REDUCING THE PUBLIC HEALTH IMPACT OF INFECTIONS CAUSED BY WATERBORNE PATHOGENS

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ABSTRACT

Many opportunistic waterborne pathogens, including *Legionella* species, non-tuberculous mycobacteria, and *Pseudomonas aeruginosa*, can thrive in hot water systems despite municipal and traditional on-site disinfection. These organisms can cause healthcare-acquired infections in immunocompromised and elderly patients. This project aimed to assess and reduce the impact of waterborne pathogens (WBPs) in these populations.

In this study I developed a LAMP based assay that is specific for *L. pneumophila* that does not cross-react with other *Legionella* species or bacteria commonly found in either water or urine samples. This assay can detect *L. pneumophila* at a concentration of 400 cfu/mL and higher in contaminated water.

Evaluation of on-site monochloramine treatment over a two year period demonstrated a significant reduction in *Legionella* and total bacterial counts. The growth of other WBPs did not increase and the negative consequences seen in municipal monochloramine addition were not observed. Using Illumina sequencing I showed that the resulting shift in water microbial ecology over the course of monochloramine treatment was immediate and not gradual over time. This sequencing analysis revealed an increase in the relative abundance of certain non-*Legionella* WBPs throughout the course of chloramination. While molecularly the relative abundance increased, the total culturable bacterial counts decreased, likely resulting in no change overall.

I conducted a different sequencing study to look at the comparison of monochloramine treated and control water sampled at the same time points. This analysis showed significant differences in the richness, evenness, and composition of microbes present, related to treatment.

A field evaluation of a new point-of-use faucet filters showed them to be effective in preventing exposure to *Legionella* for 17 weeks. While these filters did not exclude all heterotrophs, there was a significant reduction in the amount of total bacteria and the three species present in filtered samples have not been found to cause human disease.

These studies have public health significance because they aid in the rapid detection of *L. pneumophila*, the cause of most cases of Legionnaires' disease. They have also evaluated the effects of on-site monochloramine disinfection and point-of-use filtration to prevent exposure to *Legionella* and other opportunistic waterborne pathogens.

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PREFACE

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1.0 INTRODUCTION

Every year approximately 2 million patients get an infection while being cared for in the hospital [1]. In 300,000 of these individuals their disease was caused by a pathogen they obtained directly from their hospital's water supply [1]. Many of these infections are caused by *Legionella* species, most commonly *L. pneumophila*, that is regularly isolated from many water sources including that of healthcare facilities. *Legionella* species are obligate aerobic, intracellular, gram negative bacteria [2]. There are greater than 50 species and greater than 70 serogroups encompassing in the genus, almost 50% of the species have been associated with disease in humans [2]. The species that causes over 90% of disease is *L. pneumophila*, with serogroups 1, 4, and 6 being most common of its 15 serogroups [2]. *Legionella* infections can cause two distinct types of disease: Pontiac Fever and pneumonia, also known as Legionnaires' disease (LD) [2]. LD is a potentially severe bacterial pneumonia that presents 2 to 14 days after exposure to contaminated water supplies [2]. The disease is characterized by fever, progressive pneumonia, stupor, and multi-system organ failure [2]. In contrast to other bacterial pneumonias, gastrointestinal symptoms including diarrhea are more commonly present in cases of LD [2]. The case fatality rate of healthcare associated Legionnaires' disease is quite high, ranging from 38%-53%, however, community acquired disease only carries approximately 20% fatality rate [2]. This is likely due to the lack of suspicion of LD because the health care provider is unaware their hospital is colonized and the prescribing of common pneumonia antibiotics that are ineffective

against *Legionella* bacteria. Among the 20,000 to 30,000 cases of Legionnaires' disease (LD) reported annually, approximately 25% are hospital acquired. The average length of hospital stay is 10.3 days but ranges from 1 to 84 days [3]. With a total of 13,000 patients hospitalized due to the disease per year [3]. It is estimated that the total cost of each case of LD per patient is upwards of $34,000 and that the total cost of all hospitalizations is over $433,000,000 [1, 3].

This is an enormous problem for the hospitalized, immunocompromised population, especially white males over 50 who are primarily affected by the disease [4]. Other risk factors include smoking, alcoholism, immunosuppression, and chronic pulmonary disease, as these patients more frequently aspirate water into their lungs [2]. Currently 97% of clinical diagnoses are obtained using a Urinary Antigen Test [4]. These tests use monoclonal antibodies that specifically recognize most *L. pneumophila* serogroup 1 lipopolysaccharide antigens, they however, do not detect disease caused by other serogroups of *L. pneumophila* or other species of *Legionella* and can miss some *L. pneumophila* serogroup 1 bacteria that do not match the epitopes included in the test [2]. Reports indicate that *L. pneumophila* serogroup 1 alone causes from 50%-80% of LD; the remaining 20%-50% of cases cannot be detected using accepted clinical diagnostic tests and remain largely undetected [2, 4]. There is a need for improvement in rapid diagnostic technologies that would be implemented for detection of more species of *Legionella* and cases of Legionnaires' disease.

Hospital water supplies can be contaminated not only with *Legionella* species but with many other opportunistic bacterial species which can include *Pseudomonas* spp., *Stenotrophomonas maltophilia*, *Chryseobacterium* spp., and nontuberculous mycobacteria [5]. *Legionella* and *Mycobacterium* species can be fairly resistant to traditional municipal chlorine water treatment and for that reason persist and cause disease in susceptible populations [2]. The

number of undiagnosed cases of LD is most likely due to a lack of awareness of *Legionella* exposure and facility contamination. Our laboratory has determined that if more than 30% of the outlets tested have *L. pneumophila* serogroup 1 then cases of Legionnaires' disease are likely to follow [6]. However many hospitals do not test their water for *Legionella* or other waterborne pathogens. In a recent report, only 55.5% of hospitals surveyed actually test their water for the presence of bacteria [1]. In a national survey of almost 200 hospitals it was found that each had at least one case of Legionnaires' disease and that 16% had at least 5 cases [1]. In a 2004 investigation by the United States Environmental Protection Agency, 61% of the hospital water supplies contained *Mycobacterium* species [1]. These statistics suggest that more hospitals should strive to test their water and implement better water treatment methodologies to prevent acquisition of waterborne infections in their hospital.

Our objective is to reduce the public health impact of waterborne pathogens, especially *Legionella,* in the immunocompromised and hospitalized populations. The proposed research will advance our understanding of methods used to detect and control *Legionella* (Aim 1). We will also support prevention of the disease by validating water treatment methodologies (Aims 2, 3, and 4). Our aims will fill considerable gaps in the diagnosis of Legionnaires' disease and in providing pathogen-free water to hospitalized patients.

2.0 HYPOTHESES AND SPECIFIC AIMS

Overall Aim: To reduce the public health impact of waterborne pathogens by advancing our understanding of methods to detect and control *Legionella* and preventing disease by validating water treatment methodologies.

Specific Aim 1: To develop a rapid test for the presence of *Legionella* spp. and *L. pneumophila* in both pure culture bacterial solutions and environmental samples

We hypothesize that our LAMP assay can detect the presence of the genus *Legionella* as well as the especially virulent *L. pneumophila* in both pure culture bacterial solutions and water samples

Specific Aim 2: To determine the effects of monochloramine treatment on the microbial flora of a hospital's water supply over time

We hypothesize that the microbial ecology of the hot water system will change over time due to monochloramine treatment and that *Legionella* species will be eliminated

Specific Aim 3: To determine the effects of an established monochloramine system on the microbial assemblages in a hospital's hot water supply

We hypothesize that chloraminated water will have a signficantly different microbiome than chlorinated water, with *Legionella* spp. largely removed and *Mycobacterium* spp. predominating chloraminated water

Specific Aim 4: To determine the efficacy of new point-of-use faucet filters in the removal of *Legionella* and *Pseudomonas* from the hospital water supply

We hypothesize that these faucet filters will prevent patient contact with water contaminated with *Legionella* spp., *P. aeruginosa*, and total bacteria for greater than 62 days

3.0 SPECIFIC AIM 1- TO DEVELOP A RAPID TEST FOR THE PRESENCE OF *LEGIONELLA* SPP. AND *L. PNEUMOPHILA* IN BOTH PURE CULTURE BACTERIAL SOLUTIONS AND ENVIRONMENTAL SAMPLES USING THE LAMP ASSAY

3.1 INTRODUCTION

3.1.1 *L. pneumophila* specific mip primer set

The public health burden of Legionnaires' disease has been calculated to be between 20,000 to 30,000 cases reported annually with approximately 25% being hospital acquired. *Legionella* species are isolated from many water diverse sources including that of healthcare facilities [2]. The species that causes over 90% of disease in the United States is *L. pneumophila*, with serogroups 1, 4, and 6 being most common of its 15 serogroups [2]. The case fatality rate of healthcare-associated Legionnaires' disease is quite high, ranging from 38%-53%, however, community acquired disease has an approximately 20% fatality rate [2]. This high case fatality rate may be in part due to the low index of suspicion for Legionnaires' disease and suboptimal sensitivity of *Legionella* diagnostic tests.

Currently, 97% of clinical diagnoses of Legionnaires' disease are obtained using urinary antigen tests [4]. These tests use monoclonal antibodies that specifically recognize most *L. pneumophila* serogroup 1 lipopolysaccharide antigens [2]. However, these tests do not detect

disease caused by other serogroups of *L. pneumophila* or other species of *Legionella* and do not detect *L. pneumophila* serogroup 1 bacteria that do not match the epitopes included in the test [2]. *L. pneumophila* serogroup 1 alone causes about 50%-80% of Legionnaires' disease; the remaining 20%-50% of cases cannot be detected using an FDA-cleared test [2, 4]. Other serogroups of *L. pneumophila* and other species are also important in disease causation, including serogroups 4 and 6, and species *L. micdadei* and *L. longbeachae* [7]. Efforts to improve diagnosis of non-*pneumophila Legionella* species and non-serogroup 1 *L. pneumophila* serogroups are needed.

Methods of molecular detection for *Legionella* species include PCR, real time or quantitative PCR, DNA microarray, and flow cytometry [8-15]. There is a novel isothermal DNA amplification technology called Loop-mediated isothermal amplification (LAMP) [16]. LAMP is able to amplify very small quantities of DNA template and is able to recognize six regions of template using four primers. The addition of two primers can increase the amount DNA produced and can decrease amplification time [16]. LAMP has several advantages over commonly used molecular tests: it does not need thermal cycling equipment or other expensive machinery [17], it is not inhibited by direct usage of biological materials [16, 18], it does not require DNA extraction prior to use, and it does not need extended amplification times. LAMP can also be read using turbidity or direct fluorescence which may make it better suited for use as a rapid diagnostic test [17]. LAMP assays have previously been developed for many pathogens, both viral and bacterial [16, 19, 20].

This new DNA amplification technology may improve upon *Legionella* detection methods, may allow for the detection of more serogroups of *L. pneumophila*, and may even detect more cases of Legionnaires' disease. The objective of this study was to design a unique set

of LAMP primers that would be specific for all 15 serogroups of *L. pneumophila*, but would not cross-react with other species of *Legionella* or other bacteria found in water or patient specimens. This *L. pneumophila* specific LAMP assay would, therefore, be applicable to both rapid environmental detection and clinical detection.

3.1.2 *Legionella* genus specific primer set cross reaction

Every year approximately 20,000 to 30,000 cases of Legionnaires' disease (LD) are reported, with approximately 25% being hospital acquired [2]. These infections are caused by *Legionella* species, most commonly *L. pneumophila*, that are regularly isolated from many water sources including that of healthcare facilities [2]. LD is highly fatal with the fatality rate of healthcare associated cases ranging from 38%-53%, and a community acquired case fatality rate of 20% [2].

Currently 97% of clinical diagnoses of Legionnaires' disease are obtained using a Urinary Antigen Test [4]. Reports indicate that 20%-50% of cases cannot be detected using accepted clinical diagnostic tests and remain largely undetected [2, 4]. It has also been shown that approximately 8% of patients with Legionnaires' disease do not excrete antigen in their urine [21]. Because of low sensitivities and other considerations several authors suggest that urinary antigen should not be the sole diagnostic measure for cases of LD and that culture and/or other molecular methodologies should be used [7, 22].

Numerous molecular methods for *Legionella* detection have been developed including, but not limited to, PCR [8, 9], real-time PCR and quantitative real-time PCR [10-12], DNA microarray [13], flow cytometry [14, 15], and loop-mediated isothermal amplification (LAMP) [23]. These methods have been and can be applied to the identification of many other microorganisms, pathogenic and nonpathogenic, of bacterial, fungal, and viral origins.

Loop-mediated isothermal amplification (LAMP) is a novel DNA amplification technology that amplifies in isothermal conditions [16]. It is very sensitive and specific for its target DNA sequence because it utilizes four primers, recognizing six regions of template [16]. Two additional primers can be designed to increase sensitivity and decrease the time of a LAMP reaction [16]. LAMP has several benefits over traditional PCR in that it is not affected by biological inhibitors [16, 18], it does not require high temperatures or cycling [17], it takes less time than PCR, and does not require DNA extraction. These unique properties of LAMP make it well suited for rapid diagnostic testing. LAMP assays have been developed for many types of pathogens both viral and bacterial [16, 19, 20, 24-26]. It has very recently been designed for *Legionella* spp. and *L. pneumophila* using the 16S rRNA gene [23]. This study showed LAMP to be both specific and sensitive for the bacterial reference strains and environmental samples tested [23]. We decided to design our own LAMP primers for the detection of *Legionella* species and *L. pneumophila* specifically and validate the assay using a larger number of *Legionella* species, bacteria commonly found in patient urine samples, and other species of waterborne pathogens.

3.2 MATERIALS AND METHODS

3.2.1 *L. pneumophila* specific mip primer set

Primer design: The sequences for the macrophage infectivity potentiator (mip) gene of *L. pneumophila* serogroups 1, 3, 6, 7, 8, 9, 10, 12, 13, and 14 were found on GenBank (http://www.ncbi.nlm.nih.gov/nuccore) and aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). Eight template regions labeled F3, F2, LF, F1, B1,

LB, B2, and B3 were chosen with approximately 50% GC content that cover an approximately 300 nucleotide region of the gene. These regions allowed for the design of six primers F3, FIP (F1 reverse complemented + TTT + F2), LF (LF reverse complemented), B3 (B3 reverse complemented), BIP (B1 reverse complemented + TTT + B2), and LB. Primer sequences are listed in Table 3.1. Primers were synthesized by IDT and used for all subsequent LAMP and PCR reactions. The outermost F3 and B3 primers were used for PCR.

Pure culture bacterial suspensions: Pure cultures of each strain of bacteria tested (listed in Table 3.2) were grown on BCYE agar, diluted to a 3 McFarland standard turbidity (approximately 9×10^8 cfu/mL), and boiled for 15 minutes prior to use.

Environmental sample preparation: Water samples were concentrated by filtering 100 mL through a 0.2 micron filter membrane which was placed in 10 mL of the sample. From this 500 uL was centrifuged at 15,000 x g for 10 minutes, the supernatant was removed and the pellet was resuspended in 20 uL sterile water. Samples were boiled in a water bath for 10 minutes before use [27].

LAMP and PCR conditions: LAMP primers were pooled in a 1: 4: 8 (F3/B3: LF/LB: FIP/BIP) ratio. For each 25 uL LAMP reaction the following reagents were used: 2.5 uL of 10X ThermoPol Reaction Buffer (New England Biolabs), 2 uL of 10 mM deoxynucleotide mix (Sigma), 2 uL of Magnesium Sulfate ($MgSO_4$) Solution (New England Biolabs), 5 uL of Betaine solution (Sigma), 1 uL of Bst DNA polymerase, large fragment enzyme (New England Biolabs), 2.5 uL of Sterile Water (Fisher), 5 uL of the LAMP primer pool, and 5 uL of the sample. LAMP was run for 60 minutes at 61^{o}C and 10 minutes at 80^{o}C. For each 25 uL PCR reaction the following reagents were used: 2.5 uL of 10X ThermoPol Reaction Buffer (New England Biolabs), 2 uL of 1.25 mM deoxynucleotide mix (Sigma), 1.25 uL of the F3 primer, 1.25 uL of

the B3 primer, 0.25 uL of Taq DNA polymerase (New England Biolabs), 12.75 uL of Sterile water (Fisher), and 5 uL of the sample. PCR was run for 3 minutes at 94^{o}C, cycled for 30 seconds at 94^{o}C, 30 seconds at 57^{o}C, then 1 minute at 72^{o}C for 35 cycles, and 7 minutes at 72^{o}C. A 2% agarose gel was run to visualize LAMP and PCR products.

3.2.2 *Legionella* genus specific primer set cross reaction

Primer design: The sequences for 16S rRNA gene of *Legionella* species including *L. anisa*, *L. bozemanii*, *L. dresdeniensis*, L. *dumoffii*, *L. gormanii*, *L. longbeachae*, *L. pneumophila* were found on GenBank (http://www.ncbi.nlm.nih.gov/nuccore) and aligned using ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2). Eight template regions labeled F3, F2, LF, F1, B1, LB, B2, and B3 were chosen with approximately 50% GC content that cover an approximately 300 nucleotide region of the gene. These regions allowed for the design of six primers F3, FIP (F1 reverse complemented + TTT + F2), LF (LF reverse complemented), B3 (B3 reverse complemented), BIP (B1 reverse complemented + TTT + B2), and LB. Primer sequences are listed in Table 3.4. Primers were synthesized by IDT and used for all subsequent LAMP and PCR reactions. The outermost F3 and B3 primers were used for PCR.

LAMP and PCR conditions: Pure cultures of each strain of bacteria tested (noted in Figure 1) were grown on BCYE agar, diluted to a 3 McFarland standard turbidity (9 x 10^{8} cfu/mL), and boiled for 15 minutes prior to use. LAMP primers were pooled in a 1: 4: 8 (F3/B3: LF/LB: FIP/BIP) ratio. For each 25 uL LAMP reaction the following reagents were used: 2.5 uL of 10X ThermoPol Reaction Buffer (New England Biolabs), 2 uL of 10 mM deoxynucleotide mix (Sigma), 2 uL of Magnesium Sulfate ($MgSO_4$) Solution (New England Biolabs), 5 uL of Betaine solution (Sigma), 1 uL of Bst DNA polymerase, large fragment enzyme (New England

Biolabs), 2.5 uL of Sterile Water (Fisher), 5 uL of the LAMP primer pool, and 5 uL of the sample. LAMP was run for 60 minutes at 61^{o}C and 10 minutes at 80^{o}C. For each 25 uL PCR reaction the following reagents were used: 2.5 uL of 10X ThermoPol Reaction Buffer (New England Biolabs), 2 uL of 1.25 mM deoxynucleotide mix (Sigma), 1.25 uL of the F3 primer, 1.25 uL of the B3 primer, 0.25 uL of Taq DNA polymerase (New England Biolabs), 12.75 uL of Sterile water (Fisher), and 5 uL of the sample. PCR was run for 3 minutes at 94^{o}C, cycled for 30 seconds at 94^{o}C, 30 seconds at 57^{o}C, then 1 minute at 72^{o}C for 35 cycles, and 7 minutes at 72^{o}C. A 2% agarose gel was run to visualize LAMP and PCR products.

Phylogenetic analysis: A phylogram was made to compare the 16S rRNA genes of the bacterial species tested using the ClustalW2 Phylogeny website (http://www.ebi.ac.uk/Tools/phylogeny/clustalw2_phylogeny). A complete list of the species compiled is in Figure 3.3.

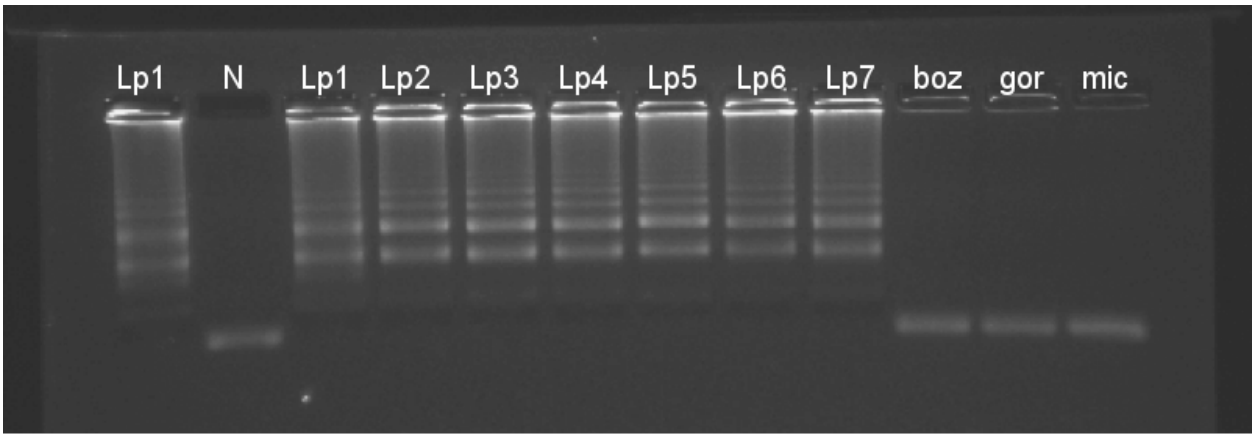
3.3 RESULTS

3.3.1 *L. pneumophila* specific mip primer set

Specificity of LAMP primers for 15 L. pneumophila serogroups: Due to the limited specificity of approved clinical tests for *Legionella* species, LAMP primers were developed based on the mip gene to selectively amplify all *L. pneumophila* serogroups. To determine the specificity of these primers for *L. pneumophila* serogroups LAMP and PCR primers were tested against pure cultures of non-*pneumophila Legionella* species, *L. pneumophila* serogroups, and bacteria commonly found in urine and water. LAMP primers detected 15 of the 15 serogroups of

L. pneumophila tested (Figure 3.1 and Table 3.2). These results suggest that this assay may be useful for clinical diagnosis as these primers can already identify more serogroups of *L. pneumophila* than urinary antigen tests, especially the clinically relevant serogroups 1, 4, and 6 (Figure 3.1). The LAMP primers failed to amplify bacteria commonly found in patient specimens, water, or other species of *Legionella* (Table 3.2). This further suggests the utility of these primers for clinical diagnosis and environmental detection as they do not cross-react with non-*L. pneumophila* bacteria. While not the primary goal of our study, PCR primers were also developed as part of the LAMP primer set. These primers were also tested for their specificity against the same pure culture bacterial solutions (Table 3.2). PCR primers were specific for all 15 *L. pneumophila* serogroups but they also amplified *L. moravica* (Table 3.2).

Sensitivity of LAMP primers for detection of L. pneumophila in environmental samples: To determine the utility of these primers for detection of *L. pneumophila* in environmental water samples, water with varying concentrations and species of *Legionella* was filter concentrated, centrifuged, and boiled then probed with the LAMP primers. Water samples were determined by standard culture methods to contain *L. pneumophila*, *L. micdadei, L. bozemanii*, and other blue-white *Legionella* species at concentrations ranging from less than 10 cfu/mL to greater than 3000 cfu/mL (Table 3.3). LAMP failed to detect *Legionella* species other than *L. pneumophila* serogroup 1, and was positive only when the concentration of *L. pneumophila* serogroup 1 was greater than or equal to 400 cfu/mL (Table 3.3).



The specificity of mip LAMP primers was tested with different serogroups of *L. pneumophila* (1-7) and other *Legionella* spp. (boz= *L. bozemannii*, gor= *L. gormanii*, mic= *L. micdadei*, N= negative control).

Figure 3.1. LAMP primers are specific for all 15 *L. pneumophila* serogroups.

Table 3.1. LAMP and PCR primer sequences designed for *L. pneumophila* detection

Primer name	Sequence of mip primers
F3[a]	5'- GATGCCACATCATTAGCTAC-3'
FIP	5'- CATAGCGTCTTGCATGCCTTTTTGCATTGGTGCCGATTTGGGGA-3'
LF	5'- GCCATTGCTTCCGGATTAAC-3'
B3[a]	5'- GCAATACAACAACGCCTGGCTTG -3'
BIP	5'- GGTTAAAGCCAATTCAGCGCCTTTGGGGAAGCCTTTTTAACTG -3'
LB	5'- GTTTCAGAAAGATTTGATGGC -3'

[a]These primers were utilized for PCR reactions

Table 3.2. LAMP primers are specific for all 15 *L. pneumophila* serogroups.

		ATCC #	LAMP	PCR
Legionella species	*L. pneumophila* sg 1	33152	+	+
	L. pneumophila sg 2	33154	+	+
	L. pneumophila sg 3	33155	+	+
	L. pneumophila sg 4	33156	+	+
	L. pneumophila sg 5	33216	+	+
	L. pneumophila sg 6	33215	+	+
	L. pneumophila sg 7	33823	+	+
	L. pneumophila sg 8	35096	+	+
	L. pneumophila sg 9	35289	+	+
	L. pneumophila sg 10	43283	+	+
	L. pneumophila sg 11	43130	+	+
	L. pneumophila sg 12	43290	+	+
	L. pneumophila sg 13	43736	+	+

Table 3.2 Continued

	L. pneumophila sg 14	43703	+	+
	L. anisa	35292	-	-
	L. birminghamensis	43702	-	-
	L. bozemannii	33217	-	-
	L. dumoffii	33279	-	-
	L. feeleii (sg 2)	ELITE	-	-
	L. fraseri (*L. pneumophila* sg 15)	35251	+	+
	L. gormanii	33297	-	-
	L. hackeliae	35250	-	-
	L. israelensis	43119	-	-
	L. jordanis	33623	-	-
	L. longbeachae	HPA	-	-
	L. maceachernii	35300	-	-
	L. micdadei	33218	-	-
	L. moravica	43877	-	+
	L. oakridgensis	HPA	-	-
	L. quateirensis	49507	-	-
	L. sainthelensi	35248	-	-
	L. santicrucis	35301	-	-
	L. wadsworthii	ELITE	-	-

Urine Bacteria	*E. coli*	25922	-	-
	K. pneumoniae	n/a	-	-
	E. faecalis	51299	-	-
	C. albicans	14053	-	-
	S. aureus	25923	-	-
	S. epidermidis	12228	-	-
	P. mirabilis	n/a	-	-

Water Bacteria	*P. aeruginosa*	27853	-	-
	S. maltophilia	51331	-	-
	A. baumannii	19606	-	-
	E. meningoseptica	13253	-	-
	A. hydrophila	35654	-	-
	M. gordonae	n/a	-	-
	M. chelonae	n/a	-	-
	M. mucogenicum/phocaicum	n/a	-	-
	M. avium	13950	-	-

Data shown are the results of LAMP and PCR testing of pure culture bacterial strains

Table 3.3. LAMP primers can detect *L. pneumophila* in environmental samples at a concentration of 400 cfu/mL and greater.

Legionella Species	Concentration in bulk	Result
micdadei	1-10 cfu/mL	-
micdadei	10 cfu/mL	-
micdadei	10 cfu/mL	-
bozemanii	20 cfu/mL	-
blue white spp.	60 cfu/mL	-
pneumophila sg 1	1-10 cfu/mL	-
pneumophila sg 1	1-10 cfu/mL	-
pneumophila sg 1	10 cfu/mL	-
pneumophila sg 1	10 cfu/mL	-
pneumophila sg 1	50 cfu/mL	-
pneumophila sg 12	70 cfu/mL	-
pneumophila sg 1	130 cfu/mL	-
pneumophila sg 1	160 cfu/mL	-
pneumophila sg 1	190 cfu/mL	-
pneumophila sg 1	250 cfu/mL	-
pneumophila sg 1	350 cfu/mL	-
pneumophila sg 1	350 cfu/mL	-
pneumophila sg 1	370 cfu/mL	-
pneumophila sg 1	400 cfu/mL	+
pneumophila sg 12	460 cfu/mL	+
pneumophila sg 1	550 cfu/mL	+
pneumophila sg 1	640 cfu/mL	+
pneumophila sg 1	650 cfu/mL	+
pneumophila sg 1	850 cfu/mL	+
pneumophila sg 1	900 cfu/mL	+
pneumophila sg 1	1750 cfu/mL	+
pneumophila sg 12	~3000 cfu/mL	+
pneumophila sg 1	>3000 cfu/mL	+

Data show the results of the LAMP assay on 28 environmental water samples

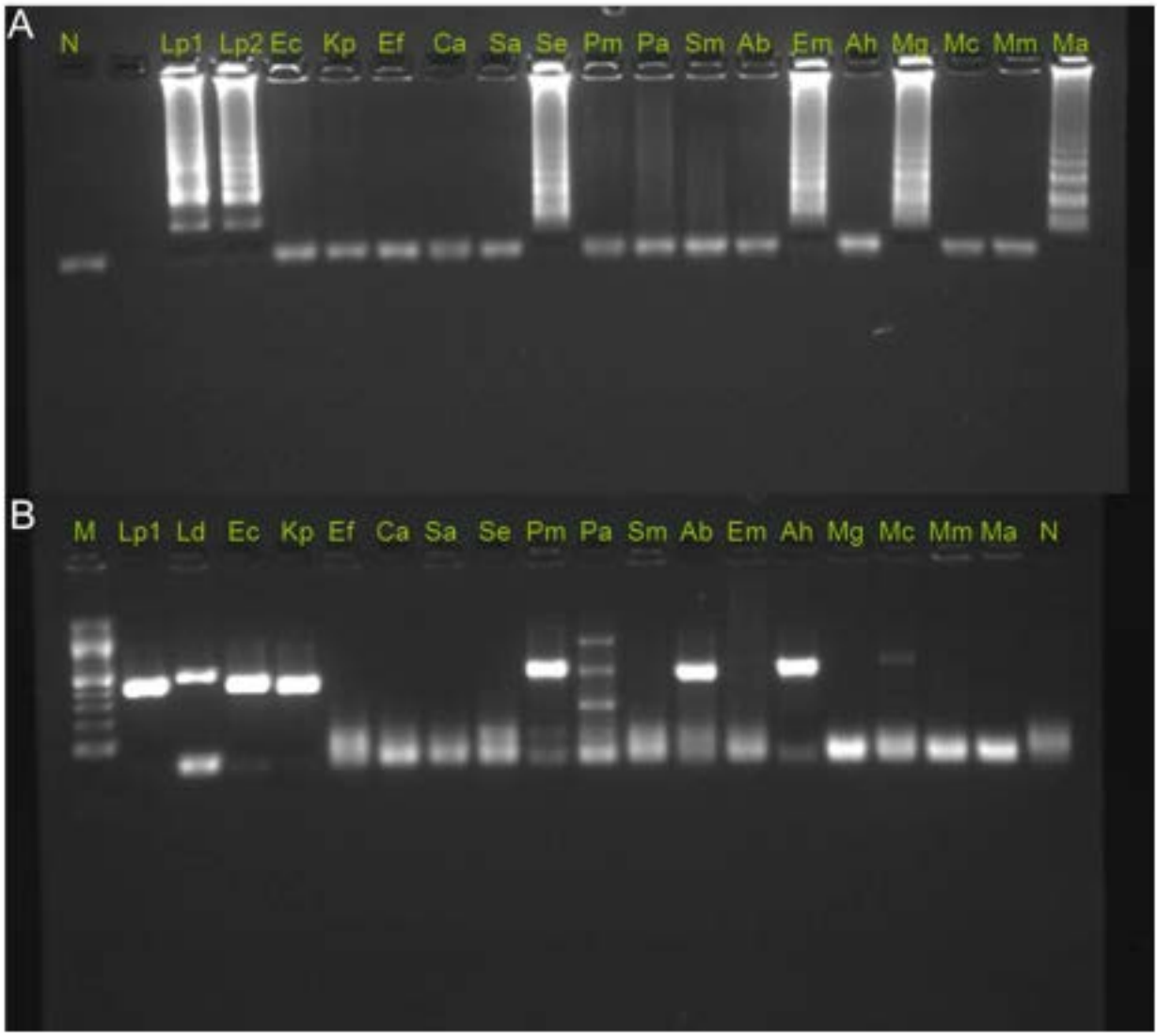
3.3.2 *Legionella* genus specific primer set cross reaction

In our attempt to design primers specific for the genus *Legionella* we noted an interesting cross-reaction using both LAMP and PCR with several non-*Legionella* species of bacteria that were non-overlapping between the assays (Figure 3.2). By LAMP the cross-reaction included the following organisms: *Staphylococcus epidermidis*, *Elizabethkingia meningoseptica*, *Mycobacterium avium*, and *M. gordonae* (Figure 3.2A). Organisms showing cross-reaction by PCR were: *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Acinetobacter baumanii*, and *Aeromonas hydrophila* (Figure 3.2B). The observation that no species that was positive by LAMP was also positive by PCR and vice versa was intriguing.

To explain this phenomenon, the 16S rRNA gene sequences of all of the bacteria tested were compared phylogenetically. The sequences of *Legionella* species as well as the other genera demonstrated a great degree of similarity, on average 86% sequence similarity (Figure 3.3). This would suggest an explanation for the observed cross-reaction of the 16S rRNA primers designed for LAMP and PCR. Interestingly, the results of the phylogram correspond directly with the results from LAMP and PCR run with these primers (Figure 3.2). The least closely related, non-*Legionella* species cross-reacted with LAMP primers (Figure 3.2A), whereas the most closely related bacterial species cross-reacted with the PCR primers (Figure 3.2B). All *Legionella* spp. LAMP and PCR primers that were designed were found to be 89-100% identical to the 16S rRNA sequences of the bacterial species tested.

Table 3.4. LAMP and PCR primer sequences designed for *Legionella* genus level detection

Primer name	Sequence of 16S rRNA primer
F3	5'-ATGCAAGTCGAACGGCAGCA-3'
FIP	5'-CCACCAACTAGCTAATCGGATTTGTAACGCGTAGGAATATGCC-3'
LF	5'-TAATCTTAAAGCGCCAGGCC-3'
B3	5'-AGGCCTTCTTCACACACGC-3'
BIP	5'-CCGATCGTCGCCTTGGTATTTAACCCTGATCCAGCAATG -3'
LB	5'-GTGGGGAATATTGGACAATGG-3'



The following bacteria were used: *Legionella pneumophila* serogroups 1 and 2 (Lp1, Lp2), *L. dumoffii* (Ld), *E. coli* (Ec), *K. pneumoniae* (Kp), *E. faecalis* (Ef), *C. albicans* (Ca), *S. aureus* (Sa), *S. epidermidis* (Se), *P. mirabilis* (Pm), *P. aeruginosa* (Pa), *S. maltophilia* (Sm), *A. baumanii* (Ab), *E. meningoseptica* (Em), *A. hydrophila* (Ah), *M. gordonae* (Mg), *M. chelonae* (Mc), *M mucogenicum/phocaicum* (Mm), *M. avium* (Ma), and a negative control (N). The 100bp DNA ladder is labeled M. Cross reactivity with 16S rRNA gene was seen for the following non-Legionella bacteria by LAMP: Se, Em, Mg, and Ma, or by PCR: Ec, Kp, Pm, Pa, Ab, and Ah.

Figure 3.2. Cross reaction of 16S rRNA primers with other bacterial species by LAMP (A) and PCR (B).

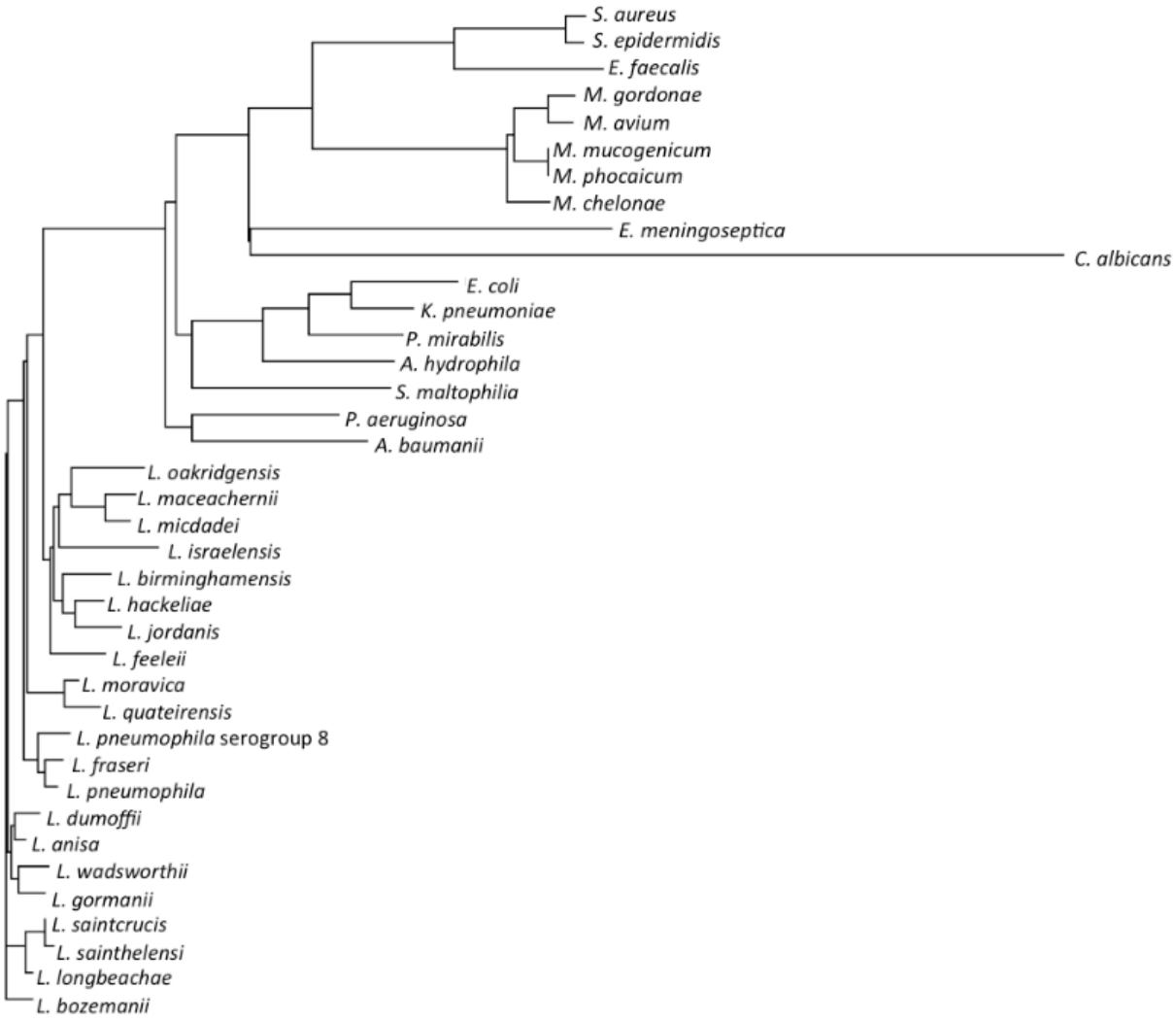


Figure 3.3. Phylogram of the genetic relatedness of 16S rRNA sequences of *Legionella* species and other bacterial species tested using both LAMP and PCR.

3.4 DISCUSSION

3.4.1 *L. pneumophila* specific mip primer set

We designed a unique set of LAMP primers, targeting the mip gene, that only detected the 15 serogroups of *L. pneumophila*. We designed and tested PCR primers that also reacted with *L. moravica*. The cross reaction with *L. moravica* would not have an effect on these PCR primers being used on clinical samples, as *L. moravica* has not yet been linked to human disease but may pose a problem in rare cases for environmental detection. We were unable to find previously published mip PCR primers that were tested against *L. moravica* so we do not know if this cross-reaction has been seen before. Our LAMP primers were able to detect concentrations of 400 cfu/mL and above in environmental water samples.

In the United States, 97% of clinical diagnoses of Legionnaires' disease are made using a urinary antigen test [4]. These tests, will miss between 20% and 50% of cases in part because the urinary antigen test only detects *L. pneumophila* serogroup 1 [2, 4]. This is a limitation as other serogroups of *L. pneumophila* including serogroups 4 and 6, and species *L. micdadei* and *L. longbeachae*, cause a significant proportion of Legionnaires' disease cases [7]. Our LAMP assay detects not only *L. pneumophila* serogroup 1, but also serogroups 2-15, which should identify *Legionella* infection in a larger number of individuals. A number of other methods of molecular detection for *Legionella* have been developed including: PCR, real time and quantitative PCR, DNA microarray, and flow cytometry [8-15]. LAMP is a promising technology for rapid pathogen detection as it does not require the specialized machinery, DNA extraction, or as much time to run as these other methods. It only requires 80 minutes of 61^{o}C amplification compared to 2-3 hours for PCR.

A different set of LAMP primers for the genus *Legionella* and *L. pneumophila* using the 16S rRNA gene was previously designed [23]. These LAMP primers were 100% specific and 100% sensitive, using environmental bacterial strains from water sources in China [23]. However, this previous report only tested the assay's specificity against nine other species of *Legionella* and did not test all of the serogroups of *L. pneumophila*. In addition, many clinically relevant pathogens found in water and urine were not tested [23]. In an attempt to make genus specific *Legionella* primers using the 16S rRNA gene, we discovered cross-reaction with other clinically and environmentally significant organisms including: *Acinetobacter baumannii*, *Aeromonas hydrophila*, *Elizabethkingia meningoseptica*, *Escherichia coli*, *Klebsiella pneumoniae*, *Mycobacterium avium*, *M. gordonae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* (J.L. Baron et al., submitted for publication). Based on the conserved nature of the 16S rRNA gene, other authors suggest avoiding the use this gene in the resolution of bacteria to the genus level and recommend protein-encoding genes be used instead [28, 29]. We utilized the protein-encoding mip gene which, in our assay, was demonstrated to be 100% specific for *L. pneumophila* serogroups.

In clinical diagnostic situations it could be beneficial to have an assay that can simultaneously detect multiple levels of bacterial identification, genus versus species versus serogroup, or multiple pathogens in the same sample, *L. pneumophila* versus *L. longbeachae*. The first strategy has been previously employed in real-time PCR detection of *Legionella* species, *L. pneumophila*, and *L. pneumophila* serogroup 1, using primers developed for the *ssrA*, *mip*, and *wzm* genes, respectively [11]. A new method for modifying LAMP primers allows for real-time, quantitative LAMP reactions for use in molecular diagnostics [30]. While this assay has not yet been applied to multiple species of *Legionella*, it can identify up to four different

bacterial pathogens in the same sample [30]. Additional LAMP primers could be designed to detect other species of *Legionella* including those that are responsible for Legionnaires' disease in different geographic areas, such as *L. longbeachae* in Australia and New Zealand. Our *L. pneumophila* specific LAMP primers and newly designed *Legionella* species LAMP primers could be multiplexed to allow for simultaneous identification of multiple *Legionella* species in one real-time LAMP reaction.

LAMP could also be adapted for environmental field use as long as water filtration could take place and a constant reaction temperature of around 60^{o}C could be maintained. While the limit of detection for our assay in environmental water samples is fairly high, around 400 cfu/mL, we believe that this is a good start towards rapid detection of *L. pneumophila* in environmental samples. However, more evaluation needs to be done to lower the limit of detection for environmental water samples. This could be attained by refining the filter concentration method or the amount of water filtered to increase *Legionella* DNA template for amplification. It should be possible to detect lower concentrations of DNA with LAMP without needing to subject samples to traditional DNA extraction methodologies. This is important as the possibility of use of LAMP as a field test would not allow for long or complicated DNA extraction procedures. This point is especially important when comparing the mip LAMP assay to our conventional PCR. Our results suggest that LAMP may be a better technique for detection *L. pneumophila* using our mip primers, in environmental water samples, and possibly in the in future patient samples, due to its specificity and potential for use in the field.

In summary, we have designed LAMP primers that are specific for all 15 *L. pneumophila* serogroups. This lays the foundation for use of our LAMP primers to increase the identification of cases of Legionnaires' disease, especially those caused by non-serogroup 1 *L. pneumophila*.

LAMP appears to be a promising assay for *L. pneumophila* detection both in clinical and environmental samples. Rapid detection of *L. pneumophila* using a LAMP-based assay may improve diagnosis in patients and initiate earlier antibiotic therapy for Legionnaires' disease. Moreover a LAMP-based assay may provide information relevant to environmental disinfection.

3.4.2 *Legionella* genus specific primer set cross reaction

This 16S rRNA sequence similarity across bacteria has been previously noted [31] and used to the advantage of molecular biologists as a method for global bacterial sequencing in many settings including water, the human body, and the air [32-39]. The small ribosomal subunit is essential for mRNA translation so it would follow that substantial alterations to this gene could not be sustained evolutionarily. These facts suggest that the use of 16S rRNA gene to develop primers to specifically distinguish whole genera of bacteria may be particularly difficult or even technically impractical. Some authors suggest also using a protein-encoding gene to resolve lower taxonomic relationships such as genus [28].

In recent years, several authors have shown higher counts of *Legionella* obtained by PCR than measured by *Legionella* specific microbiological culture or PCR positivity when culture results showed no *Legionella* presence [40-42]. They attribute this to the presence of viable but nonculturable (VBNC) *Legionella* [40], of nonviable *Legionella* [42, 43], or to the presence of *Legionella* living within amoebae [41, 42]. However, all of the bacterial species we found exhibiting cross-reactions by LAMP or PCR with *Legionella* are commonly found in both environmental water samples and clinical samples. We believe that these organisms may represent an important source of false positivity in PCR assays used for *Legionella* detection. While we do not discount the presence of VBNC, nonviable, or amoebae-living *Legionella*, we

suggest that at least some of this false positivity may be due to the presence of other bacteria, such as *P. aeruginosa*, *A. baumanii*, *A. hydrophila*, or *Mycobacterium* species, in water that are being detected through cross-reaction with the designed 16S rRNA PCR primers. Anecdotally, it has been noted that a set of primers designed for amplifying and sequencing the *Aeromonas* 16S rRNA gene are equally effective and routinely used for recovering the *Legionella* 16S rRNA gene target (R. Ratcliff, personal communication).

We propose that future LAMP and PCR primers sets made for pathogen detection be restricted to a few species or use an organism specific gene. One author has recently demonstrated that protein-encoding genes offer better *Legionella* strain identity than the 16S rRNA gene [29]. This is likely due to the relatively low percentage of informative base sites uncovered in the 16S rRNA gene, as compared to the other four genes examined. Given these findings, caution should be used when generating and validating primers for microorganisms, especially pathogenic ones. Cross-reaction with other bacterial species should be exhaustively tested to ensure the specificity of these assays, especially when the goal is to use them in environmental detection or clinical diagnosis, as these results may lead to substantial disinfection and/or healthcare costs, misdiagnosis, and even the death of infected individuals.

4.0 SPECIFIC AIM 2- TO DETERMINE THE EFFECTS OF MONOCHLORAMINE TREATMENT ON THE MICROBIAL FLORA OF A HOSPITAL'S HOT WATER SUPPLY OVER TIME USING CULTURE (2A) AND SEQUENCING (2B) METHODOLOGIES

4.1 INTRODUCTION

Drinking water distribution systems, including premise plumbing, contain a diverse microbiological population [44]. Once new pipes have been added to an existing system, microbial colonization begins rapidly, with microbial communities being established in as little as one year [33]. For the purposes of this study, the 'microbial community' is defined as planktonic microbes within the hospital hot water system during the study period. The microbial ecology of drinking water distribution systems varies widely, depending upon system parameters such as disinfection scheme [45], hydraulic parameters [46], location in the system, age of the system [47], and pipe materials [48]. Microbes are capable of corroding pipes within distribution systems, possibly releasing harmful chemicals such as lead [49-51]. It is largely believed that within a drinking water distribution system, the disinfection scheme is one of the primary factors controlling the abundance and make-up of microbes [45, 48, 52]. Additionally, the effectiveness of disinfection in removing pathogens from drinking water is mediated by the microbial ecology

of the drinking water system [44]. However, the impact of on-site disinfection on premise plumbing microbial ecology is not well understood, motivating the current study.

The complex microbial ecology of premise plumbing, hot water, systems can serve as a reservoir for opportunistic pathogens, such as *Legionella* spp. [53-56], nontuberculous *Mycobacterium* spp. [5, 57], *Pseudomonas* spp. [58, 59], *Acinetobacter* spp. [60, 61], *Stenotrophomonas* spp. [62, 63], *Brevundimonas* spp. [64], *Sphingomonas* spp. [65, 66], and *Chryseobacterium* spp. [67]. Biofilms and amoeba within the water system can protect opportunistic pathogens from disinfection [44, 68-70], and may even allow their regrowth and increase in pathogenicity [71-73]. As an example of the utility of microbial ecology-based approaches, a recent landmark microbial ecology-based study showed that biofilms in showerheads are actually enriched in opportunistic pathogens, creating the potential for an aerosol route of infection [74]. Additionally, antibiotic resistance genes have been detected in the biofilms of drinking water distribution systems [75, 76]. Each of these points highlights the necessity for a greater understanding of premise plumbing microbial ecology.

Premise plumbing systems have an approximately ten-times greater microbial load than full-scale drinking water distribution systems, due to greater water stagnation and surface area to volume ratio, among other factors [77, 78]. Premise plumbing systems of hospitals are of particular concern, as hospitals contain susceptible populations such as immunocompromised patients [79], which may not be protected by current drinking water monitoring standards [80]. To date, the majority of on-site disinfection systems have been installed in hospitals, creating a valuable testing ground to observe the impact of on-site disinfection systems on premise plumbing microbial ecology prior to more widespread application.

In addition to use in on-site systems, monochloramine as a secondary disinfectant has been advocated in the US as an effective method to reduce the production of disinfection-by-products [81, 82] and control biofilm growth within water distribution systems [83]. While monochloramine is able to penetrate biofilms better than alternative disinfectants, this may not result in a reduction in biofilm growth [50]. Additionally, chloramine treatment requires the addition of an excess of ammonia, which may cause increased growth by ammonia-oxidizing bacteria [82], such as the genera *Nitrospira* spp. and *Nitrosomonas* spp. [84]. Bacterial nitrification is known to increase the degradation rate of monochloramine [85], thereby reducing the expected longevity and effectiveness of chloramine. Denitrifying bacteria have previously been identified in chloraminated drinking water systems [86]; however, this topic has not been fully explored in the literature.

The effectiveness of chloramination in removing opportunistic pathogens in premise plumbing remains unclear [81]. Monochloramine has been proposed as a disinfection strategy for the control of *Legionella* [2, 87-89] and this disinfection strategy has been used as a method of on-site supplemental disinfection, but long-term studies have not yet been conducted [2, 87]. Recently, a culture-based study of monochloramine on-site disinfection in a hospital's hot water system for the purpose of *Legionella* control demonstrated a significant reduction in *L. pneumophila* and no change in nitrate or nitrite levels [90]. Often observed discrepancies in system performance and measures are potentially due to differing microbial ecologies or water chemistries of the systems tested. A more holistic view of system microbial ecology, such as presented in this study, may allow more efficient application of supplemental disinfection.

Despite the obvious importance of the microbial ecology of drinking water systems, there is a notable lack of studies detailing the shift in microbial diversity and composition in response

to supplemental disinfection. The objective of this study was to determine the effects of on-site monochloramine disinfection on the microbial ecology of a hospital hot water system. Both the microbial ecology of hot water systems and the response of premise plumbing microbial ecology to on-site disinfection are not currently well described in the literature This study utilizes 216 samples taken from 27 sites and pooled into five composites for two time points prior to and six time points following the addition of on-site monochloramine addition. Samples were analyzed utilizing Illumina DNA sequencing of the microbial community 16S rRNA region and results demonstrate a dynamic shift of the microbial ecology of a hospital's hot water system in response to monochloramine addition.

4.2 SPECIFIC AIM 2A- MONOCHLORAMINE CULTURE STUDY

4.2.1 Materials and Methods

Location: University of Pittsburgh Medical Center (UPMC) Mercy hospital is a 495-bed tertiary care hospital in Pittsburgh, PA. The building has 12 floors and encompasses approximately 840,000 ft^2. *Legionella pneumophila* serogroup 1 was detected in the building hot water system in the early 1990s, prompting installation of a copper-silver ionization system. Following over a decade of effective *Legionella* control, building positivity increased following a construction/renovation project in 2010. After identifying cases of hospital-acquired Legionnaires' disease among patients, the hospital elected to participate in a pilot study of the monochloramine system as an alternative disinfection technology. The copper-silver ionization

system remained active until the start of monochloramine injection (9/26/2011). At this time, the ionization system was de-activated and remained inactive for the duration of the investigation.

Monochloramine generation system: The monochloramine injection unit (Sanikill, Sanipur, Brescia, Italy) was installed on the hospital hot water system. Monochloramine generation utilized two precursor reagents: stabilized sodium hypochlorite (Enoxin) and a buffered ammonium salt solution (Zebion). Water was drawn from the hot water return and pumped into a pre-dilution loop. This loop circulated hot water through a reaction chamber where precursor chemicals were injected. A diagram illustrating system operation is shown in Figure 4.1. Monochloramine-treated water from the pre-dilution loop was injected into the facility hot water return. Reagent dosing was controlled and supervised remotely using an onboard electronic process controller. Dosage was applied proportionally based on the cold water supply volume to the hot water system as measured by flow meters.

Biological and chemical sampling was performed for five months before monochloramine injection (baseline period) while copper-silver ionization was in use and for 24 months after system start-up (post-disinfection period). Collected data were subject to statistical analysis using paired t-tests, and a p-value below 0.05 was considered indicative of a statistically significant reduction. Receiver Operating Characteristic (ROC) curves were prepared using *Legionella* and HPC data from the baseline and post-disinfection periods to evaluate the relationship between total microbial concentration and *Legionella* positivity.

Biological sample collection and analysis: Water samples were cultured for *Legionella* (ISO Standard 11731:1998 and ISO Standard 11731:2004), *Pseudomonas aeruginosa* (ASTM International Standard Test Method D5246-92), *Stenotrophomonas maltophilia* (modified ASTM International Standard Test Method D5246-92), *Acinetobacter* sp. (modified ASTM International

Standard Test Method D5246-92), nitrifying bacteria (BART™ Presence/Absence Test, Droycon Bioconcepts Inc.), mycobacteria (Middlebrook 7H10 and Trypan Blue 10/20 Medium Agars), and heterotrophic plate count (HPC) bacteria (Standard Method 9215B, Pour-Plate, R2A agar).

Baseline sampling was performed in April, May, June, and September of 2011, and the number of distal outlets sampled each month was 30, 26, 27, and 27, respectively. Samples were initially collected from 16 outlets after one week of monochloramine treatment. Following this initial sampling, samples were collected for biological analysis from 27 distal outlets that represented the complete water distribution system, and these outlets were sampled monthly for six months then bimonthly (every two months) for 18 months. Additional sampling was performed quarterly for six months after completion of the 24-month evaluation period. These samples were cultured for *Legionella* only and were not included in the statistical analyses.

Distal outlets sampled during the post-disinfection period included dual-supply sinks (18), showers (2), and sensor faucets (7). Between Months 12 and 14, one sensor faucet was replaced with a dual-supply sink. Additional sampling locations included the hot water return, two hot water tanks, and an outlet representing the closest point to the incoming cold water supply. Hot water tank samples were collected from drain lines immediately and after 30 seconds of flushing. Distal hot water samples were collected immediately upon opening each outlet. Cold water samples were collected following one minute of flushing. An immediate draw hot water sample was taken from the same outlet prior to cold water flushing and sampling. Sample collection volume was 250 mL. Outlets were flushed for one minute prior to collection of samples for physicochemical analysis.

During the evaluation, the UPMC Mercy microbiology lab also performed monthly monitoring for *Legionella* using swab samples collected from different distal outlets throughout

the hospital. Swabs were inserted into faucets and rotated 5-10 times after letting the hot water run until water was hot. Swabs were then inserted into a tube containing 10 mL of hot water from the fixture. The tube was vortexed and 0.1 mL was plated onto a selective *Legionella* agar plate (DGVP). Sampling locations were rotated monthly.

Physicochemical monitoring: Monitoring parameters included copper, silver, lead, monochloramine, total and free chlorine, nitrate, nitrite, total ammonia, pH and hot water temperature. Physicochemical monitoring was performed concurrently with biological monitoring. Parameters were assessed for compliance with maximum contaminant levels (MCLs) defined by the U.S. Environmental Protection Agency [91]. A Hach DR/890 colorimeter was used to measure chlorine (free and total), monochloramine (ppm as Cl_2), total ammonia, nitrate and nitrite (These parameters were monitored by Scott Duda). Water samples were sent to a reference laboratory (Analytics Corporation, Ashland, VA) for measurement of copper, silver and lead using atomic absorption spectroscopy.

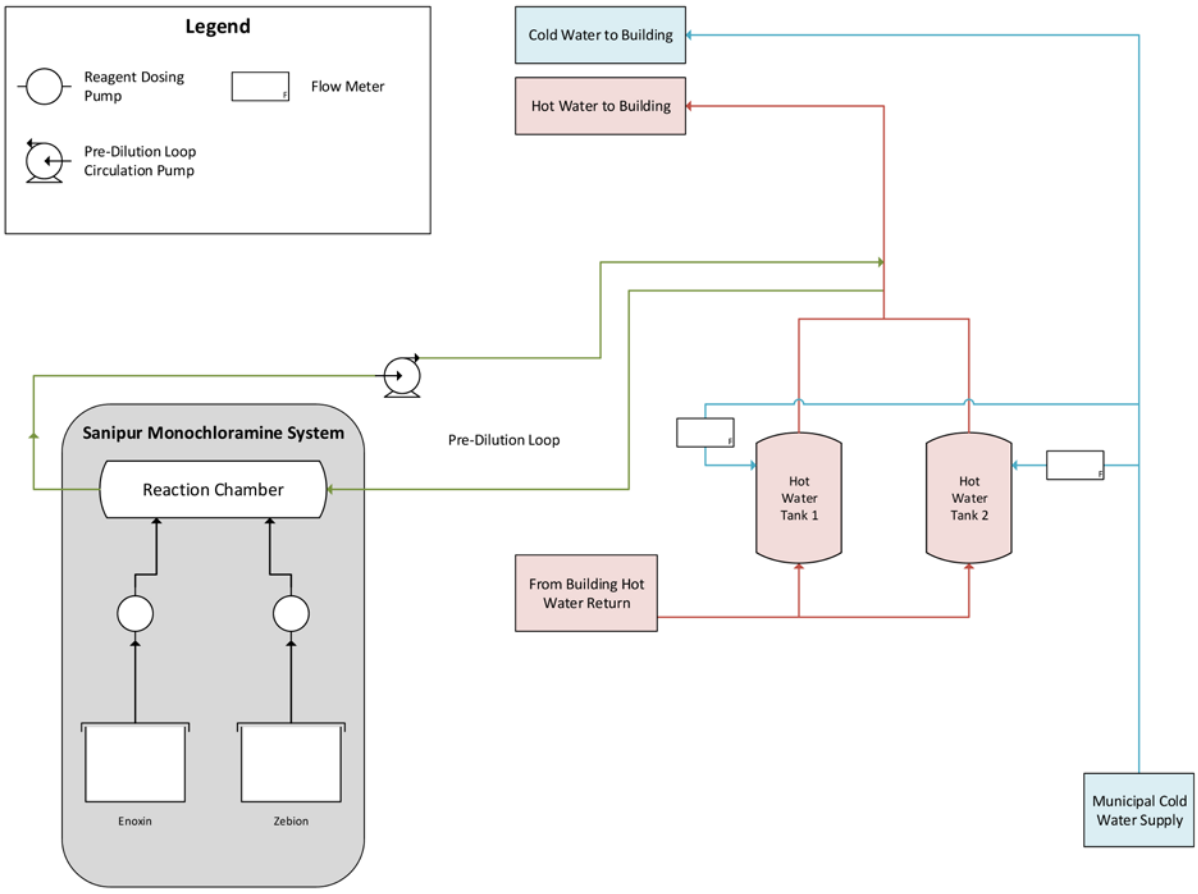
4.2.2 Results

***Legionella* positivity and HPC bacteria:** *Legionella* distal site positivity and HPC concentrations for the baseline and post-disinfection periods are shown in Figure 4.2. Sixteen samples were collected from the hot water system one week after monochloramine system start-up and showed 6% distal site positivity. Distal site positivity remained below 10% for the first eight months of the study ($p < 0.05$, Figure 4.2).

Distal site positivity increased during Months 10 and 12 to 26% and 33%, respectively. The cause of this positivity increase was investigated, and corrective actions were taken to re-

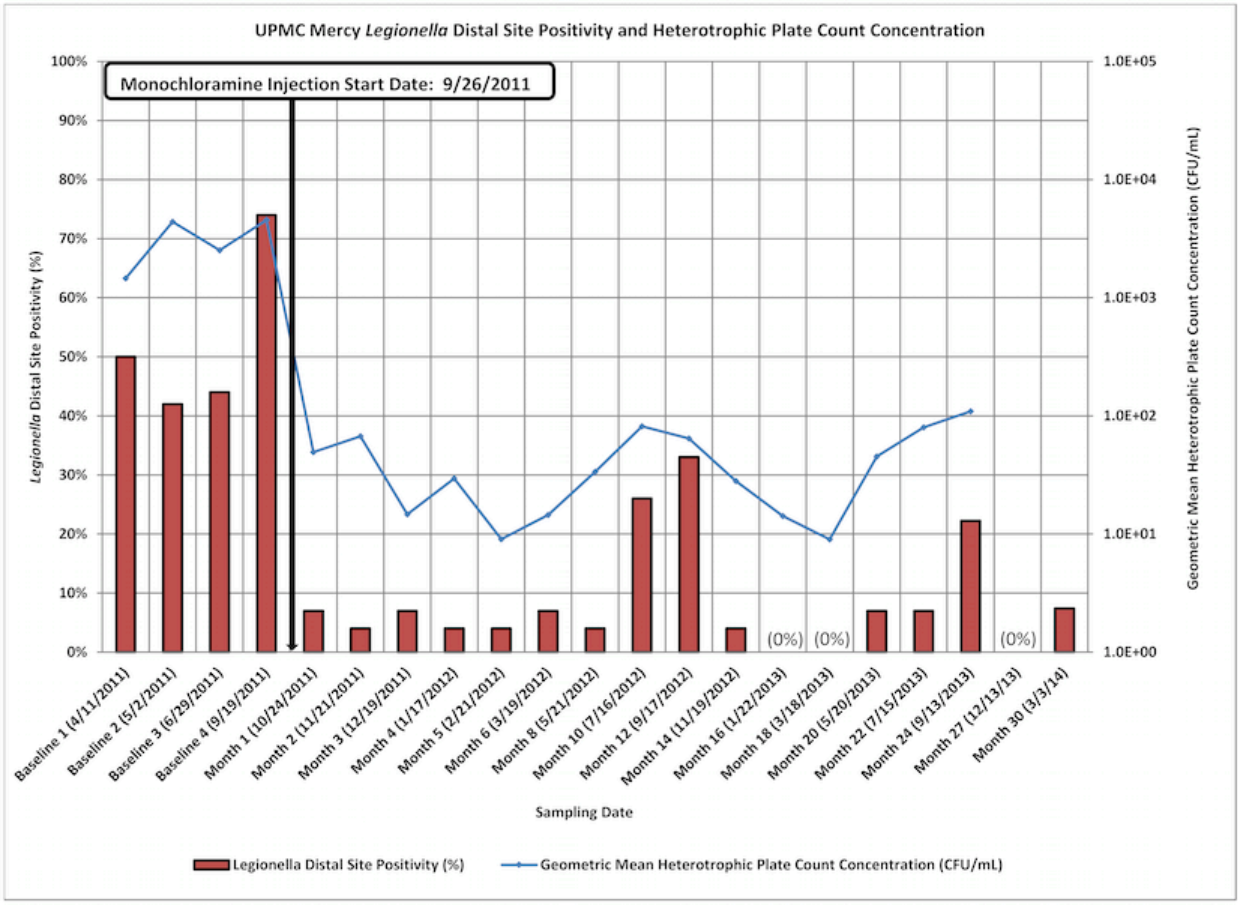
establish system efficacy. These corrective actions are discussed in further detail later in this report.

Distal site positivity decreased to 4% during Month 14 and remained below 10% until Month 24. At this time, distal site positivity rose to 22%. Corrective actions were instituted, and subsequent sample collection (Months 27 and 30) demonstrated that distal site positivity returned to below 10% (Figure 4.2).



Monochloramine was applied to the hospital hot water system only.

Figure 4.1. Schematic of the monochloramine system



A total of 27 distal outlets were tested monthly for the first six months of treatment then bi-monthly (every two months) thereafter. *Legionella* distal site positivity was significantly reduced after initialization of monochloramine injection into the hot water system. Heterotrophic plate count (HPC) bacteria were also reduced.

Figure 4.2. Results of *Legionella* and HPC distal site positivity

A shift in *Legionella* speciation was observed following monochloramine application. During the baseline period, *L. pneumophila* serogroup 1 was the dominant species isolated from distal outlets, accounting for 90% (52/58) of positive samples, and blue-white fluorescing *Legionella* species accounted for 26% (15/58). During the post-disinfection period, *L. pneumophila* serogroup 1 accounted for 49% (18/37) of positive samples, and blue-white fluorescing *Legionella* species accounted for 70% (26/37). An unidentified non-*pneumophila* *Legionella* species was isolated from 2/37 positive samples. Classification percentages do not sum to 100% since some outlets contained more than one *Legionella* species/serogroup.

Significant changes in *Legionella* concentrations (CFU/mL) at positive outlets were not observed. Most positive outlets demonstrated a concentration ≤10 CFU/mL during both the baseline and post-disinfection periods.

L. pneumophila serogroup 1 was detected in the cold water from a faucet in the hospital hot water tank room during 6/15 post-disinfection sampling months (Months 4, 5, 10, 12, 22, and 24). During each of these sampling months, *Legionella* was not detected in the immediate draw hot water sample taken from the same outlet.

During the baseline period (April, May, June, and September, 2011), a total of 108 swab samples were collected by the UPMC Mercy microbiology lab for *Legionella* culture, and 39 of these samples were positive (36%). During the 24-month post-disinfection period, 553 swab samples were collected, and none of these samples were positive (0%).

The distal outlet HPC concentration (geometric mean) was reduced from 2,900 CFU/mL during the baseline period to 32 CFU/mL during the post-disinfection period ($p < 0.05$). Analysis of the utility of HPC concentrations for prediction of *Legionella* positivity demonstrated no statistically significant predictive capacity during the baseline period (Area Under ROC Curve = 0.50). During the post-disinfection period, a "fair" statistically significant predictive capacity was observed (Area Under ROC Curve = 0.78). However, HPC concentrations >100 CFU/mL were only able to correctly classify *Legionella* positivity in 69% of samples.

Chemical parameters: Selected chemical data for the investigation are shown in Table 4.1. Nitrate, nitrite, copper and lead concentrations did not exceed their respective EPA primary MCLs during the study. Copper and silver concentrations increased during the first five months of the investigation followed by a steady decline. No significant increases in copper or silver concentrations were observed following Month 5. Temporary increases in nitrate and total

ammonia concentrations were observed during Months 6 – 10 and Months 8 – 12, respectively. Increases in total and free ammonia preceded *Legionella* distal site positivity (Figure 4.3)

Monochloramine levels were maintained between 1.0 – 4.0 ppm as Cl_2 throughout the investigation, with a target concentration of 2.0 – 3.0 ppm. Measurements reported in Table 4.1 represent the average concentration in samples taken from the hot water return and first post-injection hot water outlet on the day of each biological sampling. Routine monochloramine monitoring was performed several times per week for the first six months of evaluation and daily thereafter. Observed pH values ranged from 7.9 – 9.0, while nitrite concentrations ranged from 0.001 – 0.007 mg/L.

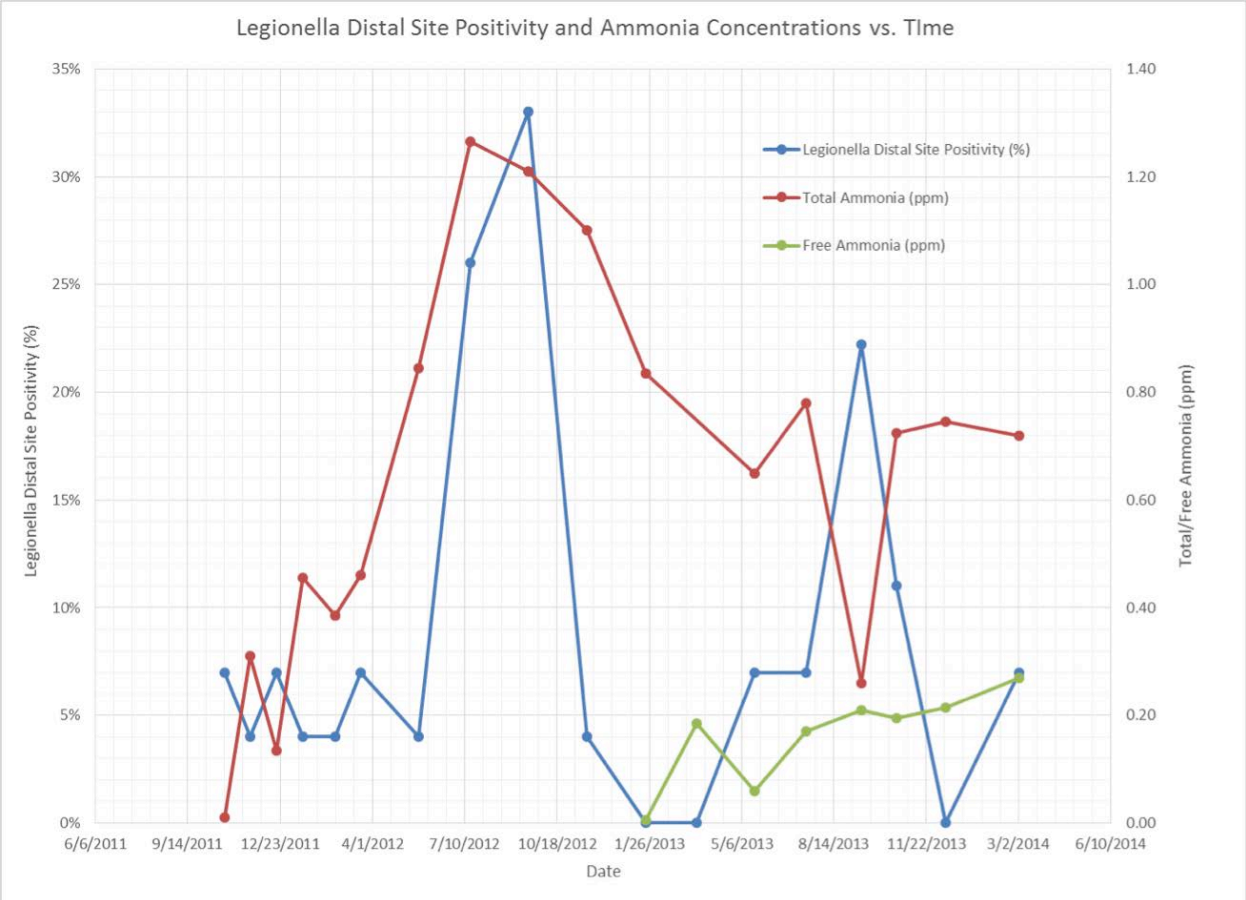
Table 4.1. Results of chemical monitoring[a] (Baseline and Months 1-24)

Sampling Month	Monochloramine (ppm as Cl_2)	Total Ammonia (ppm)	Nitrate (ppm)	Copper (ppm)	Silver (ppm)
B[b]	0.00	0.01	0.5	0.14	0.012
M1[c]	3.14	0.31	1.1	0.15	0.030
M2	0.76	0.14	0.8	0.38	0.026
M3	1.60	0.46	1.4	0.36	0.020
M4	2.58	0.39	1.3	0.74	0.035
M5	2.80	0.46	1.5	0.76	0.097
M6	2.57	0.85	6.8	0.46	0.052
M8	3.40	1.27	5.2	0.36	0.041
M10	2.76	1.21	4.8	0.12	0.025
M12	2.82	1.10	2.4	0.11	0.027
M14	3.36	0.84	1.7	0.13	0.013
M16	3.99	0.65	2.9	0.06	0.031

Table 4.1 Continued

M18	4.61	NS*	3.3	0.11	0.029
M20	3.94	0.78	3.0	0.06	0.023
M22	0.88	0.26	1.8	0.06	0.014
M24	3.10	0.73	3.7	0.08	0.026

[a] Reported chemical data are average observed valves for the hospital hot water return and first post-injection outlet measured on the day of biological sampling.
[b] B = Baseline; baseline average values were computed using data from four sampling dates taken over a five month period before initialization of monochloramine injection.
[c] M1 = Month 1, M2 = Month 2, etc
[d] NS = Not Sampled



Free Ammonia sampling did not begin until the 1/26/2013 sampling date.

Figure 4.3. Total and free Ammonia concentrations and *Legionella* distal site positivity.

The monochloramine MCL of 4.0 ppm as Cl_2 was exceeded at distal sites in the hospital on only three occasions during the investigation (Months 3, 5, and 18). These occasions were one-day events, and concentrations returned to acceptable levels the next day following performance of corrective actions. Corrective actions included cleaning/replacement of system components, adjustment of the monochloramine dosing setpoint, and cleaning/replenishment of precursor reagent tanks.

Additional microbiological monitoring: In addition to *Legionella* and HPC bacteria, other microbiological parameters analyzed during this investigation included *P. aeruginosa*, *S. maltophilia*, *Acinetobacter* spp., nitrifying bacteria, and mycobacteria (Table 4.2). No significant increase in these bacterial populations were observed during the investigation.

Table 4.2. Microbiological monitoring results for distal outlets

Microorganism	Baseline	Post-Disinfection	p-Value
Legionella spp.	56/106 (53%)	37/404 (9%)	$p < 0.05$[a]
Pseudomonas aeruginosa	2/110 (2%)	3/404 (1%)	$p > 0.05$
Stenotrophomonas maltophilia	1/110 (1%)	6/404 (2%)	$p > 0.05$
Acinetobacter spp.	0/110 (0%)	1/404 (0.4%)	$p > 0.05$
Nitrifying Bacteria	0/50 (0%)	0/240 (0%)	$p > 0.05$
Mycobacteria	10/17 (59%)	106/258 (41%)	$p > 0.05$
HPC[b]	2,900 CFU[c]/mL	33 CFU/mL	$p < 0.05$[a]

[a] Statistically significant findings.
[b] HPC results are reported in geometric mean concentration (CFU/mL) for all distal hot water samples, while all other results are reported as the ratio of positive distal hot water samples to total distal hot water samples followed by the percentage positivity.
[c] CFU = colony forming units.

4.2.3 Discussion

This was the first U.S. trial of a disinfection system for hospital water systems utilizing a new system for on-site generation of monochloramine. The study evaluated 110 baseline samples and 404 post-disinfection samples from 27 distal outlets over a 29-month period. A significant decrease in *Legionella* percent positivity was observed in the hospital hot water system following monochloramine application. The average percentage of outlets positive for *Legionella* decreased from 53% (baseline) to 9% (post-disinfection) ($p < 0.05$).

Distal site positivity below 30% has been used as an indicator of lower risk for disease transmission [92-94]. No cases of healthcare-associated Legionnaires' disease were identified during post-treatment period despite sporadic positive environmental cultures. This and other studies have demonstrated that a "zero tolerance" approach for recovery of *Legionella* from the environment is not necessary to reduce risk of illness [92].

UPMC Mercy microbiology laboratory *Legionella* culture results from swab samples demonstrated lower distal site positivity during both the baseline and post-disinfection periods. These results confirm observations from previous investigations that indicate that bulk water monitoring of *Legionella* provides greater sensitivity than monitoring using swab samples.

We observed a temporary increase in *Legionella* distal site positivity to 26—33% during Months 10—12 (7/16/12—9/17/12). Measurements taken before and during this period demonstrated increasing total ammonia and nitrate concentrations (Table 4.1), suggesting incomplete reaction of chlorine and ammonia precursors. Previous investigations have indicated that elevated ammonia concentrations can also negatively affect monochloramine biocidal efficacy [95].

We identified chlorine precursor degradation caused by high temperatures in the storage area as the cause for suboptimal reaction of the precursor chemicals. Corrective actions included draining/cleaning of storage tanks and replacement of all reagents with fresh product to ensure correct dosing proportions. Future users of this technology should be mindful of this issue. We have recommended that both *Legionella* and disinfectant concentration be monitored routinely to verify proper system operation [87]. The importance of this monitoring approach was further demonstrated in this investigation as monitoring only disinfectant concentration failed to identify periods of increased *Legionella* colonization. Routine monitoring of free ammonia is also recommended to assess precursor reagent quality.

Previous evaluations of municipal water disinfection using monochloramine observed elevated HPC concentrations following several months of continuous application [89]. We did not observe this trend during our investigation. A two-log reduction in geometric mean HPC concentrations was observed following monochloramine application (2,900 CFU/mL baseline vs. 33 CFU/mL post-disinfection, $p < 0.05$). In a previous study, we observed a similar concomitant reduction in HPC when using chlorine dioxide for hospital water system treatment [88]. Although HPC bacteria are not considered pathogenic or predictive of *Legionella* presence/absence, HPC is useful for monitoring disinfection system performance.

Municipalities using monochloramine for drinking water treatment have seen an increase in *Mycobacterium* spp. Pryor et al. reported a 23.1% increase in the percentage of sites positive for mycobacteria after beginning municipal water treatment with monochloramine [89]. We did not observe this trend in the treated hospital hot water system. The CDC has reported that no change in mycobacteria colonization was observed after monochloramine application in a hospital hot water system [96]. In our study, the percentage of distal outlets from which

mycobacteria species were isolated and their respective concentrations decreased following monochloramine treatment, but these decreases were not statistically significant ($p > 0.05$). Mycobacterial species isolated during the evaluation were identified as *M. frederiksbergense*, *M. gadium*, *M. gordonae*, with three isolates unable to be speciated. One of the unspeciated isolates was closely related to *M. rhodesiae*.

Surveillance of mycobacteria in the hospital hot water system will continue to verify that these decreases are sustained. Further studies are needed to elucidate the impact of monochloramine on nontuberculous mycobacteria in hot water systems.

This study included monitoring of both standard dual-supply faucets and electronic sensor faucets. Previous studies have indicated that sensor-activated faucets harbor *Pseudomonas* and *Legionella* to a greater degree than dual-supply faucets [97-100]. *Legionella* was detected at a total of 12/275 (4%) of standard faucets during the 24-month post-disinfection period compared to a total of 25/99 (25%) of sensor faucets ($p < 0.05$).

Sensor faucets were more likely than standard faucets to be colonized with *Mycobacterium* spp. During the post-disinfection period, 69/80 (86%) sensor faucet samples tested positive for mycobacteria compared to 35/178 (20%) standard faucet samples ($p < 0.05$). Our results are consistent with previous studies that suggest that sensor faucet design may encourage microbial colonization [97-100].

A previous investigation showed that monochloramine application in one municipal water system resulted in an increase in nitrification in water storage tanks due to proliferation of nitrifying bacteria resulting from the presence of excess ammonia [89]. During our investigation, we tested for the presence of nitrifying bacteria as a surrogate marker for nitrification. None of the 240 samples tested were positive for nitrifying bacteria.

Some researchers have reported an increase in *Pseudomonas* following municipal monochloramine application [89], while others have reported a statistically insignificant reduction in the percentage of sites positive for *Pseudomonas* spp. following monochloramine treatment of hospital hot water [90]. During our investigation, we did not observe an increase of other opportunistic waterborne pathogens such as *P. aeruginosa*, *Acinetobacter* spp., or *S. maltophilia* (Table 4.2). Our findings are consistent with those of Marchesi et al. [90]. Additional studies are necessary in water systems that have higher baseline levels of these pathogens to evaluate the impact of monochloramine treatment.

Previous investigations have indicated that municipal monochloramine application may produce elevated lead concentrations due to leaching from old lead-based plumbing components such as piping and solder. We evaluated whether monochloramine treatment may cause release of low levels of lead ions when applied to a hospital hot water system. All observed lead concentrations throughout the 24-month monitoring period were below the EPA MCL of 0.015 ppm. Lead was detected above its minimum detection limit (0.0025 ppm) but at negligible levels (<0.010 ppm) in the hot water system on three occasions throughout the post-disinfection period. Negligible levels (<0.010 ppm) were detected in the incoming cold water to the facility on two of these three occasions. In addition, we observed a transient release of low levels copper and silver ions accumulated from previous treatment with copper-silver ionization following application of monochloramine.

We have performed field evaluations of all currently available methods for disinfection of building potable water systems, including chlorine dioxide, chlorination, copper-silver ionization, and thermal eradication [87]. The results from this study demonstrate that hot water application of monochloramine successfully reduced *Legionella* positivity in the hot water

system and at outlets. As we have recommended previously, a 4-step process of validation is needed for any disinfection system [100]. This includes: (1) Verification of efficacy using laboratory studies [101-103]; (2) Anecdotal field reports of efficacy from individual institutions; (3) Controlled field trials in individual institutions [90, 96]; and (4) Successful applications in multiple institutions over a prolonged evaluation period. Monochloramine has fulfilled three of these four validation criteria [100]. Additional prospective studies of efficacy in hospitals are necessary to determine if the results reported are reproducible. Including this study, three studies have now demonstrated that monochloramine application to hospital hot water systems significantly reduces *Legionella* colonization [90, 96]. These results suggest that monochloramine is a viable option for hospitals considering disinfection for control of *Legionella*.

4.3 SPECIFIC AIM 2B- MONOCHLORAMINE SEQUENCING STUDY

4.3.1 Materials and Methods

Hospital setting: This study took place in University of Pittsburgh Medical Center (UPMC) Mercy hospital, a 495-bed tertiary care hospital complex in Pittsburgh, PA. The building has 12 floors and receives chlorinated, municipal cold water. The hospital's hot water system was being treated with the Sanikill monochloramine injection system (Sanipur, Lombardo, Flero, Italy). Monochloramine was dosed to target concentration between 1.5 and 3.0 ppm as Cl_2. Monitoring of physicochemical parameters included pH, monochloramine, total chlorine, free chlorine, total ammonia, nitrate, nitrite, copper, silver, and lead (see Table 4.3)

(These parameters were monitored by Scott Duda). A Hach DR/890 was used for all measurements except copper, silver, and lead which were sent to a reference laboratory (Analytics Corporation, Ashland, VA) [104]. Two precursor reagents (Enoxin (stabilized sodium hypochlorite) and Zebion (buffered ammonia salt solution)) were added to a pre-dilution loop supplied by the hot water return [104]. The precursors were dosed into this loop, and treated water was then injected into the circulating hot water [104]. Samples for physicochemical analysis were taken from both the hot water return and the first post-monochloramine injection outlet. Presented values are the average of measurements from the chemical concentrations put into circulation in the hot water system (first post-injection outlet) and those remaining upon return of the hot water after passage through the building (hot water return line).

Table 4.3. Physicochemical data obtained during the study

Parameter	B[a]	M1	M2	M3	M4	M5	M6
***Legionella* Distal Site % Positivity**	53%	7%	4%	7%	4%	4%	7%
Avg. HPC (log[CFU/mL])	4.15	3.87	2.64	3.01	3.76	1.55	2.68
pH	8.3	8.6	8.1	8.1	8	7.9	8.1
Monochloramine (ppm as Cl_2)	0	3.14	0.76	1.6	2.58	2.8	2.57
Total Chlorine (ppm)	0.02	2.45	0.65	1.22	2.25	2.31	2.5
Free Chlorine (ppm)	0.03	0.14	0.09	0.15	0.22	0.17	0.13
Total Ammonia (ppm)	0.01	0.31	0.14	0.46	0.39	0.46	0.85
Nitrate (ppm)	0.5	1.1	0.8	1.4	1.3	1.5	6.8
Nitrite (ppm)	0.002	0.005	0.002	0.006	0.005	0.005	0.006
Copper (ppm)	0.14	0.15	0.38	0.36	0.74	0.76	0.46
Silver (ppm)	0.012	0.03	0.026	0.02	0.035	0.097	0.052
Lead (ppm)	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	<0.0025	0.0035

[a] Baseline sampling was taken once, immediately prior to the initiation of the monochloramine generation system

Sample collection and processing: Hot water was collected from 27 sites throughout the hospital at two time points before monochloramine injection (three months and immediately prior) and monthly for the first six months of the study. Water samples were collected from a variety of locations throughout the hospital (Table 4.4). Samples were taken from hot water tanks, the hot water return line, faucets in the intensive care units, rehabilitation suites including both automatic and standard faucets, and other patient rooms on the upper floors. The faucets in the intensive care units are located on the third, fourth, and fifth floors. The faucets in the rehabilitation suites are located on floors six and seven and represent both electronic sensor faucets (automatic) and standard faucets. The final grouping of sites was from short-term use patient rooms located on floors eight, nine, ten, eleven, and twelve. At each site, hot water was flushed for one minute prior to sample collection into sterile HDPE bottles with enough sodium thiosulfate to neutralize 20 ppm chlorine (Microtech Scientific, Orange, CA). For hot water tank sampling, the drain valve was opened, allowed to flush for one minute, then sampled into sterile HDPE bottles as described above. Following sampling, 100 mL of sample water was filtered through a 0.2 µm, 47 mm, polycarbonate filter membrane (Whatman, Florham Park, NJ), placed into 10 mL of the original water sample, and vortexed vigorously for 10 seconds as described ISO Standards 11731:1998 and 11731:2004 for *Legionella* isolation. Five mL of each concentrated sample was frozen at -80^{o}C until DNA extraction.

DNA extraction, PCR, and Sequencing: Frozen water samples were thawed and pooled as described in Table 1. The 27 samples were divided into five pools including the hot water tanks and hot water return line (HWT), floors 3-5 (the intensive care units, F3), floors 6 and 7 automatic faucets (the rehabilitation suites' automatic faucets, F6A), floors 6 and 7 standard faucets (the rehabilitation suites' standard faucets, F6S), and floors 8-12 (the short-term use

patient rooms, F8). These samples were then filtered through 0.2 µm, 47 mm, Supor® 200 Polyethersulfone membranes (Pall Corporation), housed in sterile Nalgene filter funnels (Thermo Scientific; Fisher). Filter membranes were subjected to DNA extraction using the RapidWater® DNA Isolation Kit (MO-BIO Laboratories) as described by the manufacturer. PCR was performed in quadruplicate using 16S rRNA region primers 515F and 806R including sequencing and barcoding adapters as previously described [105]. These primers amplify an approximately 300 base pair region of the rRNA region spanning variable regions 3 and 4. The specificity of this primer set is considered to be well optimized and 'nearly universal' [106]; analysis of these primers against the 97% Greengenes 13.5 OTU database demonstrated a specificity of 99.9% and 98.3% for the 515f and 806r primers, respectively. Dreamtaq Mastermix (Thermo Scientific) was used and PCR product was checked on a 1% agarose gel. An independent negative control was run for each sample and primer set and all negative controls were negative for PCR amplification. PCR products were pooled and purified using the UltraClean® PCR Clean-Up Kit (MO-BIO Laboratories). Each sample then underwent additional cleaning with the Agencourt® AMPure® XP PCR purification kit (Beckman Coulter) and quantified using the QuBit® 2.0 Fluorometer (Invitrogen). Following quantification, 0.1 picomoles of each sample PCR product were pooled. The sample pool underwent two additional clean up steps with a 1.5:1 ratio of Agencourt® AMPure® XP beads followed by a 1.2:1 bead ratio (Beckman Coulter) to eliminate primer dimers. Samples were sequenced on an in-house Illumina MiSeq sequencing platform as previously described [105].

Table 4.4. Sample pool description, abbreviation, and number of pooled sites. Hot water was collected after a one-minute flush from the following locations throughout the hospital.

Sample Description	Sample Abbreviation	Number of Pooled Sites
Outlets of Hot Water Tanks 1 & 2 and the Hot Water Return line	HWT	3
Floors 3-5 patient room faucets	F3	4
Floors 6 & 7 patient room automatic faucets	F6A	7
Floors 6 & 7 patient room standard faucets and showers	F6S	7
Floors 8-12 patient room faucets	F8	6
Technical replicates of Floors 8-12 patient room faucets	F8rep	6

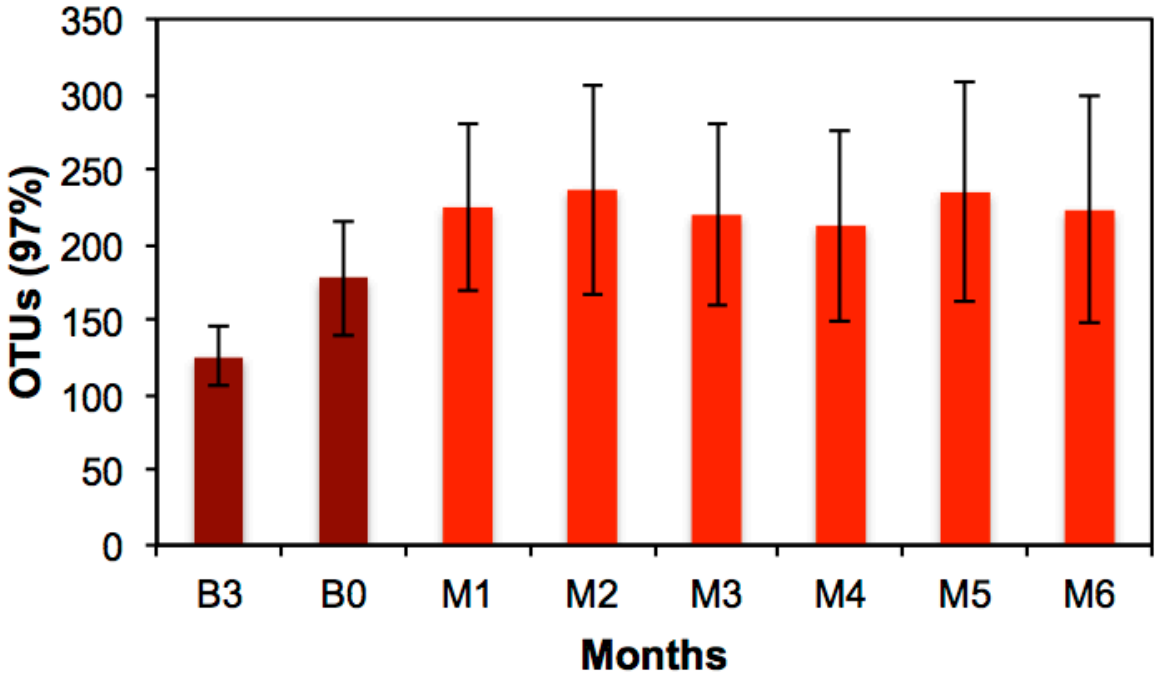
Data analysis: Data was analyzed within the MacQIIME (http://www.wernerlab.org/software/macqiime) implementation of QIIME 1.7.0 [107]. Sequences were parsed based upon sample-specific barcodes and trimmed to a minimum quality score of 20. Operational taxonomic units (OTUs) at 97% were then picked against the Greengenes 13.5 database using UCLUST [108] for taxonomic assignment. Following assignment, 7,000 successfully assigned sequences from each sample were chosen at random to allow for even downstream analyses and even cross-sample comparison. Observed OTUs were defined as observed species whereas unassigned sequences were removed from subsequent analyses (closed reference OTU picking). Alpha-diversity evenness was calculated using the 'equitability' metric within QIIME. Beta diversity analyses were conducted by UNIFRAC analysis [109]. OTUs were also open-reference picked, where unassigned sequences are placed in the taxa "other" and therefore not removed. Open-reference OTU picking did not result in a shift in any fundamental conclusions with the exception of the increase in the genus *Stenotrophomonas* spp. following monochloramine addition; closed-reference OTU picking is presented for higher-quality taxonomic assignment (The aforementioned data analysis was

performed by Dr. Kyle J. Bibby). Morisita-Horn indices were calculated as previously described [110, 111]. Sequences are available on MG RAST under accession numbers 4552832.3 to 4552878.3.

4.3.2 Results

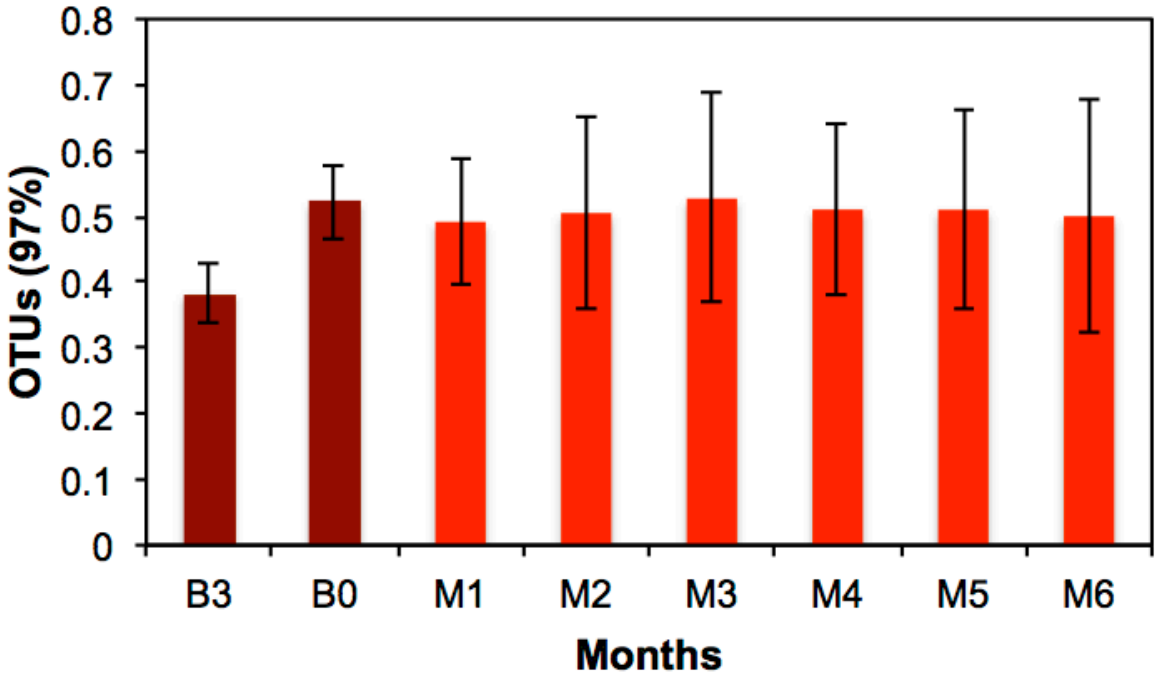
Sequence Data: Sequencing reads were split by sample-specific barcodes, trimmed to a minimum quality score of 20, and placed into OTUs at 97% through comparison with the Greengenes 13.5 coreset. For each sample, 7,000 sequences with assigned taxonomy were selected to allow for even comparison across samples. Two types of OTU picking were done for this study: closed reference (sequences were compared to a reference set of sequences for OTU clustering, any sequences not matching one of these pre-defined sequences were discarded) and open reference (sequences were compared to each other for OTU picking, sequences not mapping to the reference database were grouped as ‘other).

Alpha Diversity: Alpha diversity (number of observed OTUs) of samples treated with monochloramine was significantly higher than samples from the baseline months (Figure 4.4). Prior to treatment, the average number of observed OTUs at 97% similary was 151.2 ± 39.7, whereas during treatment the average number of observed OTUs was 225.2 ± 61.2 ($p < 0.001$) (Figure 4.4). This shift was not associated with a statistically significant loss of sample evenness (Figure 4.5). The same statistical trends in alpha diversity were observed for open-reference picked OTUs (Figure 4.5).



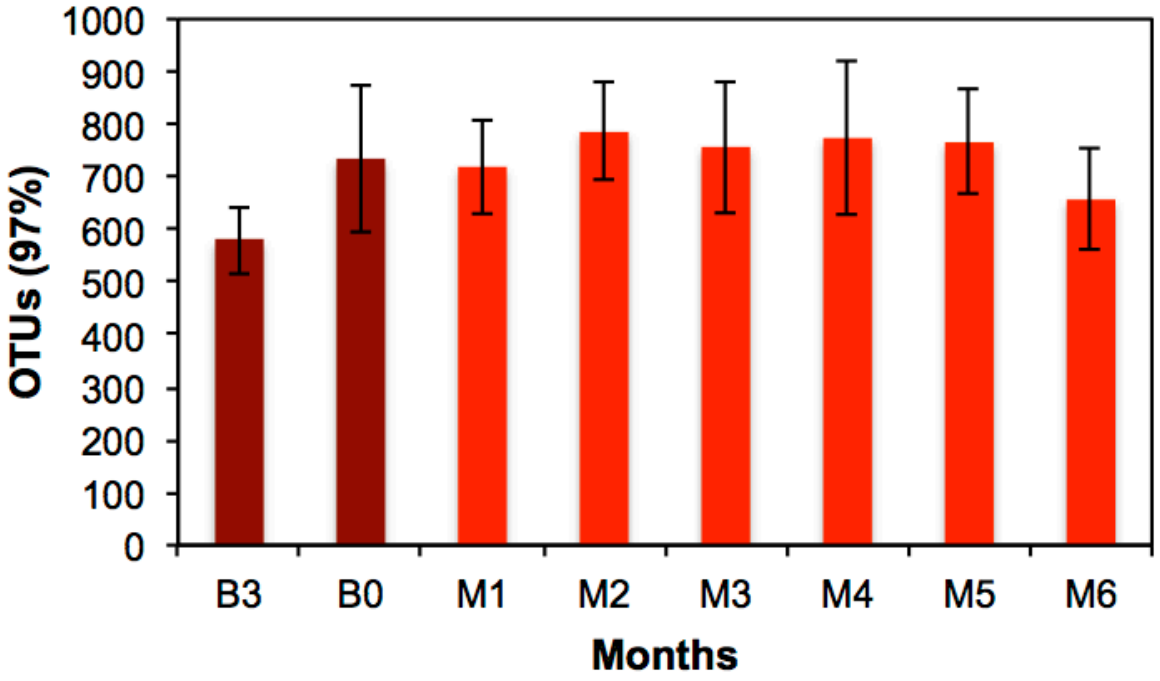
Bars represent standard deviation. Each sample pool was normalized to 7,000 sequences. Samples from B3 and B0 represent those taken three months and immediately prior to monochloramine treatment, respectively. Samples from M1, M2, M3, M4, M5, and M6 were taken monthly during the first six months of treatment.

Figure 4.4. Comparison of the number of OTUs (97% similarity) for each month.



No statistically significant different was observed for samples taken prior to or following monochloramine addition.

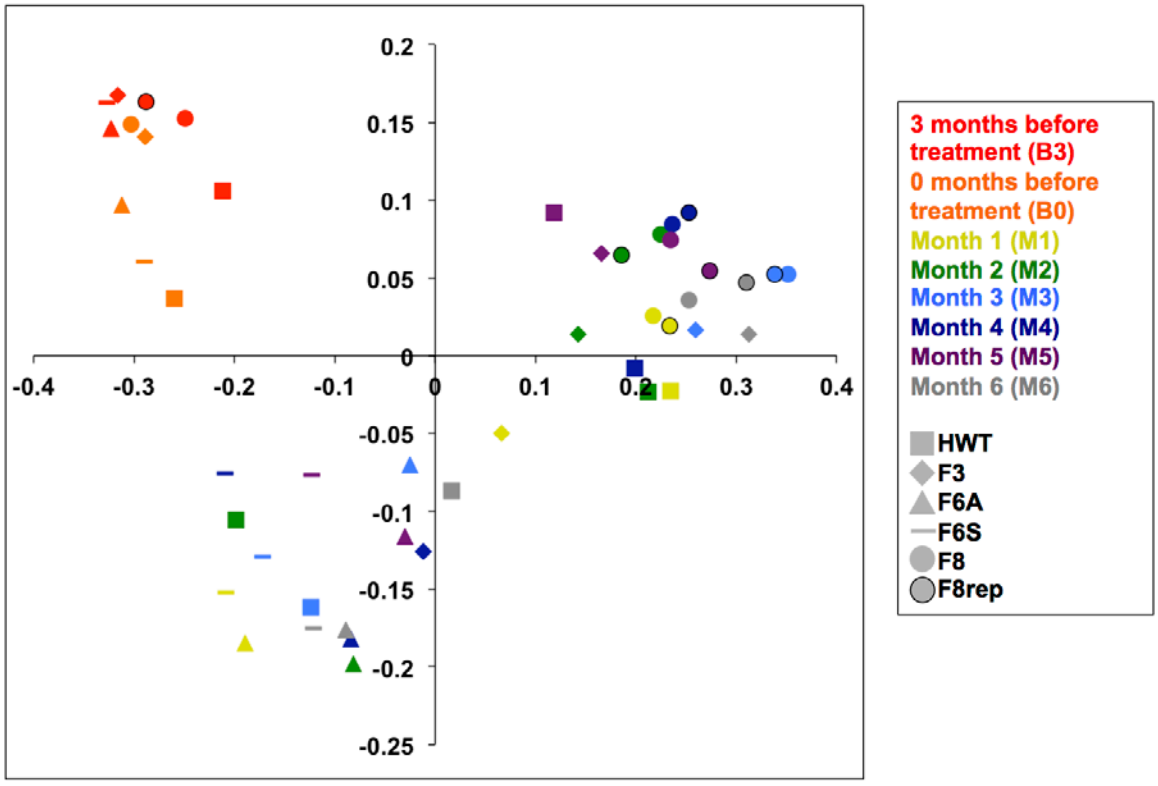
Figure 4.5. Sample evenness for closed-reference OTU picking.



Statistically significant different was observed for samples taken prior to or following monochloramine addition (p=0.046).

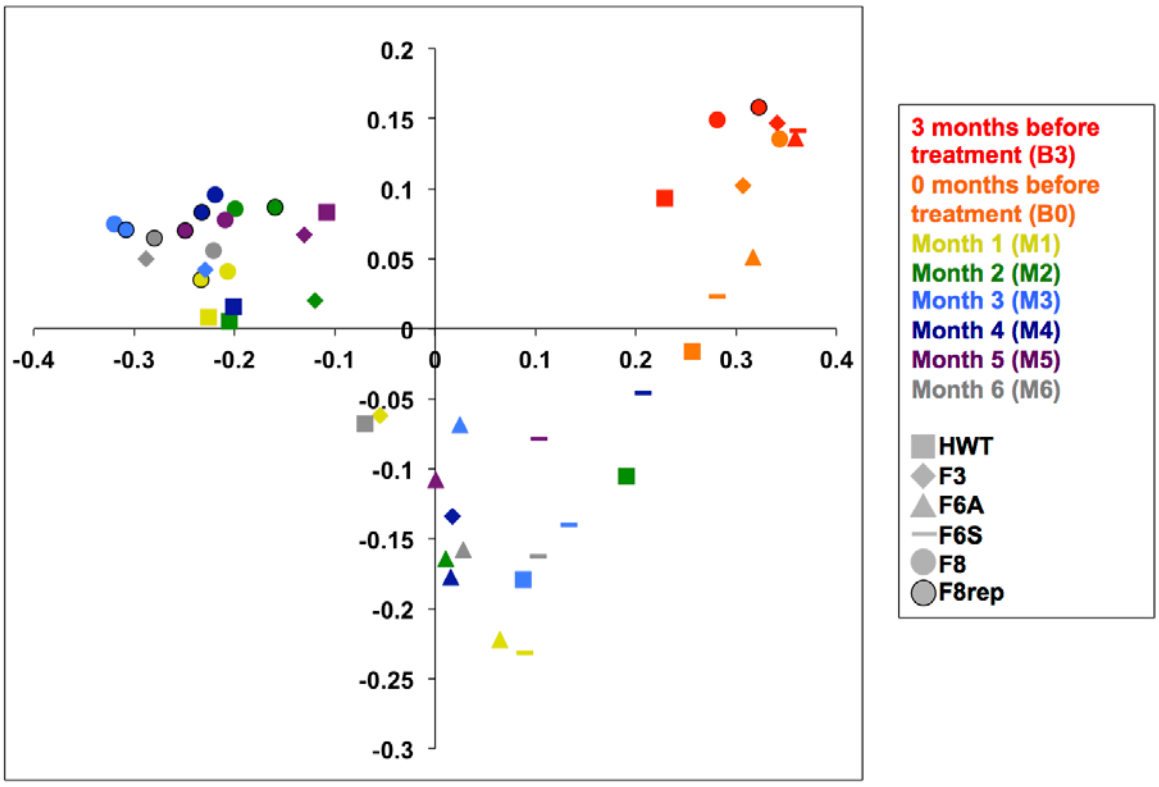
Figure 4.6. Alpha diversity for open-reference OTU picking.

Beta Diversity: Beta diversity (sample interrelatedness) was analyzed using weighted UNIFRAC [109]. The principal coordinate analysis (PCoA) plot from this analysis is shown in Figure 4.7. Samples from the first two months prior to treatment cluster together whereas those following disinfection tend to cluster by sample site more strongly than sample time (Figure 4.7). The same trend was observed for open-reference picked OTUs (Figure 4.8).



Samples that cluster more closely together share a greater similarity in microbial community structure. Colors represent months sampled whereas shapes represent sample pool. Samples from B3 and B0 represent those taken prior to monochloramine treatment. Whereas samples from M1, M2, M3, M4, M5, and M6 are the first six months of treatment.

Figure 4.7. PCoA analysis of samples pools.



Samples from before monochloramine treatment clustered together whereas following treatment samples clustered by location more so than month of treatment.

Figure 4.8. PCoA analysis for open-reference OTU picking.

Taxonomic Comparison: Figure 4.9 shows the phyla-level taxonomy for each of the sample pools. Phyla <1.3% relative abundance for this figure are listed as 'minor phyla'. Prior to treatment, samples from all locations were similarly structured, predominantly comprised of Betaproteobacteria, with lesser quantities Firmicutes, Bacteroidetes, Alphaproteobacteria, and Gammaproteobacteria (Figure 4.9 Panels A-E). Following initiation of treatment (M1) there was a shift away from the predominance of Betaproteobacteria and towards a greater relative abundance of Firmicutes, Alphaproteobacteria, Gammaproteobacteria, and minor fractions of

Cyanobacteria and Actinobacteria (Figure 4.9 Panels A-E). The same taxonomy trends were observed for open-reference picked data (Figure 4.10 Panels A-E).

The samples from the hot water tank (HWT) from pre-treatment months (B3 and B0) were approximately 60% Betaproteobacteria with approximately 35% Firmicutes, Bacteroidetes, Alphaproteobacteria, and Gammaproteobacteria in aggregate (Figure 4.9 Panel A). Following treatment the amount of Betaproteobacteria was reduced to approximately 20% and the amount of Firmicutes, Alphaproteobacteria, and Gammaproteobacteria subsequently increased to comprise an average of 78% of the total relative abundance (Figure 4.9 Panel A).

The profile of samples from the lower floors of the hospital (intensive care units, F3) was slightly different than those of the hot water tank samples but a similar trend was observed (Figure 4.9 Panel B). Over 65% of pre-treated samples were Betaproteobacteria with Firmicutes, Bacteroidetes, Alphaproteobacteria, and Gammaproteobacteria accounting for a combined 20% of bacteria identified (Figure 4.9 Panel B). Following treatment the amount of Betaproteobacteria and Bacteroidetes decreased to an average of 23% relative abundance, while the abundance of Firmicutes and Alphaproteobacteria increased sharply to approximately 68% (Figure 4.9 Panel B).

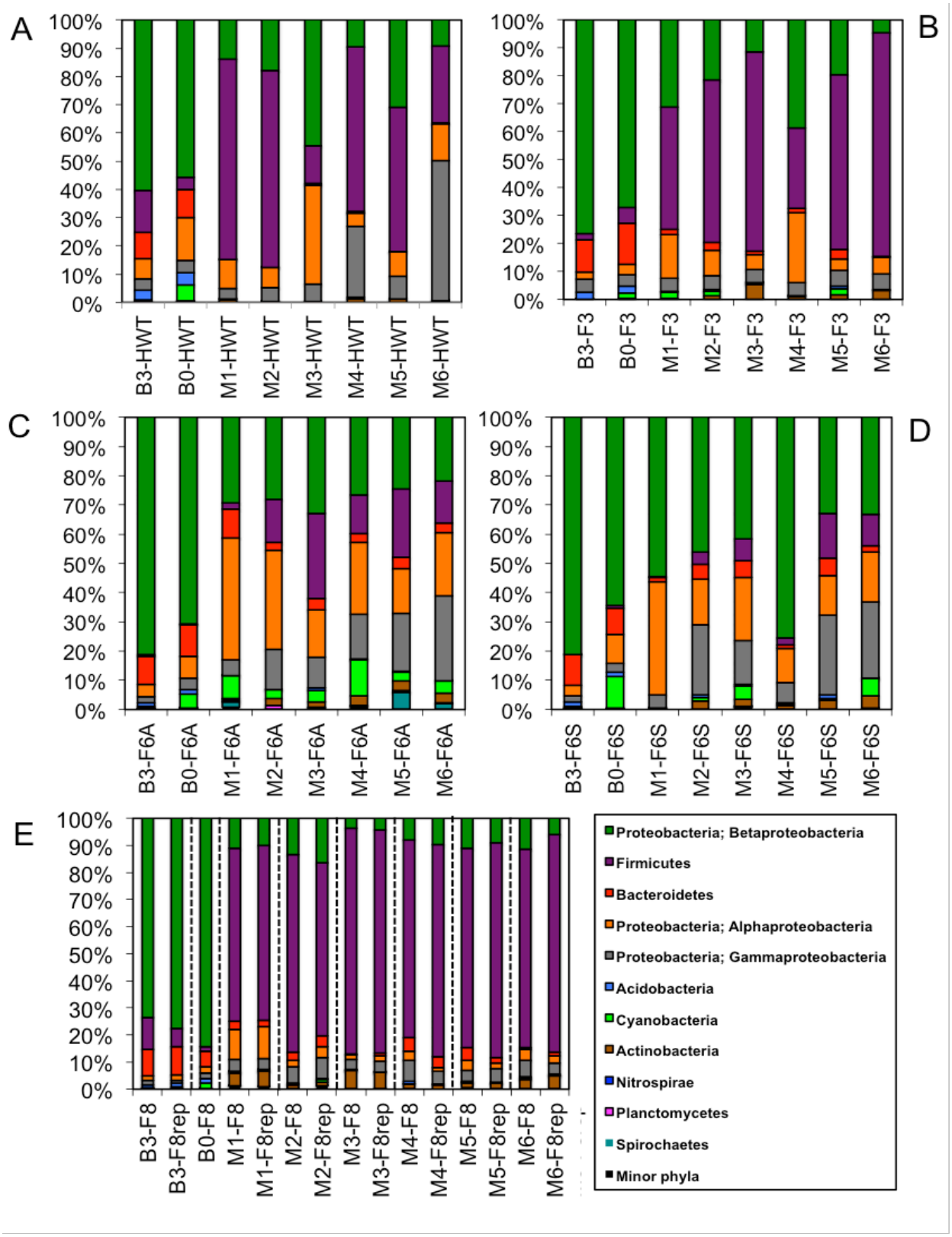
In spite of being from the same rooms, the taxonomic composition of samples from F6A and F6S differed after treatment (Figure 4.9 Panels C and D). Prior to treatment both the automatic (F6A) and standard faucets (F6S) in the rehabilitation suites contained 65-80% Betaproteobacteria, with Bacteroidetes, Alphaproteobacteria, Gammaproteobacteria, and Cyanobacteria accounting for the other 20-35% of major phyla (Figure 4.9 Panels C and D). However, after treatment the automatic faucets (F6A) saw a 50% reduction in the total relative abundance of Betaproteobacteria and became enriched in Firmicutes, Alphaproteobacteria,

Gammaproteobacteria, Actinobacteria, and Spirochaetes (Figure 4.9 Panel C). The standard faucets (F6S) on the other hand lost only 26% of their Betaproteobacteria, but also saw an increase in members of the Firmicutes, Alphaproteobacteria, Gammaprotobacteria, and Actinobacteria phyla from an average of 10% before treatment to 46% after monochloramine addition (Figure 4.9 Panel D).

Samples from the upper floors of the hospital (short-term use patient rooms, F8) prior to treatment resembled most of the other baseline samples with over 70% Betaproteobacteria and approximately 20% of Firmicutes, Bacteroidetes, Alphaproteobacteria, Gammaproteobacteria, Acidobacteria, and Cyanobacteria (Figure 4.9 Panel E). Following monochloramine treatment the Betaproteobacteria were reduced from approximately 70% to 10% and were replaced by Firmicutes, which increased from 7% in the baseline to 74% of the relative abundance after treatment (Figure 4.9 Panel E). There was only a slight increase, from 2% to 9% relative abundance, in the amount of Gammaproteobacteria and Actinobacteria present (Figure 4.9 Panel E).

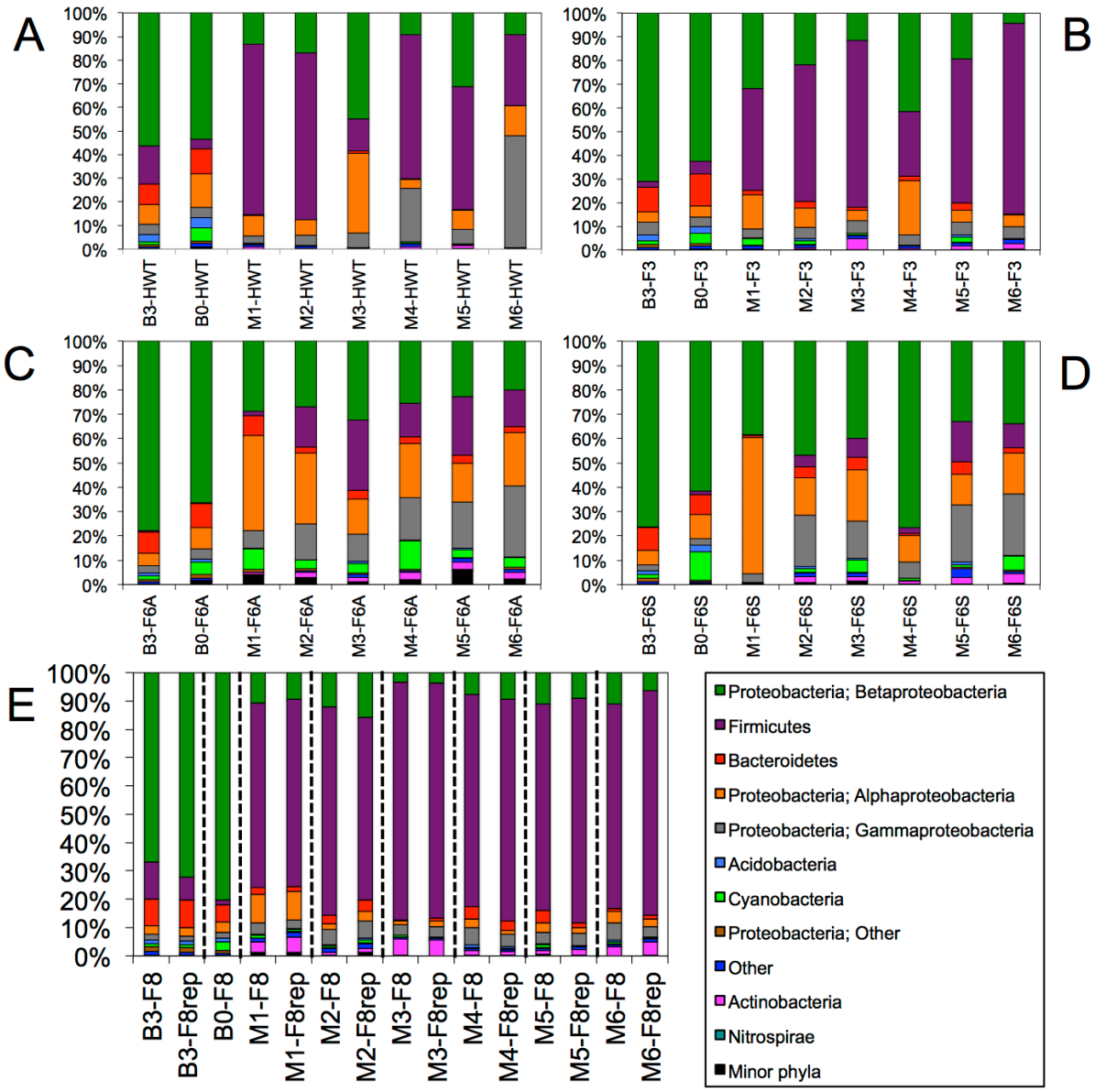
Sample Replicates: Separately amplified and barcoded technical replicates of sample pool F8 for 7 of the 8 sample pools were also sequenced to verify technical reproducibility. There is no replicate for month B0. UNIFRAC analysis demonstrated that the replicates from each month cluster very closely (Figure 4.7). All of the samples from F8 in samples M1-M6 and their replicates (circles and outlined circles) clustered together in the upper-right hand quadrant (Figure 4.7). Morisita-Horn analyses of the taxa found in F8 samples and replicates demonstrate high levels of bacterial community similarity, ranging from 0.990 (M2) to 0.9998 (M3). These results further validate the technical reproducibility of the methodology (Figure 4.9 Panel E) [110, 111]. The open-reference picked UNIFRAC analysis and taxonomy also show replicates to

have similar profiles to their original samples (Figure 4.9 and Figure 4.10 Panel E). Morisita-Horn analyses of these samples show high levels of community similarity ranging from 0.991 (M2) to 0.9992 (M1).



HWT (hot water tank samples) (Panel A), F3 (floors 3-5) (Panel B), F6A (floors 6 and 7 automatic faucets) (Panel C), F6S (floors 6 and 7 standard faucets) (Panel D), F8 (floors 8-12) and F8rep (replicate barcoded PCRs of samples from floors 8-12) (Panel E). Samples from B3 and B0 represent those taken prior to monochloramine treatment. Whereas samples from M1, M2, M3, M4, M5, and M6 are the first six months of treatment. Black lines in Panel E separate pairs of replicates.

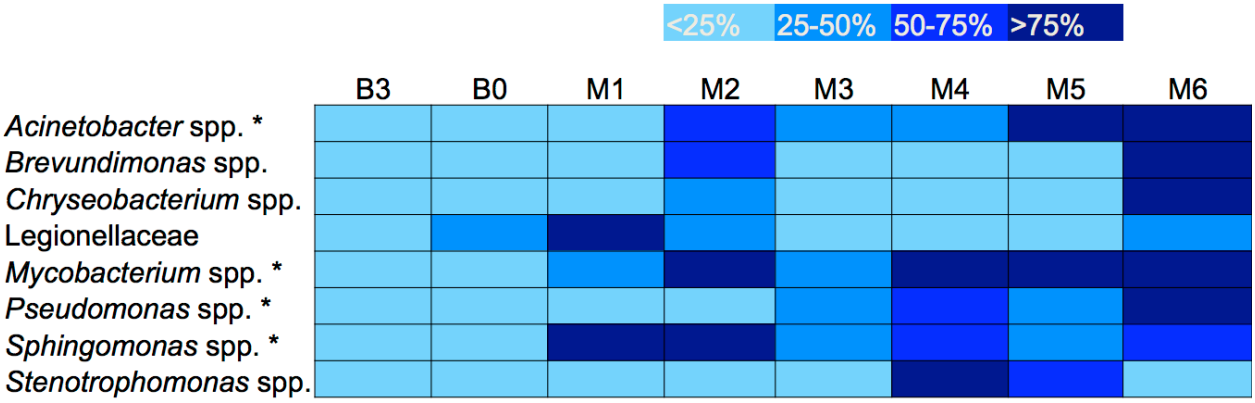
Figure 4.9. Taxonomic assignments of sequences from closed-reference data picking.



HWT (hot water tank samples) (Panel A), F3 (floors 3-5) (Panel B), F6A (floors 6 and 7 automatic faucets) (Panel C), F6S (floors 6 and 7 standard faucets) (Panel D), F8 (floors 8-12) and F8rep (replicate barcoded PCRs of samples from floors 8-12) (Panel E) for open-reference OTU picking. Samples from B3 and B0 represent those taken prior to monochloramine treatment. Whereas samples from M1, M2, M3, M4, M5, and M6 are the first six months of treatment. Black lines in Panel E separate pairs of replicates.

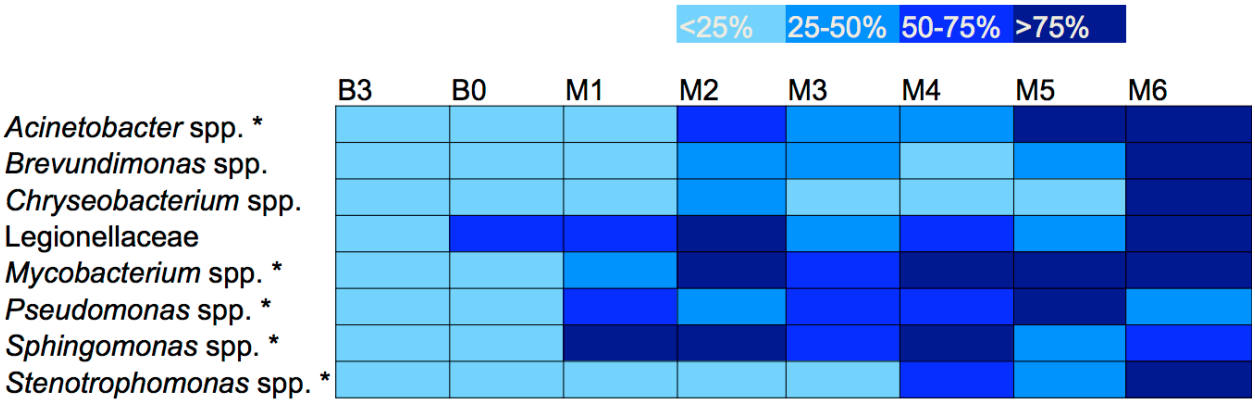
Figure 4.10. Taxonomic assignment of sequences from open-reference OTU picking.

Genera Containing Opportunistic Pathogens: Sequence data was further analyzed to observe the change in genera containing opportunistic pathogens of interest during treatment. Genera analyzed were: *Legionella* spp., *Pseudomonas* spp., *Acinetobacter* spp., and *Stenotrophomonas* spp. (Gammaproteobacteria group); *Brevundimonas* spp. and *Sphingomonas* spp. (Alphaproteobacteria group); *Chryseobacterium* spp. (Bacteroidetes group); and non-tuberculous *Mycobacterium* spp. (Actinobacteria group). These genera are of special interest as some to all of the species contained within them are pathogens; however, the nature of short-read 16S rRNA region sequence analysis is such that species-level pathogens cannot be definitively identified. Trends demonstrated by this analysis could be used to direct future analyses targeting opportunistically pathogenic organisms more specifically. Analysis of the relative abundance of each of these organism groups over time shows a statistically significant increase in relative abundance for *Acinetobacter* ($p = 0.0054$), *Mycobacterium* ($p = 0.0017$), *Pseudomonas* ($p = 0.031$) and *Sphingomonas* ($p = 0.034$) as treatment progressed (Figure 4.11). *Brevundimonas*, *Chryseobacterium*, Legionellaceae, and *Stenotrophomonas* did not demonstrate a statistically significant increase in abundance following treatment (Figure 4.11). The open-reference picked data demonstrated an increase in the same opportunistic pathogen containing genera as the closed-reference picked data, *Acinetobacter* ($p = 0.004$), *Mycobacterium* ($p = 0.002$), *Pseudomonas* ($p = 0.015$), and *Sphingomonas* ($p = 0.025$), but also showed a significant increase in the genera *Stenotrophomonas* ($p = 0.03$) (Figure 4.12).



Samples color coded into four groupings calculated by 25% of the maximum relative abundance for each organism. Months with the least relative abundance are lightest in color, whereas months with the highest relative abundance are darkest. * denotes a statistically significant increase in the relative abundance of this organism following treatment.

Figure 4.11. Relative abundance of different genera of opportunistic waterborne pathogens

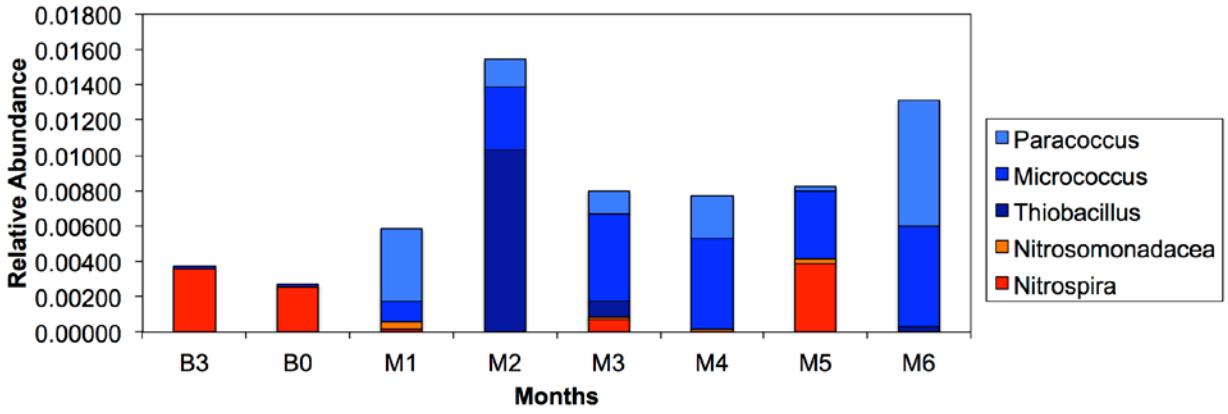


Samples color coded into four groupings calculated by 25% of the maximum relative abundance for each organism. Months with the least relative abundance are lightest in color, whereas months with the highest relative abundance are darkest. A statistically significant increase in *Acinetobacter* spp., *Mycobacterium* spp., *Pseudomonas* spp., *Sphingomonas* spp., and *Stenotrophomonas* spp. was observed following treatment.

Figure 4.12. Relative abundance of waterborne pathogen containing genera for open-reference OTU picking

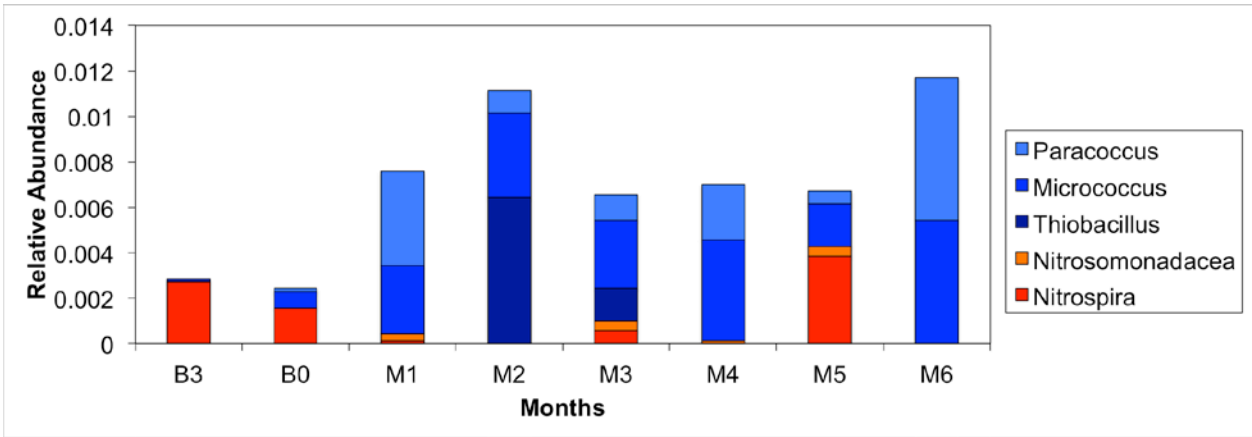
Nitrification and Denitrification: Additionally, we investigated the shift in relative abundance of representative genera associated with nitrification and denitrification (Figure 4.13). There was no statistically significant difference in the potential nitrifiers *Nitrospira* and Nitrosomonadaceae, before (mean = 0.0015 ± 0.0018) and after treatment (mean = 0.0005 ± 0.0011) ($p = 0.175$). Other nitrifier-containing genera such as *Nitrosococcus*, *Nitrobacter*,

Nitrospina, or *Nitrococcus*, were not identified in any samples. The total relative abundance of genera containing denitrifiers (*Thiobacillus, Micrococcus,* and *Paracoccus*) underwent a statistically significant increase in the relative abundance of denitrifying bacteria before (mean = 0.00005 ± 0.000074) and after treatment with monochloramine (mean = 0.0029 ± 0.0029) ($p = 0.026$). Other denitrifier-containing genera *Rhizobiales* and *Rhodanobacter* were not identified in any samples. The same trends in nitrifying and denitrifying bacteria were observed in open-reference picked data (Figure 4.14). Following initiation of monochloramine treatment, both *Legionella* distal site positivity and average HPC decreased significantly ($p < 0.05$) (Table 4.3) [104]. Concentrations of nitrate, nitrite, copper, and lead did not exceed their EPA maximum contaminant levels (Table 4.3) [104]. Total chlorine, free chlorine, and total ammonia concentrations increased upon initiation of monochloramine injection and mirrored the variability of monochloramine levels (Table 4.3) [104].



No other genera associated with nitrification (*Nitrosococcus*, *Nitrobacter*, *Nitrospina*, or *Nitrococcus*,) or denitrification (*Rhizobiales* and *Rhodanobacter*) were found in any of our samples. The x-axis represents sampling months with months B3 and B0 being before monochloramine treatment and months M1-M6 representing the first six months of treatment. The y-axis represents the relative abundance.

Figure 4.13. Relative abundance of genera containing nitrifying and denitrifying bacteria.



No other nitrifying bacteria (*Nitrosococcus*, *Nitrobacter*, *Nitrospina*, or *Nitrococcus*,) or denitrifying bacteria (*Rhizobiales* and *Rhodanobacter*) were found in our samples. The x-axis represents sampling months with months B3 and B0 being before monochloramine treatment and months M1-M6 representing the first six months of treatment. The y-axis represents the relative abundance.

Figure 4.14. Relative abundance of genera containing nitrifying and denitrifying bacteria for open-reference OTU picking.

4.3.3 Discussion

Our study objective was to examine the shift in the microbial ecology of a hospital hot water system associated with the introduction of on-site monochloramine addition. To evaluate the shift in microbial community structure we sampled 27 sites in a hospital and pooled samples into 5 groups for 8 sample time points. Sites were pooled based on their location and use in the hospital and faucet type (automatic versus standard). This study took place during the first U.S. trial of the Sanikill on-site monochloramine generation system (Sanipur, Brescia, Italy) [112-114]. These samples were subjected to DNA extraction, 16S rRNA region barcoded PCR, and Illumina sequencing to analyze the response of the microbial ecology to the addition of monochloramine.

The microbial population shift in response to monochloramine addition was immediate. The number of OTUs observed (alpha diversity) significantly increased following monochloramine treatment (Figures 4.4 and 4.6). It is possible that the overall loss of dominance of dominant microbial groups (e.g. Betaproteobacteria) allowed for a greater number of other bacterial species to grow, thereby increasing the alpha diversity. Samples taken before monochloramine treatment were comprised of similar microbial populations of microbes and samples taken after treatment were distinct from baseline samples (Figures 4.7, 4.8, 4.9, and 4.10). All treated samples clustered independently from the pre-treated samples and were themselves grouped more by their location in the hospital than the month in which they were taken post-treatment. Interestingly, it appears that following monochloramine treatment the location of sampling matters more in sample similarity (Beta-diversity) than does the month they were taken (Figures 4.7 and 4.8). Microbial communities from the lower floors' intensive care units (F3) and the upper floors' short term patient rooms (F8) were more similar than to the floors 6 and 7's rehabilitation suites (F6A and F6S) automatic and standard faucet samples. These sites were located in single patient rooms in rehabilitation units and may experience as much use as some locations on the lower and upper floors, which include the trauma burn unit, the intensive care unit (ICU), the neonatal ICU, and the cardiovascular ICU. The HWT samples from earlier months of treatment closely resembled floors 6 and 7 (F6A and F6S) whereas the HWT microbial ecology from the later months was more related to the lower (F3) and upper floors (F8).

We investigated the possible differences in microbial ecology between automatic and standard faucets as it has been previously demonstrated that opportunistic pathogens, including *Legionella* [100] and *Pseudomonas aeruginosa* [58], are detected more frequently and in greater

concentrations in automatic faucets. It has been suggested that the reason for the differences between automatic and standard faucets could be due to water flow, temperature, and structural issues. The automatic faucets may have diluted monochloramine concentrations due to low flow and poor flushing [58, 100]. Automatic faucets also contain mixing valves, which are made of materials such as rubber, polyvinylchloride, and plastic, which more easily support the growth of biofilms [58, 100]. Potentially due to these biofilms, the increased colonization can persist even following disinfection with chlorine dioxide [100]. We observed a differential reduction in the abundance of Betaproteobacteria following treatment. The automatic faucets lost 50% of their relative abundance of Betaproteobacteria whereas the standard faucets only saw an average 26% reduction.

There was an overall shift towards less Betaproteobacteria, and more Firmicutes, Alphaproteobacteria, Gammaproteobacteria, Cyanobacteria and Actinobacteria after monochloramine treatment. This selection may be due to the resistant nature of some the bacteria found in these phyla; these characteristics include endospore formation, unique cell wall structure, adaptation to survive low nutrient conditions, nitrogen fixation, and general environmental stress tolerance [115, 116]. A previous microbial ecology study of a simulated drinking water distribution system treated with monochloramine demonstrated a different trend, with an increase in specific genera within the Actinobacteria, Betaproteobacteria, and Gammaproteobacteria phyla [45]. The dissimilarity of these studies may be due to the fact that the latter occurred in a cold water system whereas our study was in a hot water supply.

Several waterborne pathogen-containing genera were examined for changes in relative abundance due to monochloramine treatment. The relative abundance of a few of these waterborne pathogen-containing genera examined, including *Acinetobacter*, *Mycobacterium*,

Pseudomonas, and *Sphingomonas*, showed an increase after monochloramine treatment. This occurred despite a previously observed statistically significant reduction in culturable *Legionella* and total bacteria following treatment (Table 4.3) [112-114]. Other studies have described an increase in some of these organisms including *Legionella*, *Mycobacterium*, and *Pseudomonas* in chloraminated water [45, 117] as well as biofilms treated with monochloramine [118]. Feazel et al. previously demonstrated that *Mycobacterium* spp. can be enriched in showerhead biofilms compared to the source water [74]. An increased relative abundance of *Mycobacterium* spp. due to monochloramine treatment is of concern, as these microorganisms may pose a specific threat of aerosol exposure to immunocompromised patients who reside in buildings with an increased abundance of these organisms in hot water [74].

Previous studies have found an increase in nitrification in chloraminated systems, which effectively decreased monochloramine concentration [85, 117]. This chemical decay led to higher levels of *Legionella* spp., *Mycobacterium* spp., and *P. aeruginosa* at earlier water ages than in the chlorinated simulated distribution systems in one study [117]. A change in potentially nitrifying bacteria was not observed in the culture-based portion of this study [112-114], consistent with our molecular observations. Concentrations of nitrate and nitrite remained fairly stable throughout the study months, with the exception of a spike in nitrate levels in M6 (Table 4.3) [104]. We observed a statistically significant increase in genera associated with denitrification in monochloramine treated samples. This finding is consistent with a previous study that found high levels, up to 200,000 cfu/mL, of potentially denitrifying bacteria in a chloraminated system even after regular flushing [86]. The highest relative abundance of bacterial genera associated with denitrification occurred during M6 when there was a spike in nitrate concentrations (Table 4.3) [104]. However, in months 1 and 2 there was also a large

number of these bacteria present with fairly low nitrate concentrations, suggesting that some other factor might be important in their abundance. We do not believe that these trends were due to seasonality in our study as microbiological data were largely consistent across the study period. However, the possibility for seasonal effects cannot be excluded.

The incidence of reported Legionnaires' disease cases increased threefold from 2000 to 2009 [4]. This fact, coupled with an increasingly elderly and immunocompromised population [4], has lead to an increased concern about *Legionella* and other opportunistic waterborne pathogens. Additionally, the American Society of Heating, Refrigerating, and Air-Conditioning Engineers (ASHRAE) has recently proposed Standard 188P for the prevention of legionellosis associated with premise plumbing systems [119]. This standard serves to reduce the risk of *Legionella* infections through a risk management approach [119]. For these reasons, secondary on-site disinfection has become progressively important to protect patients in hospitals and long-term care facilities. An increased understanding of the influence of on-site disinfection on premise plumbing microbial ecology is necessary to maximize effectiveness and to limit undesired side effects.

A notable increase in the genus *Alicyclobacillus* spp. (Firmicutes phylum) was observed following monochloramine treatment, from an average of 4.1±4.5% of the microbial population prior to treatment to an average of 40.9±27.1% following treatment ($p < 0.001$). This genera is comprised primarily of spore-formers that are of concern in food spoilage [120], and has previously been detected in drinking water [121]. The abundance of *Alicyclobacillus* spp. suggests a potentially dominant role in chloraminated hot water system microbial ecology worthy of future investigation.

This study demonstrates that there exists the potential for unwanted consequences of supplemental disinfectant addition for the removal of *Legionella* such as the potential enrichment of other waterborne pathogens, including *Acinetobacter*, *Mycobacterium*, *Pseudomonas*, and *Sphingomonas*. Understanding the impact of supplemental disinfection on water system microbial ecology is necessary to maximize disinfectant effectiveness and to ensure that supplemental disinfectant does not select for alternative opportunistic pathogens. A recent review emphasizes not only the role of disinfectants but also other system factors that may impact microbial ecology such as temperature, pipe material, organic carbon, presence of automatic faucets, and point-of-use filtration [122]. The authors suggest a probiotic approach to opportunistic pathogen control which would either add microbes that can outcompete these pathogens, remove key species, or using engineering controls to favor benign organisms that are antagonistic to opportunistic pathogens [122]. This systematic, probiotic, approach to premise plumbing opportunistic pathogen management is an inventive concept for dealing with the diverse microbial ecology of these systems, but requires a greater understanding of the drivers of premise plumbing microbial ecology, such as provided in this study.

In conclusion, we observed a shift in the microbial ecology of a hospital's hot water system treated with on-site chloramination. This shift occurred immediately upon treatment. Prior to treatment, the bacterial ecology of all samples was dominated by Betaproteobacteria; following treatment, members of Firmicutes and Alphaproteobacteria dominated. Differences in community composition were seen in different locations within the hospital as well as between automatic and standard faucets. This suggests that water from different locations and outlet types should be sampled to get a better, more thorough picture of the microbiota of a system. There was an increase in the relative abundance of several genera containing opportunistic waterborne

pathogens following the onset of monochloramine treatment, including *Acinetobacter*, *Mycobacterium*, *Pseudomonas,* and *Sphingomonas* and genera associated with denitrification. The benefits and risks of each supplemental disinfection strategy should be evaluated before implementation in any building, especially in hospitals, long term care facilities, and other buildings housing immunocompromised patients. This work demonstrates the effects of a supplemental monochloramine disinfection system on the microbial ecology of premise-plumbing biofilms. Given the importance of premise-plumbing microbial ecology on opportunistic pathogen presence and persistence, understanding the driving influence of supplemental disinfectants on microbial ecology is a crucial component of any effort to rid premise-plumbing systems of opportunistic pathogens. As additional facilities turn to on-site water disinfection strategies, more long-term studies on the effects of disinfectants on microbial ecology in premise plumbing are needed as well as those evaluating a probiotic approach to opportunistic pathogen eradication.

5.0 SPECIFIC AIM 3- TO DETERMINE THE EFFECTS OF AN ESTABLISHED MONOCHLORAMINE SYSTEM ON THE MICROBIAL ASSEMBLAGES IN A HOSPITAL'S HOT WATER SUPPLY USING 454 PYROSEQUENCING

5.1 INTRODUCTION

Contamination of a hospital's hot water supply with waterborne pathogens such as *Legionella* can be the source of infection for hospitalized patients [5]. The case fatality rate of healthcare associated Legionnaires' disease can be quite high, ranging from 38%-53% [2]. Supplemental disinfection of the water distribution system in the healthcare facility is an effective approach to prevention of this mode of transmission [2, 87]. Many options for disinfection exist including: copper-silver ionization, chlorine dioxide, point-of-use-filtration, hyperchlorination, and UV light; however each of these methods has benefits and shortfalls [2, 87].

Water treatment with monochloramine has been used at the municipal level but is a new strategy for supplemental disinfection at the building level and has not been evaluated in long-term studies [2, 87]. A recent study in Italy evaluated the use of monochloramine in one hot water network of a hospital's hot water distribution system [90]. They found that monochloramine significantly reduced the levels of *L. pneumophila* without a major change in nitrite and nitrate concentrations but had no effect on *P. aeruginosa* [90]. However, the total

microbial composition in hospital water supplies treated with monochloramine, in contrast to those with no secondary disinfection, remains largely unknown.

Culture-based protocols for assessing microbial populations require organism specific conditions and make population studies complicated and expensive. High throughput sequencing technologies provide an approach to identify many types of bacteria in parallel. This approach can characterize entire microbial populations in biofilms, water, and aerosols of water distribution systems and hospitals [32, 34, 74, 123-126]. These methods identify the presence of bacterial taxa by sequencing segments of their DNA in a culture-independent manner.

We sought to use high throughput sequencing to investigate the effects of monochloramine on the bacteria of a hospital's hot water system. Our study is the first to assess the changes in bacterial assemblages due to on-site chloramination in a hospital's hot water system using high throughput sequencing. Characterization of the selective pressures of monochloramine on bacterial populations may yield new information to assess the risks and benefits of this disinfection strategy based upon changes in bacterial assemblages, including the populations of waterborne pathogens.

5.2 MATERIALS AND METHODS

Hospital setting and Monochloramine system: This study was conducted in University of Pittsburgh Medical Center (UPMC) Mercy hospital. The complex consists of a 12-story, 495-bed tertiary care facility and an 11-story administrative building. Both facilities are supplied by the same chlorinated municipal water source but have independent circulating hot water systems. The hospital's hot water system had been treated using a monochloramine generation system

since September 2011 (Sanipur, Lombardo, Flero, Italy) [112-114]. The administrative building received no supplemental water treatment and served as an appropriate physically adjacent control.

Sample collection and water processing: Immediate-draw (or "first-catch") hot water samples were collected in sterile Nalgene High Density Polyethylene (HDPE) bottles (Thermo Scientific; VWR) from seven sites (six faucets and the hot water tank) from each of the two buildings monthly in May, June, and July 2012. Due to low microbial biomass in the monochloramine treated building two liters of water were collected, whereas one-liter samples were collected from the untreated control building. In addition, a second sample was collected from each outlet after a one-minute water flush to assess the differences in microbial populations at the site versus upstream in the pipe. The temperature of each sample was taken using an infrared thermometer (MiniiiIR Traceable; Control Company; Fisher Scientific). The monochloramine and free chlorine concentrations of the treated and control samples were tested using a Hach DR/890 Colorimeter using Monochlor F Reagent (Hach) and DPD Free Chlorine Reagent (Hach), respectively.

Collected water was filtered through 0.2 µm, 47 mm, Supor® 200 Polyethersulfone membrane disc filters (Pall Corporation) housed in sterile, single-use Nalgene filter funnels (Thermo Scientific; Fisher). Filter membranes were folded and stored at -80^{o}C until DNA extraction.

Samples of the adjacent sink or hot water tank were collected in sterile HDPE bottles with enough sodium thiosulfate to neutralize 20 ppm of chlorine (Microtech Scientific) for enumeration of *Legionella* and total bacteria. Culturing for *Legionella* and total heterotrophic

bacteria was performed according to standard methods using BCYE and DGVP agar plates for *Legionella* [127] and R2A media for total bacteria [128].

DNA extraction and PCR: Genomic DNA was extracted from filter membranes using a bead-beating, phenol-chloroform extraction as described previously [129, 130]. Test PCR was performed for confirmation of successful extraction using universal 16S ribosomal RNA primers 515F and 1391R [35, 130]. A 1% agarose gel was used to confirm the presence of an appropriate PCR product.

Barcoding, Pooling, and Sequencing: DNA was amplified in triplicate with barcoded bacterial PCR primers 8F and 534R that included adaptors for the Roche 454 sequencing platform [131]. A negative PCR control was performed for each barcode, and PCR was repeated for any sample where the control was positive. Amplicons were pooled after normalization of DNA concentration using the Invitrogen SequalPrep Kit [132] (The barcoding and pooling was performed by Dr. Mark Stevens) and sequenced using the Roche 454 FLX Titanium platform per manufacturer's instructions (University of Pittsburgh Genomics and Proteomics Core Laboratories). Sequence data was submitted to NCBI and is available under accession number SRP035587.

Data analysis: Sequence reads were assigned to sample of origin using the bar code sequence added during PCR and screened for basic quality defects (short sequences < 200 nucleotides [nt] in length; > 1 nt ambiguity, best read with quality ≥ 20 over a 10 nt moving window) by the software program BARTAB [133]. Potential chimeras identified with Uchime (usearch6.0.203_i86linux32) [134] using the Schloss Silva reference sequences [135] were removed from subsequent analysis. Filtered sequences (308,799 sequences; average 4,173 sequences/sample) were aligned and classified with SINA (1.2.11) [136] using the 244,077

bacterial sequences in Silva 111NR [137] as reference configured to yield the Silva taxonomy (tax_slv). Sequences with identical taxonomic assignments were clustered to produce Operational Taxonomic Units (OTUs) (The aforementioned data analysis was performed by Drs. J. Kirk Harris and Charles Robertson). The software package Explicet (v2.9.3) (www.explicet.org) [138] was used to compute ecological statistics (e.g. Relative Abundance, the number of species observed (Sobs), Morisita-Horn, ShannonH, and Good's mean) and compose figures. Alpha diversity statistics were calculated using Explicet at the rarefication point of 824 sequences. Good's coverage was > 97% for all libraries. P-values were computed in Explicet via Two-part analysis [139]. Comparison of temperature and chemical measures by ANOVAs and t-tests was performed using http://www.vassarstats.net.

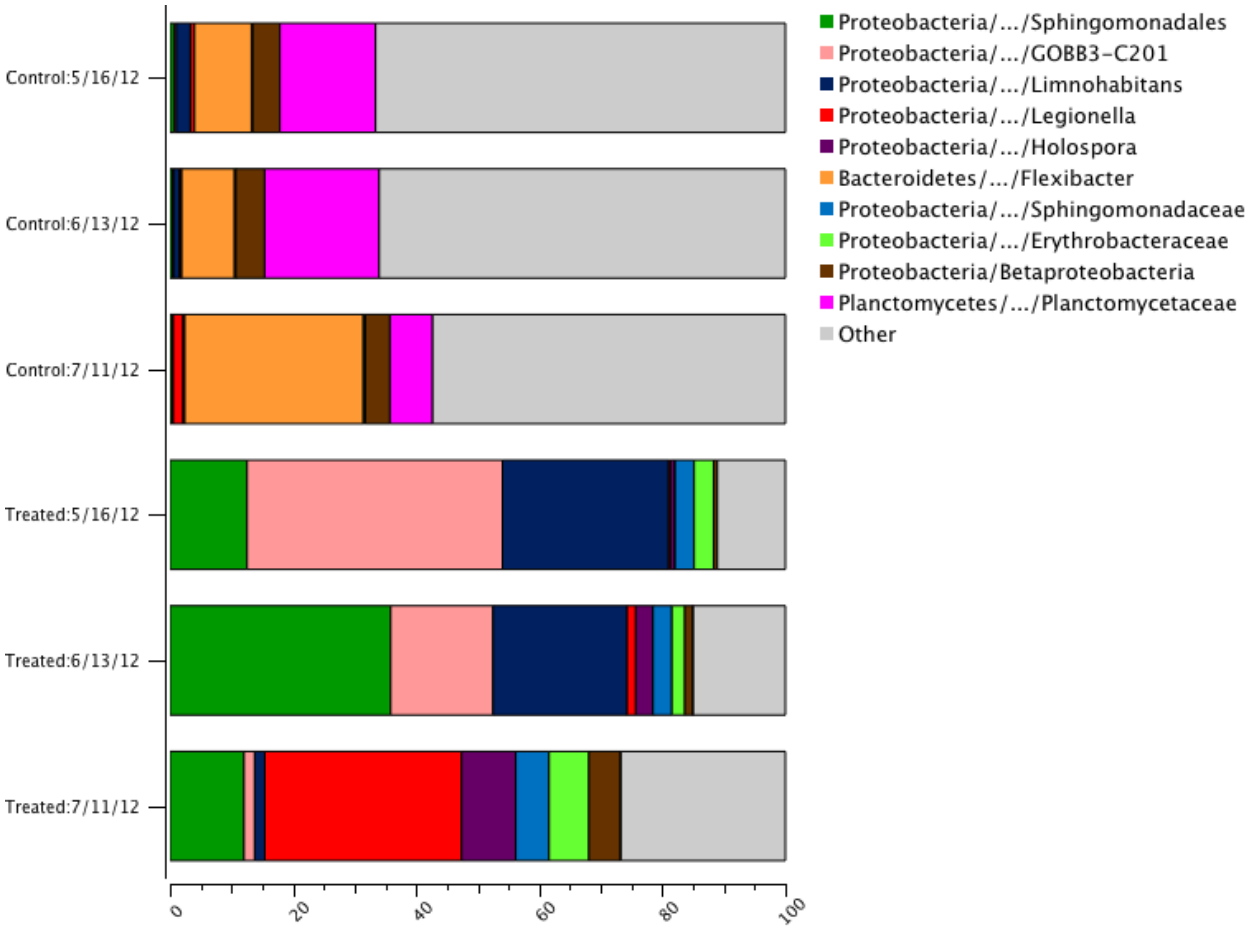
5.3 RESULTS

Taxonomic composition: Monochloramine treated water contained different microbial assemblages than control water (Figure 5.1 and Figure 5.2). Treated samples mostly contained bacteria from the order Sphingomonadales and *Limnohabitans* while the control samples contained *Flexibacter* and the family Planctomycetaceae (Figure 5.1 and Figure 5.2). The control samples contained mostly organisms not found in the top ten treated taxa present based on abundance (Figure 5.1 and Figure 5.2). The monochloramine treated samples had lower bacterial richness compared to the control building samples as fewer organisms accounted for more of the total relative abundance (Figure 5.1 and Figure 5.2) and there were less taxa observed overall (Figure 5.3). The top ten taxa in the treated building accounted for approximately 75-90%

relative abundance clustered by month whereas the top ten taxa in the control building water only accounted for 35-45% relative abundance by month (Figure 5.1).

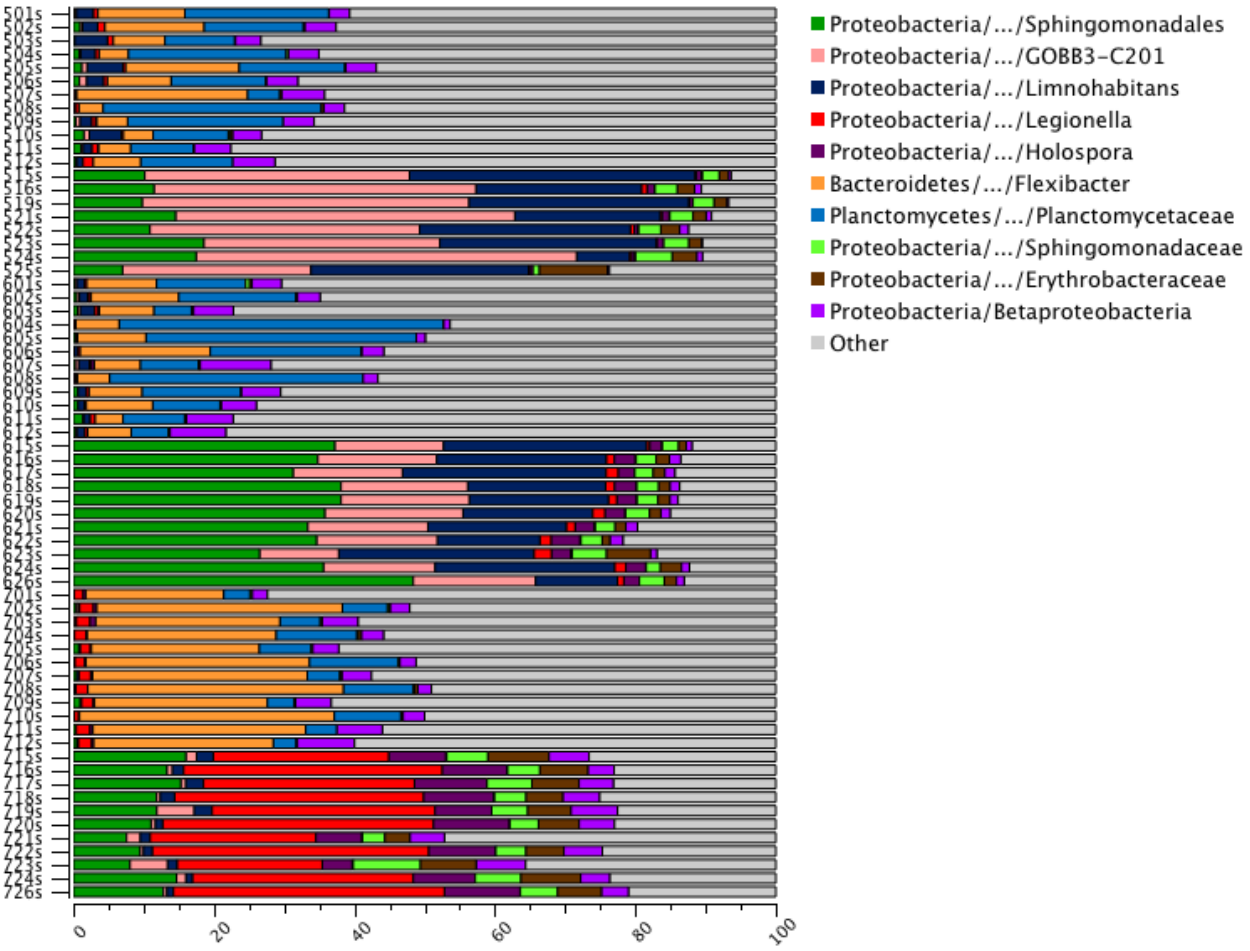
An increase in the relative abundance of *Legionella* was observed in the monochloramine treated samples collected in July and to a lesser extent in the controls from July (Figure 5.1 and Figure 5.2). The bacterial richness in July decreased overall from that of May and June in the control samples whereas in the treated samples the richness increased as the monochloramine degraded (Figure 5.3). A similar trend was seen with the bacterial diversity in these samples as measured by Shannon diversity index (Figure 5.4). The control samples were more diverse than the treated with a slight reduction in Shannon diversity index in July (Figure 5.4). The treated samples had less bacterial diversity overall with an increase in diversity as the monochloramine degraded, although this did not result in a level of diversity that matched the control samples (Figure 5.4).

The *Legionella* and total bacterial counts, obtained by culture, were greatly reduced in the treated samples versus the controls for each sampling month (Table 5.1).



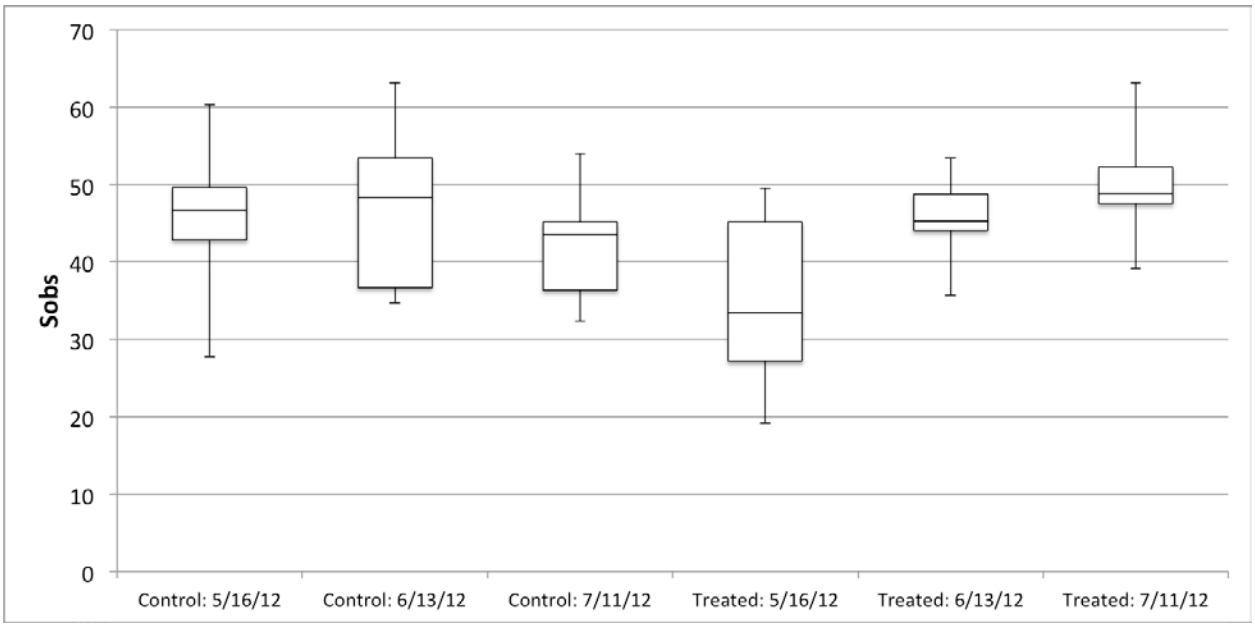
Taxonomic assignments of bacteria on monthly sampling occasions from the control building and treated building. The x-axis represents relative abundance of each taxonomic grouping. The y-axis represents sample type (control or treated) pooled by month.

Figure 5.1. Top ten taxa present in control and treated samples grouped by month.



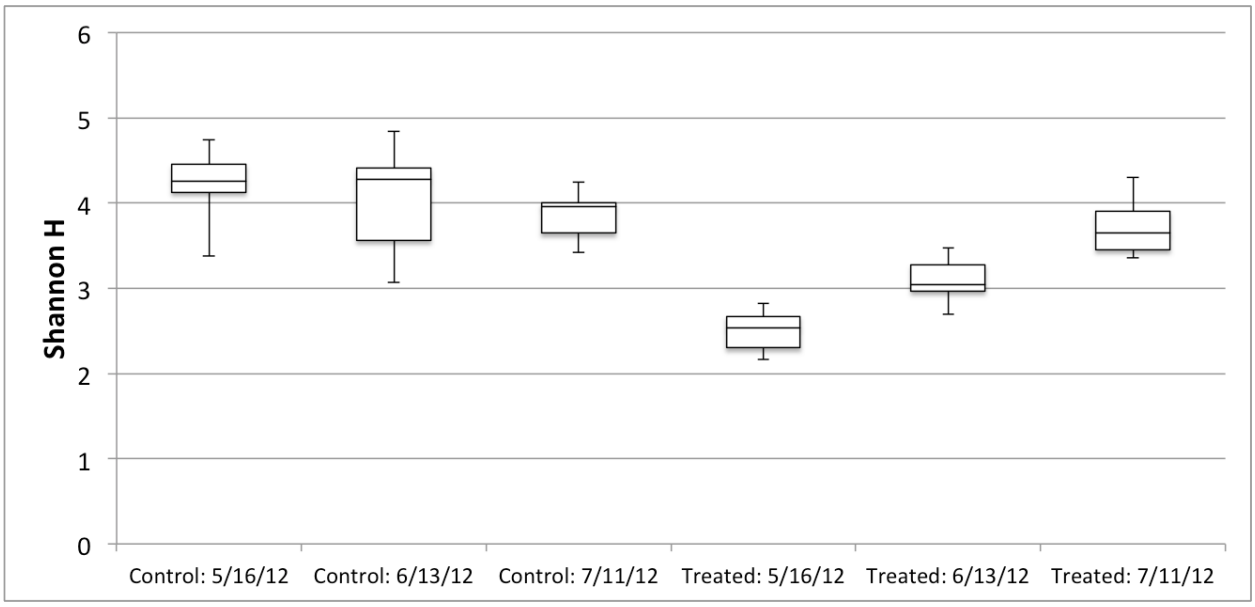
Taxonomic assignments of all bacteria by individual sample. The x-axis represents relative abundance of each taxonomic grouping. The y-axis represents individual samples. The first digit represents the month sampled (5 is May) and the second two digits represent sample site (01-12 are control samples; 15-26 are treated samples).

Figure 5.2. Top ten taxa present in control and treated samples.



Bacterial species richness measured in samples for each sampling month arranged along the x-axis with the 3 control sampling events followed by the 3 treated sampling events. The y-axis represents the number of species observed. The boxes show the median, 25th and 75th percentile with the error bars showing the minimum and maximum values.

Figure 5.3. Bacterial species richness in control and treated samples.



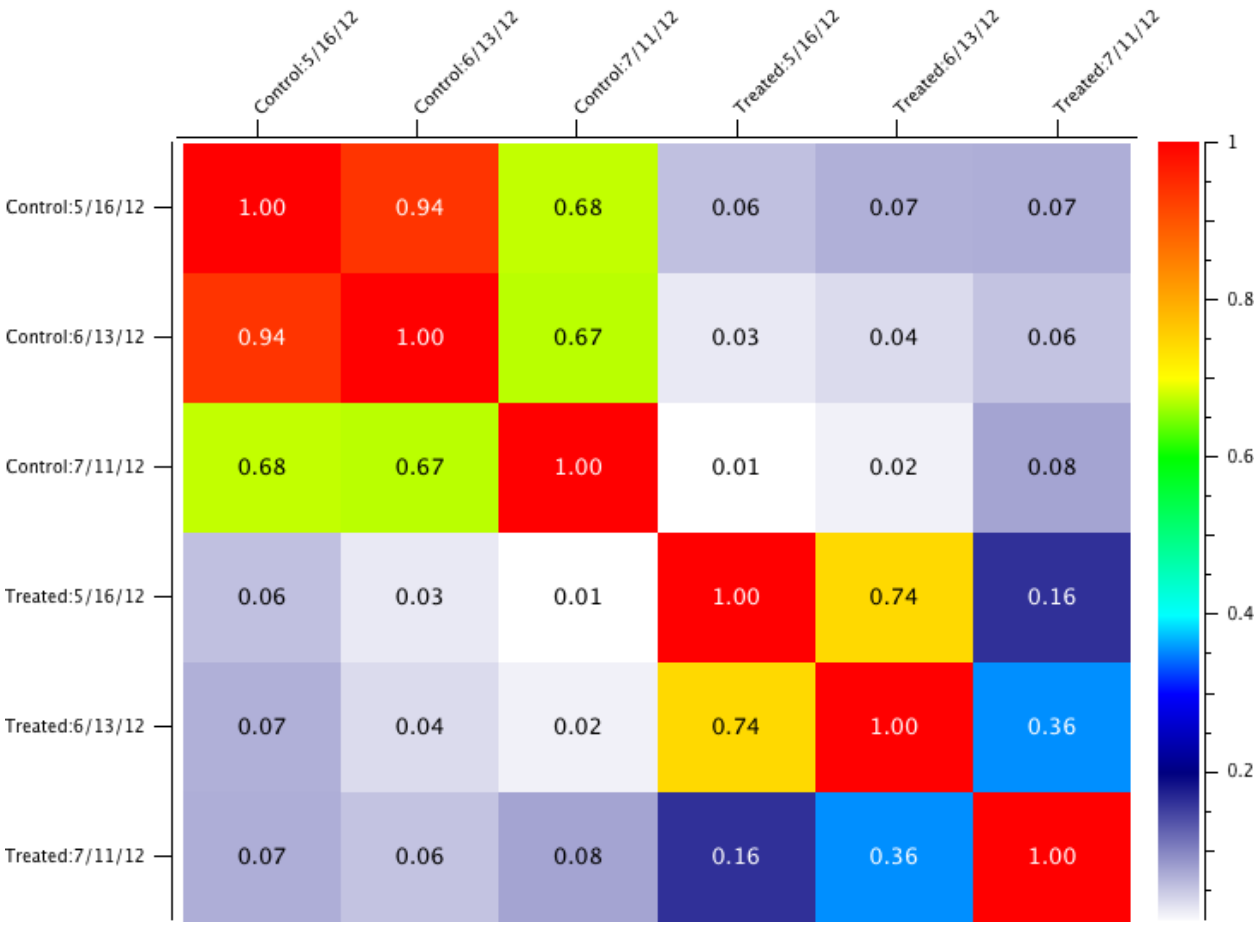
Bacterial species diversity measured in samples for each sampling month arranged along the x-axis with the 3 control sampling events followed by the 3 treated sampling events. The y-axis represents the Shannon diversity index. The boxes show the median, 25th and 75th percentile with the error bars showing the minimum and maximum values.

Figure 5.4. Bacterial species diversity in control and treated samples.

Table 5.1. *Legionella* and total bacteria colony forming units from immediate and post flush samples collected in the treated and control building.

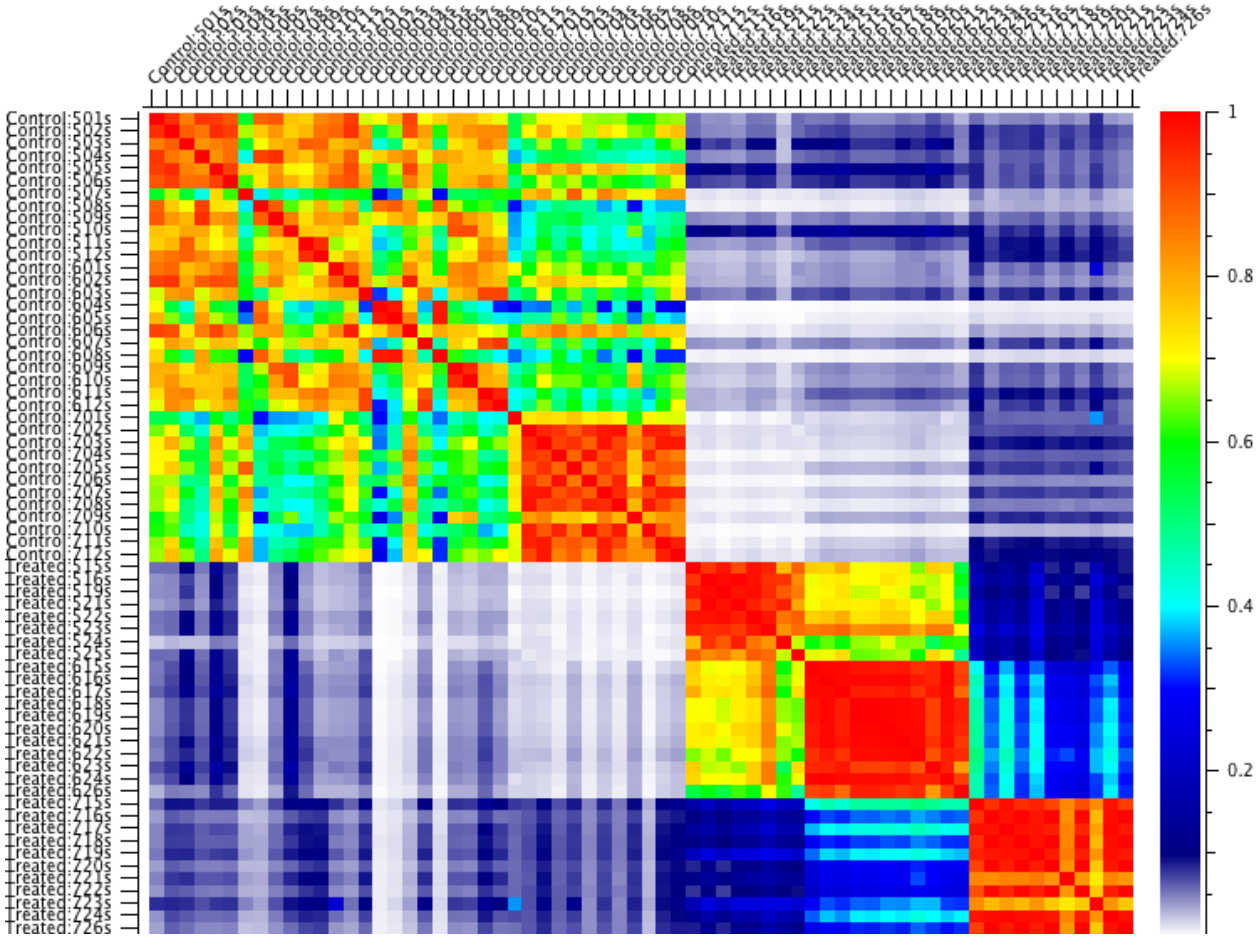
	Legionella (cfu/mL)				Total bacteria (cfu/mL)			
	Control		Treated		Control		Treated	
	Immediate	Flush	Immediate	Flush	Immediate	Flush	Immediate	Flush
May	20	18	0	0	1397	140	2	1
June	28	13	0	0	1237	198	28	1
July	39	29	1	0	2045	249	60	1

Sample dissimilarity: The bacterial taxa found collectively in monochloramine treated and control samples were very dissimilar (Figure 5.5 and Figure 5.6). Pairwise comparisons between treated and control samples showed distinct assemblages with a Morisita-Horn Index (MHI) below 0.09 (Figure 5.6). Figure 5.5 summarizes these data by pooling the libraries generated for each month in the treated and control building, whereas Figure 5.6 shows all the data compared by sample. While MHI was very high within the two buildings for the May and June time points, treated and control water samples collected in July had slightly reduced MHI (Figure 5.5). However, MHI did not decrease to the level of the comparison between the treated and control samples, showing that the monochloramine treatment still caused an alteration in the microbial community (Figure 5.5 and Figure 5.6). A similar result is seen when comparing all samples individually with high MHI when comparing control samples from within chloraminated or control building samples (Figure 5.6). The MHI decreased in July for both control and chloraminated samples, although the MHI values did not decrease to the same level as the comparisons of control and treated individual samples (Figure 5.6).



Morisita-Horn statistical pair-wise comparisons of bacterial sequence sets from pooled samples collected from the control vs. treated building each month. Heat bar at right indicates MH similarity level, with MH = 0 indicating no similarity between samples (cool colors), and MH = 1 indicating complete similarity between samples (hot colors). Samples are arranged along the ordinates such that all control samples are clustered together according to month, followed by all treated samples according to month.

Figure 5.5. Community similarity of control and treated samples grouped by month.

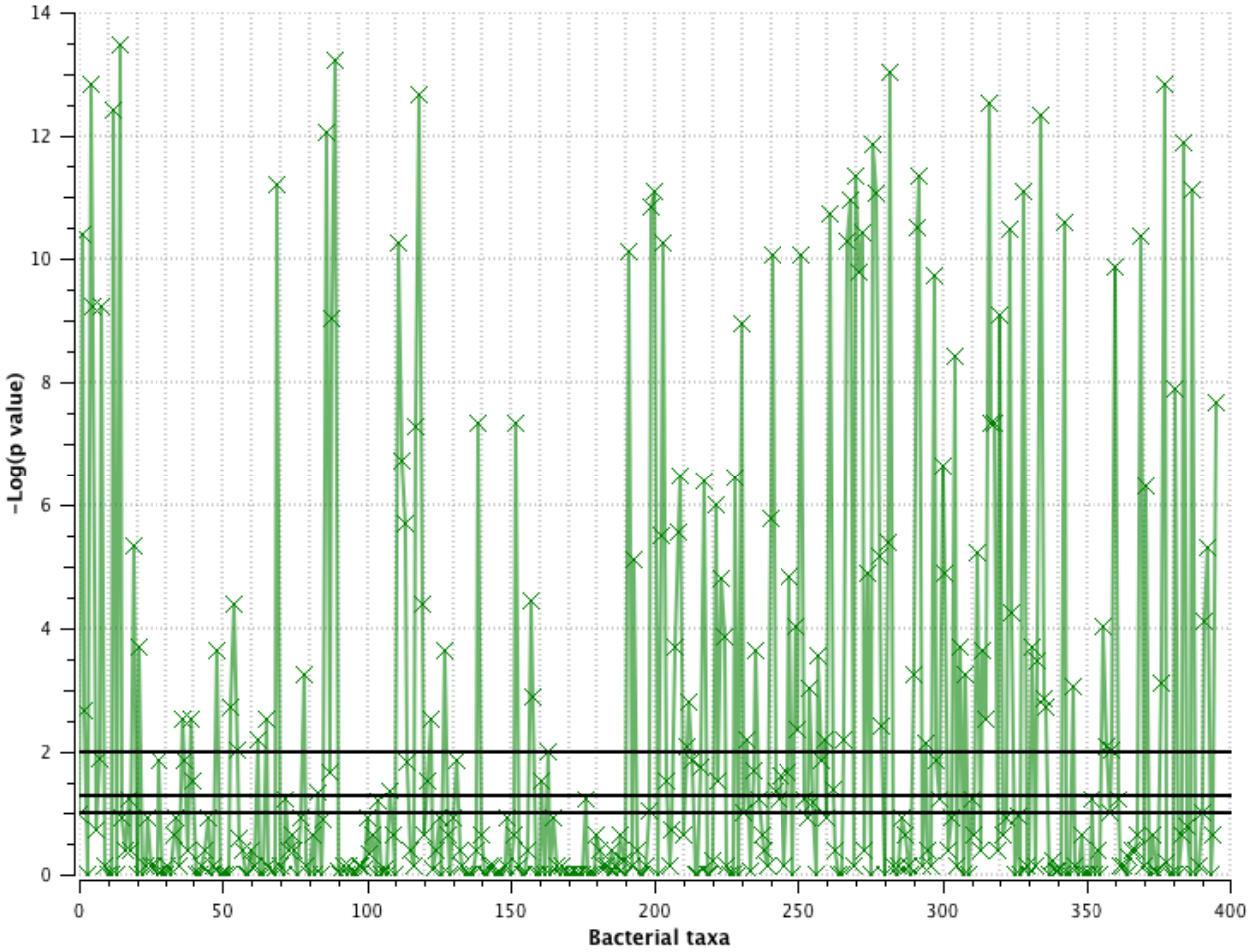


Morisita-Horn statistical pair-wise comparisons of bacterial sequence sets from each sample. Heat bar at right indicates MH similarity level, with MH = 0 indicating no similarity between samples (cool colors), and MH = 1 indicating complete similarity between samples (hot colors). Samples are arranged along the ordinates such that all control samples are clustered together according to the time series, followed by all treated samples according to the time series.

Figure 5.6. Community similarity of control and treated samples.

Comparison of treated and untreated taxa: A comparison of bacteria identified in monochloramine treated and control water demonstrates highly significant differences in relative abundance and prevalence between the treated and control building for 140 bacterial taxa identified in this study (Figure 5.7). Among organisms present at high relative abundance in both treated and control samples, there was a significant increase in Sphingomonadales and

Limnohabitans in treated samples with a concurrent reduction in *Flexibacter*, the family Planctomycetaceae, and Nitrosomonadaceae.

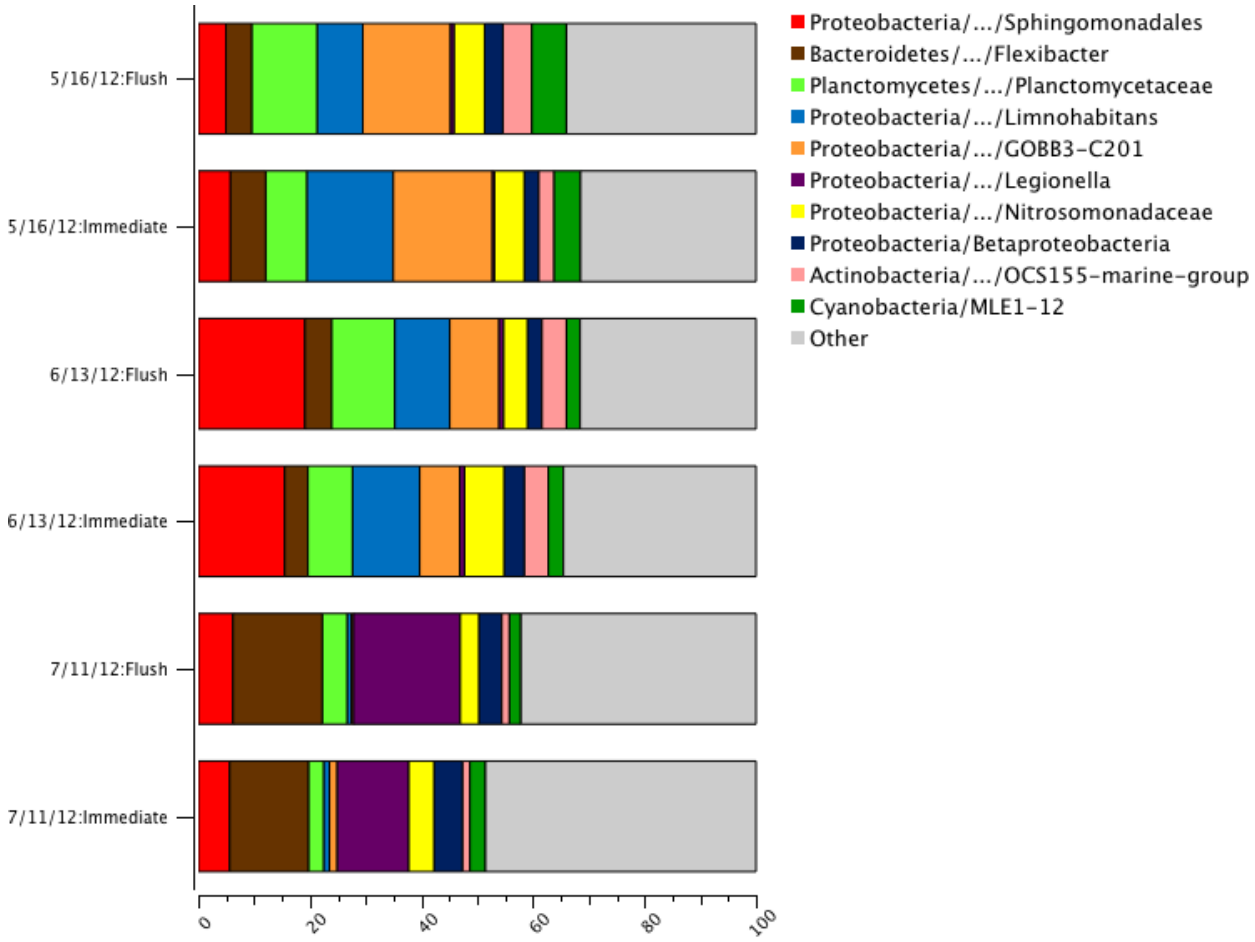


Manhattan plot displaying p-values for comparisons of bacterial taxa abundance between the control and treated sample types. The x-axis represents each bacterial taxon arranged alphabetically. The y-axis displays the negative log of p-values. Black lines represent p-value thresholds of 0.10, 0.05, and 0.01, from bottom to top, respectively.

Figure 5.7. Comparison of the abundance of bacterial taxa between control and treated samples.

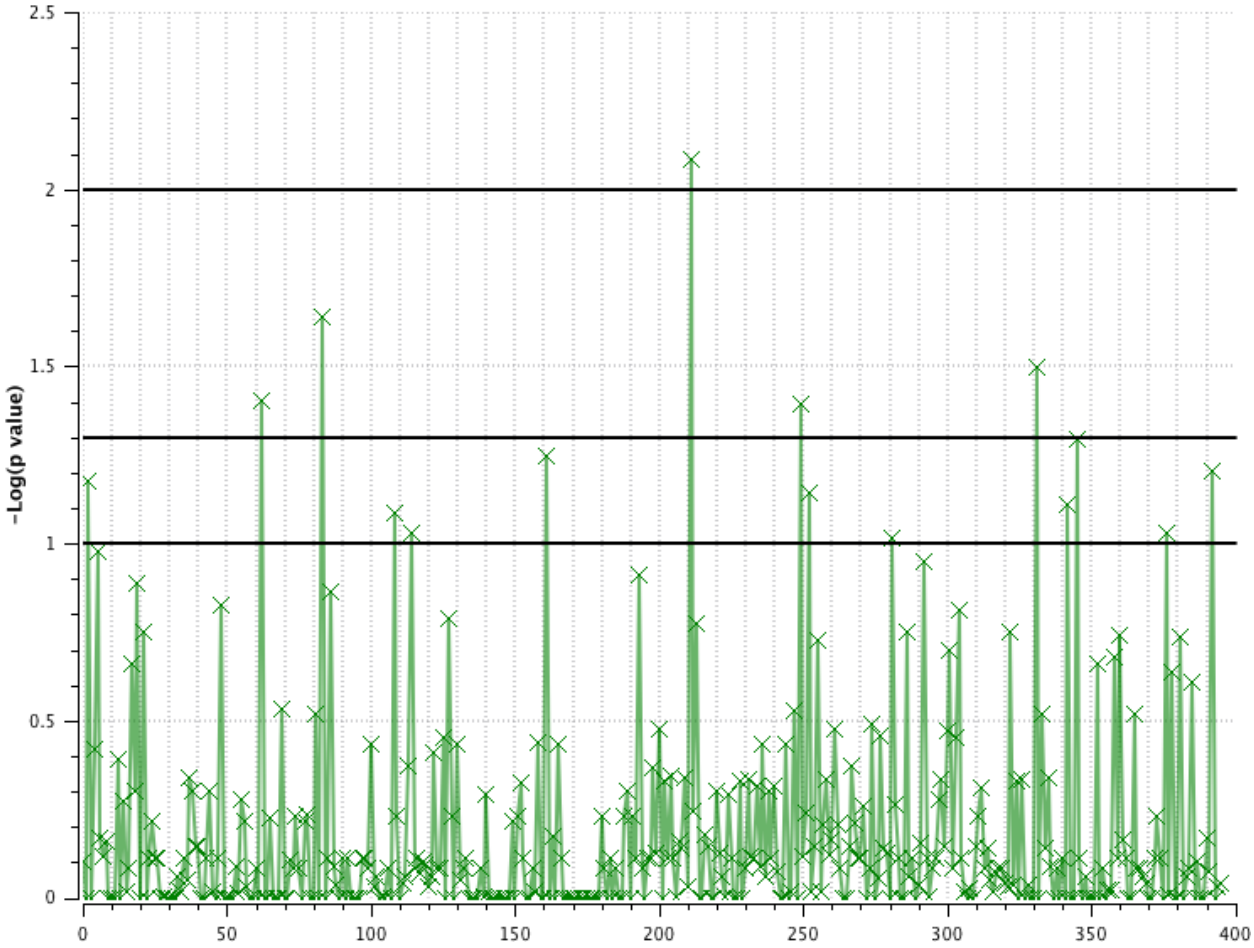
Comparison of immediate and flushed taxa: Comparison of the top ten bacterial taxa present, for each month's sampling, in the immediate and flushed samples shows an almost indistinguishable similarity in both the top organisms present as well as their relative abundance in a given month (Figure 5.8). There were very few significant differences in bacterial

composition between immediate and flush samples for particular months or sites (Figure 5.9). The culturable total bacterial concentrations decreased in the samples flushed for one minute compared to the samples collected immediately for each sampling month (Table 5.1). The culturable *Legionella* concentrations did not differ greatly between immediate and flushed samples (Table 5.1).



Taxonomic assignments of bacteria for each sampling month clustered into immediate and flushed samples. The x-axis represents abundance of each taxonomic grouping. The y-axis represents sample type (immediate-catch or post one-minute flush) pooled by month.

Figure 5.8. Top ten taxa present in immediate and flushed samples grouped by month.



Manhattan plot displaying p-values for comparisons of bacterial taxa abundance between the immediate and flushed sample types. The x-axis represents each bacterial taxon arranged alphabetically. The y-axis displays the negative log of p-values. Black lines represent p-value thresholds of 0.10, 0.05, and 0.01, from bottom to top, respectively.

Figure 5.9. Comparison of the abundance of bacterial taxa between immediate and flushed samples.

Temperature and chemical measures: The average temperatures of the control and treated immediate samples were highly similar ($p > 0.05$) (Table 5.2). This was also the case for the control and treated flush samples; however, the control flushed samples were slightly warmer than the treated ($p > 0.05$) (Table 5.2). There were no significant differences in free chlorine concentrations over time ($p > 0.05$) or between immediate and post flush samples ($p > 0.05$) (Table 5.2). There was no difference in monochloramine concentration between immediate and flushed samples ($p > 0.05$), however there was a statistically significant reduction in

monochloramine concentration in July ($p < 0.01$) (Table 5.2). Despite this reduction in monochloramine concentration all measured values fall well within the manufacturer's suggested effective monochloramine concentration range.

Table 5.2. Temperature and chemical concentrations of treated and control water for both immediate and post flush samples

	Temperature (^{o}C)				Chemical concentrations			
	Control		Treated		Control[a]		Treated[b]	
	Immediate	Flush	Immediate	Flush	Immediate	Flush	Immediate	Flush
May	34.5	48.7	34.4	43.5	0.023	0.030	2.15	2.42
June	31.3	47.4	37.2	43.9	0.023	0.022	2.29	2.23
July	29.9	45.4	32.2	44.1	0.017	0.024	1.99	2.02

[a] For control samples free chlorine was measured and reported as ppm of Cl_2
[b] For treated samples monochloramine was measured as ppm of Cl_2

5.4 DISCUSSION

The purpose of this study was to determine the selective pressures exerted on microbial populations by monochloramine addition to a hospital's hot water supply. To accomplish this we collected 84 hot water samples over a three-month period from seven sites in each of two buildings: a control administrative building and a hospital treated with on-site monochloramine injection. These buildings were chosen because they both receive the same incoming cold water but differ in secondary disinfection. This study took place eight months into the first evaluation of a commercially available monochloramine generation system applied to a hospital's water system in the United States [112-114]. These samples were analyzed using 454 pyrosequencing

of amplified Small Subunit-rRNA for bacterial identification and subsequent comparison of the effects of monochloramine on the microbial assemblages in hot water.

The differences in bacterial composition and relative abundance between monochloramine treated and control samples were dramatic. There was practically no overlap in the bacterial taxa present in the treated and control waters (Figure 5.1). We found this difference to be statistically significant, by two-part statistical analysis, and due to the monochloramine addition. There was a reduction in both *Legionella* and total bacteria culturable in the treated samples. A difference in relative bacterial abundance, richness, and community composition between immediate and flushed samples was not found in this study. There was, however, a reduction in total bacterial counts in flushed samples versus their immediate-catch counterparts but interestingly the overall community compositions were equivalent.

Chemical water treatment can have unintended consequences. In the case of chlorine, corrosion and selection for chlorine tolerant bacteria such as *Legionella* have been observed [140]. Both chemical and microbiological changes have been noted in municipal water systems using monochloramine. These include increased ammonia, nitrate, and nitrite concentrations as well as increases in total bacteria and mycobacterial species [89].

We did not find evidence of these undesirable consequences of monochloramine treatment when applied to a hospital hot water system. Neither 454 pyrosequencing nor culture-based methods showed increases in mycobacterial relative abundance or load, and total bacterial concentrations actually decreased significantly by approximately 1 order of magnitude during our previous study [114]. Interestingly, we saw an approximate 10-fold reduction in the relative sequence abundance of *Mycobacterium* spp. by 454 pyrosequencing compared to the control. This relative reduction was also observed in the culture-based portion of the Duda et al. study.

In our study, and consistent with studies in municipal and simulated water systems, [32, 45, 117, 125] we found monochloramine treatment exerted strong selective pressures on the microbes in these waters. Several of these previous studies have shown an increase in *Mycobacterium* spp. after treatment [45, 117, 125], but we did not observe this effect. It is possible that the difference in behavior of mycobacteria in response to monochloramine may be due to the fact that our study took place in a hot water system instead of a municipal cold water distribution system or that the incoming cold water was already chlorinated.

Unlike a study of a drinking water distribution system [32], we saw more members of the order Sphingomonadales in chloraminated water than chlorinated water. This group includes *Sphingomonas paucimobilis*, a waterborne pathogen, and although we were unable to confirm the presence of this species, the genus *Sphingomonas* was identified. There was a decrease in the presence of bacteria that oxidize ammonia or nitrite, Nitrosomonadaceae and *Nitrospira* respectively, in the chloraminated samples.

Another sequencing study has described nitrification in a chloraminated simulated distribution system [117]. The results in culture studies are mixed with a municipal system observing nitrification in storage tanks [89], but an investigation performed using a hospital hot water system did not find any variation in levels of nitrate or nitrite [90]. In our previous culture-based study of this system we saw fairly stable levels of nitrate, nitrite, and total ammonia until March when nitrate and total ammonia increased [114]. This was slightly before the increase in *Legionella* presence observed in July (see description below) [114]. There were no nitrifying bacteria isolated by culture during the previous culture study [114].

We serendipitously sampled in July during a time of sub-optimal monochloramine precursor reagent dosing. A shift in the microbiological populations was observed showing a

dramatic increase in the relative abundance of *Legionella* from 1.4% in June to 32% in July by 454 pyrosequencing (Figure 5.1). In a previous related study, culture of 27 water outlets in the hospital one week later showed distal site positivity increased from 4% in May to 26% in July [114]. There was no increase in the colony forming units of *Legionella* recovered from these time points, which remained at 1-10 CFU/mL on average [114].

Upon investigation, it was found that the chlorine precursor solution used to form the monochloramine on-site had degraded and the excess of ammonia precursor reagent impacted the efficacy of the monochloramine solution injected into the hot water system. This altered solution proved inadequate in preventing *Legionella* growth. While the monochloramine concentrations measured did decrease in July, they still fell well within the manufacturer's recommended levels. This suggests that the total ammonia concentrations may be helpful in determining the effectiveness of treatment since monochloramine levels, as measured by ppm as Cl_2, did not alone predict the rebound of *Legionella*.

Legionella rebound after disinfection has been demonstrated previously. In a pilot-scale domestic water loop system, virtually all disinfection strategies showed *Legionella* rebound after 4-5 days following the use of ozone, electro-chlorination, chlorine dioxide, monochloramine, chlorine, or copper-silver ionization [141]. Other studies found re-colonization 11 days following chlorine dioxide treatment [142] and one month following thermal eradication [143]. A study of a nuclear power plant cooling circuit found rebound of *L. pneumophila* following chloramination within approximately one month [144]. With incorrect dosing of monochloramine precursor reagents, as happened in the system in the present study, it is clear that bacterial regrowth can occur quickly and seems to explain the dramatic increase in *Legionella* spp. presence that occurred in July. An alternative, and possibly compounding, explanation is that some *Legionella*

were present in the incoming water. This is supported by the identification of *Legionella* in the control samples from the month of July, whereas *Legionella* were not observed in previous months. Some seasonality has been noted for *Legionella* presence in water, especially in the warmer summer months with many more cases of Legionnaires' disease occurring in summer and fall than winter or spring [145, 146]. It is therefore important, as with any disinfection system, to monitor levels of chemical disinfectants as well as levels of *Legionella* especially during the summer when weather is warm and more of these bacteria may be isolated from the water supply [146].

Monochloramine disinfection has been shown to result in the presence of a viable but not culturable (VBNC) form of *Legionella* [40]. Our results demonstrate that 454 pyrosequencing showed low relative abundance of *Legionella* when cultures showed little or no viable *Legionella,* and higher relative abundance when *Legionella* recolonized the system during a period of sub-optimal monochloramine dosing. These results suggest that viable non-culturable forms did not represent a large proportion of the microbial population when the water was treated with monochloramine. However, as with other chemical disinfectants, *Legionella* remains present in the system and is capable of recolonizing in a relatively short period (days to weeks) when disinfectant is not maintained at the effective concentration.

This study shows the positive effects of monochloramine on reducing *Legionella* presence as well as a lack of some of the issues with chloramination seen in municipal water supplies. Future studies should look at change over time in these systems as monochloramine is added. Also, it may be beneficial to look at the incoming water for *Legionella* presence as well as longitudinally at the system over different seasons to see how this affects the microbial communities in this type of system.

In conclusion, this is the first study to identify the effects of on-site monochloramine treatment on bacterial communities in a hospital hot water system. Overall, we saw little to no similarity between assemblages in treated and untreated control waters. There was also a reduction in bacterial richness and diversity in monochloramine treated communities. This may have led to the relative ease for *Legionella* to recolonize the system once the disinfectant was not dosed with the correct proportion of chlorine to ammonia. It is unknown whether the bacterial populations (taxa) now in abundance due to monochloramine treatment will have a negative impact on plumbing or represent a new risk of infection for hospitalized patients. Ongoing studies will elucidate these long-term effects. Next-generation sequencing may be beneficial to this end as it becomes more affordable and faster to perform.

6.0 SPECIFIC AIM 4- TO DETERMINE THE EFFICACY OF NEW POINT-OF-USE FAUCET FILTERS IN THE REMOVAL OF *LEGIONELLA* AND *PSEUDOMONAS* FROM THE HOSPITAL WATER SUPPLY

6.1 INTRODUCTION

The water distribution system of hospitals is an important reservoir for waterborne pathogens, including *Legionella* species, *Pseudomonas aeruginosa*, *Acinetobacter* species, nontuberculous *Mycobacterium*, *Stenotrophomonas maltophilia*, and fungi, such as *Aspergillus* species [147]. These organisms may persist despite widespread disinfection of the water distribution system using any number of techniques and chemicals, such as chlorine dioxide, copper silver ionization, hyperchlorination, ultraviolet light, or super heating [142, 147, 148]. At even low levels, these organisms may pose a threat to certain patient populations, including those in bone marrow transplant units, hematology/oncology units, or solid organ transplant units [147]. Filters can be used in addition to or in the place of systemic disinfection.

Numerous studies have investigated the efficacy of point-of-use filters installed in high risk areas to prevent the transmission of waterborne pathogens to their immunocompromised hosts [147-153]. Different models of filters have been shown to be efficient in removing *Legionella* species [147, 148, 153], *P. aeruginosa* [149, 153], *P. aeruginosa* and *S. maltophilia* [150], *Mycobacterium* species [151, 153], and fungi [152, 153]. However, in six of these seven

studies, the filters were only rated for 1 week [147] or 2 weeks of continuous use [149-153]. In addition, flow restriction has been reported to further shorten the duration of use for the filters [7]. The utility of point-of-use filters as a tool for infection prevention would improve and be less cost-prohibitive if the filters maintained efficacy and flow for longer periods of time. We evaluated a new extended use 62-day point-of-use faucet filter. The purpose of our study was to provide an objective field evaluation of this filter in eliminating *Legionella*, *Pseudomonas*, and total bacteria in water from faucets over a period that met and exceeded the manufacturer's 62 days of approved use.

6.2 MATERIALS AND METHODS

The location for the study was a cancer center in northwestern Pennsylvania that was colonized by *L. pneumophila* serogroup 1. Point-of-use filters (QPoint) (Pall Medical Corporation, East Hills, NY) were installed on 5 faucets. Five faucets without filters served as control sites.

Samples were collected weekly from May 8th 2013 through August 28th 2013. Approximately 250 mL of water was collected after the hot water valve was turned on and flushed for 1 minute. Samples were collected in HDPE bottles with enough sodium thiosulfate to neutralize 20 ppm of chlorine (Microtech Scientific). Prior to sampling, the faucets equipped with filters were wiped with an antiseptic wipe to remove bacteria from the external surface of the filter.

Culturing for *Legionella* was done using BCYE and DGVP agar plates (ISO Standard 11731:1998 and ISO Standard 11731:2004), for *Pseudomonas* using MPAC agar (ASTM International Standard Test Method D5246-92), and total bacteria using an R2A pour plate

methodology (Standard Method 9215B). Representative isolates of bacteria recovered from the faucets with filters were speciated by DNA sequencing (MIDI Labs, Newark, DE).

Rainfall data was analyzed from June 1st 2013 to August 14th 2013. Data was obtained from the National Climatic Data Center website at www.ncdc.noaa.gov and was from the nearest weather station to the cancer center.

ANOVA was used to compare *Legionella* and total bacterial counts between filtered and non-filtered sites using Stata version 13.0.

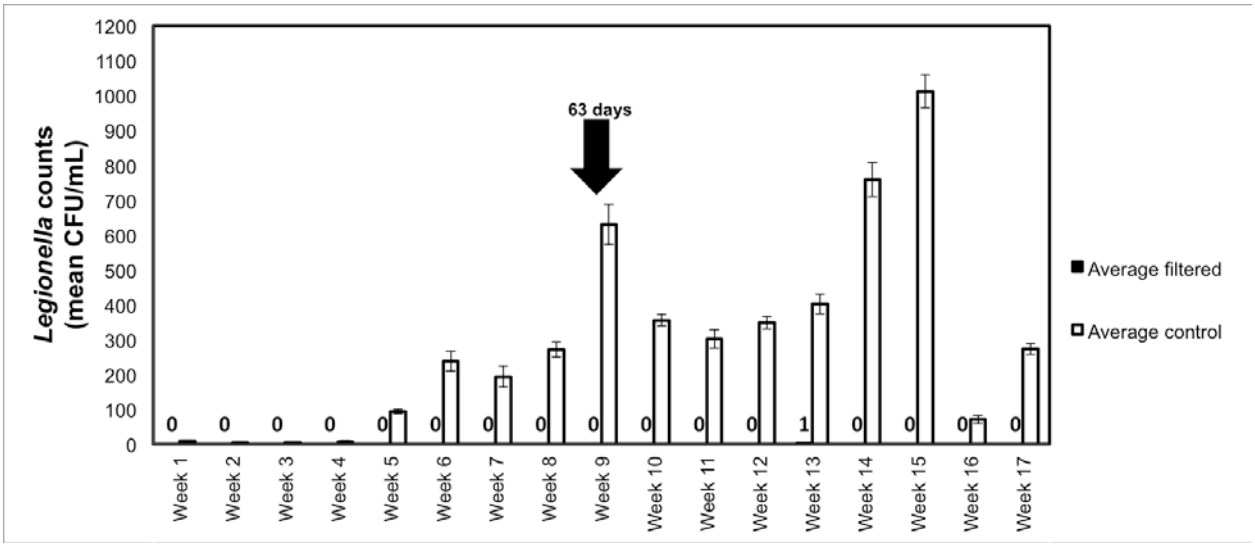
6.3 RESULTS

***Legionella*:** No *Legionella* were recovered from water samples collected from faucets with filters over the entire 17-week period for 4 of 5 faucets with filters (Figure 6.1). Water obtained from one faucet on week 13 was positive for *Legionella* at 1-10 CFU/mL (one colony on the plate) (Figure 6.1). *Legionella* was not recovered from this fixture in weeks 14-17. Control faucets had on average 292.4 CFU/mL of *Legionella* during the study, ranging from 1-10 CFU/mL to 1150 CFU/mL (Figure 6.1). This reduction in *Legionella* was statistically significant ($p < 0.0001$).

Other Bacteria: Filters completely excluded heterotrophic plate count (HPC) bacteria (total bacteria) from samples for the first two weeks (Figure 6.2). During these two weeks, the average log reduction in HPC bacteria in filtered samples was 4.35 (3.93 and 4.77; in weeks 1 and 2, respectively) compared to the controls. This was followed by an average 1.86 log reduction in filtered samples for the remainder of the study (range 1.31 to 2.47). The filters significantly reduced the amount of total bacteria in these water samples ($p < 0.0001$). *P.*

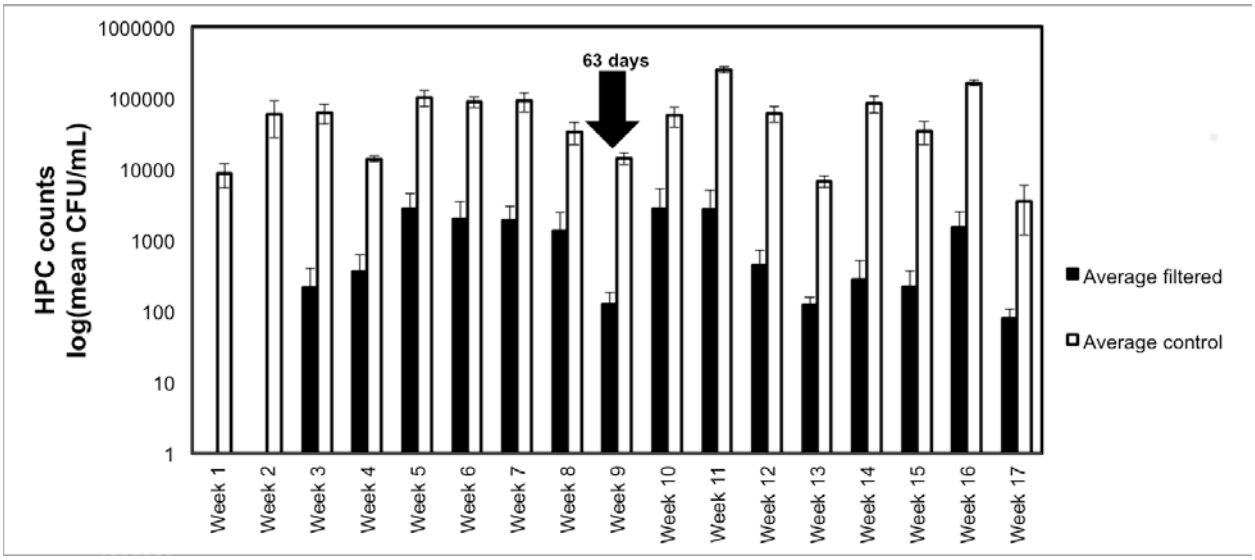
aeruginosa was not isolated from this water supply, so no conclusions can be made about the efficacy of these filters with respect to this organism.

Flow Restriction: Prior to the study the total suspended solids (TSS) were measured and found to contain 2.40 mg/L at 0.2 microns or larger. Despite this level and size of particulates, adequate flow was observed throughout the study and found to be unrestricted even at 17 weeks.



Data from weekly sampling and culturing of *Legionella* spp. from filtered faucets and control faucets is shown over a period of 17 weeks (119 days). Values are averages of 5 sites. Arrow indicates 63 days, one day past the proposed maximum usage by the manufacturer. Error bars represent standard error of the mean.

Figure 6.1. Faucet filters prevent exposure to *Legionella.*



Data from weekly sampling and culturing of heterotrophic plate count (HPC) bacteria from filtered faucets and control faucets is shown over a period of 17 weeks (119 days). Values are averages of 5 sites. Arrow indicates 63 days, one day past the proposed maximum usage by the manufacturer. Error bars represent standard error of the mean.

Figure 6.2. Faucet filters significantly reduce the total amount of bacteria recovered.

6.4 DISCUSSION

Legionella and other opportunistic pathogens multiply in hospital water systems and pose a threat to patients despite receiving treated water from municipal water treatment plants [142, 147, 148]. Additional secondary disinfection measures are sometimes necessary to prevent healthcare-acquired infections. Disinfection methods include chemical (chlorine, chlorine dioxide, copper-silver ionization, monochloramine) and physical (UV light and point-of-use filtration). Since systemic disinfection cannot completely eliminate *Legionella* from all fixtures, point-of-use filters have been used to further protect high-risk patients.

While point-of-use (POU) filters have been successful at preventing exposure to *Legionella* and other waterborne pathogens, their use has been limited due to relatively short

recommended duration of use, flow restrictions and cost. The purpose of this study was to provide a field evaluation of the efficacy a new point-of-use faucet filter both in excluding waterborne pathogens from water and also evaluating the number of weeks they could maintain this exclusion. To accomplish this, we sampled 10 faucets (5 with filters installed and 5 without) in a cancer center for 17 weeks and analyzed for the presence of *Legionella*, *Pseudomonas*, and total bacteria.

The faucet filters removed *Legionella* from the hot water throughout the course of the study (Figure 6.1). There was one positive water sample (1-10 CFU/mL) from one filtered site recovered during week 13. There was no further breakthrough through 17 weeks of testing.

Complete bacterial exclusion was achieved for the first two weeks of this study (Figure 6.2). Thereafter, heterotrophic plate count (HPC) bacteria were isolated on the R2A culture media. This is consistent three previous studies where total bacteria were seen consistently within 14 days of use [147, 151, 153]. Explanations for the presence of total HPC bacteria have included external contamination of the filter housing or that growth occurred within the filter. Our data suggests an alternative explanation. External contamination is unlikely due to the fact that the outside of the filter was sanitized with an antibacterial wipe prior to sample collection. The bacteria that we isolated were atypical small gram-negative rods and represented a limited number of colony types and included *Hydrogenophaga* species. Due to its small dimensions (0.24 ± 0.01 μm wide by 2.48 ± 1.04 μm long) and flexibility, this organism has been shown previously to consistently pass through 0.2 μm filters [154]. We could not find any reference to human diseases caused by this organism, so their presence may be inconsequential from an infection control perspective. The other two organisms isolated have similar characteristics to *Hydrogenophaga*, but we were unable to speciate them by DNA sequencing.

This study also provided an opportunity to observe changes in *Legionella* positivity over a 17-week period. The study began in May (Spring) and ended in late August (Summer). Initially the hot water samples from the control faucets had very low concentrations of *Legionella* (1-10 CFU/mL). The concentration increased beginning in June. There were spikes in the *Legionella* counts from the control faucets in weeks 9 (July) and 14 (August), increasing from an average of 271 to 630 cfu/mL and from 402 to 758 cfu/mL, respectively. We investigated possible causes of these increases, such as hydrant flushing or disruption of water service due to work on water mains by the water authority, or unusual weather conditions. No flushing or maintenance occurred during these time intervals. Rain events in this area during the week before each spike were compared to an average level of rain for the period between June 1st 2013 and August 14th 2013. The week between the week 8 and week 9 samplings had 3 of 5 reported days with higher than the average levels of rainfall. The second interval, between weeks 13 and 14, had 1 of 7 reported days with higher than average rainfall. The observed increase in *Legionella* concentration may have been due to increased rainfall or may represent a normal increase due to seasonal changes in temperature affecting the microbial ecology and water treatment. Several studies have found either *L. pneumophila* in rainwater [155] or a link between rain events and cases of Legionnaires' disease [156, 157]. Hot weather and rainfall can lead to increased sediment and bacterial presence in drinking water, which can allow for an increase *Legionella* replication and decrease the effectiveness of chlorine in killing these and other organisms [156]. Further study is necessary to investigate the influence of these ecological changes on *Legionella* growth in hospital water systems.

These new filters differ from previously designed faucet filters in that they contain a 30 μm prefiltration layer, a 1 μm Supor membrane and a 0.2 μm Supor membrane. Previous filter

models only contain two layers, a 1 μm prefiltration layer and a 0.2 μm Supor membrane. These alterations may lead to better performance and less clogging than other faucet filters due to the prefiltration of larger particles and two layers of Supor membrane.

In summary, these new faucet filters prevented *Legionella* exposure for longer than the 62 days recommended by the manufacturer. All but one sample were free of *Legionella* for 90 days, plus an additional four weeks. We do not recommend use beyond the manufacturer's suggested guidelines, but our results suggest that failure may not occur for some time beyond 62 days. The new 62 day point-of-use filter has the advantage of requiring half the number of change-outs than the previous 30-day filters and could be a cost effective method of preventing exposure to opportunistic waterborne pathogens in hospitals with high-risk patients.

7.0 OVERALL CONCLUSIONS AND IMPLICATIONS TO PUBLIC HEALTH

7.1 CONCLUSIONS

In conclusion, our studies have lead to the development of a LAMP primer set that is specific for the 15 serogroups of *L. pneumophila*. This assay can detect as little as 400 cfu/mL and higher of this species in contaminated water samples. We were unable to design primers that were specific for the genus *Legionella* based on the 16S rRNA of this organism due to cross reaction of primer sequences with other genera of bacteria. This is a particularly interesting finding that may explain, at least in part, some of the false-positivity that has been seen previously with molecular assays used in environmental water samples. The cross reaction with other waterborne organisms may lead to further evaluation of the specificity of current molecular technologies for detecting *Legionella*.

We also evaluated the application of an on-site monochloramine generation system over time by microbiological culturing and sequencing. There was an overall significant reduction in both culturable *Legionella* and total bacterial counts whereas other WBPs did not show change over time. Through Illumina sequencing we saw an immediate shift in the microbiology of this system upon initiation of treatment. Over time there was an increase in the relative abundances of *Acinetobacter*, *Mycobacterium*, *Pseudomonas*, and *Sphingomonas*. This increase in WBP relative abundance was not seen in the culture data, as there was no increase in any WBPs surveyed. This difference between the culture study and the molecular study may due to the

significant reduction in culturable total bacteria. While the relative abundance increased, the total counts decreased potentially resulting in no overall change.

During this larger study, we examined the differences between monochloramine treated samples and those from an attached building that received no secondary disinfectant (control). This study eliminated the confounding factors of time and building location, as these samples were taken on the same day and the buildings receive the same incoming cold water. Thus there would not be a difference in the source water due to season or the control building not being attached to the treated building. In this study we found a difference in relative bacterial abundance, diversity, and community composition in treated and control samples. We did not see an increase in *Mycobacterium* species or nitrifying bacteria as had been seen in municipal (cold) water systems.

Both the culture study and molecular studies were important in assessing the microbial ecology of the hospital hot water system treated with monochloramine. The culture study allowed us to examine certain waterborne pathogens we were specifically surveying, for which we had appropriate culture media. The sequencing studies allowed us to obtain a broader picture of the microbes present in the hot water system and how these organisms were dynamically affected by monochloramine treatment. It was interesting to see that the 454 pyrosequencing study showed an increase in *Legionella* relative abundance in samples taken approximately one week prior to samples taken for *Legionella* culturing that showed increased positivity. It is possible the increase in DNA was present even earlier than one week prior to culturable bacteria being found. As next-generation sequencing technologies become more affordable they could be used to survey, in real time, changes to the relative abundance of *Legionella* and other

waterborne pathogens. This would allow for faster remediation while culturing takes place and confirms the results of an increase in the presence of opportunistic pathogens in water systems.

Our point-of-use faucet filter study demonstrated efficacy in preventing *Legionella* exposure for the manufacturer suggested 62 days, through our 119 days of study. There was not an exclusion of all bacteria by these filters but there was an almost 2 log reduction in total bacterial counts in filtered samples. Only three species were able to penetrate these filters and were unusually thin gram-negative rods. None of these species have been found to cause disease in humans, however.

We believe that these aims have filled considerable gaps in the detection of *Legionella*, the cause of Legionnaires' disease, and in the quest of providing pathogen-free water to hospitalized patients.

7.2 FUTURE DIRECTIONS

In this study we have developed a LAMP assay that detects *L. pneumophila* and not other *Legionella* species or other bacteria commonly found in water or urine specimen. While the 15 serogroups of *L. pneumophila* are a significant cause of Legionnaires' disease, other species of *Legionella* are important in the U.S. and especially other countries such as Australia and New Zealand. It would be beneficial to have a *Legionella* genus level primer set but we were unable to design one using the 16S rRNA gene. It is possible that another gene may be more promising for this application since the 16S rRNA gene is conserved throughout all bacteria. If it is not possible to do this, it may be important to design LAMP assays specific for other disease causing species of *Legionella*. This would be beneficial in places such as Australia and New Zealand

where a larger proportion of their Legionnaires' disease cases are caused by *L. longbeachae*. Future directions for this project could include testing the *L. pneumophila* specific assay on clinical samples to determine its effectiveness on correctly identifying *Legionella* contamination in sputum, blood, and urine samples. The limit of detection of this assay for environmental water samples is currently at 400 cfu/mL. While this is a good start we would like to reduce this to 100 cfu/mL or even lower if possible. This could be accomplished by further concentrating the water samples tested or by extracting DNA from the water samples prior to their use in the LAMP assay.

The effects of monochloramine as a secondary disinfectant are promising in the control of *Legionella* on the building level. However, there may be unwanted consequences in regards to the abundance of other opportunistic waterborne pathogens in response to this treatment. Our studies need to be repeated in other water systems with different water qualities in different states and countries to get a better idea of the impact of on-site monochloramine generation systems in other settings. The amount of organic carbon present in source water may affect the efficacy of monochloramine in municipal systems and its effect at the building level is unknown. Both culture and sequencing based studies should be performed to get a better idea of the overall microbial shifts in response to treatment with monochloramine. In the future these studies should take place over several full calendar years to see the effects of seasonality on the microbiology of these systems. More studies are needed to determine the long term effects of the bacterial populations selected for by monochloramine on the plumbing and risk of infection for hospitalized patients. This is especially important in the case of *Mycobacterium* species as they pose a significant threat to immunocompromised patients and were found by one of our studies to be enriched due to monochloramine treatment. It is interesting to note that one of our

sequencing studies saw an increase in the relative abundance in *Mycobacterium* over six months of treatment, whereas the other saw a 10-fold reduction overall in *Mycobacterium* in control versus treated samples. An important difference in these studies is the season in which they were conducted. The time course study (Illumina sequencing) took place in late fall and through winter while the control versus treated study (454 pyrosequencing) took place in summer. It is possible that the samples taken during the winter are more similar to municipal cold water systems in that the temperature of the source water would be considerably colder than the hot water system it is fed into. In municipal cold water systems the amount of *Mycobacterium* spp. has been seen to increase with monochloramine treatment, as was the case with our Illumina sequencing study. Several species of mycobacteria grow more readily in colder temperature waters and may represent a larger proportion of the total mycobacteria in municipal water treated with monochloramine. However, due to sequencing depth, we are unable to identify specific species of *Mycobacterium* in the samples. This further emphasizes the need for the incoming water to be characterized to determine not only the effects of season on the municipal cold water makeup but also what microbes are selected for by the hot water system in general and then further selected for by monochloramine addition.

Point-of-use filtration can be a good alternative to whole system disinfection if only some patients are at risk and can provide extra protection for these patients since systemic chemical treatment does not remove all bacteria from water supplies. Our study should be repeated in other facilities with differing water quality to determine the filters' effectiveness in other settings. Further investigation should be done into the bacteria that were able to pass through the filters to determine if this is acceptable or if the filters need to be redesigned to exclude all bacteria. New studies should be done to determine the costs and benefits of this type of WBP

prevention strategy. Due to the longer life of these filters the manpower needed to replace them will be decreased and this will reduce, but not eliminate, the risk of human error in regards to the timing of their replacement. This may lead to greater acceptance of point-of-use filtration and a reduction in the cost of their use.

7.3 IMPLICATIONS TO PUBLIC HEALTH

Every year approximately 300,000 hospitalized patients are infected by pathogens found in their hospital's water supply. *Legionella* species, specifically *L. pneumophila*, are a large cause of infections in these populations. Legionnaires' disease is a severe bacterial pneumonia that can cause death in 38-53% of healthcare acquired cases. This disease is a large burden on the healthcare industry leading to approximately 13,000 hospitalizations per year and costing over $433,000,000. Disease caused by this and other waterborne pathogens is preventable. On average slightly over half of hospitals actually test their water for bacteria. This number needs to increase substantially.

Our LAMP assay can detect the presence of *L. pneumophila*, the cause of over 50-80% of Legionnaires' disease cases, in pure culture samples and in water. This assay is important because when doctors are aware that their water system is contaminated they should have a greater suspicion of hospital-acquired pneumonia being caused by *Legionella*. Our assay could aid in both aspects of infection prevention. First, our LAMP primers could be used to test the water to detect the presence of *Legionella*. Second, after further validation, our assay could potentially be used to test the patient for *L. pneumophila*. An increased suspicion of the disease and early diagnosis can lead to a reduction in the high mortality rate of Legionnaires' disease.

An important method of disease prevention is disinfection of the water supply. We evaluated two methods of disinfection including monochloramine application and point-of-use filtration. In all three of our monochloramine studies we saw a reduction in *Legionella* counts and relative abundance during effective treatment. However several other important waterborne pathogens increased in relative abundance in our sequencing studies. While *Legionella* species are an important and fatal cause of infection, some of these other WBPs are even less easily diagnosed and treated by antibiotics than *Legionella*. The presence and survival of these opportunistic pathogens suggests that more research needs to be done in the field of water microbial ecology to determine better ways to deliver pathogen-free water to citizens and especially hospitalized patients.

The use of monochloramine itself does have an impact to public health and to the water treatment industry as well. Monochloramine usage has a number of benefits to more widely used chemical disinfectants including: 1) faster biocidal activity and deeper biofilm penetration than copper silver ionization, chlorine dioxide, and chlorine, 2) fewer hazardous byproducts than chlorine, 3) more chemical residual stability than chlorine dioxide or chlorine, and 4) ability to measure all necessary parameters in the field, unlike copper silver ionization. However, there are also disadvantages to the use of monochloramine, as was seen in our study, including: 1) persistence of other waterborne pathogens, 2) possible nitrification of storage tanks, 3) necessity of on-site generation, and 4) leaching of lead from old piping materials.

There may exist public concern as to the consumption of drinking water disinfectants. In our study design, the possibility of consumption of monochloramine containing water is very low. This is due to the fact that only the hot water was treated with monochloramine, since; in general only cold water is consumed. Also, the levels of monochloramine that were dosed into

the system were within the EPA mandated limits (below 4 ppm) and generally within the 2 to 3 ppm range. Studies conducted to test the effects of normal (2 ppm) and high-level (15 ppm and 200 ppm) consumption of monochloramine in male adults and male rats have been conducted and show the treated water to be non-toxic and well tolerated even at high levels [158, 159].

There is also concern that waterborne organisms may develop resistance to monochloramine treatment. This is not just an issue with monochloramine treatment but the majority of chemical-based water disinfection strategies as well. Some methods of resistance to monochloramine have been observed in *E. coli* including: 1) a reduction in the abundance of cell membrane permeases [160], 2) an increase in genes associated with iron acquisition and iron, sulfur, and cysteine metabolism [160, 161], 3) an increase in cell wall stress/repair and oxidative stress/metabolism genes [160, 161], and 4) an increase in the potential for protective biofilm formation [160, 161]. These resistance mechanisms are not unique to monochloramine disinfection and can occur due to other biological and chemical stressors, including other water treatment strategies.

Point-of-use filtration can be used alone or to supplement secondary disinfection for specific at risk populations. As these filters are redesigned to perform for longer periods of time they may be a more cost-effective way to provide water without WBPs and maybe, eventually, provide sterile water to individuals. While disinfection comes at some monetary cost, there will be a substantial reduction in healthcare costs overall due to these preventable infections and the needless loss of human lives.

These projects fit into the larger fields of public health microbiology and microbial ecology. On average people spend approximately 90% of their time indoors where many microbes live in the air, on surfaces, and in water. In the past there was not much concern or

effort given to characterization of these microorganisms. However, there has been a push to understand the microbiology of hospitals and other built environments due to the risk of illness from opportunistic pathogens that may infect susceptible individuals and a general lack of knowledge of the microbial makeup of engineered environments. New technological advances, such as high-throughput DNA sequencing, and specific funding for study of the "Microbiology of the Built Environment" are allowing for characterization of the microbes present in these ecosystems. Some of this study has resulted in the development of new guidelines for building water systems, especially those to try to prevent *Legionella* colonization of the systems and subsequent Legionnaires' disease. In the future, hospitals will likely need to address these issues of microbial water quality and may even be required to provide water to patients that meet new, higher standards aimed at reducing waterborne pathogen infections. The studies completed evaluating monochloramine disinfection in a hospital's hot water supply represent a step towards better understanding the effects of widespread chemical disinfection on the microbial ecology of hot water. These projects and future ones will have a great impact on public health microbiology and characterization of the hospital microbiome. This shift in thinking about the built environment as an important microbial ecosystem represents a new paradigm in microbiology and will have broader implications in infection control, civil and environmental engineering, and public health.

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