example aqueous H_2 concentration calculation:
For copper and CPVC rig H_2 analysis, approximately 20 mL of water was collected in 40 mL glass vials, with air-tight needle septum sampling port. Samples were collected, allowed to equilibrate for approximately 1 hour, and then 10 mL of headspace was drawn out of the 40 mL sampling vial (with 20 mL water and 20 mL headspace). Hydrogen concentration was measured by gas chromatography, and reported in volumetric parts per billion (ppb_v). To convert ppb_v measured in the headspace to aqueous concentration:
Example calculation for 2500 ppb_v reported in the headspace.

1. Convert ppb_v to mol/L in air:

$$\frac{mol}{L}H_2 = X\ ppb_b \left(\frac{1\ L\ H_2}{1\ ppb_v \times 10^9\ L\ air}\right)\left(\frac{P}{RT}\right)$$

$$2500\ ppb_b \left(\frac{1\ L\ H_2}{1\ ppb_v \times 10^9\ L\ air}\right)\left(\frac{K\ mol\ H_2 \times 1\ atm}{0.0826\ L\ H_2\ atm \times 298\ K}\right) = 1.02 \times 10^{-7}\frac{mol}{L}H_2$$

2. Calculate aqueous H_2 concentration in the water (mol/L):

$$[H_2]_{aq} = K_H \times P_{H_2}$$

$$[H_2]_{aq} = 7.8 \times 10^{-4}\frac{mol}{L\ atm} \times \left(\frac{2500}{10^9} \times 1\ atm\right) = 1.95 \times 10^{-9}\frac{mol}{L}H_2$$

3. Calculate total H_2 in container:

$$Total\ H_2 = ([H_2]_{gas} + [H_2]_{aq}) \times V$$

$$Total\ H_2 = \left(1.02 \times 10^{-7}\frac{mol}{L}H_2 + 1.95 \times 10^{-9}\frac{mol}{L}H_2\right) \times 0.02\ L = 2.07 \times 10^{-9}\ mol\ H_2$$

4. Assuming all H_2 present in from the original 20 mL water sample, calculate original H_2 concentration in the 20 mL of water sampled:

$$[H_2]_{aq,origina;} = \frac{Total\ H_2}{Original\ Volume} = \frac{2.07 \times 10^{-9}\ mol\ H_2}{0.02\ L} = 1.03 \times 10^{-7}\ M\ H_2$$

Appendix 2 – *Legionella* cultivation media
Legionella base agar was prepared according to manufacture specifications in 20 mL 100 mm sterile plates (Legionella Agar from Dot Scientific Manufacturer Neogen - http://foodsafety.neogen.com/pdf/Acumedia_pi/7728_pi.pdf). After sterilization at 121 °C for 15 minutes, 0.4 g/L L-Cysteine (Sigma-Aldrich), 3 g/L ammonium-free glycerin (BioRad), 80,000 units/L Polymyxin B (Enzo Life Sciences), 0.001 g/L vancomycin (P Biomedicals LLC), and 0.08 g/L cycloheximide (Sigma Aldrich) were added when the agar had cooled to approximately 50 °C.
For sample preparation for the CPVC rig, 10 ml of sample was centrifuged at 5000 X g for 30 minutes. Supernatant was discarded and pellet was suspended in 1 ml of sterile water. 0.1 ml was spread onto media described above in triplicate and incubated at 37 °C

for 2-4 days. Potential *Legionella* colonies were isolated via streaking onto media described above, incubated at 37 °C for 3 days, and then re-streaked onto the *Legionella* base agar with no L-cysteiene supplement. Those without growth on the L-cysteine negative plates were considered to be *Legionella*. Isolates were confirmed using end-point PCR with the *Legionella* spp. and *L. pneumophila* primers used for the quantitative polymerase chain reaction assays. The quantification limit for culturable *Legionella* was 20 CFU/mL (corresponding to having 20 countable colonies on each 0.1 mL plated after 10X concentration).

For the SGWHs, 0.1 or 1 mL was plated directly in triplicate, followed by the same procedure outlined above to confirm *Legionella* growth. The quantification limit for culturable *Legionella* was 200 CFU/mL (corresponding to having 20 countable colonies on each 0.1 mL volume plated) or 20 CFU/mL mL (corresponding to having 20 countable colonies on each 1 mL volume plated).

APPENDIX I – QUANTITATIVE POLYMERASE CHAIN REACTION ASSAYS

Sample preparation:

From the CPVC rig, approximately 0.5 L of first-flush water was collected directly from the influent, recirculating lines, and each distal tap at the end of regular stagnation periods for each use condition. For SGWHs, approximately 0.4 L was collected from each reactor. Exact volumes filter-concentrated was recorded and applied to each individual sample. Water was filtered through sterile 0.22-µm pore-size mixed cellulose ester filters (Millipore, Billerica, MA). Filters were fragmented and subjected to DNA extraction.

For biofilm sampling from the CPVC rig, 65 cm^2 of the influent, recirculating line, and ends of the distal tap pipes accessible by threaded union connections were swabbed using sterile cotton-tip applicators (Fisherbrand, Fisher Scientific, UK).

DNA was extracted directly from the fragmented filters and cotton swabs using a FastDNA Spin Kit (MP Biomedicals, Solon, OH) according to the manufacturer protocol. Field, trip, and equipment negative controls consisting of pre-sterilized (via filtration through 0.22 um pore-size filter and autoclaving 15 min at 121 °C) water in identical sampling bottles were included each time samples were collected.

Detailed Sampling Procedure:

All pipes were sampled during their regularly scheduled flush to avoid disrupting the usual patter of flow/stagnation in the system. To illustrate: High use pipes would flush at 8 AM, 4 PM, and 12 AM everyday. Medium use pipes would flush at 12 PM to stagger the addition of cold influent water to “shock” the systems on Mon, Wed, and Fri. Low use pipes would flush at 12 PM on Tuesday. So for sampling, High and Medium use pipes would sampled one day, and Low use pipes sampled the next day.

On the day of sampling, only water needed for the samples was collected to minimize the effects that flushing one pipe would have on the results of collecting samples from another pipe. We aimed to collected water that was continuously mixed in the tank/recirculating lines and water that was stagnant/slowly exchanging with the recirculating line in the distal taps. Previous estimates of the convective mixing gradient in these systems (See Rhoads et al., 2016) indicate that the volume exchanged while sampling occurred in the pipes with mixing (<2 minutes total) would be negligible.

For sampling order: when recirculating line samples were desired, we sampled the recirculating line first (this did introduce ~1.5 L of additional “fresh” cold water into the recirculating line in the 81 L water heater and recirculating line reservoir; we assumed this amount is negligible). Then we sampled the upward oriented pipes at once, one right after another. We did not allow the water to flush for the regular water change length, but instead cut off the water to only collect that volume that is necessary for our samples (3×~0.5L ≈ 1.5 L). We immediately then sampled the downward oriented pipes with no

mixing, again only collecting the volume of water necessary for our samples (~1.5 L). This was done to minimize the impact that sampling would have on the samples.

The regularly scheduled water changes of the high use pipe were allowed to proceed as scheduled, again, to not interrupt the regular flow/stagnation paper.

Assays:

Gene markers for *Legionella* spp., *L. pneumophila*, and *M. avium*, were enumerated by quantitative polymerase chain reaction (q-PCR) assays using previously established methods (Wang et al., 2013). In brief, all q-PCR assays were performed in 10 µL reaction mixtures containing SsoFast Probes or Evagreen Supermix (Bio-Rad, Hercules, CA), 250 or 400 nM primer, and 93.75 nM probe (Taqman assay only) with 1 µL of DNA template. DNA extracts (diluted 1:10 to minimize inhibition), a negative control, 10-fold serial dilutions of standards, and a positive spike into randomly selected sample DNA matrix were included in triplicate wells with each q-PCR run. The quantification limit (QL) for all q-PCR assays ranged from 50 to 100 gene copies/reaction and was implemented as appropriate for each run. Samples yielding threshold cycles ≥ QL in at least two q-PCR triplicate wells were considered quantifiable. Samples with only one triplicate above the QL threshold cycle or samples otherwise below the QL were re-analyzed undiluted to increase the QL of the assays. On each re-run plate, standard DNA template was spiked into the experimental DNA matrix to confirm that amplification reactions were not inhibited in undiluted samples. If inhibited, the sample was marked as below the QL. All values are reported as log(gene copies/mL + 1).

qPCR standards were PCR-amplified from environmental samples using traditional PCR and cloned using the TOPA TA Cloning Kit (Invitrogen, Carlsbad, CA). Cloned plasmids were linearized via PCR-amplification with M13 primers and Sanger sequenced to confirm template gene sequence.

R^2 values ranged from 0.983-0.996, 0.988-0.999, 0.970-0.995 for *Legionella* spp., *L. pneumophila*, and *M. avium* respectively. Amplification efficiencies ranged from 77.9-110%, 79.9-90.5%, and 80.9-97.5% for *Legionella* spp., *L. pneumophila*, and *M. avium* respectively.

APPENDIX J – FLOW CYTOMETRY

Bacterial cells in 0.5 mL aliquots of well mixed samples were stained with 5 µL of SYBRs Green I diluted 1:100 in TRIS buffer then incubated in the dark for 15 min before measurement. Where necessary, samples were diluted just before measurement in nanopure water (with background 1-3 event/µL).

Previous work has concluded that total cell counts is a reliable and useful parameter to monitor general microbial content of drinking water. It has correlated well with ATP in drinking water distribution systems (in the absence of treatments that would lead to large quantities of extra-cellular ATP such as ozonation). See:

Hammes, F., Berney, M., Wang, Y., Vital, M., Köster, O., & Egli, T. (2008). Flow-cytometric total bacterial cell counts as a descriptive microbiological parameter for drinking water treatment processes. Water Research, 42(1), 269-277.

Liu, G., Van der Mark, E. J., Verberk, J. Q. J. C., & Van Dijk, J. C. (2013). Flow cytometry total cell counts: a field study assessing microbiological water quality and growth in unchlorinated drinking water distribution systems. BioMed research international, 2013.

To confirm efficacy of this method prior to application in our lab, we conducted two bench-top measurments.

1. Confirmation of a known culture

E. Coli Strain K5808 was grown in 100 mL LB broth according to manufactures recommendations overnight at 37 °C for 12-18 hours to a concentration of approximately 10^9 CFU/mL. The grown culture was then diluted in nanopure water and run on the flow cytometer according to the procedure described above in 10-fold serial dilutions from $1{:}10^3$ to $1{:}10^6$. This was done in duplicate:

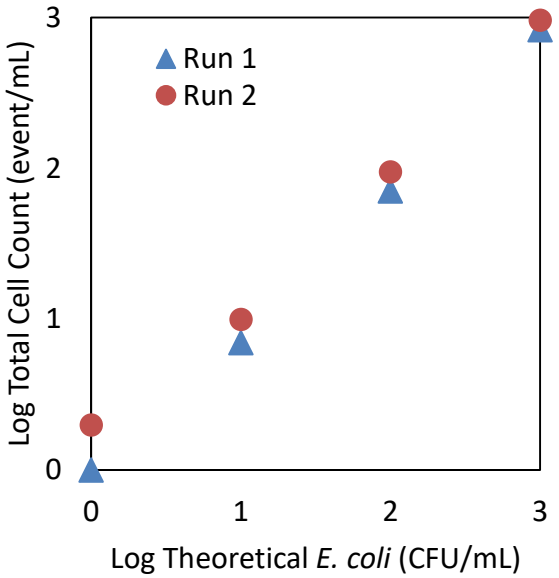


Fig. J.1 Serial dilutions of *E. coli* pure culture

2. Relative comparisons in environmental samples

To confirm that relative comparisons within our environmental sample matrix would hold, we also did serial dilutions of environmental samples in a similar manner as the *E. coli* pure culture. Theoretical cell count was calculated based on the measured value of the raw undiluted sample and dilution performed. For instance, the raw sample measured 3303 events/uL. For the first 1:1 dilution, the theoretical cell count is 1651.5 events/uL (3303 events/uL divided by 2) and the measured count is 1459 events/uL (11% error). Note on the graph below, slope is 1.01 and R^2 is 0.997, suggesting good relative comparisons of bacterial cells in a similar background matrix.

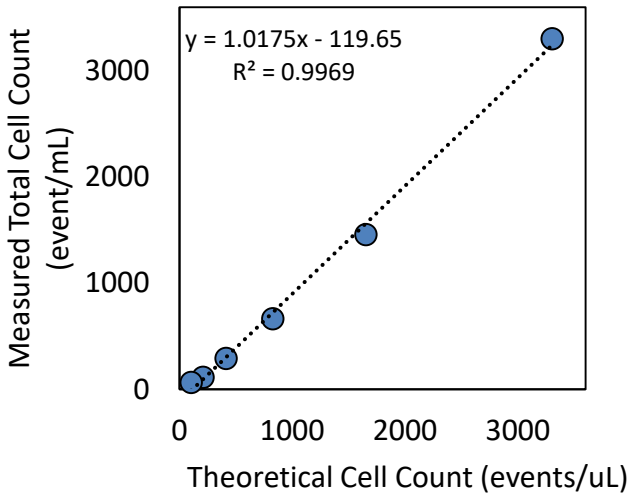


Fig. J.2. 1:1 dilution series of a SGWH sample.

APPENDIX K – CALCULATION FOR UNEXPLAINED H2 IN SYSTEM WITH COPPER PIPE AND RECIRCULATION RELATIVE TO WITH CUPRIC IONS (SEE TABLE 7.2)

There was more H2 produced in the system with copper pipe and recirculation relative to the system with cupric ions:

$$\frac{2146\ nM\ H_2}{1.9\ nM\ H_2} = 1129\ X\ more\ H_2$$

And the anode rod corroded faster:

$$\frac{15.1\ \%\ anode\ weight\ loss\ per\ year}{9.6\ \%\ anode\ weight\ loss\ per\ year} = 1.57\ X\ faster\ anode\ weight\ loss$$

If anode weight loss corresponds directly to H2 evolution, then there should be only 1.57X less H2 in the system with 9.6% weight loss relative to the system with 15.1% weight loss. And we could predict how much H2 the system with 9.6% anode weight loss should have:

$$\frac{2146\ nM\ H_2}{1.57} = 1366\ nM\ H_2\ predicted$$

But there was only 1.9 mM, so a large portion of the H2 generated in the system with higher weight loss is not explained only by the anode rod corroding:

$$\frac{1366\ nM\ H_2\ predicted}{1.9\ nM\ H_2\ accounted\ for\ by\ measurement} = 719\ X\ more\ H_2\ not\ explained$$

Thus, our hypothesis is that the form of copper (i.e., from copper pipe vs dosed as cupric) impacted the nature of the anode rod corrosion to favor the H2 evolution pathway. This increased the rate (as evidenced by the higher weight loss) and increased the evolution of H2 (as evidenced by the much more H2 produced). The impact of the deposition corrosion is likely dependent on the amount of copper that deposits and localized water chemistry parameters at the anode rod surface (I.e., dissolved oxygen and pH)

CHAPTER 8. CONCLUSIONS, FUTURE WORK, AND LESSONS LEARNED

Chapters 1-3 highlight the fact that OPs are native members of drinking water microbial communities and can proliferate in any domestic plumbing system when design and/or operational conditions are favorable for their growth. The work in Flint, MI represents an aging and failing infrastructure, under poor operating conditions, while the work in green buildings represents the cutting edge of new building designs. Although there are obvious and well-documented drawbacks to older systems with respect to OP growth, the results emphasize there are overarching design and operational parameters that can dictate when and how OPs grow. Chapters 5 and 6, in particular, expanded on this idea by exploring temperature as a driving factor for OP proliferation. The results showed there are circumstances where flow is both favorable and detrimental to OPs, and can be mediated or exacerbated by basic system design (i.e., pipe orientation) and operation (i.e., water use frequency) parameters.

Corrosion and water chemistry also emerged as critical overarching themes of this work. At one extreme, Chapter 1 outlined the massive impacts that corrosive water can have on all aspects of water distribution systems from damaging the physical integrity of pipes to shifting microbial communities to support OPs. This work highlights the importance of water chemistry in light of the fact that U.S. drinking water infrastructure is nearing its design life span. Water chemistry and corrosion control will be especially important moving forward to maintain distribution system integrity while solutions are sought for upgrading or replacing the aging pipes in place. In addition, as utilities seek out additional water supplies to meet future demand, such as direct potable water reuse, it will be important to carefully assess the impacts new sources may have on the existing infrastructure. Chapter 7 explored a more nuanced relationship between corrosion and OP growth, and investigated past discrepancies in the literature regarding the effects of copper on the growth of *Legionella*. While every impact of copper on *Legionella* growth has been documented in scientific literature, from complete inhibition of *Legionella* growth to actually facilitating its replication, this work presented mechanistic, water chemistry-driven potential explanations for the discrepancies.

Potential follow-up work and future directions of research from each of these overarching themes that deserve the attention of researchers in this field are briefly presented below.

CHLORINE DECAY

Accelerated chlorine decay observed in this and previous work is highly concerning. Maintaining a disinfectant residual within cold domestic plumbing is a critical factor for controlling OP growth. While hydraulic based chlorine demand models in main distribution systems have been presented (Rossman, 2002; Clark, 2015), there is little work investigating the practical range of chlorine demand in domestic plumbing. There is likely a highly variable rate of decay in individual building systems that are dependent on specific plumbing components and configurations, water use patterns and frequency, water use flow rates, background water chemistry (e.g., type of disinfectant applied, levels organic matter) and background water microbiology (e.g., types of levels of specific bacteria such as nitrifying or denitrifying bacteria). Exploring these factors and developing well-defined hydraulic models is a priority for future research.

BEST MANAGEMENT PRACTICES FOR GREEN BUILDINGS

For off-grid buildings applying rainwater collection practices, enough water must be stored to endure typical fluctuations in rainfall patterns. In the off-grid office building monitored in Chapter 3, the estimated average water age was up to 6 months. To avoid water sitting stagnant in the cistern before use, a portion of the stored water was circulated twice daily through the treatment system (GAC filtration and UV disinfection) and entire plumbing system. However, high gene numbers of OPs were still detected in this building. Typical rainwater cistern designs include a plug-flow design, wherein water flows through the treatment system one time and is distributed to the point-of-use. There is limited scientific evidence that one approach is better, and no head-to-head comparisons studying flow regimes in this type of system.

The unique water chemistry of pure rainwater (i.e., low pH and no alkalinity) also presents a challenge in limiting unwanted health risks. For instance, the water chemistry of untreated raw rainwater can be very conducive to lead leaching from in-line brass devices (Edwards et al., 1999). Identifying and subsequently finding solutions to these unique problems is a priority for future work regarding sustainable and safe water goals. This may include a national analysis of rainwater quality by region, to identify regional trends and chemistries that could be supportive of OP growth.

For buildings with high water age due to low water demand that are connected to municipal supply with secondary disinfectant residuals, the current temporary solution to water age related problems is to flush water through the system to introduce new "fresh" water and purge out the old "bad" water. While this has been demonstrated in one building, where as little as 1% of the total daily demand of the building was wasted to maintain water quality within the building (Nguyen et al., 2012), wasting water is inherently against the goals of sustainable building practices. There are research opportunities, which are closely tied with the chlorine demand modelling research topic above, in optimizing the water waster approach and finding alternative uses for the water that is flushed. For instance, in the building from Nguyen et al. (2012), water was wasted at only one location and had the desired effect. However, in more complex or larger buildings, flushing is typically recommend on several floors of the buildings and at various locations. Future work could focus on identifying a logical framework to approach optimizing flushing protocols.

Green building rating systems and green building plumbing codes also play a role in encouraging building owners to design their buildings to green standards. Their impact on water quality, by specifying or promoting use of water- and energy-saving technologies, sustainable materials, or alternative water sources, is largely unaccounted for in the rating systems reward structures or new green building codes. Developing strategies to quantitatively account for water age in design decision making would be beneficial to promoting green buildings systems that at least consider impacts of design decisions on water quality.

FLOW RATE

Several field studies have indicated very high levels of *Legionella* and *P. aeruginosa* in low flow rate devices, such as hands-free faucets (Syndnor et al., 2012; Zingg and Pittet, 2012; Charron et al., 2015; Halabi et al., 2001; Leprat et al., 2003; Merrer et al., 2005). Others have also observed

stagnation to trigger proliferation of *L. pneumophila* (Ciesielski et al., 1984; Harper, 1988; Muraca et al., 1987; Stout et al., 1986; Liu et al., 2006). This work has shed some insight into the effects of flow. For instance, the impact of flow frequency is dependent on the water quality (i.e., temperature, chlorine residual, nutrients) being delivered to distal pipes. However, the impact of flow rate within distal pipes has not been systematically studied. An initial survey of the literature in this area highlight the following hypotheses that deserve research:

- Biofilm in low flow pipes may remain more intact than biofilms in high flow rate pipes, allowing OPs to colonize and proliferate
- Biofilm and sediment accumulation in low flow pipes are more conducive to OPs because they react with and consume disinfectant residuals more quickly than biofilms and sediment in high flow rate pipes
- Temperature profiles in hot water pipes with low flow are more conducive to OPs because not enough hot water is drawn to increase water temperatures within the pipe enough to prevent or limit biofilm formation

BUILDING SCALE TEMPERATURE PROFILES

This work clearly indicated that temperature profiles within individual distal lines can undermine thermal controls applied to the water heater reservoir and main recirculation branches. One study has suggested a temperature diagnostic tool to focus monitoring efforts and has outlined a decision tree to assess and remediate problem areas (Bedard et al., 2015). Future directions for this work include identifying practical extremes of temperature profiles in high risk buildings (as defined by ASHRAE 188) and evaluating the effectiveness of the tool presented by Bedard et al., and iterating as necessary to improve it.

INTERACTIVE EFFECTS OF WATER CHEMISTRY AND COPPER

In Chapter 7, copper released from copper pipes, but not dosed as soluble cupric ions triggered the release of excess elemental hydrogen into the water as a result of deposition corrosion onto the water heater anode rod. In addition, when cupric ions were dosed directly to the pilot-scale rigs, the overall efficacy of the cupric ions as a disinfection mechanism were not as dramatic as expected.

Copper chemical speciation and solubility is likely to be a key, but largely unstudied, factor governing its toxicity towards *Legionella* (and other OPs) as well as influencing the deposition corrosion mechanism. Chemistry of cupric ion (Cu^{2+}), as the dominant thermodynamically favored copper species in potable water plumbing systems has been examined in cold water, but little work has been done at higher temperatures more relevant to the *Legionella* growth range because hot water is not regulated by the Lead and Copper Rule (i.e., consumers are simply advised not to drink hot water). Cuprous ion (Cu^{1+}) toxicity to *Legionella* has not been reported; however, it is hypothesized that it is more readily deposited (i.e., plated) onto less noble metals (zinc, aluminum, iron) than is Cu^{2+}, which can accelerate corrosion of relevant plumbing components (i.e., galvanized iron, steel, water heater anodes) and cause release of nutrients such as hydrogen (H_2) and iron to the water, as was observed in this work.

It is essential that we develop a fundamental understanding of copper speciation and solubility under relevant warm and hot water conditions in premise (i.e., building) plumbing. Current knowledge gaps undermine the potential to reliably tap into the natural antimicrobial properties of copper pipes for controlling OPs. In particular, understanding of the factors governing Cu^{+1}/Cu^{+2} speciation, their impacts on OP growth, and interactions with the broader microbial community is needed.

LESSONS LEARNED

Pilot-scale systems that simulate real-world plumbing conditions are useful research tools. They permit examination of practical controls that are implemented in full-scale systems under realistic conditions that are more likely to translate to real buildings systems than are bench-scale systems. However, these systems present an element of risk when pursing these types of long-term experiments if they do not provide the results you plan on them providing. While designing, constructing, and operating my systems, there are several lessons I have learned, that I would like to pass on to anyone else pursuing such an endeavor:

- Be ambitious with the design of the system, but know the cost associated with your ambition. With the required experimental replicates and multiple sampling events per condition for statistical power, it is easy to imagine how the design of a system can become complex. For my systems: 2 temperature settings × 2 pipe orientations × 3 water use frequencies × 3 for triplicate pipes = 36 pipes. I wouldn't recommend going much higher than this due to the logistics (and honestly, will power) of sampling that many conditions accurately, with the appropriate controls, and maintaining a high standard for the quality of your work. There were many times while doing the work I wished I hadn't included water use frequency as a variable because it essentially tripled the amount of work. At times, this many conditions seemed overwhelming (especially while writing up the first round of results), but in reflecting on the findings in each chapter, and the specific caveats I identified based on water use pattern, there is a high potential this work would not have been as impactful without it. So go for it, just know what you're getting into.
- Having said that, there seems to be a trade-off between experimental replicates and conditions being tested. For the research questions we were asking, it was more important for me to test all these conditions simultaneously because the very basic hypothesis was that they all interacted. If experimental replicates are more important than adding new conditions, or gain you more in terms of how impactful your conclusions are, this is worthwhile trade-off to make.
- Prototype your system (or changes to your existing system) on spare parts before implementing your design on the actual experimental apparatus. This piece of advice will also help with the previous note about realizing what you are getting into – you can determine how difficult it will be to run the system before you commit to a long-term experiment. The test-run also allows you to work out kinks in the design and/or operation of the system. For instance, when dosing copper to the experimental system in Chapter 7, I hooked up the chemical dosing pump to an extra water heater to make sure that the cupric sulfate in the stock solution remained soluble over time, that I could achieve my copper targets consistently without under or (more importantly) over shooting, and determine what I could expect in terms of routine maintenance needs. Prototyping will cost you mainly time (the Edwards lab is well-endowed with spare plumbing that, as far as I can tell, will

never be used and therefore was frequently subject to my pilfering), and will not solve all of your problems, but will allow you to get up the learning curve before you make a change to your main experimental apparatus.

- Chemistry and physics come before biology. For this work, we obviously value the biological results the most, but the biology has very little chance of working the way you expect if the chemistry and physics are off. It is always a good idea to make sure your temperature profiles, chlorine, ammonia, TOC, pH, DO, etc. are doing about what you would expect before you spend the time, money, and effort on biological sampling. If the chemistry is not right, neither will the biology be. After some initially frustrating results, I adopted the policy that I would nail down physical and chemical trends before I ever thought about qPCR. I was happier for it.
- Be productive while your system adapts to new experimental conditions. For my work, we wanted to test near steady-state results. This meant that after making a change to the systems, it was usually our practice to allow the system to adjust to the new condition before sampling again. While it is important to make sure your system is performing how think it should right after a switch, if you have extended down time, take advantage of it. For me, this meant field sampling (Chapter 3), volunteering on other projects, reading literature, writing backgrounds, getting trained on new techniques, etc.
- Use one lab notebook for each project, and always keep that notebook with/near that project in the lab. I have been very fortunate to have help from undergraduate researchers with my work, but I did not have the experience to see how frustrating each one having their own lab notebook would be. Going back to examine data from a specific time period of my work usually involves looking through at least four notebooks. Keep the project in one place, and do not let it leave to lab.
- Be a control freak about notetaking. Again, while I'm very fortunate to have had the help I have had, each of my undergraduate students had a different style of short-hand, different threshold for type and depth of information that should be recorded, etc. If/when you get help with your research, or for just yourself at the beginning of your degree, spend time to think about developing a system that will work for you so you can return three years later and answer simple questions about why trends in your work may have been occurring. You have to be able to understand and discern what was going on.
- Transfer things to whatever spreadsheet software you use promptly. This will 1) create an electronic format of the data that can be backed up and saved in multiple locations, 2) as you enter data (I recommend you do this, and not have an undergraduate do it), you familiarize yourself with the trends and can probably tell if something is not right – the sooner you catch it the better, and 3) allow you to up-date your collaborates (read: advisors) intelligently and promptly so they know what is going on with your work. They have a knack for asking good questions about your experiment, and often when you have answers in the moment, while you're sitting in the room together, it leads to cool insights.

REFERENCES

Rossman, L.A. (2002) EPANET 2 User's Manual, Water Supply and Water Resources Division, National Risk Management Research Laboratory, Cincinnati, OH45268.

Clark, R. (2015). The USEPA's distribution system water quality modeling program: a historical perspective, *Water and Environment Journal*, 29, 320-330.

Fisher, I., Kastl, G., Sathasivan, A., (2011). Evaluation of suitable chlorine bulk-decay models for water distribution systems. *Water Research*, 45, 4896–4908.

Monteiro, L., Figueiredo, D., Dias, S., Freitas, R., Covas, D., Menaia, J., Coelho, S. (2013). Modeling of chlorine decay in drinking water supply systems using EPANET MSX, 12th International Conference On Computing and Control For The Water Industry, CCWI 2013, Perugia, ITALY, September 2013, Vol. 70, 1192-12004 Modelling section

Seyoum, A., Tanyimboh, T., Siew, C. (2013). Assessment of water quality modelling capabilities of EPANET multiple species and pressure-dependent extension models, *Water Science and Technology-Water Supply*, 13(4), 1161-1166.

Kim, H., Hwang, I., Kwon, D. (2015). Prediction of water quality in piping system of bank filtrate, *Desalination and Water Treatment*, 54(4-5), 1393-1400.

Helbling, D., VanBriesen, J. (2009). Modeling Residual Chlorine Response to a Microbial Contamination Event in Drinking Water Distribution Systems, *Journal of Environmental Engineering* 135(10), 918-927.

Siew, C. Tanyimboh, T.T. (2010) Pressure-dependent EPANET extension: pressure-dependent demands. Proceedings of the 12th Annual Water Distribution Systems Analysis Conference, WDSA 2010, September 12-15, Tucson, Arizona.

Ladd, J. (2005). An Evaluation and Pressure-Driven Design of Potable Water Plumbing Systems, Master's Thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Sydnor, E.R., Bova, G., Gimburg, A., Cosgrove, S.E., Perl, T.M., Maragakis, L.L. (2012). Electronic-eye faucets: Legionella species contamination in healthcare settings. Infection Control and Hospital Epidemiology, 33(3), 235-240.

Zingg, W., Pittet, D. (2012). Electronic-Eye Faucets—Curse or Blessing? Infection Control & Hospital Epidemiology, 33(03), 241-242.

Charron, D., Bédard, E., Lalancette, C., Laferrière, C., Prévost, M. (2015). Impact of electronic faucets and water quality on the occurrence of Pseudomonas aeruginosa in water: A multi-hospital study. Infection Control & Hospital Epidemiology, 36(03), 311-319.

Halabi, M., Wiesholzer-Pittl, M., Schoberl, J., Mittermayer, H. (2001). Non-touch fittings in hospitals: a possible source of Pseudomonas aeruginosa and Legionella spp. The Journal of Hospital Infection, 49(2), pp. 117-21.

Leprat, R., Denizot, V., Bertrand, X., Talon, D. (2003). Non-touch fittings in hospitals: a possible source of Pseudomonas aeruginosa and Legionella spp. The Journal of Hospital Infection, 53(1), pp. 77.

Merrer, J., Girou, E., Ducellier, D., Clavreul, N., Cizeau, F., Legrand, P., Leneveu, M. (2005). Should electronic faucets be used in intensive care and hematology units? Intensive Care Medicine, 31(12), pp. 1715-1718.

van der Mee-Marquet, N., Bloc, D., Briand, L., Besnier, J.M., Quentin, R. (2005). Non-touch fittings in hospitals: a procedure to eradicate Pseudomonas aeruginosa contamination. The Journal of Hospital Infection, 60(3), pp. 235-9.

Yapicioglu, H., Gokmen, T.G., Yildizdas, D., Koksal, F., Ozlu, F., Kale-Cekinmez, E., Mert, K., Mutlu, B., Satar, M., Narli, N., Candevir, A. (2012). Pseudomonas aeruginosa infections due to electronic faucets in a neonatal intensive care unit. Journal of Pediatrics and Child Health, 48(5), pp. 430-4.

Ciesielski, C.A., Blaser, M.J., Wang, W.L. (1984). Role of stagnation and obstruction of water flow in isolation of *Legionella pneumophila* from hospital plumbing. *Applied and environmental microbiology*, 48(5), 984-987.

Harper, D. (1988). Legionnaires' disease outbreaks—the engineering implications. *Journal of Hospital Infection*, 11, 201-208.

Muraca, P., Stout, J.E., Yu, V.L. (1987). Comparative assessment of chlorine, heat, ozone, and UV light for killing *Legionella pneumophila* within a model plumbing system. *Applied and Environmental Microbiology*, *53*(2), 447-453.

Stout, J.E., Best, M.G., Yu, V.L., Rihs, J.D. (1986). A note on symbiosis of *Legionella pneumophila* and Tatlockia micdadei with human respiratory flora. *Journal of applied bacteriology*, 60(4), 297-299.

Liu, Z., Lin, Y.E., Stout, J.E., Hwang, C C., Vidic, R.D., Yu, V.L. (2006). Effect of flow regimes on the presence of *Legionella* within the biofilm of a model plumbing system. *Journal of applied microbiology*, 101(2), 437-442.