Relationship Between Organic Carbon and Opportunistic Pathogens in Simulated Premise Plumbing Systems

Krista Ann Williams

A Thesis submitted to the Faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Master of Science

In

Environmental Sciences & Engineering

Marc Edwards, Chair

Joseph Falkinham, III

Amy Pruden

August 4, 2011

Keywords: *L. pneumophila, M. avium, A. polyphaga,* premise plumbing, hot water heaters

Relationship Between Organic Carbon and Opportunistic Pathogens in Simulated Premise Plumbing Systems

Krista Ann Williams

ABSTRACT

Consumer exposure to opportunistic pathogens in potable water systems poses a significant challenge to public health as manifested by numerous cases of pneumonia, non-tuberculosis lung disease, and keratitis eye infections. Water utilities have extensive understanding in control of heterotrophic and coliform bacteria re-growth in water distribution systems via disinfection, control of assimilable organic carbon (AOC), and biologically degradable organic carbon (BDOC). However, little is known about the effect of AOC on the proliferation of heterotrophic bacteria and pathogens within premise plumbing. This thesis is the first systematic examination of opportunistic pathogen persistence and amplification in simulated glass water heaters (SGWH) as a function of influent organic matter concentration. The role of plumbing conditions that may internally generate AOC is critically examined as part of this evaluation.

Strong correlations were often observed between influent organic matter and heterotrophic bacteria in effluent of SGWH as indicated by 16S rRNA gene abundance (average R^2 value of 0.889 and 0.971 for heterotrophic organisms and 16S rRNA respectively). The correlation was strongest if water turnover was more frequent (every 48-72 hours) and decreased markedly when water changes were less frequent (stagnation up to 7 days). No simple correlations were identified between the concentration of pathogenic bacteria (*L. pneumophila*, *M. avium, A. polyphaga,* and *H. vermiformis*) and AOC, although correlations were observed between *M. avium* and TOC over a limited range (and only for a subset of experiments). Indeed, there was little evidence that *Legionella* and *Acanthamoeba* proliferated under any of the conditions tested in this work.

Parallel experiments were conducted to examine the extent to which factors present in premise plumbing (e.g. sacrificial magnesium anode rods, cross-linked polyethylene, nitrifying bacteria, and iron) could influence water chemistry and influence growth of bacteria or specified pathogens. Although these factors could strongly influence pH, dissolved oxygen concentrations, and levels of organic matter

(e.g. iron, magnesium, nitrifying bacteria), there was no major impact on effluent concentrations of either heterotrophic bacteria or premise plumbing pathogens under the conditions investigated.

While additional research is needed to confirm these findings, at present, there is no evidence of correlations between organic matter and pathogen concentrations from SGWH under conditions tested. Substantial effort was also invested in attempting to identify SGWH and oligotrophic nutrient conditions that would consistently support *L. pneumophila* and *A. polyphaga* amplification. A review of the literature indicates no prior examples of large scale amplification of these microorganisms at nutrient levels commonly found in synthesized potable water. It is likely that a complex combination of abiotic and biotic factors (i.e. micronutrients, necrotrophic growth, ambient water temperature, disinfectant type and dose, plumbing materials, water usage patterns), which are not yet fully understood, control the amplification and viability of these pathogenic organisms in premise plumbing systems.

ACKNOWLEDGEMENTS

I would like to thank Dr. Marc Edwards for his guidance, encouragement, support, and friendship throughout my graduate studies. I also want to thank the members of my committee, Dr. Amy Pruden and Dr. Joseph Falkinham III for their excellent advice and supervision during this investigation. In addition, I would like to acknowledge Myra Williams and Amanda Martin for their assistance in collecting and processing data. Additionally, I would like to thank all the members of the Pruden/Edwards research group for their laboratory assistance, advice, and friendship. I would especially like to thank Jeff Parks, Jody Smilie, Sheldon Masters, Chad McKinney, Simoni Triantafyllidou, Hong Wang, Yanjun Ma, Maria Virginia Prieto Riquelme, Rachel Kistler, and Kelly Roberti.

Finally, thank you to my family and friends, for their encouragement, constant support, and assistance during this process.

Table of Contents

List of Figures

List of Tables

CHAPTER 1: *Legionella* in Potable Water Distribution Systems

Krista Williams, Amanda Strickhouser, Amy Pruden and Marc Edwards

Department of Civil and Environmental Engineering, Virginia Tech, Blacksburg, Virginia

ABSTRACT

Legionella, the causative agent of Legionnaires' Disease, affects thousands of people each year in the United States. Several aspects of *Legionella* occurrence relative to potable water treatment and premise plumbing are examined, including practical aspects of microbiology, laboratory methods for its detection, enumeration, environmental factors that affect survival, and colonization of potable water systems.

1.1 INTRODUCTION

Legionella was first identified in 1977 through an investigation of circumstances at a 1976 American Legion convention that caused 182 illnesses and 29 deaths (Fraser et al., 1977). The bacterium responsible for the deaths was isolated and named *Legionella pneumophila* (AWT, 2003). Upon further investigation, the Centers for Disease Control and Prevention (CDC) found *Legionella* in fifty-year old tissue samples preserved from earlier pneumonia-associated deaths; additional analysis led to identification of *Legionella* sub-species including *Legionella micdadei*, *Legionella pneumophila*, and *Legionella bozemanii* (McDade, 2002).

The number of *Legionella* cases detected in the United States and reported to the CDC in 2005 was 2,301, or approximately 8 cases per million people, which is comparable to the rate of 10 cases per million reported in Europe (WHO 2005, CDC 2007). Due to systematic under-reporting and poor detection of *Legionella* cases, the CDC estimates that 8,000-18,000 people are hospitalized each year in the United States due to Legionnaires' disease; while the Occupational Safety and Health Administration (OSHA) estimates over 10,000-50,000 cases occur each year (OSHA, 2009). The fatality rate for untreated immuno-suppressed patients may be as high as 40-80%, but with detection and proper treatment fatality rates drop to 5-30%. In contrast, in individuals who are not immuno-suppressed the

mortality rate is 10-15% and total estimated deaths per year in the U.S. is about 4,000 (OSHA, 2009). The direct healthcare cost of nosocomial (i.e. acquired in hospital) Legionellosis in America is estimated to be over $34 million (McCoy 2005). The CDC estimates of hospital acquired Legionellosis comprise around to 25-45% of the reported cases. Additionally, both European and North American studies estimate that infection from *Legionella* accounts for 2-15% of community-acquired pneumonia requiring hospitalization (Gobin et al., 2009).

1.2 MICROBIOLOGY

General Microbiology

Legionellae are Gram-negative rods that range from 0.3 to 0.9 µm in width and 2 to over 20 µm in length (Garrity, 2005). Unlike most Gram-negative bacteria, *Legionella* cell walls contain high amounts of branched-chain cellular fatty acids and ubiquinones with side chains of 9-14 isoprene units that interfere with cell staining (Garrity 2005, WHO). *Legionellae* are urease-negative, catalase-positive, heterotrophic, aerobic, and transitionally motile (Garrity, 2005). When motile, they possess one or more straight, curved, polar or lateral flagella. *Legionellae* utilize amino acids as a principle metabolic source, do not oxidize or ferment carbohydrates, and require L-cysteine-HCl and iron salts for growth amongst other essential nutrients. Additionally, it has been documented that *Legionellae* can persist in a wide range of temperatures. In a survey of lakes and rivers, Fliermans et al., found *Legionella* at temperatures between 5.7 and 63 °C (Fliermans et al., 1981). There are presently 52 identified species of *Legionella*, 25 of which have been associated with Legionnaires' disease and/or Pontiac fever (Gobin et al., 2009). *Legionella* species are divided into serogroups; however, most infections are associated with *L. pneumophila*, especially serogroups 1, 4, and 6 (OSHA, 2005). Recent studies have estimated that 85% of Legionellosis is attributed to *L. pneumophila* sterogroup 1 (Gobin et al., 2009)

Life Cycle

Legionella possesses a two phase lifecycle involving both infectious and replicative stages. During the infectious phase *Legionellae* search for a host cell to infiltrate. *Legionella* cells are only infectious when the bacterium is a short, thick rod that is flagellated, stress resistant, sodium sensitive, and does not have the ability to replicate (Steinert et al., 2002). Once the bacterium finds a host, it penetrates through the organism through coiling phagocytosis and resides within the phagosome (McCoy 2005;

Figure 34). *Legionellae* are effectual in inhibiting the phagosome-lysosome fusion, thereby avoiding bacterial lysis. As free living organisms within the host, the bacterium enters a replicative (exponential) phase, which is characterized by a long, filamentous rod structure that is non-flagellated, sodium resistant, stress sensitive, and has the ability to replicate (Steinert et al., 2002). While residing in the host, *Legionellae* propagate and effectively overpopulate the phagosome, completely overwhelming the organism in as little as 48 hours (McCoy 2005). Subsequently, the protozoan host undergoes necrosis, bursting and releasing *Legionellae* into the environment to find other hosts by reentering the infectious phase of their life cycle (Figure 34). Without a host, *Legionella* growth has only been observed in nutrient rich laboratory media (Fields et al., 2002), which may better simulate the environment present inside the host organism relative to normal aquatic environments.

There are several known protist hosts *Legionellae* can infect including 14 species of amoebae, two species of ciliated protozoa, and one species of slime mold (Fields et al., 2002). *Legionella* hosts are also known to cause infection. As examples, *Acanthamoebae* can infect contact lens wearers and cause severe keratitis and *Naegleria fowleri* can cause fatal meningoencephalitis (Wynter-Allison et al., 2005, CDC 1992a, CDC 2003, Cogo et al., 2004, CDC 1986, CDC 1987). The protozoan hosts are imperative to the survival and propagation of *Legionella* in the human lung cell. Virulent and nonvirulent strains are phagocytized in alveolar macrophages. However, only virulent strains are able to multiply and inhibit the fusion of phagosomes with lysosomes (Horwitz et al., 1993). This process eventually leads to the death of the host cell and replication of *Legionella* as discussed above. Interestingly, studies have found that *Legionella* undergoes active replication inside *A. castellanii* (a host organism) at temperatures above 25 °C by activating the CsrA regulator gene. However, at temperatures below 20 °C *A. castellanii* effectively digests *L. pneumophila* and is eliminated from the protist through encystations (Ohno et al., 2008). Further investigations are needed to examine the role temperature and how it pertains to this parasitic relationship.

1.3 LEGIONELLOSIS

Legionnaires' Disease

Legionnaires' disease is a bacterial pneumonia caused by several species of *Legionella* (OSHA). Symptoms typically appear 2 to 14 days after exposure to the bacteria (CDC 2005a). Initial symptoms

can include a slight fever, headache, aching joints and muscles, malaise, loss of appetite, and in some cases gastrointestinal problems such as nausea, vomiting, and diarrhea (OSHA, Harrison and Taylor 1988). In the initial phase, there is no upper respiratory tract involvement, but as the disease progresses the symptoms become pneumonia-like and can include a high fever, cough, difficulty breathing, chills, and chest pains. As with other bacterial pneumonias, Legionnaire's disease principally affects the lower respiratory tract causing lesions to appear in the bronchioles and alveolar ducts (Gobin et al., 2009). Nervous system symptoms including disorientation or confusion are also present in about 25% of cases.

Legionnaires' disease is transmitted through inhalation or aspiration of aerosols containing *Legionella* (OSHA). Examples of water sources implicated in the causation of Legionnaire's disease include cooling towers, domestic hot water systems, humidifiers, spas, and dental water lines (OSHA). Additional research has detected *Legionella* in ice machines; however this source has never been confirmed as a source of infection (Stout et al., 1985b). Some recent papers have placed more emphasis on aspiration as the primary mode of transmission (EPA 1999, Yu 1993). Humans do not re-release *Legionella* to the environment in high quantities and there is currently no evidence of person-to-person transmission or of human infection from animals, thus human infection is not considered to be part of the *Legionella* life cycle (EPA 2001).

Legionella infects human macrophages in an analogous manner to pathogenic protozoan cells. The bacteria enter and deposit in the lower airways of the lungs (EPA 2001). Alveolar macrophages phagocytize the *Legionellae* and the bacteria grow rapidly within the phagosomes. The phagosome does not fuse with lysosomes or become acidic (Fields et al., 2002). The cells then lyse, releasing the bacteria and further infecting the patient (EPA 2001). There are a few minor differences in the way in which *Legionella* infect human cells verses protozoa. These distinctions include the requirement for actin polymerization, the role of host cell protein synthesis, and the induction of programmed cell-death or apoptosis (Fields et al., 2002).

The general population is resistant to *Legionella* infection, as evidenced by the low incidence of Legionnaires' disease despite widespread environmental detection of the bacterium (EPA 2001). However, certain risk factors can increase a person's susceptibility to contracting the disease including heavy smoking, alcoholism, cancer, bone-marrow and organ transplants, chronic renal failure, and age

over 50 in males (AWT 2003, EPA 2001,WHO 2007). Additional risk factors are believed to be due to increased exposure, such as hospitalized patients who require ventilators or intubation. Legionnaires' disease rarely occurs in patients with HIV, possibly due to the precautions taken to prevent infection in this population (EPA 1999). In the general population, the human immune system is usually effective for preventing infection, although people without risk factors can also acquire the disease.

The risk factors for Legionnaires' disease are often associated with high rates of nosocomial infections. In a study of Legionnaires' disease in England and Wales, it was found that 8% of cases were hospital-acquired (Joseph et al., 1994). Determination of nosocomial acquired Legionellosis is considered definitive if the person was hospitalized 10 days before the onset of symptoms (Hoffman et al., 2008). The 40-50% death rate for nosocomial infections is much higher than the average Legionnaires' mortality rate (NIH 2007; Hoffman et al., 2008). Legionnaires' disease is also often acquired during travel, perhaps due to exposure through hotel water or whirlpool spas (EPA 1999). However, detection and quantification of travel-related disease outbreak is difficult due to the incubation time of the disease, low attack rate, and the number of potential water sources encountered (Fields et al., 2002).

To be diagnosed with Legionnaires' disease, a patient must first be diagnosed with pneumonia based on a chest x-ray by a physician in addition to positive laboratory results through culturing or antigen testing. It is impossible to differentiate Legionnaires' disease from other pneumonias using a chest x-ray, and follow-up laboratory testing is necessary (OSHA). However, subsequent testing is often not completed unless the patient has risk factors for Legionnaires' disease. Antibiotic treatment of the disease consists of quinolones such as ciprofloxacin, levofloxacin, moxifloxacin, or gatifloxacin, and macrolides such as azithromycin, clarithromycin, or erythromycin (NIH 2007). β-lactam drugs such as penicillin and ampicillin are not effective against *Legionella* because they do not penetrate phagosomes and lysosomes. Furthermore, several *Legionella* species also produce β-lactamase which inactivates penicillin (McCoy 2005).

Proper diagnosis and early treatment are imperative factors in the reduction of mortality (Heath et al., 1996). Mortality rates are diminished to 5-10% if patients are treated with appropriate antibiotics, and there is significant potential to achieve even lower mortality rates as hospitals are increasingly utilizing urine antigen testing, which provides more rapid diagnosis (Gobin et al., 2009). Currently, there is no

vaccine available for Legionnaires' disease although patients who have contracted the disease appear to have developed a natural resistance to future infection. Additional studies are needed to determine the underlying factors involved and the duration of natural immunity (Environmental Health Directorate, 2006).

Pontiac Fever

In many cases, a person exposed to *Legionella* may develop Pontiac fever instead of Legionnaires' disease. Pontiac fever is a flu-like illness that is acute and self-limiting. Studies suggest the illness may not be due to the infection and replication of *Legionella*, but instead is a hypersensitivity response to antigens (OSHA, 2009). Unlike Legionnaires' disease, the incubation period is one to three days and recovery typically occurs within 2 to 5 days without antibiotics. Approximately 90 percent of people who are exposed to *L. pneumophila* will contract Pontiac fever.

Animal Infections

It is not known whether animals are infected with *Legionella* in the natural environment; however, animals have been used in laboratory studies to isolate the bacterium (EPA, 2001). Legionnaires' disease progression in humans is similar to that in guinea pigs. Berendt et al., 1980 determined that the median lethal dose of aerosolized *L. pneumophila* serogroup 1 is 1.4×10^5 cells, and intake of as few as 5 colony forming units into the lungs will infect 100% of guinea pigs.

1.4 LABORATORY METHODS

Environmental Sampling Procedure

Environmental samples should be collected in sterile polypropylene containers (CDC 2005b, OSHA). The CDC states a minimum of 1 L should be collected, however OSHA recommends 250 mL – 1 L. OSHA also recommends that the system should not be flushed before collecting samples. Any chlorine present should be neutralized utilizing 0.5 mL of 0.1N sodium thiosulfate for each liter of disinfectant present in the water (CDC 2005b). While the CDC does not specify timing for the sodium thiosulfate addition, it is advised to have it present in the bottle prior to sample collection (McCoy 2005).

Legionella is often associated with growth in biofilms. Analysis for biofilm collection involves collecting swab samples from inside pipes after removing the aerator or showerhead. For this procedure, CDC recommends sterile polyester swabs with wooden shafts (CDC, 2005b). After sampling, swabs are submerged in 3-5 mL of water to prevent drying during transport (CDC 2005b). Stout et al. recommends that samples should be stored at 2-8 °C both before and after processing, unless the sample is of hot water in which case it should be processed within 24 hours (Stout et al., 1998b). Conversely, OSHA, states that samples should not be refrigerated but should be stored at room temperature and processed within 2 days, whereas CDC recommends refrigeration for any samples not processed within 72 hours (OSHA, CDC 2005b).

Culture Methods

Legionella was first isolated using guinea pigs and embryonated chicken eggs (McDade et al., 1977). The first culture medium used to isolate *Legionella* was Mueller-Hinton agar supplemented with 1% hemoglobin and 1% IsoVitaleX. This medium has undergone several revisions (Table 1). Charcoal can be added to inactivate exogenous hydrogen peroxide to which *Legionella* is extremely sensitive (Hoffman et al., 1983). Sodium chloride used in the first culture medium, was later found to inhibit the growth of virulent *Legionella pneumophila* (Catrenich and Johnson 1989). Thus, this salt source was later replaced with yeast extract. The ACES buffer and α-ketoglutarate increase the recovery of *Legionella* on solid media (Edelstein 1981). Additionally, adding 1.0% bovine serum albumin (ABCYE) to the medium has been shown to enhance the growth of *Legionella micdadei* and *Legionella bozemanii* (Morrill et al., 1990). Buffered Charcoal Yeast Extract Agar with Albumin (BCYEα) is currently considered to be the standard medium for *Legionella* isolation, but other media have been developed such as *Legionella* blood agar, a transparent medium, and a chemically defined medium (Dennis et al., 1981, Armon and Payment 1990, Ristroph et al., 1981, Warren and Miller 1979. While these media were developed with the intention of enhancing isolation and detection of *L. pneumophila* as compared to BCYEα, the net improvement in *Legionella* detection limits (if any) has not been established.

Water samples typically contain low levels of *Legionella* and therefore require sample concentration. Filter concentration and centrifugation are two methods used for this purpose. Filter concentration is accomplished by drawing the 1 L sample through a sterile 0.2 µm polycarbonate filter, resuspending the filter in 10 mL of the original sample, and vortexing to release bacteria from the filter (CDC 2005b). This

method can be used to concentrate *Legionella* (and other bacteria) by a factor of up to 100, but if the original sample is larger than 1 L, greater concentration is possible. In centrifugation, the water sample is centrifuged and the supernatant decanted. *L. pneumophila* concentration has been analyzed using centrifugation at 1,000 g for 10 minutes, 3800 g for 30 minutes, and 8150 g for 15 minutes (Ta et al., 1995; Boulanger and Edelstein, 1995). In each of these cases, filtration was demonstrably more effective at concentrating *Legionella* than centrifugation. However, regardless of concentration method, less than 50% of viable *Legionella* are usually recovered (McCoy, 2005).

If samples are plated directly, 0.1 mL of the sample is placed onto both BCYE and a selective BCYE media and spread with a sterile glass rod or sterile disposable plastic spreader (CDC 2005b). If a swab sample was taken, the swab is drawn down the center of the medium and streaked perpendicularly with an inoculating loop (ACHD 1997). Culture media should be incubated between 35 and 37 °C in a humid atmosphere with colonies appearing after 3 to 5 days. Although the CDC recommends incubation with 2.5% CO_2, *L. gormanii* is the only *Legionella* species that exhibited improved growth under this condition (CDC 2005b, Garrity 2005). Thus, earlier evidence reported by Pine et al., 1979 indicating improved growth at 5% CO_2 should not be generalized.

Improving Detection for *L. pneumophila*

To improve the detection of *Legionella* in environmental and clinical samples, it is sometimes critical to selectively inhibit other bacteria that interfere with *Legionella* enumeration on culturing media. Methodologies to accomplish this selectivity include addition of antibiotics and heat or acid pretreatments. For example, supplementing anisomycin inhibits yeasts, cycloheximide inhibits fungi, cefamandole and vancomycin inhibit staphylococci, and glycine helps to inhibit general environmental flora, but not respiratory flora (Stout 1998b, Harrison and Taylor 1988). Furthermore, glycine can be inhibitory to some non-*pneumophila* species, especially *L. gormanii* (Calderon and Dufour 1984). Several different selective *Legionella* media have been developed and studies have compared performance of these media under different circumstances (Table 2, Table 3).

Legionella is relatively acid and heat resistant compared to other bacteria, thus controlled exposure to heat and acid allow survival of *Legionella* while inactivating many competitors. Heat pretreatment involves exposure to a 50 °C water bath for 30 minutes followed by rapid cooling, which is especially

effective at reducing pseudomonads, coliforms, and micrococci while only slightly affecting *L. pneumophila* (Dennis et al., 1984a). Other heat pretreatments involve placing a sample in a 55 °C water bath for 15 minutes or a 60 °C water bath for 3 minutes (WHO, Wilkinson 1987, Edelstein et al., 1982b). There are several methods for acid pretreatment (Table 4). Each method involves the creation of an acid-buffer solution (AB) from hydrochloric acid and potassium chloride, and later mixing the AB with the sample in a specific ratio ranging from 1:1 to 1:9. The pH of the AB solution is adjusted to 2.0-2.5 before sterilization or combination with the environmental sample. After adding to the environmental sample, the mixture is allowed to react for 3 to 30 minutes before plating directly. However, other methods recommend that the acid be neutralized with potassium hydroxide before plating (Harrison and Taylor, 1988). When a swab sample is pretreated with the acid mixture, the swab is submersed in the acid-buffer solution and then vortexed (Stout, 1998b). Experimental evidence has shown that acid pretreatments have a negative effect on the growth of *L. wadsworthii*, but does not appear to have a statistically significant effect on other *Legionella* species (Calderon and Dufour, 1984).

Heat enrichment is another technique utilized that is designed to improve detection of *Legionella*. This method involves incubating samples at 35 °C and culturing every 2 weeks for up to 6 weeks. Effectively, the *Legionella* can multiply within protozoan cells and reach a detectable level (CDC, 2005b). Thus, this methodology is useful for recovering *Legionella* from samples that initially appear to be negative, but the method is not suitable to determining the initial concentration. There has also been significant research performed on protozoan hosts cells to determine intracellular pathogenic growth rates (Steinert et al., 1994). This information may be useful in developing a future highly sensitive method for estimating initial *Legionella* concentrations.

When considering sample processing, it would be very time consuming to concentrate, acid pretreat, heat pretreat, and use the range of available selective media on every sample. Depending on the circumstances, one method may be more effective than another, and pretreatments are not always necessary. General guidance documentation is utilized to help laboratories decide which method(s) to use on a given sample (Table 5).

Cultures should be examined after a minimum of 3 days of incubation using a dissecting microscope. Negative cultures should be monitored for 7-14 days (CDC 2005b, Stout 1998b, OSHA). *Legionella* colonies appear round and convex with younger colonies having a ‘ground-glass’ appearance (CDC

2005b, Harrison and Taylor 1988). Colony edges are typically pink or purple with younger colonies having blue or green edges. As the colony grows older it becomes smoother and may appear gray. Examination with an ultraviolet light (366 nm) can help distinguish between species (Harrison and Taylor 1988). *L. bozemanii, L. gormanii, L. dumoffii, L. anisa, L. cherrii,* and *L. parisiensis* fluoresce white, *L. rubrilucens* and *L. erythra* fluoresce red, and *L. pneumophila* is a dull green or yellow. Suspected *Legionella* colonies should be streaked onto both BCYE agar and either BCYE agar without L-cysteine [BCYE(-)] or blood agar (CDC 2005b). A colony that grows on BCYE and not BCYE(-) is considered presumptive *Legionella*. To confirm that a culture is *Legionella,* it should be tested using the direct fluorescent antibody (DFA) or slide agglutination test (SAT).

Molecular Methods

In addition to their lack of standardization, culture methods are expensive, time-consuming process requiring over a week to obtain results. Polymerase Chain Reaction (PCR) techniques in general are expected to enable better detection of *Legionella* residing within a protozoan host (Figure 33), which is a significant advantage for molecular techniques versus standard plating methods. Molecular techniques, targeting DNA or RNA, produce results much faster, typically after one or two days (McCoy, 2005). The 16S rRNA gene is generally used to detect various *Legionella* spp., while the macrophage infectivity potentiator (*mip*) gene is a common target for *L. pneumophila*. In a side by side interlaboratory investigation, Joly et al., 2006 found that the *mip* gene provided greater specificity for *L. pneumophila* than the 16S rRNA gene.

PCR can be used to determine the presence or absence of *Legionella*; however, it cannot directly differentiate between viable and non-viable bacteria. Effectively, *Legionella* killed by disinfectant or otherwise inactivated are indistinguishable from live organisms that could infect humans. Reverse transcription PCR (RT-PCR) targets RNA and therefore is more indicative of active bacteria, because RNA from dead organisms degrades rapidly in the environment. Unfortunately RNA based methods are generally impractical for drinking water because of the low abundance and poor recoverability of RNA from dilute water samples. A new and promising approach for selectively detecting and quantifying live cells was recently reported that circumvents the need for RNA isolation (Delgado-Viscogliosi et al., 2009). This method uses the acid-binding dye ethidium monoazide bromide (EMA), which selectively

penetrates the membranes of non-viable cells and renders the DNA unsuitable for PCR. However, this method has not yet been applied to the detection of opportunistic pathogens.

Another potential drawback of PCR-based analysis is the potential for inhibition, because the presence of compounds such as rust or some divalent cations (calcium, magnesium, silver, or copper) can compete and hasten the destruction of DNA, thereby causing false negative results (Miskowski, Maiwald et al., 1994). However, inhibitor controls can be run which can determine if PCR inhibitors are present in a sample (e.g., Wellinghausen et al., 2001; Joly et al., 2006). In a study of 260 samples from hot water systems and cooling tower water samples in France, Joly et al., determined that PCR inhibition prevented analysis of only 2.7% of samples, while a study of 77 hospital water samples found 1.3% inhibition (Wellinghausen et al., 2001). In rapidly evaluating a patient with Legionellosis, PCR from respiratory samples can serve as an efficient means of diagnosis. However, PCR inhibition can cause false negative results from biological specimens.

Respiratory samples in particular may contain potent inhibitors including viscous polysaccharides, leukocytes, erythrocytes, hemoglobin, proteases, and cell detritus (Kern et al., 2009). Other research has implicated organic matter (humic, fulvic, and humin) as a considerable source of PCR inhibition (Hill et al., 2010). Inhibition in sample analysis may be reduced by the utilization of more robust polymerase enzymes or PCR facilitators. Supplementary evidence has shown that bovine serum albumin is effective in facilitating PCR by binding to inhibiting compounds in analysis of biological specimens. In addition these methodologies, the gp32 protein, casein, betaine, PVP, amides, glycerol, organic solvents, polymers, and non-ionic detergents have been implicated in PCR enhancement (Hill et al., 2010).

Quantitative real-time PCR (qPCR) can be used to enumerate *Legionella* by quantifying the number of *Legionella* 16S rRNA or *mip* genes present in a sample. The resulting output is in genome units (GU) per liter, which will correlate with colony forming units (CFU) per liter if *Legionella* in protozoans or non-viable *Legionella* are insignificant. Non viable bacteria are defined as bacteria that are actively metabolizing but not undergoing binary fission. Because the latter forms of *Legionella* are typically significant, qPCR often detect more *Legionella* than culturing methods (Joly et al., 2006; Wellinghausen et al., 2001; Yaradou et al., 2007).

Interestingly, two different studies have found that the correlation between CFU and GU determined by qPCR is not consistent across sample type (Joly et al., 2006; Yaradou et al., 2007). In particular, water cooling tower samples appear to have little or no correlation to CFU, compared to a strong correlation observed in some hot water samples. Joly et al., suggest that this may be a result of higher diversity of *Legionella* and other bacterial species in cooling waters, which could interfere with the methods. Considering that differences in results have also been reported by different labs performing the same analysis, this underscores the importance of each lab validating and optimizing the methods for each sample type (Joly et al., 2009). While PCR is not currently the standard method for *Legionella* testing, OSHA states that it may eventually be accepted as the method of choice following further confirmation testing (OSHA). The growing availability of commercial kits and instruments that perform sample concentration, DNA extraction, and PCR (e.g., GeneSystems, Bruz, France) will likely improve the accessibility of molecular detection technologies in the future.

Detection limit remains a challenge for qPCR enumeration of *Legionella* mainly because of the upstream sample processing and concentration steps, rather than qPCR itself. Wellinghausen et al., (2001) were able to detect a single copy of the gene in the qPCR assay. However, when sample processing and storage were considered in another study, a practical detection limit of 250 CFU/L was reported (Joly et al., 2006). In that work the upstream sample procedure was fairly extensive and consisted of filtering 1L of sample water onto a 0.45 µm polycarbonate filter, detaching the cells from the filter by sonication in 5 mL of water, storing a 1 mL subsample at -20 °C, and thawing and centrifugation prior to extraction. However, in spite of the higher detection limit when processing is considered, the high-throughput nature of PCR is a major advantage over culturing. For this reason Villari et al., 1998 proposed a method in which samples are analyzed using PCR and, if negative, samples were concentrated using filtration and then testing using culture methods. This method has the advantage of fast detection if *Legionella* concentrations are high, with potential positive detection using culture methods that may have greater sensitivity along with the ability in detecting live pathogens. Additionally, Joly et al., 1985 concluded that qPCR is generally reliable for detecting *Legionella* when a significant health risk is posed.

Air Detection Methods

The two most successful methods for detecting airborne *Legionella* involve capturing cells via gas impingement to a liquid and or gas impingement on a solid medium using an Anderson sampler (CDC

2005b). For gas impingement to a liquid, the CDC recommends sterile 0.25% yeast extract broth as the collection liquid. A comparative study including phosphate-buffered saline, Page's saline, 2% yeast extract broth, and buffered yeast extract (BYE) determined that BYE was the most efficient (Ishimatsu et al., 2001). However, due to the high air velocity some cells may be destroyed using the liquid impingement method. The Anderson sampler is useful because it can estimate the amount of *Legionella* that would enter the lungs. For increased likelihood of detection without interference from other bacteria, two Anderson samplers should be run simultaneously with one containing BCYE media and the other containing a selective *Legionella* medium (CDC, 2005b). Air sampling is generally not a recommended method for measuring potential exposure if other methods are available, presumably due to a high probability of false-negative results (DHS, 2007). The air sampling methodology is important to mechanistically understanding disease transmission.

1.5 ENVIRONMENTAL FACTORS

Premise Plumbing

Legionella is a natural inhabitant of the aquatic environment and is present in almost 100% of surface waters in the United States (Fliermans et al., 1981). Furthermore, *Legionellae* can survive drinking water treatment and enter the distribution system (Lee and Jones-Lee 1993b). Studies have shown that 0 to 37% of residential water supplies, 4 to 89% of buildings, 41 to 75% of hotels, 12 to 93.7% of hospitals, 37 to 47% of cooling towers, 5.9 to 36% of spas, 89% of emergency eyewash showers, and 25 to 68% of dental-unit waters are colonized with *Legionella* (Table 6).

Hot water tanks in particular are especially prone to *Legionella* colonization. Although the recommended temperature to control *Legionella* is 60 °C as outlined by the Canadian Standing Committee on Building and Plumbing Services, scalding can occur within six seconds at this temperature (SCBPS 2005, CPSC). Thus, the United States Consumer Product Safety Commission has recommended 49 °C as a safety measure to prevent scalding. However, this relatively low temperature may allow *Legionella* proliferation within these tanks (CPSC). The SCPBS seeks to address this issue by recommending that water heaters be set at 60 °C and that subsequent control measures be taken, such as installing a temperature regulator ensuring the temperature supplied to distal sites will not exceed 49 °C (SCBPS 2005).

Many studies of potable water supplies have found that temperatures below a certain level are positively associated with *Legionella* colonization; however, this level varies between 50 and 60 °C (Flannery et al., 2006; Arnow and Weil 1984; Borella et al., 2005). When water heater thermostats are set at 60 °C, electric water heaters can still potentially become colonized with *Legionella* due to spatial positioning of heating elements. In electric water heaters, the lower heating element is positioned 15-20 cm above the bottom of the tank (Joly 1985), which is much cooler than the bulk water.

In contrast, typical gas water heaters have heating elements positioned at the bottom which eliminates temperature stratification and reducing the likelihood of *Legionella* proliferation. Over time, the bottom of hot water tanks can also collect sediment, which may serve as additional resources for *Legionella*, heterotrophs, and host organisms (Stout et al., 1985a). *Legionella* has been recovered from gas and instantaneous water heaters, although not to the extent of electric water heaters (Lee et al., 1988; Alary and Joly, 1991; Joly, 1985).

In order to link occurrence of Legionnaires' disease with the potable water supply, studies should associate the same stereo group and subtype of *Legionella* found in the patient's sputum with those present in the water source (OSHA). This method has been utilized in several different studies and provides strong evidence that household water can be a source of the Legionnaires' disease (Stout et al., 1987; Stout et al., 1992b; Chen et al., 2002). In 2000-2002, 37.9% of Legionnaires' disease cases in Europe were believed to be community-acquired (Joseph, 2004). In a study of 20 community-acquired cases of Legionnaires' disease in Pittsburgh, Pennsylvania, 8 (40%) were linked to potable water as the infection source (Stout et al., 1992b). Furthermore, a study in the United Kingdom estimated that 12% of *Legionella* cases were due to drinking water (VROM 2004).

Chemical Factors

Studies have attempted to correlate *Legionella* occurrence in potable water systems with presence in environmental samples. For example, copper in potable water is believed to exert an inhibiting action on *Legionella* (Zacheus and Martikainen 1994, Leoni et al., 2005, Marrie et al., 1994, States et al., 1985). Other investigations have found copper pipes limit *Legionella* colonization, but that iron pipes are effective in promoting it (van der Kooij et al., 2005, Rogers et al., 1994, Zeybek and Cotuk 2002). These studies were completed on tap water in model water systems by testing both the water and the biofilm

in addition to analyzing the water in actual hotel water systems, and correlating it to the closest pipe material. In two surveys in Italy, copper levels above 50 µg/L were correlated with lower *Legionella* colonization (Borella et al., 2005, Borella et al., 2004). One survey noted that six times less *Legionella* was present in water samples containing greater than 50 µg/L copper. In contrast, a study by Mathys et al., 2008 found a positive correlation between copper and *L. pneumophila* proliferation in hot water systems and another study found no effect over the long-term.

Some studies have indicated that iron is positively correlated with *Legionella*, while others have found that it is negatively correlated (Borella et al., 2004; States et al., 1985; Zacheus and Martikainen 1994; Marrie et al., 1994). Certainly *Legionellae* need iron to grow in culture media, and virulence is significantly reduced in iron-limited conditions (McCoy, 2005; James et al., 1995). Evidence from James et al., 1995 has indicated that 3.3 µM Fe^{3+} was required for optimum growth. Recent research has suggested that replicative ability of *Legionella* within macrophages is extremely dependent on the uptake and utilization of iron (Cianciotto et al., 2007). Human host cells treated with iron chelators will not support the endosymbiotic growth of *Legionella*. In addition, patients with high iron levels as well as smokers have been found to have an increased susceptibility to Legionnaires disease (Cianciotto, 2007), and States et al. found that very high levels of iron (>50 ppm) had a toxic effect in samples of hot water tanks with pH between 7.14-7.76 (States et al., 1985).

Zinc concentrations both above 200 µg/L and below 100 µg/L have been correlated with lower *Legionella* concentrations, suggesting that the optimal amount of zinc for *Legionella* growth might be between 100 and 200 µg/L (Borella et al., 2005, Borella et al., 2004). Stainless steel with zinc and silver ions have reduced concentrations of *L. pneumophila* within 2 hours of exposure (Rusin et al., 2002).

The effects of a range of other trace ions and nutrients on *L. pneumophila* growth have also been examined. Manganese levels below 3 µg/L are associated with lower *Legionella* levels. However, above 10 µg/L there seems to be a negative correlation (Borella et al., 2004; Zacheus and Martikainen, 1994). In a recent study by Bargellini et al., 2011, correlations above 6 µg/L of Mn were found to promote *L. pneumophila* amplification. Additionally, a study of Italian hotels demonstrated that magnesium had a negative correlation with *Legionella*, while study of Pittsburgh water supplies indicated that there was

no correlation (Borella et al., 2005; Stout et al., 1992a). Reeves et al., 1980 determined that low concentrations of magnesium (approximately 0.83 μM) resulted in consistently enhanced growth.

Conflicting results have been found regarding the association of calcium and *Legionella*, with three studies showing positive, one negative, and another no correlation (Zacheus and Martikainen 1994, Marrie et al., 1994, Asada et al., 2001, Stout et al., 1992a, Borella et al., 2005). In investigating correlations between nitrate, nitrite, and *Legionella,* one study found a positive correlation and another found a negative (Zacheus and Martikainen 1994; Marrie et al., 1994). Negative correlations have also been found with sodium, barium, chloride, and hardness; positive associations have been ascertained between potassium, phosphate, sulfate, and TOC (Zacheus and Martikainen 1994; Marrie et al., 1994; States et al., 1985; Ortiz-Roque and Hazen 1987). In a study by Rogers et al., *L. pneumophila* was discovered to be most prevalent in biofilms on plastics at 40°C, where its abundance was accounted up to 50% of the microbiota present. This experiment was performed under conditions of a base filter sterilized tap water with a mixed inoculum from sludge at the bottom of a calorifier implicated in an outbreak of Legionnaires' disease (Rogers et al., 1994).

The pH range believed to support the growth of *Legionella* is between 5.0 and 8.5, and naturally occurring *L. pneumophila* have been shown to multiply between pH 5.5 and 9.2 (OSHA, Wadowsky et al., 1985). *Legionella* have been recovered from lakes and rivers between pH 5.5 and 8.1 (Fliermans et al., 1981). Research has indicated that *Legionellae* are able survive in dissolved oxygen concentrations between 0.3 and 9.6 ppm. However, in one study, an environmental isolate of *L. pneumophila* grew between 6.0 to 6.7 mg/L and not 1.7 to 2.2 mg/L (Wadowsky et al., 1985). Subsequent studies by Mauchline et al., 1992 suggested that *L. pneumophila* can behave as a microaerophile in chemically defined media, with maximal growth at 0.32 mg/L as O_2.

Legionellae are well-adapted to surviving in harsh oligotrophic and environmental conditions. Studies have shown that after 48 hours of nutrient deprivation, the infectivity of *Acanthamoeba castellanii* by *L. pneumophila* is not significantly different than if it had been grown to log phase on BCYE plates (Steinert et al., 1994). Within protozoan amoebae, *L. pneumophila* effectively accumulates intracellular reserves of poly-3-hydroxybutyrate (PHB) which promotes long term survival for up to 600 days under nutrient poor conditions (James et al., 1999). In a study by Steinert et al., 1997, it was discovered that *Legionella*e

cells are capable of entering a viable but non culturable state (VBNC) with the inability to replicate and colonize on traditional plating media. The addition of *A. castellanii* to the dormant cells resulted in resuscitation of *Legionella* to a culturable state. The loss of culturability of cells is known to be induced by low nutrient condition and accelerated at elevated temperatures (37 °C compared to 4 °C) (Hussong et al., 1987). There are several reported environmental factors that may contribute to the development of the VBNC state such as nutrient starvation, incubation outside of normal temperature ranges, fluctuations in salt concentrations, higher oxygen concentrations, food heavy metals, and exposure to white light (Oliver, 2009). Additionally, it has been surmised that H_2O_2 production by cells may also induce the VBNC metabolic state, possibly due to the inability of the cell to detoxify harmful metabolites. This issue was also explored by Garcia et al., 2007 in attempting to understand the effects of disinfection on *A. polyphaga* and *L. pneumophila*. Results of the study showed that seven strains of *L. pneumophila* reverted to a VBNC state upon exposure to 256 ppm NaOCl solution. After a period of 22 and 46 hours after treatment exposure, samples of chlorine treated samples containing non-culturable *Legionella* were co-cultured with *A. polyphaga*. Non-culturable *Legionella* were able to be resuscitated through phagocytosis through the amoeba. In absence of the amoeba, samples taken at the 22 and 46 hour periods did not experience regrowth. Intracellular *L. pneumophila* within *A. polyphaga* in this study was resistant up to 1024 ppm NaOCl treatment exposure (Garcia et al., 2007).

In examining the effects of temperature on the growth of *L. pneumophila*, it was discovered that at lower temperatures (5 and 24 °C), *L. pneumophila* could survive in tap water for up to 299 days with the maximum survival rate (80%) occurring at 5 °C in sterilized tap water (Hsu et al., 1984). Supplementary studies have indicated that at low temperatures (4 to 20 °C), *Legionella* can survive at up to 3% sodium chloride, while at higher temperatures (30 and 37 °C), sodium chloride concentrations above 1.5% resulted in a greater than 2 log reduction of *Legionella*. Levels of sodium chloride between 0.1 and 0.5% have been shown to enhance *Legionella* survival (Heller et al., 1998). Another investigation demonstrated that while *L. pneumophila* could not be cultivated in hot spring water (with salt concentrations consistent with seawater), the bacterium maintained metabolic activity (Ohno et al., 2003).

Amino acids serve as the principle carbon source for *Legionella* and several studies have sought to determine which amino acids are required for growth. George et al., found that *L. pneumophila*

Bloomington-2 and Los Angeles-1 strains required arginine, cysteine, isoleucine, threonine, valine, methionine, serine, and phenylalanine or tyrosine for growth. Tesh and Miller attained similar results, except instead of phyenylalanine or tyrosine they discovered that glutamic acid was required (George et al.,1980; Tesh and Miller, 1981). Additionally, Tesh et al. found that the only amino acids used for energy were glutamate, serine, threonine, and tyrosine (Tesh et al., 1983). All of these amino acids are present in the BCYE growth medium, but they are present only at low concentrations in potable water (Chinn and Barrett, 2000; Chellam and Xu, 2004). More recently, Hoffman et al., 2008 reported arginine, isolucine, leucine, valine, methoine, serine, and theonine are required to support *L. pneumophila* growth refuting earlier claims. Genome analysis linked the biosynthetic pathways for all these amino acids with the exception of arginine (Hoffman et al., 2008). Finally, it important to note that amino acids can potentially destroyed by reactions with free chlorine in potable water systems (Hureiki et al., 1994).

Potential Symbiotic and Competitive Interactions with Other Bacteria

Legionellae are known to have a symbiotic relationship with several other bacteria on culture media. *Flavobacterium breve*, *Aeromonas, Pseudomonas vesicularis, P. paucimobilis, P. maltophilia*, and *Vibrio fluvalis* are known to stimulate growth of *Legionella* when grown on media not containing cysteine, indicating that they can produce the cysteine necessary for *Legionella* growth (Wadowsky and Yee 1983; Toze et al., 1990). A study from Toze et al. indicated that up to 32% of heterotrophic bacteria from chlorinated drinking water are inhibitory to *Legionella* species (Toze et al., 1990). Additional bacterial species can pose significant inhibitory effects to *Legionella* growth. *Aeromonas hydrophila, Aeromonas salmonicida, Flavobacterium meningosepticum, Pseudomonas aeruginosa,* and some *Staphylococcus* and *Bacillus* species are known to inhibit *Legionella* on culture media (Paszko-Kolva et al., 1993; Rowbotham,1983). Recent evidence has implicated the possibilities of *L. pneumophila* control through the use of *Bacillus subtilis* BS 104 (Temmerman et al., 2007), *P. aeruginosa*, *P. fluorescens*, *P. putida*, *Burkholderia cepacia*, *Aeromonas hydrophila*, and *Stenotrophomonas maltrophilia* (Guerrieri et al., 2008). In the study from Temmerman et al., 2007, a key finding was BS 104 induction of *L. pneumophila* cellular lysis. This evidence indicates possible production of inhibitory metabolic byproducts from *Bacillus subtilis* BS 104 or a decline in *L. pneumophila* due to adverse ambient growth conditions. Guerrieri et al., 2008 attained evidence that *L. pneumophila* was inhibited by bacterocin-like substances (BLS) produced from heterotrophic species due to a bactericidal effect.

In addition to growth on culture media, *Legionella* growth in water can be influenced by other organisms present. Stout et al., 1985a found that environmental bacteria and sediment both improved the survival of *Legionella pneumophila* in environmental samples. However, it remains unclear whether the environmental bacteria in this study include host organisms such as amoebae. Although they can compete for nutrients, non-*Legionella* bacteria have not been found to compete with *Legionella* for uptake by protozoan hosts (Declerck et al., 2005). Biofilm matrices can provide nutrient sources and sheltering within a complex microenvironment. Thus, some researchers have proposed that biofilms may be able to support the replication of *L. pneumophila* without the assistance of a protozoan host (Hoffman et al., 2008; Valster et al., 2010; Guerrieri et al., 2008)

Additionally, microbial biomass has been implicated in supporting *L. pneumophila* amplification through necrotrophic growth (Temmerman, et al., 2007). This is in accordance with findings from Stout et al., which provided evidence indicating *Legionella* require both organic matter and saprophytic microbial association for survival (Stout et al., 1985). In a study by Murga et al., *L. pneumophila* was investigated in the context of a biofilm reactor composed of *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Hartmannella vermiformis*, and a *Flavobacteirum*-like microorganism isolated from water containing *Legionella*. Studies found that *L. pneumophila* persisted in these biofilms with and without the presence of *H. vermiformis*. Even so, without the addition of amoebae host *H. vermiformis, L. pneumophila* was unable to replicate (Hoffman et al., 2008). As an important note, the model utilized does not effectively represent the complexity of diverse biofilms that may potentially support *L. pneumophila* growth. Investigations from Kupier et al., 2004 have also suggested that *Legionella* proliferation is unable to occur without the addition of a protozoan host cell (Kuiper et al., 2004). Additional research and evidence is needed to determine if *Legionellae* possess a means to propagate independently of host cells within biofilms (Hoffman et al., 2008).

Although the effects of heterotrophic bacteria on *Legionella* replication remained unclear (Murga et al., 2001), in a study in Finnish apartments it was found that microbial biomass, thermophilic bacteria at 50 °C, and fungi at 20 °C were all positively correlated with *Legionella* (Zacheus and Martikainen, 1994). OHSA has noted that algae, *Pseudomonas,* and flavobacteria provide essential nutrients for *Legionella* growth. *P. aeruginosa* has been shown to support the replication of *L. pneumophila* in *Naegleria lovaniensis* and *Acanthamoeba castellanii* (OSHA, Declerck et al., 2005), but it has also been

hypothesized that *Pseudomonas* can compete with *Legionella* for growth. In two surveys, Leoni et al., found a negative correlation between *Legionellae* and *P. aeruginosa* in swimming pools in Italy and Borella et al., found *Legionella pneumophila* serogroup 1 to be associated with lower *Pseudomonas* prevalence in private homes in Italy (Leoni et al., 2001b; Borella et al., 2004).

Assimilable organic carbon (AOC) is low-weight dissolved organic carbon that is capable of being utilized by heterotrophic microorganisms in biofilms. Although AOC represents only 0.1-10% of the total organic carbon (TOC) in water, it is regarded as a principle factor in controlling heterotrophic bacteria and biological water stability (Hammes et al., 2005). As *Legionella* cells are also classified as heterotrophic organisms, there may be an increased likelihood of *Legionella* at higher AOC in premise plumbing systems (Hammes et al., 2005; LeChevalier et al., 1991). Additionally, there are several mechanisms by which AOC creation in premise plumbing has been theorized, including nitrification, hydrogen (H_2) oxidation from magnesium anode rods, increased biodegradability of sorped organics, and leaching of organics from PEX and premise pipelines (Tare et al., 2005; Zhang et al., 2009; Camper et al., 2004; Haddix et al., 2003).

Physical Factors

There are several different physical attributes that will affect *Legionella* colonization. The presence of older pluming infrastructure is positively correlated with *Legionella* colonization, and in Pittsburgh, it was found that city residences had a higher prevalence of *Legionella* than suburban residences (Alary and Joly 1991; Marrie et al., 1994; Borella et al., 2004; Borella et al., 2005; Lee et al., 1988). Research has also indicated that larger hospitals and buildings greater than 10 stories have an increased prevalence of *Legionella* (Marrie et al., 1994; Flannery et al., 2006).

In addition to building features, rubber and silicone within the distribution system also can support *Legionella* growth in theory, by providing a more suitable surface for attachment than other distribution system components (Schofield and Locci, 1985; Schofield and Wrigh, 1984). Stagnation and interruptions in water service increase the prevalence of *Legionella*, possibly due to lower disinfectant residuals (Storey et al., 2004; Flannery et al., 2006). Turbulent conditions can cause large assemblages of *Legionella* to become detached and move into the bulk water (Storey et al., 2004).

1.6 AIREBORNE *LEGIONELLA*

As *Legionella* is an aquatic pathogen, humidity represents a significant parameter relative to human exposure; humidity is a deterministic factor in the persistence and suspension of aerosols. A study by Hambleton et al., 1984 found that *L. pneumophila* aerosols stayed in the air and survived better at 65% relative humidity as compared to 30%, 55%, or 90%. Alternatively, Dennis and Lee found that *L. pneumophila* was least stable at 60% relative humidity, most stable at 90%, and had intermediate stability at 30% (Dennis and Lee, 1988). In a second study from Dennis and Lee, 1984b, a shower bath was studied in which the initial relative humidity was 55% and the relative humidity after 15 minutes was 100%. *Legionella* were recovered from the air for up to 15 minutes after showering. Fisman et al. examined weather patterns in an attempt to correlate Legionellosis to changes in humidity. As a result of the study, it was found that Legionellosis was acutely associated with rainy, humid periods. Additionally, reported outbreaks were also coincided with periods of heavy rainfall (Fisman et al., 2005)

Deloge-Arbarkan et al. found that the concentration of *Legionella* in the water was not correlated with their airborne abundance in typical showers, implying that setting hazard guidelines based on water concentration will be difficult considering that delivered dose exposure depends on specifics of the local environmental conditions and possibly shower nozzle design (Deloge-Arbarkan et al., 2007). Parker et al. found that when waterborne pathogens are aerosolized they are concentrated by an enrichment factor (concentration of cells per droplet volume divided by the concentration of cells in the bulk suspension per equivalent volume) (Parker et al., 1983). For *Legionella pneumophila* strain Philadelphia 1, the enrichment factor was found to be 1,230, and for the Philadelphia 2 strain it was 2,560. In addition, *Mycobacterium*, another water pathogen which can become aerosolized, the concentration within aerosols was dependent on growth medium composition, formation of cell aggregates, age of culture, concentration of salt, and species and strain of the bacterium (Falkinham 1989). Understanding aerozolization and concentration of *Legionella* remains of significant mechanistic importance in waterborne disease outbreaks.

Similar factors may affect the concentration of *Legionella* within aerosols and might be important to human infection under specific circumstances. For example, some water saving shower heads mix air into the water to form bubbles in the shower (E-co Shower). According to the mechanism of Parker et al., *Legionella* could potentially be concentrated in the water droplets aerosolized from such

showerheads (Parker et al., 1983). A recent study by Perkins et al. investigated the implementation of membrane-integrated showerheads as a method of pathogen control in hospitals. The average total bacterial counts found were 2.2 x 10^7 cells/liter in shower water and 3.4 x 10^4 cells/m^3 in shower aerosol, and these counts were reduced to 6.3 x 10^4 cells/liter (representing 99.6% removal efficiency) and 8.9 x 10^3 cells/m^3 (representing 82.4% removal efficiency) using membrane integrated showerheads (Perkins et al., 2009). Evidence remains unclear if this methodology is effective in preventing nosocomial infections and diseases. Additional research and epidemiological evidence is needed to establish if this method could be utilized as a viable control measure towards *L. pneumophila*.

1.7 PREVENTION OF LEGIONNAIRES' DISEASE

Risk Assessment

There are conflicting views about whether sampling for *Legionella* is of value when no Legionnaires' disease cases have been reported. The Allegheny County Health Department (ACHD) in Pennsylvania recommends that hospitals should sample yearly if no transplants are performed and more often if they are (ACHD 1997). That work recommended that at least ten distal sites (water fountains, showers, faucets, etc.) should be tested if there are less than 500 beds, and two distal sites should be tested for every 100 beds if total beds are more than 500. A study was done on these guidelines by Squier et al. to evaluate the effectiveness of the ACHD guidelines in the effectiveness of preventing Legionellosis. Due to these implementations the number of health-care acquired Legionnaires disease experienced a significant decline due to improved testing and diagnostics (Squier et al., 2005). The CDC, however, does not recommend routine sampling unless the hospital performs transplants (CDC, 2004). OSHA only recommends an investigation if there is a probable diagnosis of Legionnaires' disease or a probable basis for suspecting that the water contains *Legionella* (OSHA, 2009).

A level-one investigation consists of an overview of the water system, an inspection of the facility, and a recommendation of control actions based on the results of the walk-through. A level-two investigation consists of the same as level-one but also a second walk-through where water samples are collected, initiation of an employee awareness program, and a review of worker absences (OSHA, 2009). If more than one case of Legionnaires' disease has been diagnosed, a level-two investigation should be implemented, but a level-one investigation is otherwise warranted. If the outbreak is considered to be ongoing, control measures should be immediately implemented (OSHA, 2009).

Several action levels have been published to assist hospitals and building managers when *Legionella* is detected in the water system. OSHA recommends prompt cleaning and/or biocide treatment when *Legionella* levels in cooling towers exceed 100 CFU/mL, domestic water exceed 10 CFU/mL, or a humidifier exceed 1 CFU/mL (OSHA). Immediate cleaning and/or biocide treatment and limitation of employee exposure is recommended at levels above 1,000 CFU/mL for cooling towers, 100 CFU/mL for domestic water, or 10 CFU/mL for humidifiers. Biocide treatment recommended by OSHA is either continuous chlorination above 1 mg/L or the addition of bromine. An additional study by Stout et al. has examined the affects of continued clinical and environmental surveillance in the absence of Legionellosis outbreaks. *Legionella* species were isolated from 14 of the 20 hospitals in the test study (Stout et al., 2007) and 30% of distal outlet sources tested positive for the presence of *L. pneumophila*. This study provides evidence that continued environmental surveillance in the absence of disease outbreak is a viable strategy for mitigating nosocomial Legionellosis.

Pathcon Laboratories has also developed suggested action criteria with five different stages ranging from reviewing the routine maintenance program to immediate cleaning and/or biocide treatment (Morris and Shelton 1998). These levels are based on the water source and concentration of *Legionella*. However, the infective dose for humans has not been determined and may be as low as a single organism (EPA 2001, WHO). Instead of criteria based on water source and *Legionella* concentration, the Allegheny County Health Department recommends disinfection when at least 30% of distal sites contain *Legionella* or there is a case of Legionnaires' disease (Squier et al., 2005).

Disinfection

There are several methods currently utilized for disinfection within water distribution systems for *Legionella*. These methods can be divided into two categories: systemic and focal (EPA, 2001). Systemic methods disinfect the entire system, while focal methods only disinfect a portion of the system.

Some methods for systemic disinfection include: super heating and flushing, chlorination, chloramination, chlorine dioxide, bromine, and copper-silver ionization. The super heat and flush method involves heating hot water tanks to 70 °C and flushing for 30 minutes, ensuring the temperature at each outlet is at least 60 °C (ACHD 1997). Studies have found discrepancies in the time before recolonization using this method. One study found *Legionella* reproliferation occurring within weeks to

months (Lin et al., 1998). Another study found *L. pneumophila* recolonization after day 7, and achieving 66% of original concentration by day 9 (Ragull et al., 2005). However, if the hot water temperature is consistently maintained at 60 °C, bacterial regrowth can be delayed. A 90% reduction of *Legionella* can be achieved at 45 °C for 2500 minutes, 50 °C for 380 minutes, 60 °C for less than 5 minutes, and 70 °C for less than 1 minute. However, a recent study from Allegra et al. has suggested temperature adaptation of *Legionella* after in vitro heat treatment at 70 °C for 30 minutes. Results of that study found the mean percentage of viable and VBNC cells varying from 4.6 to 71.7%. These findings suggest *L. pneumophila* strains can acquire heat-resistance after prolonged exposure (Allegra et al., 2010).

Legionellae are more resistant to chlorine than most bacteria. Even indicator organisms such as *Escherichia coli* can be reduced in 40 to 68 times less contact time than free-living *Legionella* (WHO). Shock hyperchlorination temporarily increases the chlorine level to 20 – 50 mg/L and replaces the water after 1 – 2 hours (Lin et al., 1998). Furthermore, this study suggested that this method can effectively control *L. pneumophila* for up to 2 – 5 months. In continuous hyperchlorination, the chlorine level is maintained at 2 – 6 mg/L to control *L. pneumophila*. Although 0.4 mg/L of free chlorine controls planktonic *Legionella*, continuous hyperchlorination is needed to penetrate and inactivate biofilm bacteria. High levels of chlorine can be corrosive to plumbing systems (Sarver et al., 2011), and one university reported 30 times more leaks during 3 years of continuous chlorination (Rutala and Weber 1997). Chlorination is not effective against some *Legionella* host organisms, which may provide a reservoir for the bacterium during periods of shock hyperchlorination. For example, *Acanthamoebae* cysts can survive free chlorine concentrations up to 50 mg/L, and *Hartmannellae* support the growth of *Legionellae* up to 4 mg/L (Kilvington and Price 1990). Recent studies have also shown that *L. pneumophila* replicated from *H. vermiformis* possess greater chlorine resistivity than cells replicated from *A. castellani* (Chang et al., 2009), as those from *H. vermiformis* withstood temperatures up to 53°C and 4mg/L of free chlorine. This evidence has extreme implications for hot water heater systems, as the efficacy of free chlorine in extremely reduced under these environmental conditions (Chang et al., 2009).

Chloramine apparently has many advantages over chlorine. Several studies have reported that hospitals using chloramine are less likely to be colonized by *Legionella* than those using free chlorine (Kool et al.,

1999a, Kool et al., 1999b). The reasoning behind this could be attributed to the stability and better biofilm penetration of chloramines as compared with free chlorine (Lin et al., 2000).

Chlorine dioxide penetrates biofilms better than chlorine and is also a more effective oxidizer (WHO). ClO_2 is a chemical disinfectant that reacts with organic and inorganic compounds through one-electron transfer. A study was undertaken by Zhang et al. to investigate the efficiency to introducing chlorine dioxide as a means of *Legionella* control in two hospital water systems. In both systems distal water outlet testing positive for the presence of *Legionella* decreased from 60% ≤10% after chlorine dioxide treatments (Zhang et al., 2009). Shock treatment of chlorine dioxide (50 – 80 mg/L for 1 hour) followed by continuous treatment (3 – 5 mg/L) greatly reduces microbial biofilm (Walker et al., 1995). Thomas et al. found that chlorine dioxide was more efficient than chlorine, chloramines, ozone, or copper-silver ionization, however there have not been many other investigations on this topic (Thomas et al., 2004).

Bromine also has suspected advantages relative to chlorine in that it is less corrosive, less dependent on the pH of the water, and produces less disinfectant by-products (OSHA). As long as bromine levels are maintained between 0.5 – 1.5 mg/L free halogen, it is an effective disinfectant (Thomas et al., 1999). However, bromine is not as effective as chlorine for reducing *Legionella* when compared on a mass basis.

In recent years, copper-silver ionization has become a principle disinfection method for water distribution systems worldwide. This technique entails channeling water through pipelines and applying low potential electricity to silver and copper electrodes (Cachafeiro et al., 2007). In effect, ions are released into the water are electro-statically attracted to negatively charged cell membranes. These tension forces alter cellular permeability, denaturing proteins, and ultimately result in lysis of the cell (ACHD, 1997). Evidence has shown this method to be extremely effective in controlling pathogen proliferation including *Legionella* cells (Cachafeiro et al., 2007). However, the utilization of this method alone will not achieve pathogen eradication within the distribution system, therefore making it ineffective for long-term disinfection. Studies on these ionization systems have produced intriguing results. Research has shown *Legionella* recolonization can occur within 2 months after the treatment period ends (Liu et al., 1994). Stout et al. found that copper-silver ionization is more effective than the superheat and flush method (Stout et al., 1998a). Rohr et al. found that while continuous copper-silver

ionization initially decreased *Legionella* from 40,000 to 7 CFU/L, after three years the concentration was 10,000 CFU/l (Rohr et al., 1999). Copper-silver ionization may also be less effective at higher pH levels due to the lower levels of Cu^{+2} and precipitation of copper solids (Lin et al., 2002). Landeen et al. found that concentrations of 400 and 40 μg/L for copper and silver, respectively, resulted in a 3-log reduction in *L. pneumophila* (Landeen et al., 1989). However, concentrations of 800 and 80 μg/L resulted in a significantly better inactivation rate. At higher levels, (40 μg/L) the ions have a synergistic effect, but at lower levels (20 μg/L) they only have an additive effect (Lin et al., 1996).

Ozone, ultraviolet light, and instantaneous heating are three types of focal disinfection used to control *Legionella*. Ozone is more effective than chlorine in controlling *L. pneumophila* concentrations, however it undergoes rapid decomposition and its utilization is much more costly (Heuner et al., 1997, EPA 2001). More than 99% of *L. pneumophila* can be killed within 5 minutes with 0.1 to 0.3 mg/L ozone added to the system (Domingue et al., 1988). Another technique currently employed is disinfection with ultraviolet light which treats the water with 254 nm light. This method disrupts DNA synthesis by producing thymine dimers (Lin et al., 1998). To prevent recolonization for several months, prefiltration is required. Ultraviolet lights should also be installed at the point of use (Kim et al., 2002). A 5 log decrease in *L. pneumophila* has been observed after 20 minutes with 30,000 μW-s/cm^2 ultraviolet light (Muraca et al., 1987). Instantaneous heating is accomplished by flash heating the water to 88 °C and then blending it with cold water to lower the temperature (Lin et al., 1998). This system is effective for killing planktonic *Legionella*. However, if instantaneous heating is installed in a system with established biofilms it may not be effective.

1.8 CONCLUDING DISCUSSION

Scientific research has made considerable progress in understanding and mitigating *Legionella* proliferation in both hot water systems and in distribution systems. It is unclear the extent to which improved infrastructure and mechanisms for controlling pathogen re-growth could reduce risk for waterborne disease from this source. Understanding all the factors for *Legionella* amplification is paramount in achieving safe conditions for consumers and improving public health. Additional investigations of *Legionella* in consumer homes as a function of water system operation, plumbing materials, and water use patterns are warranted.

REFERENCES

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

Alary, M. and J. R. Joly (1992). "Factors contributing to the contamination of hospital water distribution systems by *Legionellae*." Journal of Infectious Diseases **165**: 565-569.

Allegheny County Health Department (ACHD) (1997). Approaches to Prevention and Control of *Legionella* Infection in Allegheny County Health Care Facilities.

Armon, R. and P. Payment (1990). "A transparent medium for isolation of *Legionella pneumophila* from environmental water sources." Journal of Microbiological Methods **11**: 65-71.

Arnow, P. M. and D. Weil (1984). *Legionella pneumophila* Contamination of Residential Tap Water. *Legionella*: Proceedings of the 2nd International Symposium. C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski. Washington, D.C., American Society for Microbiology**:** 240-241.

Asada, T., O. Masayoshi, T. Kimura. (2001) "Bactericidal Effect of Calicum Oxide on Calcined Shell Calcium on *Legionella pneumophila* " Journal of Health Science **47**(4): 418-418

Association of Water Technologies (AWT) (2003). "*Legionella* 2003."

Atlas, R. M., J. F. Williams, et al.,(1995). "*Legionella* contamination of dental-unit waters." Applied and Environmental Microbiology **61**(4): 1208-1213.

Berendt, R. F. (1980). "Survival of *Legionella pneumophila* in aerosols: effect of relative humidity." Journal of Infectious Diseases **141**: 689-689.

Bollin, G. E., J. F. Plouffe, et al.,(1985a). "Aerosols containing *Legionella pneumophila* generated by shower heads and hot-water faucets." Applied and Environmental Microbiology **50**: 1128-1131.

Bollin, G. E., J. F. Plouffe, et al.,(1985b). "Difference in virulence of environmental isolates of *Legionella pneumophila*." Journal of Clinical Microbiology **21**: 674-677.

Bopp, C. A., J. W. Sumner, et al., (1981). "Isolation of *Legionella* spp. from environmental water samples by low-pH treatment and use of a selective medium." Journal of Clinical Microbiology **13**: 714-719.

Borella, P., M. T. Montagna, et al.,(2004). "*Legionella* infection risk from domestic hot water. " Emerging Infectious Diseases **10**(3): 457-464.

Borella, P., M. T. Montagna, et al., (2005). "*Legionella* contamination in hot water of Italian hotels." Applied & Environmental Microbiology **71**(10): 5805-5813.

Boulanger, C. A. and P. H. Edelstein (1995). "Precision and accuracy of recovery of *Legionella pneumophila* from seeded tap water by filtration and centrifugation." Applied and Environmental Microbiology **61**(5): 1805-1809.

Brennen, C., R. M. Vickers, et al., (1987). "Discovery of occult *Legionella* pneumonia in a long stay hospital: results of prospective serological survey." Br.Med.J.(Clin.Res.Ed). **295**: 306-307.

Camper, A. (2004) "Involvement of humic substances in regrowth" Int. J. Food Microbiol. **92**: 355-364.

Cachafeiro, S. P., Naveira, I. M., Garcia, I. G. (2007)**.** Is copper–silver ionisation safe and effective in controlling *Legionella*? Journal of Hosp Infect **67**, 209–216

Calderon, R. L. and A. P. Dufour (1984). Media for Detection of *Legionella* spp. in Environmental Water Samples. *Legionella*: Proceedings of the 2nd International Symposium. C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski. Washington, D.C., American Society for Microbiology**:** 290-292.

Canadian Standing Committee on Building and Plumbing Services (SCBPS) (2005). "Report of the SCBPS: Hot Water Delivery in Buildings."

Catrenich, C. E. and W. Johnson (1989). "Characterization of the selective inhibition of growth of virulent *Legionella pneumophila* by supplemented Mueller-Hinton medium." Infection and Immunity **57**: 1862-1864.

Centers for Disease Control and Prevention (CDC) (1986). "*Acanthamoeba* Keratitis Associated with Contact Lenses -- United States." Morbitiy and Mortality Weekly Report **35**(25): 405-408.

Centers for Disease Control and Prevention (CDC) (1987). "*Acanthamoeba* Keratitis in Soft-Contact-Lens Wearers." Morbitiy and Mortality Weekly Report **36**(25): 397-398, 403-404.

Centers for Disease Control and Prevention (CDC) (1992a). "Primary Amebic Meningoencephalitis -- North Carolina, 1991." Morbitiy and Mortality Weekly Report **41**(25): 437-440.

Centers for Disease Control and Prevention (CDC) (1992b). "Procedures for the recovery of *Legionella* from the environment."

Centers for Disease Control and Prevention (CDC) (2003). "Primary Amebic Meningoencephalitis -- Georgia, 2002." Morbitiy and Mortality Weekly Report **52**(40): 962-964.

Centers for Disease Control and Prevention (CDC) (2004). "Guidelines for Preventing Health-Care--Associated Pneumonia, 2003." Morbitiy and Mortality Weekly Report **53**(RR03): 1-36.

Centers for Disease Control and Prevention (CDC). (2005a). "Legionellosis." from http://www.cdc.gov/ncidod/dbmd/diseaseinfo/legionellosis_g.htm.

Centers for Disease Control and Prevention (CDC) (2005b). "Procedures for the Recovery of *Legionella* from the Environment."

Centers for Disease Control and Prevention (CDC) (2007). "Summary of Notifiable Diseases - United States, 2005." Morbitiy and Mortality Weekly Report **54**(53).

Challacombe, S. J. and L. L. Fernandes (1995). "Detecting *Legionella pneumophila* in water systems: a comparison of various dental units." J.Am.Dent.Assoc. **126**(5): 603-608.

Chang, C.W., Kao, C.H., Liu, Y.F. (2009). "Heterogeneity in chlorine susceptibility of *Legionella pneumophila* released for *Acanthamoeba* and *Hartmannella*"Journal of Applied Mircrobiology **106** (1): 97-105

Chellam, S. and W. Xu (2004). "Amino Acids in Drinking Water Supplies: Occurrence, Bulk Diffusivities, Disinfection By-Product Formation, and Control." Environmental Institute of Houston 2003 Annual Report: 29-30.

Chen, Y. S., W. R. Lin, et al.,(2002). "Residential water supply as a likely cause of community-acquired Legionnaires' disease in an immunocompromised host." European Journal of Clinical Microbiology & Infectious Diseases **21**(10): 706-709.

Chinn, R. and S. Barrett (2000). Occurrence of amino acids in drinking water sources. Natural Organic Matter and Disinfection By-Products; Characterization and Control in Drinking Water. S. E. Barrett, S. W. Krasner and G. L. Amy, American Chemical Society**:** 96-108.

Cianciotto N.P. (2007) Iron acquisition by *Legionella pneumophila*. Biometals 20: 323–331

Clescerl, L. S., A. E. Greenberg, et al., (1999). 9260 J. *Legionella*. Standard Methods for the Examination of Water and Wastewater, American Public Health Association**:** 9-106 - 9-109.

Cogo, P. E., M. Scaglia, et al.,(2004). "Fatal Naegleria fowleri Meningoencephalitis, Italy." Emerging Infectious Diseases **10**(10): 1835-1837.

Declerck, P., J. Behets, et al., (2005). "Impact of non-*Legionella* bacteria on the uptake and intracellular replication of *Legionella pneumophila* in *Acanthamoeba* castellanii and Naegleria lovaniensis." Microbial Ecology **50**(4): 536-549.

Deloge-Abarkan, M., T.-L. Ha, et al.,(2007). "Detection of airborne *Legionella* while showering using liquid impingement and fluorescent in situ hybridization (FISH)." Journal of Environmental Monitoring **9**(1): 91-97.

Dennis, P. J., C. L. Bartlett, et al., (1984a). Comparison of Isolation Methods for *Legionella* spp. *Legionella*: Proceedings of the 2nd International Symposium. C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski. Washington, D.C., American Society for Microbiology**:** 294-296.

Dennis, P. J. and J. V. Lee (1988). "Differences in aerosol survival between pathogenic and non- pathogenic strains of *Legionella pneumophila* serogroup 1." J.Appl.Bacteriol. **65**: 135-141.

Dennis, P. J., J. A. Taylor, et al.,(1981). "Phosphate buffered, low sodium chloride blood agar medium for *Legionella pneumophila* " Lancet **2**: 636-636.

Dennis, P. J., A. E. Wright, et al., (1984b). "*Legionella pneumophila* in aerosols from shower baths." J.Hyg.(Lond) **93**: 349-353.

Delgado-Viscogliosi, P., Solignac, L., Delattre, J.M. 2009. Viability PCR, a Culture-Independent Method for Rapid and Selective Quantification of Viable *Legionella pneumophila* Cells in Environmental Water Samples. Appl. Environ. Microbiol. **75**(11): 3502-3512.

Domingue, E. L., R. L. Tyndall, et al., (1988). "Effects of three oxidizing biocides on *Legionella pneumophila* serogroup 1." Applied & Environmental Microbiology **54**(3): 741-747.

E-co Shower. "E-co Shower Evolving." Retrieved October 14, 2007, from http://users.bigpond.net.au/ecoshower/system.htmL.

Edelstein, P. H. (1981). "Improved semiselective medium for isolation of *Legionella pneumophila* from contaminated clinical and environmental specimens." Journal of Clinical Microbiology **14**: 298-303.

Edelstein, P. H. (1982a). "Comparative study of selective media for isolation of *Legionella pneumophila* from potable water." Journal of Clinical Microbiology **16**: 697-699.

Edelstein, P. H., J. B. Snitzer, et al.,(1982b). "Enhancement of recovery of *Legionella pneumophila* from contaminated respiratory tract specimens by heat." Journal of Clinical Microbiology **16**: 1061-1065.

Environmental Health Directorate (2006). Legionnaires Disease Environmental Health Guide. from http://www.public.health.wa.gov.au/cproot/1451/2/Legionnaires_Disease.pdf

Falkinham, J. O., III (1989). Factors influencing the aerosolization of mycobacteria. Climate and Health Implications of Bubble-Mediated Sea-Air Exchange. E. C. Monahan and M. A. Van Patten. Groton, CT, Sea Grant Marine Science Institute, University of Connecticut.

Feeley, J. C., R. J. Gibson, et al.,(1979). "Charcoal-yeast extract agar: primary isolation medium for *Legionella pneumophila*." Journal of Clinical Microbiology **10**: 437-441.

Feeley, J. C., G. W. Gorman, et al., (1978). "Primary isolation media for Legionnaires disease bacterium." Journal of Clinical Microbiology **8**: 320-325.

Fields, B. S., R. F. Benson, et al., . (2002). "*Legionella* and Legionnaires' disease: 25 years of investigation." Clinical Microbiology Reviews **15**(3): 506-526.

Fisman DN, Lim S, Wellenius GA, Johnson C, Britz P, Gaskins M, et al., (2005) "It's not the heat, it's the humidity: wet weather increases Legionellosis risk in the greater Philadelphia metropolitan area. Journal of Infectious Diseases.**192**: 2066–73

Flannery, B., L. B. Gelling, et al., (2006). "Reducing *Legionella* colonization in water systems with monochloramine." Emerging Infectious Diseases **12**(4): 588-596.

Fliermans, C. B., W. B. Cherry, et al.,(1981). "Ecological distribution of *Legionella pneumophila* " Applied and Environmental Microbiology **41**: 9-16.

Fraser, D. W., T. R. Tsai, et al.,(1977). "Legionnaires' disease: description of an epidemic of pneumonia." New England Journal of Medicine **297**: 1189-1197.

Garcia, M. T., S. Jones, et al., (2007). "*A. polyphaga* resuscitates viable non-culturable *L. pneumophila* after disinfection." Environmental Microbiology. **9**(5): 1267-1277.

Garrity, G. M., E.M. Elder et al., (1981). " Serological and genotypic diversity among serogroup 5-reacting environmental *Legionella* isolates. " Journal of Clinical Microbiology **15:** 646-643

George, J. R., L. Pine, et al., (1980). "Amino acid requirements of *Legionella pneumophila*." Journal of Clinical Microbiology **11**: 286-291.

Gobin , I., Newton, P.R, Hartland, E.L, et al., . (2009). "Infections caused by nonpneumophila species of *Legionella*" Reviews in Medical Microbiology **20**:1–11

Goetz, A. M., J. E. Stout, et al., . (1998). "Nosocomial legionnaires' disease discovered in community hospitals following cultures of the water system: seek and ye shall find." American Journal of Infection Control **26**(1): 8-11.

Gorman, G. W., J. C. Feeley, et al., (1985). "*Legionella anisa*: a new species of *Legionella* isolated from potable waters and a cooling tower." Applied and Environmental Microbiology **49**: 305-309.

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

Haddix, P.L., Shaw, N.J., LeChevallier, M.W. (1994) "Characterization of bioluminescent derivatives of assimilable organic carbon test bacteria." Appl. Environ. Microbiol. **70**(2): 850-854.

Hambleton, P., P. J. Dennis, et al., (1984). Survival of Airborne *Legionella pneumophila*. *Legionella*: Proceedings of the 2nd International Symposium. C. Thornsberry, A. Balows, J. C. Feeley and W. Jakubowski. Washington, D.C., American Society for Microbiology**:** 301-302.

Hammes F.A. and Egli, T. (2005) "New method for assimilable organic carbon determination using flow-cytometric enumeration and a natural microbial consortium as inoculum." Environmental Science and Technology, 39, 3289 – 3294.

Harrison, T. G. and A. G. Taylor (1988). A Laboratory Manual for *Legionella*. Great Britain, John Wiley & Sons Ltd.

Heath, C. H., D. I. Grove, et al., (1996). "Delay in appropriate therapy of *Legionella* pneumonia associated with increased mortality." European Journal of Clinical Microbiology & Infectious Diseases **15**(4): 286-290.

Hedges, L. J. and D. J. Roser (1991). "Incidence of *Legionella* in the Urban Environment in Australia." Water Research **25**(4): 393-399.

Heller, R., C. Holler, et al., (1998). "Effect of salt concentration and temperature on survival of *Legionella pneumophila*." Lett.Appl.Microbiol. **26**(1): 64-68.

Heuner, K., J. Hacker, et al., (1997). "The alternative sigma factor sigma28 of *Legionella pneumophila* restores flagellation and motility to an Escherichia coli fliA mutant." Journal of Bacteriology **179**(1): 17-23.

Hill, V.R., J. Vinje, et al., (2010). "Sample preparation methods for molecular techniques for drinking water." Water Research Foundation. Subject Area: Water Quality.

Hoffman, P. S, H. Friedman, M Bendinelli (2008). *Legionella pneumophila*: Pathogenesis and Immunity. New York: Springer Science & Business Media, LLC

Hoffman, P. S., L. Pine, et al., (1983). "Production of superoxide and hydrogen peroxide in medium used to culture *Legionella pneumophila*: catalytic decomposition by charcoal." Applied and Environmental Microbiology **45**: 784-791.

Horwitz MA (1993). Toward an understanding of host and bacterial molecules mediating pathogenesis. In: Barbaree JM, Breiman RF and Doufur AF, eds. *Legionella: current status and emerging perspectives*, Washington, DC, American Society for Microbiology, 55–62.

Hsu, S. C., R. Martin, et al., (1984). "Isolation of *Legionella* species from drinking water." Applied and Environmental Microbiology **48**: 830-832.

Hureiki, L., J. P. Croue, et al., (1994). "Chlorination studies of free and Combined amino acids." Water Research **28**(12): 2521-2531.

Ishimatsu, S., H. Miyamoto, et al., (2001). "Sampling and detection of *Legionella pneumophila* aerosols generated from an industrial cooling tower." Annals of Occupational Hygiene **45**(6): 421-427.

James, B. W., W. S. Mauchline, et al., (1995). "Influence of iron-limited continuous culture on physiology and virulence of *Legionella pneumophila*." Infection and Immunity **63**(11): 4224-4230.

Joly, J. (1985). "*Legionella* and domestic water heaters in the Quebec City area." Can.Med.Assoc.J. **132**: 160-160.

Joly, J. R., M. Boissinot, et al., (1984). "Ecological distribution of *Legionella* in the Quebec city area." Can.J.Microbiol. **30**: 63-67.

Joly, P., P. A. Falconnet, et al., (2006). "Quantitative real-time *Legionella* PCR for environmental water samples: data interpretation." Applied & Environmental Microbiology **72**(4): 2801-2808.

Joseph, C. A., J. M. Watson, et al.,(1994). "Nosocomial Legionnaires' disease in England and Wales, 1980-92." Epidemiology & Infection **112**: 329-345.

Joseph, C. A. and I. European Working Group for *Legionella* (2004). "Legionnaires' disease in Europe 2000-2002." Epidemiology & Infection **132**(3): 417-424.

Kern,M. S. Bohm, et al., (2009). " Inhibition of *L. pneumophila* PCR in respiratory samples: A quantitative approach." J.Appl.Bacteriol. **79:** 189-193.

Kilvington, S. and J. Price (1990). "Survival of *Legionella pneumophila* within cysts of *Acanthamoeba* polyphaga following chlorine exposure." J.Appl.Bacteriol. **68**: 519-525.

Kim, B. R., J. E. Anderson, et al., (2002). "Literature review--efficacy of various disinfectants against *Legionella* in water systems. [Review] [68 refs]." Water Research. **36**(18): 4433-4444.

Kool, J. L., D. Bergmire-Sweat, et al., (1999b). "Hospital characteristics associated with colonization of water systems by *Legionella* and risk of nosocomial legionnaires' disease: a cohort study of 15 hospitals." Infection Control & Hospital Epidemiology **20**(12): 798-805.

Kool, J. L., J. C. Carpenter, et al., (1999a). "Effect of mono disinfection of municipal drinking water on risk of nosocomial Legionnaires' disease." Lancet **353**(9149): 272-277.

Kuchta, J. M., S. J. States, et al., (1983). "Susceptibility of *Legionella pneumophila* to chlorine in tap water." Applied and Environmental Microbiology **46**: 1134-1139.

Kuiper, M. W., B.A. Wullings et al., (2004). "Intracellular Proliferation of *L. pneumophila* in *H. vermiformis* in Aquatic Biofilms Grown on Plasticized Polyvinyl Chloride." Applied & Environmental Microbiology **70:** 6826-6833

Kusnetsov, J. M., P. J. Martikainen, et al., (1993). "Physical, chemical and microbiological water characteristics associated with the occurrence of *Legionella* in cooling tower systems." Water Research **27**(1): 85-90.

Landeen, L. K., M. T. Yahya, et al., (1989). "Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila*." Applied and Environmental Microbiology **55**: 3045-3050.

LeChevalier, M.W., Schulz, W., Lee, R.G. (1991) "Bacterial nutrients in drinking water. Applied and Environmental Microbiology." **57**: 857

Lee, G. F. and A. Jones-Lee (1993b). Public Health Significance of Waterborne Pathogens, G. Fred Lee & Associates.

Lee, T. C., J. E. Stout, et al., (1988). "Factors predisposing to *Legionella pneumophila* colonization in residential water systems." Archives of Environmental Health **43**: 59-62.

Lee, T. C., R. M. Vickers, et al., (1993a). "Growth of 28 *Legionella* species on selective culture media: a comparative study." Journal of Clinical Microbiology **31**: 2764-2768.

Leoni, E., L. G. De, et al., (2005). "*Legionella* waterline colonization: detection of *Legionella* species in domestic, hotel and hospital hot water systems." Journal of Applied Microbiology **98**(2): 373-379.

Leoni, E. and P. P. Legnani (2001a). "Comparison of selective procedures for isolation and enumeration of *Legionella* species from hot water systems." Journal of Applied Microbiology **90**(1): 27-33.

Leoni, E., P. P. Legnani, et al., (2001b). "Prevalence of *Legionella* spp. in swimming pool environment." Water Research. **35**(15): 3749-3753.

Lin, Y. E., V. L. Yu, et al., (2000). "Discussion of "Monochloramine and Legionnaires' disease". Journal of the American Water Works Association **92**(10): 88-90.

Lin, Y. S., J. E. Stout, et al., (1998). "Disinfection of water distribution systems for *Legionella*" Seminars in Respiratory Infections **13**(2): 147-159.

Lin, Y. S., R. D. Vidic, et al., (2002). "Negative effect of high pH on biocidal efficacy of copper and silver ions in controlling *Legionella pneumophila* " Applied & Environmental Microbiology **68**(6): 2711-2715.

Lin, Y. S. E., R. D. Vidic, et al., (1996). "Individual and COMBined effects of copper and silver ions on inactivation of *Legionella pneumophila*." Water Research **30**(8): 1905-1913.

Liu, W. K., D. E. Healing, et al., (1993). "Monitoring of hospital water supplies for *Legionella*." J.Hosp.Infect. **24**: 1-9.

Liu, Z., J. E. Stout, et al.,(1994). "Controlled evaluation of copper-silver ionization in eradicating *Legionella pneumophila* from a hospital water distribution system." Journal of Infectious Diseases **169**: 919-922.

Mauchline, W. S., R. Araujo et al., (1992). "Physiology and morphology of *L. pneumophila* in continuous culture at low oxygen concentration." Journal of General Microbiology **138:** 2371-2380

Maiwald, M., K. Kissel, et al., (1994). "Comparison of polymerase chain reaction and conventional culture for the detection of *Legionella*s in hospital water samples." J.Appl.Bacteriol. **76**: 216-225.

Marrie, T., P. Green, et al., (1994). "*Legionella*ceae in the potable water of Nova Scotia hospitals and Halifax residences." Epidemiology & Infection **112**: 143-150.

Mathys, W., J. Stanke, M. Harmuth, et al., (2008) "Occurrence of *Legionella* in hot water systems of single-family residences in suburbs of two German cities with special reference to solar and district heating." Int J Hyg Environ Health **211**:179–185

McCoy, W. F. (2005). Preventing Legionellosis. London, IWA Publishing.

McDade, J. E. (2002). Legionnaires' Disease 25 Years Later: Lessons Learned. *Legionella*. R. Marre, Y. Abu Kwaik, C. Bartlett et al,. Washington, D.C., ASM Press**:** 1-10.

McDade, J. E., C. C. Shepard, et al., . (1977). "Legionnaires' disease: isolation of a bacterium and demonstration of its role in other respiratory disease." N.Engl.J.Med. **297**: 1197-1203.

Miller, R. D. and K. A. Kenepp (1993). Risk Assessments for Legionnaires Disease Based on Routine Surveillance of Cooling Towers for *Legionellae*. *Legionella*: Current Status and Emerging Perspectives. J.

M. Barbaree, R. F. Breiman and A. P. Dufour. Washington, D.C., American Society for Microbiology**:** 40-43.

Miller, R. D. and D. A. Koebel (2002). Prevalence of *Legionella* in Whirlpool Spas: Correlation with Total Bacterial Numbers. *Legionella*. R. Marre, Y. Abu Kwaik, C. Bartlett et al,. Washington, D.C., ASM Press**:** 275-279.

Miller, R. (2008) Preventing *Legionella*: Common Disinfection Techniques. from http://earthwiseenvironmental.com/editable/uploads/File/Earthwise_Spring08_Article.pdf

Miskowski, D. "An Overview of *Legionella* Analyses." Retrieved October 14, 2007, from http://www.*Legionella*testing.com/pdf/*Legionella*Analyses.pdf.

Morrill, W. E., J. M. Barbaree, et al., (1990). "Increased recovery of *Legionella* micdadei and *Legionella bozemanii* on buffered charcoal yeast extract agar supplemented with albumin." Journal of Clinical Microbiology **28**: 616-618.

Morris, G. K. and B. G. Shelton (1998). "*Legionella* Bacteria in Environmental Samples: Hazard Analysis and Suggested Remedial Actions." Pathcon Laboratories.

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

Murga, R., T.S. Forster, E. Brown, J.M., et al., (2001). "The role of biofilms in the survival of *Legionella pneumophila* in a model potable water system" Microbiology **147**: 3121–3126.

Ohno, A., N. Kato, et al., (2003). "Factors influencing survival of *Legionella pneumophila* serotype 1 in hot spring water and tap water." Applied & Environmental Microbiology **69**(5): 2540-2547.

Ohno A., N. Kato et al., (2008). "Temperature-dependent parasitic relationship between *Legionella* pneumophila and a free-living amoeba (*Acanthamoeba* castellanii). Applied & Environmental Microbiology. **74**: 4585–4588.

Oliver, J.D. (2009). "Recent findings on the viable but nonculturable state in pathogenic bacteria." FEMS Microbiology Reviews. **34**: 415-425

Ortiz-Roque, C. M. and T. C. Hazen (1987). "Abundance and distribution of *Legionella*ceae in Puerto Rican waters." Applied and Environmental Microbiology. **53**: 2231-2236.

Occupational Safety and Health Administration (OSHA) (2009). "Legionnaires Disease " http://www.osha.gov/dts/osta/otm/legionnaires/index.htmL

Perkins S.D., Mayfield, JD, Fraser, V. et al., (2009). "Potential Pathogenic Bacteria in Shower Water and Air of a Stem Cell Transplant Unit."Appl Environ Microbiol. **75**: 5363–5372.

Parker, B. C., M. A. Ford, et al., (1983). "Epidemiology of infection by nontuberculous mycobacteria. IV. Preferential aerosolization of Mycobacterium intracellulare from natural waters." The American Review of Respiratory Disease **128**(4): 652-656.

Pasculle, A. W., J. C. Feeley, et al., (1980). "Pittsburgh pneumonia agent: direct isolation from human lung tissue." Journal of Infectious Diseases **141**: 727-732.

Paszko-Kolva, C., P. A. Hacker, et al., (1993). "Inhibitory Effect of Heterotrophic Bacteria on the Cultivation of *Legionella dumoffii*." *Legionella*: Current Status and Emerging Perspectives. J. M. Barbaree, R. F. Breiman and A. P. Dufour. Washington, D.C., American Society for Microbiology**:** 203-205.

Pine, L., J. R. George, et al., (1979). "Development of a chemically defined liquid medium for growth of *L. pneumophila*." Journal of Clinical Microbiology **9:** 615-626

Pringler, N., P. Brydov, et al., (2002). Prevalence of *Legionella* in Whirlpool Spas: Correlation with Total Bacterial Numbers. *Legionella*. R. Marre, Y. Abu Kwaik, C. Bartlett et al., . Washington, D.C., ASM Press**:** 298-301.

Ragull, S., M. G. Nunez et al., (2007). "*Legionella pneumophila* in Cooling Towers: Fluctuations in Counts, Determination of Genetic Variability by Pulsed-Field Gel Electrophoresis (PFGE), and Persistence of PFGE patterns." Applied and Environmental Microbiology. **73**(16): 5382-5384

Reeves M. W., L. Pine, et al., (1980). "Metal requirements of *L. pneumophila*." Journal of Clinical Microbiology. 13(4): 688-695

Reinthaler, F. F., J. Sattler, et al., (1993). "Comparative study of procedures for isolation and cultivation of *Legionella pneumophila* from tap water in hospitals." Journal of Clinical Microbiology **31**: 1213-1216.

Ristroph, J. D., K. W. Hedlund, et al., (1981). "Chemically defined medium for *Legionella pneumophila* growth." Journal of Clinical Microbiology **13**: 115-119.

Roberts, K. P., C. M. August, et al., (1987). "Relative sensitivities of environmental *Legionellae* to selective isolation procedures." Applied and Environmental Microbiology **53**: 2704-2707.

Rogers, J., A. B. Dowsett, et al., (1994). "Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora." Applied and Environmental Microbiology **60**: 1585-1592.

Rohr, U., M. Senger, et al., (1999). "Four years of experience with silver-copper ionization for control of *Legionella* in a German university hospital hot water plumbing system." Clinical Infectious Diseases **29**(6): 1507-1511.

Rowbotham, T. J. (1983). "Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae." Journal of Clinical Pathology **36**: 978-986.

Roy, C. (2009), "*Legionella pneumophila*: an intracellular pathogen of phagocytes", in Finlay, B. (ed.), *Molecular Basis of Bacterial Infection:* , The Biomedical & Life Sciences Collection, Henry Stewart Talks Ltd, London (online at http://hstalks.com/bio)

Rutala, W. A. and D. J. Weber (1997). "Uses of Inorganic Hypochlorite (Bleach) in Health-Care Facilities." Clinical Microbiology Reviews **10**(4): 597-610.

Rusin, P., K. Bright and C. Gerba (2003). "Rapid reduction of *Legionella pneumophila* on stainless steel with zeolite coatings containing silver and zinc ions." Letters in Applied Microbiology **36** (2): 69–72

Schofield, G. M. and R. Locci (1985). "Colonization of components of a model hot water system by *Legionella pneumophila*." J.Appl.Bacteriol. **58**: 151-162.

Schofield, G. M. and A. E. Wright (1984). "Survival of *Legionella pneumophila* in a model hot water distribution system." J.Gen.Microbiol. **130**: 1751-1756.

Squier, C. L., J. E. Stout, et al., (2005). "A proactive approach to prevention of health care-acquired Legionnaires' disease: the Allegheny County (Pittsburgh) experience." American Journal of Infection Control **33**(6): 360-367.

Stanwick, R. S., M. E. K. Moffatt, et al., (1981). "Hot tap water scalds in Canadian children." Canadian Medical Association **125**: 1250-1253.

States, S. J., L. F. Conley, et al., (1985). "Effects of metals on *Legionella pneumophila* growth in drinking water plumbing systems." Applied and Environmental Microbiology **50**: 1149-1154.

Steinert, M., U. Hentschel, et al., (2002). "*Legionella pneumophila*: an aquatic microbe goes astray." FEMS Microbiology Reviews. **26**(2): 149-162.

Steinert, M., M. Ott, et al., (1994). "Studies on the uptake and intracellular replication of *Legionella pneumophila* in protozoa and in macrophage-like cells." FEMS Microbiology Ecology **15**: 299-308.

Storey, M. V., J. Langmark, et al., (2004). "The fate of *Legionellae* within distribution pipe biofilms: measurement of their persistence, inactivation and detachment." Water Science & Technology **49**(11-12): 269-275.

Stout, J. E. (1998b). "Culture Methodology for *Legionella* Species." HC Special Report 308.

Stout, J. E., Y. S. Lin, et al., (1998a). "Controlling *Legionella* in hospital water systems: experience with the superheat-and-flush method and copper-silver ionization." Infection Control & Hospital Epidemiology **19**(12): 911-914.

Stout, J. E., V. L. Yu, et al., (1985a). "Ecology of *Legionella pneumophila* within water distribution systems." Applied and Environmental Microbiology **49**: 221-228.

Stout, J. E., V. L. Yu, et al., (1985b). "Isolation of *Legionella pneumophila* from the cold water of hospital ice machines: implications for origin and transmission of the organism." Infect.Control. **6**: 141-146.

Stout, J. E., V. L. Yu, et al., (1987). "Legionnaires' disease acquired within the homes of two patients. Link to the home water supply." JAMA **257**: 1215-1217.

Stout, J. E., V. L. Yu, et al., (1992b). "Potable water as a cause of sporadic cases of community-acquired legionnaires' disease." N.Engl.J.Med. **326**: 151-155.

Stout, J. E., V. L. Yu, et al., (1992a). "*Legionella pneumophila* in residential water supplies: environmental surveillance with clinical assessment for Legionnaires' disease." Epidemiology & Infection **109**: 49-57.

Stout, J.E., R.R. Muder, S. Mietzner, et al., (2007) "Role of environmental surveillance in determining the risk of hospital-acquired legionellosis: a national surveillance study with clinical correlations." Infect Control Hosp Epidemiol **28** 818–824

Swanson M.S., Hammer B.K., 2000. "*Legionella pneumophila* pathogesesis: a fateful journey from amoebae to macrophages." Annu. Rev. Microbiol. **54**: 567-613

Ta, A. C., J. E. Stout, et al., (1995). "Comparison of culture methods for monitoring *Legionella* species in hospital potable water systems and recommendations for standardization of such methods." Journal of Clinical Microbiology **33**(8): 2118-2123

Tarre, S., Green, M. (2004) "High-rate Nitrification at Low pH in Suspended- and Attached-Biomass SGWH" Appl. Environ. Microbiol. **70** (11): 6481.

Temmerman, R., Vervaeren, H., Noseda, B., Boon, N., and Verstraete, W. (2007)" Inhibition of *Legionella pneumophila* by *Bacillus* sp. Engineering Life Sciences" **7:**497-503

Tesh, M. J. and R. D. Miller (1981). "Amino acid requirements for *Legionella pneumophila* growth." Journal of Clinical Microbiology **13**: 865-869

Tesh, M. J., S. A. Morse, et al., (1983). "Intermediary metabolism in *Legionella pneumophila*: utilization of amino acids and other compounds as energy sources." Journal of Bacteriology **154**: 1104-1109

Thomas, W. M., J. Eccles, et al., (1999). "Laboratory observations of biocide efficiency against *Legionella* in model cooling tower systems." ASHRAE Trans **105**: SE-99-3-4

Thomas, V., T. Bouchez, et al., (2004). "Amoebae in domestic water systems: resistance to disinfection treatments and implication in *Legionella* persistence." Journal of Applied Microbiology **97**(5): 950-963.

Toze, S., L. I. Sly, et al., (1990). "Inhibition of growth of *Legionella* species by heterotrophic plate count bacteria isolated from chlorinated drinking water." Current Microbiology **21**: 139-143.

United States Consumer Product Safety Commission (CPSC) "Tap Water Scalds."

United States Environmental Protection Agency (EPA) (1999). "*Legionella*: Human Health Criteria Document."

United States Environmental Protection Agency (EPA) (2001). "*Legionella*: Drinking Water Health Advisory."

United States National Institutes of Health (NIH). (2007). "Legionnaire's disease." from http://www.nlm.nih.gov/medlineplus/ency/article/000616.htm.

United States Occupational Safety & Health Administration (OSHA). "OSHA Technical Manual." from http://www.osha.gov/dts/osta/otm/otm_iii/otm_iii_7.htmL.

Yaradou, D.F., Hallier-Soulier, S., Moreau, S., et al., (2007) "Integrated real-time PCR for detection and monitoring of *Legionella pneumophila* in water systems." Appl. Environ. Microbiol. 73(5): 1452-1456

Valster, R.M., B.A. Wullings, et al., (2010). »Detection of Protozoan Hosts for *L. pneumophila* in Engineered Water Systems by Using a Biofilm Batch Test « Applied and Environmental Microbiology. **76**(21): 7144-7153

van der Kooij, D., H. R. Veenendaal, et al., (2005). "Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene." Water Research **39**(13): 2789-2798.

Vickers, R. M., A. Brown, et al., (1981). "Dye-containing buffered charcoal-yeast extract medium for differentiation of members of the family *Legionella*ceae." Journal of Clinical Microbiology **13**: 380-382.

Vickers, R. M., V. L. Yu, et al., (1987a). "Determinants of *Legionella pneumophila* contamination of water distribution systems: 15-hospital prospective study." Infect.Control. **8**: 357-363.

Vickers, R. M., J. E. Stout, et al., (1987b). "Manual of culture methodology for *Legionella*." Seminars in Respiratory Infections **2**: 274-279.

Victoria Australia Department of Human Services (DHS). (2007). "*Legionella*." from http://www.health.vic.gov.au/environment/*Legionella*/index.htm.

Villari, P., E. Motti, et al., (1998). "Comparison of conventional culture and PCR methods for the detection of *Legionella pneumophila* in water." Letters in Applied Microbiology **27**(2): 106-110.

VROM (2004). International Congress on the problem and prevention of *Legionella* in Europe. Aquatech, Amsterdam, Netherlands.

Wadowsky, R. M., R. Wolford, et al., (1985). "Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water." Applied and Environmental Microbiology **49**: 1197-1205.

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

Wadowsky, R. M. and R. B. Yee (1983). "Satellite growth of *Legionella pneumophila* with an environmental isolate of Flavobacterium breve." Applied and Environmental Microbiology **46**: 1447-1449

Wadowsky, R.M., T.M Wilson et al., (1990).“Multiplication of *Legionella* spp in Tap Water Containing *Hartmannella vermiformis*” Applied and Environmental Microbiology **57**: 1950-1955

Walker, J. T., C. W. Mackerness, et al., (1995). "Control of *Legionella pneumophila* in a hospital water system by chlorine dioxide." J.Ind.Microbiol. **15**(4): 384-390

Warren, W. J. and R. D. Miller (1979). "Growth of Legionnaires disease bacterium (*Legionella pneumophila*) in chemically defined medium." Journal of Clinical Microbiology **10**: 50-55

Wellinghausen, N., C. Frost, et al., (2001). "Detection of *Legionellae* in hospital water samples by quantitative real-time LightCycler PCR." Applied & Environmental Microbiology **67**(9): 3985-3993

Wilkinson, H. W. (1987). Hospital-laboratory diagnosis of *Legionella* infections. Atlanta, Ga., U.S. Dept. of Health and Human Services, Public Health Service, Centers for Disease Control.

World Health Organization (WHO). "*Legionella*." from http://www.who.int/water_sanitation_health/dwq/admicrob4.pdf.

World Health Organization (WHO) (2005). "Legionellosis." from http://www.who.int/mediacentre/factsheets/fs285/en/.

Wynter-Allison, Z., J. L. Morales, et al., (2005). "*Acanthamoeba* infection as a cause of severe keratitis in a soft contact lens wearer in Jamaica." American Journal of Tropical Medicine and Hygiene **73**(1): 92-94.

Yu, V. L. (1993). "Could aspiration be the major mode of transmission for *Legionella*?" American Journal of Medicine **95**: 13-15.

Zacheus, O. M. and P. J. Martikainen (1994). "Occurrence of *Legionellae* in hot water distribution systems of Finnish apartment buildings." Can.J.Microbiol **40**: 993-999.

Zeybek, Z. and A. Cotuk (2002). Relationship between Colonization of Building Water Systems by *Legionella pneumophila* and Environmental Factors. *Legionella*. R. Marre, Y. Abu Kwaik, C. Bartlett et al., . Washington, D.C., ASM Press**:** 305-308.

Zhang, Y., M. Edwards, A. Pinto, N. Love, A. Camper, M. Rahman, H. Baribeau. (2009b). “Effect of Nitrification on Corrosion in the Distribution System.” AWWARF Final Report.

CHAPTER 1: APPENDIX A

Table 1: Modifications to initial *Legionella* culture media:

Revision	Medium Name	Reference
Replace IsoVitaleX with L-Cysteine Replace hemoglobin with soluble ferric pyrophosphate	Feeley-Gorman (F-G) Agar	Feeley *et al.,* 1978
Replace starch with charcoal Replace acid hydrolysate of casein with yeast extract	Charcoal-Yeast Extract Agar	Feeley *et al.,* 1979
Addition of ACES buffer	Buffered Charcoal Yeast Extract (BCYE)	Pasculle *et al.,* 1980
Addition of α-ketoglutarate	BCYEα	Edelstein 1981

Table 2: Selective media for *Legionella* isolation

Medium	Reference	Medium Contents								
		P U/ml	Cy mg/L	V mg/L	G	D mg/L	Cep mg/L	Co mg/L	A mg/L	Cef mg/L
PCV	CDC 2005b	100	80	5						
GVPC	Dennis *et al.,* 1984a	100	80	5	0.3%					
DGVP	Vickers *et al.,* 1987b	50		1	0.3%	10				
PAV	CDC 1992b	100		5					80	
GPAV	CDC 1992b	100		5	0.3%				80	
BMPAα	Edelstein 1981	80							80	4
MWY	Edelstein 1982a	50		1	0.3%	10			80	
GVP	Wadowsky and Yee, 1981	100		5	0.3%					
CCVC	Bopp *et al.,* 1981		80	0.5			4	16		
PAC	Vickers *et al.,* 1987b	80							80	4

P = Polymyxin B, Cy = Cycloheximide, V = Vancomycin, G = Glycine, D = Dyes (Bromocresol Blue and Bromothymol Purple), Cep = Cephalothin, Co = Colistin, A = Anisomycin, Cef = Cefamandole

Table 3: Comparison of selective media

Media compared	Results	Reference
CCVC, WY~, MWY, EPA-CCVC^, EPA-WY^, EPA-MWY^	For high concentrations of *Legionella*, EPA-CCVC and MWY had the best overall recovery; For low concentrations of *Legionella*, CCVC and EPA-CCVC; had the highest frequency of *Legionella* isolations	Calderon and Dufour 1984
BCYE, PAV, PAC	*L. pneumophila* growth was significantly better than non-*pneumophila* species on all media tested; 3 of 28 *Legionella* species grew poorly on PAV; 11 of 28 *Legionella* species did not grow on PAC	Lee et al., 1993a
BCYE, GVPC, MWY	Highest recovery frequency on GVPC; MWY medium gave a lower percentage of positive samples, but not statistically different than MWY using the chi-squared test	Leoni and Legnani 2001a
MWY, BMPAα, GVPC	No significant difference	Reinthaler et al., 1993
BCYE, DGVP, PAV, GPAV, GVPC	Recovery greatest with DGVP; Non-*Legionella* bacteria grew on BCYE and PAV	Ta et al., 1995
BCYE, BCYE with dyes	On BCYE with dyes, *Legionella pneumophila* is pale green, *Tatlockia micdadei* (*Legionella micdadei*) is blue-gray, and *Fluoribacter* spp. is bright green	Vickers et al., 1981
BCYEα*, BMPAα*, MWY	Best recovery from MWY	Edelstein 1982a
CCVC, GPV, BMPAα	CCVC least inhibitory to non-*Legionellaceae* with no acid wash; After acid wash all media were the same	Joly et al., 1984

~ WY is MWY agar without anisomycin or dyes

^ substitutes proteose peptone no. 3 for yeast extract

* Acid wash used

Table 4: Methods for acid-buffer pretreatment

Acid-Buffer (AB) Mixture	AB pH	Volume AB: Volume Sample	Reaction Time (min)	Reference	Notes
25 mL 0.2 M KCl 3.9 ml 0.2 M HCl	2.2	1:9	5	Bopp *et al* 1981	Most effective when using centrifuged sample
			4	Edelstein 1981	
			15	Reinthaler *et al* 1993	
		1:2	Not specified	Kuchta *et al* 1983	
		1:1	3; 15 if needed	Ta *et al* 1995	
			10	Miller and Kenepp 1993	Use filter-concentrated sample
	2.0 initially; 2.2 after dilution	1:1	15	Roberts *et al* 1987	Add KOH neutralizer
	2.0	1:1	15	Gorman *et al* 1985	Add KOH neutralizer
		1:9	4	Gorman *et al* 1985	Use centrifuged sample
	2.5	1:9	10	Bollin *et al* 1985a	Use centrifuged sample
		1:2	5	Bollin *et al* 1985b	Used for organ specimens
25 mL 0.2 M KCl 3.9 mL 0.2 M HCl 100 ml distilled water	2.2	1:9	30	Rowbotham 1983	
0.005 M HCl 0.04 M KCl			4	Zacheus and Martikainen 1994	Use filter-concentrated sample
18 parts 0.2 M KCl 1 part 0.2 M	Not specified	1:1	15; 30 if needed	CDC 2005b	

HCl					

Table 5: Guidelines for selective methods

Reference	Sample Source	Pretreatment	Culture medium
CDC 2005b	High total bacterial count water (swabs and non-potable water)	Acid or heat	BCYE, PCV, GPCV, and PCV(-)*
	Moderate total bacterial count water (hot water tanks, some non-potable water)	Direct culture	
	Low total bacterial count water (most potable water)	Concentration by filtration or centrifugation	
Stout 1998b	Cooling towers	Acid pretreatment and direct culture	BCYE, DGVP, and CCVC
	Hot water tanks	Direct culture	BCYE and DGVP
	Water fixtures	Filter concentration and direct culture	BCYE and DGVP
	Clinical Specimens	Direct culture; Acid pretreat if necessary	BCYE, PAV, and PAC
Clescerl et al., 1999	High bacterial count water	Dilute 2x with sterile tap water or phosphate buffer; Plate acid-treated and non-acid-treated sample	BCYE and GPVA or CCVC
	Low bacterial count water	Concentration by filtration or centrifugation; Plate acid-treated and non-acid-treated sample	

*PCV(-) is PCV without cysteine and is used as a control

Table 6: *Legionella* colonization frequencies from various sources

Sampling Location	Colonization Frequency	Geographical Area	Reference	Notes
Residential Water	37%	Quebec City, Canada	Alary and Joly 1991	Electric water heaters
	12%			Faucets
	15%			Shower heads
	10.9%	Pittsburgh, USA	Lee et al., 1988	
	6%; range within 6 areas 0-22%	Pittsburgh, USA	Stout et al., 1992a	
	37%	Chicago, USA	Arnow and Weil 1984	
	22.6%	Italy	Borella et al., 2004	
Buildings	30%	Finland	Zacheus and Martikainen 1994	
	37%	New South Wales, Australia	Hedges and Roser 1991	Warm water systems
	89%	Copenhagen, Denmark	Pringler et al., 2002	Hot water systems
	60%	San Francisco, USA	Flannery et al., 2006	Chlorine Disinfectant
	4%			Monochloramine Disinfectant
Cooling Towers	37%	New South Wales, Australia	Hedges and Roser 1991	
	47%	Not specified	Kusnetsov et al., 1993	
Spa	36%	New South Wales, Australia	Hedges and Roser 1991	
	5.9%	San Diego, USA	Miller and Koebel 2002	
Dental-Unit Waters	68%	Not specified	Atlas et al., 1995	
	25%	Not specified	ChallaCOMBe and Fernandes 1995	
Hotels	75%	Italy	Borella et al., 2005	
	60.9%	Italy	Leoni et al., 2005	
	41%	Istanbul, Turkey	Zeybek and Cotuk 2002	
Hospitals	50%	Not specified	Brennen et al., 1987	Hot water tanks
	45%			Distal sites
	68%	Quebec, Canada	Alary and Joly 1992	
	93.7%	Italy	Leoni et al., 2005	

	83%	Not specified	Geotz et al., 1998	
	12%	England & Scotland	Liu et al., 1993	
	60%	Pennsylvania, USA	Vickers et al., 1987a	
Emergency Eye Wash Stations	76.9%	Not Specified	Paszko-Kolva et al., 1989	
Emergency Showers	46.2%	Not Specified	Paszko-Kolva et al., 1989	

CHAPTER 2: EFFECT OF ORGANIC MATTER ON PATHOGEN GROWTH IN SIMULATED HOT WATER HEATER SYSTEMS.

Krista Williams, Amy Pruden, Joseph Falkinham and Marc Edwards

Virginia Tech, 418 Durham Hall; Blacksburg, VA 24060

ABSTRACT

Potential relationships between organic carbon concentrations and pathogenic bacteria (*L. pneumophila*, *M. avium, A. polyphaga,* and *H. vermiformis*) were examined in small-scale simulated water heaters with long stagnation events up to 7 days. Strong correlations were observed between organic carbon concentrations versus total 16S rRNA genes and heterotrophic plate counts (HPC) in systems undergoing regular 80% water changes (Average R^2 values of 0.971 and 0.889 for 16S rRNA and HPC analysis respectively). However, no linear or consistent correlations were identified between any of the pathogenic bacteria and added total organic carbon (TOC). *M. avium* occasionally correlated with organic carbon concentrations over limited ranges of TOC (0-1,000 µg/L); however, this correlation was weak or non-existent without regular water changes (occurring 3 times per week). Stagnation events generally resulted in slight amplification of *Legionella*, but overall, there was only limited evidence of amplification for these pathogens in the synthesized potable water and premise plumbing conditions tested.

2.1 INTRODUCTION

The growth of opportunistic pathogens in premise plumbing systems thought to be controlled by various factors such as source water, chemical/biological treatments, distribution system design, plumbing material selection, and overall system operation (Chapter 1). Manipulation of the water chemistry through dosing of chloramine exemplifies a viable and effective community-wide approach as a *Legionella* abatement strategy (Kool et al., 1999a). However, for other waterborne pathogens such as *M. avium,* the use of chloramine may be less effective or even increase overall incidence of the organism (Moore et al., 2006). A plausible option for regrowth control encompassing a wide range of opportunistic pathogens in premise plumbing is the removal of essential nutrients, such as organic carbon, from the source water (van der Kooij, 1992; Camper et al., 2003; LeChevalier et al, 1991).

Although removal of organic carbon as a control strategy for biofilm and pathogen regrowth has never been explicitly tested in premise plumbing, correlations have been observed between AOC and higher re-growth potential for heterotrophic and coliform bacteria in water distribution systems (LeChevalier et al, 1991). Simple guidelines were established indicating AOC concentrations over 50 µg/L were associated with higher microbial abundance and corrosive processes (LeChevalier et al, 1991). Extension of similar relationships might be observed for HPC and opportunistic pathogens including *Acanthamoeba polyphaga*, *Legionella pneumophila,* and *Mycobacterium avium* in simulated hot water systems. Additionally, conceptualizing these relationships may provide an avenue for sustainable and comprehensive control of a broad array of pathogenic organisms in premise plumbing systems.

Key distinctions of premise plumbing, as compared to main distribution lines, alter the likelihood of finding correlations between microbial growth and nutrient concentrations. Specifically, infrequent water flow in building plumbing systems, versus nearly continuous flow in water mains, may increase the probability of inducing nutrient limitations via reduced mass transfer and nutrients available to biofilms. On the other hand, investigations with ultrapure water systems indicate that microbial regrowth can sometimes be extensive (>10^4 CFU/mL) in systems with frequent stagnation events, even though virtually all essential and trace nutrient concentrations are near their practically achievable minimums (US Filter, 2001; Kayser et al., 1975).

This investigation sought to understand the relationship between the concentration of organic matter and microbial growth in simulated premise plumbing hot water systems over a period of several months. Other than utilizing long stagnation events (48 hours) and absence of chlorine/chloramine residual to maximize potential pathogen proliferation (Clark et al., 2004; Mathys et al., 2009), other factors thought to increase the likelihood of microbial re-growth (e.g., iron deposits, nitrifying bacteria, and PVC pipelines) were excluded in order to establish a baseline relationship between pathogens and ambient organic carbon concentration (Figure 1).

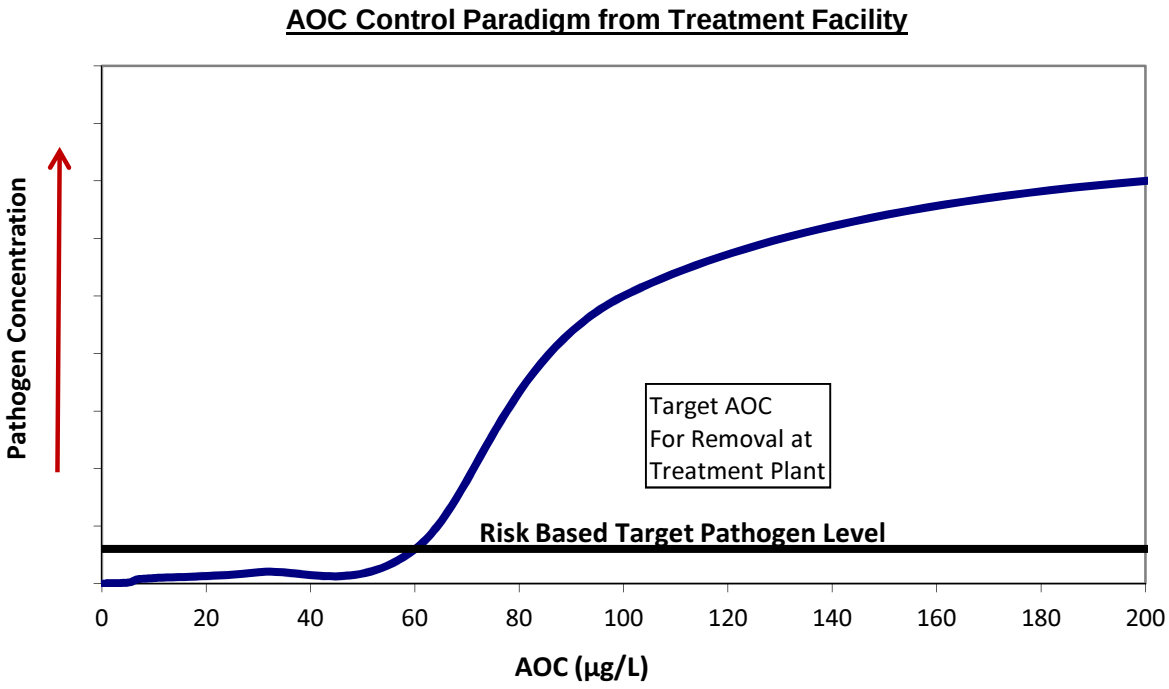

Figure 1: Hypothetical control of pathogens and other microorganisms by manipulating availability of organic matter (e.g., assimilable organic carbon (AOC)) from the source water. If a threshold of AOC (or other indicators of organic matter) limiting pathogen growth was identified, water utilities might be able to reduce the incidence of waterborne disease by targeting low levels of AOC in effluent distribution water. Figure adapted and modified from Edwards et al., 2009.

2.2. MATERIALS AND METHODS

The experimental design utilized simulated glass water heaters (SGWH) in conjunction with established synthesized potable water chemistry in a bench scale simulation of hot water heaters (Table 7). A synthesized baseline water was modified with addition of 0, 5, 50, 300, 1,000, 3000, 10,000, and 30,000 µg/L as TOC from a stock solution of ozonated fulvic acids that was isolated from a lake by XAD resin. Targeted levels of TOC (and corresponding levels of AOC) were tested in triplicate making 24 SGWH total (=8 levels of organic matter X 3, Figure 2).

The SGWH consisted of 120 mL French square glass bottles (Quorpak) with polytetrafluoroethylene (PTFE) caps. The total calculated surface area to volume ratio for the SGWH in this experiment was calculated at 1.24 cm^{-1} versus approximately 0.05 cm^{-1} for a typical 40 gallon water heater. Before use in experiments the bottles were acid washed, rinsed in reagent grade nanopure water and baked in a muffle oven at 550 °C.

Figure 2: Experimental SGWH Setup; 24 SGWH run in triplicate with TOC concentration from 0-30,000 µg/L. Full SGWH volume (100mL) was changed (80mL was poured out) and refilled 3 times per week to simulate a low-use water system.

Table 7: Norton et al., 2004 Synthetic Tap Water Recipe with indicated reagent grade salts added to purified water

Constituents	Concentration
$MgCl_2$ x $6H_20$	10.7mg/L
$CaSO_4$	27 mg/L
$NaHCO_3$	100 mg/L
Na_2HPO_4	0.45 mg/L
$(NH_4)_2SO_4$	1 mg/L
K_2HPO_4	0.3 mg/L
KH_2PO_4	0.7mg/L

Synthesized oligotrophic water was initially used as the baseline water in order to reduce the role of unknown variables while also enabling a systematic investigation of the effect of organic carbon. Unfortunately, the synthetic potable water was unable to support *L. pneumophila* growth. To date, no literary evidence has been found documenting *Legionella* growth in a synthetic tap water medium. Ultimately, three variations in the baseline water were employed (i.e., 0 µg/L added TOC) as follows (See Table 10 for experiment summaries):

- **Water Source 1**: Nanopure water with UV carbon destruction and Norton et al., 2004 recipe (Table 1) to create synthesized tap water. (Dates Utilized: 12/1/2009- 8/3/2010, Experiments: A1 & B1)
- **Water Source 2**: A mixture of 90% water source 1 mixed with 10% Blacksburg tap water that had been circulated through a GAC filter. (Dates Utilized: 8/3/2010-1/30/2011, Experiments A2 & B2)
- **Water Source 3**: A mixture of 90% water source 1 that had been purified using Reverse Osmosis (RO) mixed with 10% source water from Blacksburg tap water that had been circulated through a GAC filter. Trace manganese, iron, and zinc were added along with a stock solution of trace amino acids at levels encountered in potable water systems (Dates Utilized: 1/30/2011-5/25/11, Experiments A3, A4, B3, and B4).

The above water sources were inoculated with the stock fulvic acid (ozonated to 50% reduction in overall UV_{254}) with a final TOC of 1240 mg/L (Table 8). At various points *L. pneumophila*, *M. avium*, and *A. polyphaga* were inoculated into the SGWH as listed in Table 10.

Table 8: Total Organic Carbon from Baseline Water Sources. N/A = not tested. TOC in the baseline water (0 µg/L added TOC) and the organic stock solution were measured routinely. Other concentrations are calculated based on added volumes of 1240mg/L NOM stock solution.

Condition	TOC Water Source 1 (µg/L)	TOC Water Source 2 (µg/L)	TOC Water Source 3 (µg/L)
0 µg/L	460 (n=9)	1,145 (n=3)	507 (n=3)
5 µg/L	465	1,150	512
50 µg/L	510	1,195	557
300 µg/L	760	1,445	807
1,000 µg/L	1,460	2,145	1,507
3,000 µg/L	3,460	4,145	3,507
7,000 µg/L	N/A	8,145	7,507
10,000 µg/L	10,460	N/A	N/A
15,000 µg/L	N/A	16,145	15,507
30,000 µg/L	30,460	N/A	N/A

14.5% of the TOC present in the fulvic acid stock solution was present as BDOC (Table 9).

Table 9: BDOC Calculation of Fulvic Acid Stock Solution

Sample	Average Δ TOC	Standard Deviation Δ TOC	Average Initial TOC	Standard Deviation Initial TOC	BDOC/TOC	% BDOC	% Standard Deviation BDOC
7K	1.35	0.23	9.21	0.49	0.147	14.67	1.69
15K	2.33	1.27	16.3	1.05	0.143	14.31	6.9

Each start date listed in Table 10 references the initial inoculation period in which pathogenic bacteria were added into the SGWH systems. The first phase of the study (described in this chapter) examines oligotrophic, idealized water conditions in which no premise plumbing deficiencies are present. The second phase of the investigation examines plumbing deficiencies and potential AOC generation mechanisms and is discussed in Chapter 3.

Table 10: Summary of experimental methodologies and iterations for SGWH. Each start date indicates initial inoculation.

Experiment Title	Start Date	Condition	Water/Experiment
B1	3/18/2010	SGWH with Deficiencies (see Chap 3)	• Norton et al., 2004 synthetic tap, Water Source 1 • initial addition 10mL from Blacksburg hot water heater • 37°C SGWH temperature • 3 water changes per week
A1	4/23/2010	SGWH with added AOC	**SGWH Condition Change:** • Norton et al., 2004 synthetic tap, Water Source 1 • 10mL from Blacksburg hot water heater • 37°C SGWH temperature • 3 water changes per week
A2	9/27/2010	SGWH with added AOC	**SGWH Condition Change:** following incubator increase to 50°C • 10% GAC filtered Tap • 90% WRF synthetic tap (Norton et al., 2004), Water Source 2 • Temperature set to 32°C temperature following suggestion from Dr. Ashbolt (EPA). • 3 water changes per week
B2	9/27/2010	SGWH with Deficiencies (see Chap 3)	**SGWH Condition Change:** following incubator increase to 50°C • 10% GAC filtered Tap • 90% WRF synthetic tap (Norton et al., 2004), Water Source 2 • Temperature set to 32°C temperature following suggestion from Dr. Ashbolt (EPA). • 3 water changes per week
A3	3/21/2011	SGWH with added AOC	**SGWH Condition Change:** • 10% GAC filtered Tap, 90% WRF synthetic tap (Norton et al., 2004), Water Source 3 • Amino Acid addition Table 20 • pH adjustment to 7.5; DO adjustment to 4.0mg/L • Addition of Mg, Zn, and Fe • Inoculation with *H. vermiformis* • 1mm diameter glass beads • 32°C SGWH temperature

			• 1 water changes per week
B3	3/21/2011	SGWH with Deficiencies (see Chap 3)	**SGWH Condition Change:** • 10% GAC filtered Tap, 90% WRF synthetic tap (Norton et al., 2004), Water Source 3 • Amino Acid addition Table 20 • pH adjustment to 7.5, DO adjustment to 4.0mg/L • Addition of Mg, Zn, and Fe • Inoculation with of *H. vermiformis* • 1mm diameter glass beads • 32°C SGWH temperature • 1 water change per week
A4	4/19/11	SGWH with added AOC	**Performed identically to previous (3/21/11)**
B4	4/19/11	SGWH with Deficiencies (see Chap 3)	**Performed identically to previous (3/21/11)**

Quantification of Microorganisms

Deoxyribonucleic Acid (DNA) extraction

For general quantification of microorganisms in the SGWH effluent, composite samples from the triplicate SGWH were collected in a 250 mL sterile nalgene bottle and subject to DNA extraction. 200 mL of this sample was concentrated onto 47 mm diameter 0.22 µm pore size mixed cellulose ester filters (Whatman) using vacuum filtration. The filter membranes were then torn into 6 pieces using sterile tweezers and added to Lysing Matrix A extraction tubes from the FastDNA kit. DNA extraction proceeded utilized as detailed by FastDNA® SPIN Kit protocol (MP Biomedicals, Solon, OH) as recommended, except the FastPrep® (MP Biomedicals) instrument was set at a speed setting of 4.0 for 20 seconds to reduce the possibility of DNA shearing. Following the extraction process, DNA from SGWH effluent was re-suspended in 100 µL of DNA Elution Solution (DES) and preserved in a -22 °C freezer prior to quantification by qPCR using the specified protocols listed below.

Quantitative Polymerase Chain Reaction (qPCR)

qPCR was the principle methodology of bacterial quantification for this research experiment as detailed for specific targets below. All assays utilized a Bio-Rad CFX96 real time system (Hercules, CA).

Legionella Assay

A SsoFast supermix® (Bio-Rad, Hercules, CA) qPCR assay was developed for *L. pneumophila* targeting the macrophage infectivity potentiator (*mip*) gene, which only contains a single copy within *L. pneumophila* cells. The forward primer LmipF (AAAGGCATGCAAGACGCTATG) and reverse primer LmipR (GAAACTTGTTAAGAACGTCTTTCATTTG) amplified a 78 base pair region within this specific gene. The Taqman Probe (TGGCGCTCAATTGGCTTTAACCGA) was labeled at the 5' end with FAM reporter dye and TAMRA quencher located at the 3' end, respectively (Nazanian et al., 2008). Each real-time PCR reaction contains 10 µl of 2×SsoFast supermix (Bio-Rad), 250 nM of each primer, 93.75 nM of probe and 1 µl DNA template for a total volume of 20 µl. The thermal cycle profile consists of initial incubation for 2 minutes at 95 °C, followed by 40 cycles of 5 seconds at 95 °C (denaturing) and 10 seconds at 60° C (annealing and extension, plate read). Negative controls (consisting of template DNA replaced by molecular grade nanopure water) and 10-fold serial dilutions of known amounts of positive control DNA were included in triplicate for each qPCR assay and each sample was analyzed in triplicate.

***Hartmannella* Assay**

The *H. vermiformis* qPCR assay was adapted from the method from by Kuiper et al., 2006 using an Evagreen® (Bio-Rad, Hercules, CA) assay. The forward primer Hv1227F (TTACGAGGTCAGGACACTGT) and reverse primer Hv1728R (GACCATCCGGAGTTCTCG) target the 18S rRNA gene of *H. vermiformis*. The final reaction volume was 20 µl and contained10 µl of 2× EvaGreen® PCR Supermix, 400 nM of each primer, and 1 µl DNA template. The qPCR reaction consisted of the following steps: initial incubation for 2 min at 98° C and then 40 cycles of 5 seconds at 98° C (denaturing), 10 seconds at 64 °C (annealing) and 8 s at 72° C (extension, plate read). Negative controls (consisting of template DNA replaced by molecular grade nanopure water) and 10-fold serial dilutions of known amounts of positive control DNA are included in triplicate for each qPCR assay, and each sample was tested in triplicate.

***Acanthamoeba* Assay**

A SsoFast supermix® (Bio-Rad, Hercules, CA) qPCR assay for *Acanthamoeba* quantification was developed based upon the primers and probes designed by Riviere et al., 2006. The primers TaqAcF1 (CGACCAGCGATTAGGAGACG) and TaqAcR1 (CCGACGCCAAGGACGAC) amplified position 1267–1362 of the *Acanthamoeba* 18S rRNA gene, which distinguishes *Acanthamoeba* from 10 other genera. The Taqman probe (TGAATACAAAACACCACCATCGGCGC) was labeled with 6-FAM. Each real-time PCR reaction contained 10 µl of 2×SsoFast supermix (Bio-Rad), 250 nM of each primer, 93.75 nM of probe

and 1 µl DNA template for a total volume of 20 µl. The thermal cycle profile consists of initial incubation for 2 minutes at 95° C, followed by 40 cycles of 5 seconds at 95° C (denaturing) and 10 seconds at 60° C (annealing and extension, plate read). Negative controls (consisting of template DNA replaced by molecular grade nanopure water) and 10-fold serial dilutions of known amounts of positive control DNA are included in triplicate for each qPCR assay, and each sample was tested in triplicate.

M. avium **Assay**

The *M. avium* qPCR assay was developed using an Evagreen® (Bio-Rad, Hercules, CA) assay. The forward primer MycavF (AGAGTTTGATCCTGGCTCAG) and reverse primer MycavR 9 (ACCAGAAGACATGCGTCTTG) delimited a 180-bp DNA fragment of the 16S rRNA gene in *M. avium* cells and their specificities were verified using Primer-Blast. The final reaction volume contained 20 µl containing 10 µl of 2× EvaGreen® PCR Supermix, 400 nM of each primer, and 1 µl DNA template. Each qPCR reaction consists of the following steps: initial incubation for 2 min at 98° C and then 40 cycles of 5 seconds at 98° C (denaturing), 5 sec at 62° C (annealing), and 5 sec at 65° C (extension, plate read). Negative controls (consisting of template DNA replaced by molecular grade nanopure water) and 10-fold serial dilutions of known amounts of positive control DNA are included in triplicate for each qPCR assay, and each sample was tested in triplicate.

16S rRNA Assay

A SsoFast supermix ® (Bio-Rad, Hercules, CA) qPCR assay was developed for the 16S rRNA gene (Smith et al., 2006). The forward primer 1396F (CGGTGAATACGTTCYCGG) and reverse primer 1492R (GGWTACCTTGTTACGACTT) were utilized to amplify this specific gene. The 16S rRNA Taqman Probe (CTTGTACACACCGCCCGTC) was labeled at the 5’ end with FAM reporter dye and TAMRA quencher located at the 3’ end, respectively. Each real-time PCR reaction contains 10 µl of 2×SsoFast supermix (Bio-Rad), 250 nM of each primer, 93.75 nM of probe and 1 µl DNA template for a total volume of 20 µl. The thermal cycle profile consists of initial incubation for 2 minutes at 95 °C, followed by 40 cycles of 5 seconds at 95 °C (denaturing) and 10 sections min at 60 °C (annealing and extension, plate read). Negative controls (consisting of template DNA replaced by molecular grade nanopure water) and 10-fold serial dilutions of known amounts of positive control DNA are included in triplicate for each qPCR assay, and each sample is tested in triplicate.

Inhibition

Significant qPCR inhibition was observed due to co-extracted inhibitory substances from SGWH. Substances in Routine QA/QC overcame inhibition through dilution of samples to the point that spike and recovery was not significantly different than 100%. In general, a 10x dilution with molecular grade nanopure was found to be sufficient to eliminate inhibition (Chapter 2: Appendix A, Chapter 3: Appendix A).

Plating Methodologies

M. avium

In processing effluent SGWH water samples, 0.1 mL of each sample was diluted and spread directly on M7H10 agar containing 0.5% (vol/vol) glycerol and 10% (vol/vol) oleic-albumin (M7H10+GOA). Subsequently, plates were inverted, incubated at 37°C for up to 3 weeks, and enumerated.

A. polyphaga/ H. vermiformis

For effluent water samples, 10 µl was added to a Petroff-Hauser Counting Chamber and count amoeboid cells at 100-magnification; a note was made whether amoeba cells were trophozoites or encysted. The limit of detection limit for this assay is approximately 5 x 10^4 amoeba/mL using most probable number (MPN) methodology.

L. pneumophila

0.1 mL of each effluent SGWH sample was diluted and spread directly on BCYE agar containing L-cysteine. If overgrown, 1 mL of water sample was mixed with 1 mL of 0.2 M KCl-HCl solution and incubated at room temperature for 15 min (acid-treatment) before spreading. Plates were incubated at 37°C and examine daily for *Legionella* colonies for up to 7 days prior to enumeration. Possible *Legionella* colonies were picked and streaked for isolation on BCYE agar lacking L-cysteine. If colonies were cultivated on plates lacking L-cysteine, results were discarded as being non *Legionella* cells.

Heterotrophic Plate Count (HPC)

An R2A agar (Difco) was utilized to enumerate HPC to monitor the amount of heterotrophic bacteria present in SGWH effluent from each sample. 0.1 mL of each sample was diluted and spread directly on

R2A agar until dry. Subsequently, plates were inverted and incubated at room temperature for one week before colony enumeration (APHA,1985).

Total Organic Carbon (TOC) and Biodegradable Dissolved Organic Carbon (BDOC) Measurement

A Sievers 800 TOC analyzer was used following Standard Method 5310A (APHA, 1985). BDOC of the stock was analyzed utilizing the method of Servais et al., 1987 with tripicate dilution of the organic matter stock solution to 3,000, 7,000, and 15,000 µg/L as TOC. The samples were mixed with 10% V/V GAC filtered Blacksburg tap water to provide nutrients, inoculated with 1% V/V aliquots from the simulated water heater dosed with 1,000 µg/L TOC and incubated at 32°C. DOC in samples was quantified weekly over a 30 day period after passage through pre-rinsed 0.7 µm pore size glass fiber filters (Whatman) to remove large aggregates and protozoan cells, wasting the first 10 mL of filtrate and collecting the next 10 mL for analysis. BDOC in the stock solution was quantified based on the overall change in TOC during the 30 day incubation (Table 9).

Assimilable Organic Carbon (AOC) Measurement

The method of Weinrich et al., 2009 was used to quantify AOC with cross-checking using Standard Method 9217A (APHA, 1985). Standard curves for the growth bioluminescent strains of *Spirillium sp.* strain NOX and *Pseudomonas fluorescens* P-17 were obtained using acetate carbon standards in the range 0-1,000 µg/L with a stock mineral salt buffer. Aliquots of samples and standards were inoculated with either 10^4 viable cells of P-17 or NOX per mL, and 300 µL aliquots were taken for analysis on microplates at least 2 times a day for a total of 7 days. A photon counting luminometer was utilized (Spectra Max L, Molecular Devices, Sunnyvale, CA). Subsequent plate count measurements were performed spreading 0.1 mL of sample diluted with mineral salt buffer.

2.3 EXPERIMENTAL METHODOLOGY

First Phase of Testing: Experiment A1

The SGWH were rinsed in reagent grade nanopure water and baked in a muffle oven at 550 °C as discussed above. Clean SGWH were filled to volume (100 mL) with the specified amount of TOC to achieve targeted final concentrations (5, 50, 300, 1,000, 3000, 7000, and 15,000 µg/L) versus the 0 µg/L added TOC as the control solution (Water Source 1). On 4/23/10, 5 mL from a Blacksburg hot water heater sample was added to each of the SGWH to inoculate a normal water heater flora. The SGWH

were then incubated at 37 °C for 1 week. On 5/5/10, a 1 mL aliquot of *Acanthamoeba polyphaga* was added to each SGWH after an 80 mL (80%) water change and delivering a dose of 542 gene copies/mL (Aliquots were obtained from ATCC medium 2373 PYG from Dr. Falkinham's laboratory in Blacksburg, VA. Additional aliquots were obtained over the course of this investigation from this laboratory). After an additional week of stagnation, a 1 mL aliquot of *M. avium* (5.84 x 10^4 gene copies/mL) and *L. pneumophila* (5.09 x 10^9 gene copies/mL) were inoculated (Cultivation of *M. avium* was performed using Middlebrook7H9 (M7H9) broth under 37 °C incubation for 7 days; *L. pneumophila* was cultivated using ATCC medium 1099 CYE under incubation at 37 °C for 7 days. These methods were repeated throughout experiments performed in Chapter 2 & 3). *A. polyphaga* was in a stationary phase before inoculation to encourage phagocytosis of heterotrophic and pathogenic microorganisms. *L. pneumophila* was inoculated at exponential phase to increase growth potential within the simulated systems; *M. avium* culture was inoculated at stationary phase as per prior experimentation. After inoculation 80% of the SGWH volume was changed 3 times per week starting on 5/18/10 (Day 0 of experiment, after 8 days of stagnation).

First Set of Changes to Baseline: Experiments A2, B2

A set of experimental changes was implemented in an attempt to increase the likelihood of *Legionella* amplification in the SGWH. Primarily, the use of 10% Blacksburg tap water recirculated through a GAC filter in the baseline water was incorporated. The intention of this modification was to introduce inorganics, nutrients and microorganisms commonly found in a potable water system to the simulated water heaters as well as reduce experimental control (Water Source 2). The temperature of the water heater was reduced from 37 to 32° C to better mimic conditions at the bottom of electric water heaters and attempt to increase protozoan survival and encourage cellular phagocytosis of *L. pneumophila*. Re-inoculation was completed with 1 mL aliquots as before but with slightly different concentrations including *A. polyphaga* (1 x 10^3 amoeba/mL or 9.84 x 10^4 gene copies/mL) on 9/17/10, *M. avium* (2.6 x 10^5 CFU/mL or 7.15 x 10^5 gene copies/mL), and *L. pneumophila* on 9/27/10 (3.2 x 10^3 CFU/mL or 5.96 x 10^6 gene copies/mL). Formal water changes for the SGWH were restarted on 10/11/10 (Day 0 of experiment, after 25 days of stagnation) as before.

Second Set of Changes to Baseline: Experiments A3-A4, B3-B4

A second set of changes was made to the experimental conditions in an additional attempt to improve the ability of *L. pneumophila* to amplify in the SGWH. *H. vermiformis* was considered as a potential better host to *L. pneumophila* (Kuiper et al, 2002; Hoffman et al., 2008) and had naturally colonized the simulated glass water heaters (SGWH) presumably via the GAC filtered Blacksburg, VA water. The surface area of the SGWH was increased by an additional 235.8 cm^2 ± 5.89 cm^2 through addition of glass beads to encourage biofilm formation and increase biomass. The ambient pH was reduced from 8.6 to 7.5 to via use of small additions of CO_2 and the initial dissolved oxygen was lowered to 4.0 mg/L to create a microaerophilic environment better suited to *L. pneumophila* according to Wadosky et al., 1985 (Table 11). Finally, a suite of amino acids typically found in tap water was added as per investigations from Strickhouser, 2007 (Table 20), and three strains of *L. pneumophila* isolated from potable tap water and shower sources were inoculated to introduce bacteria more conducive of surviving in typical premise plumbing environments.

Table 11: Summary of Modifications: Second Set of Changes to Baseline Water

Modifications	**SGWH Series Utilized in Experiments A3-A4, B3-B4**
Biological	ATCC *L. pneumophila* strains from tap water and shower head (ATCC 33733, 33734, and 33823 respectively)
	Addition of *H. vermiformis* instead of *A. polyphaga* in the hopes of inducing phagocytosis and enhance more protozoan representation
	Adjust dissolved oxygen to 4.0 mg/L with concentrated N_2
Chemical	Addition of Manganese 6 µg/L
	Addition of Zinc 375 µg/L
	Addition of Iron 42 µg/L
	Adjust bulk water pH to 7.5
	Addition of Amino Acids commonly found in tap water; see Table 20
Surface Area	Addition of 5 grams of 1mm diameter glass beads (approximate additional surface area= 235.8 cm^2± 5.89 cm^2)

H. vermiformis was inoculated in all SGWH in this modified research phase on 3/18/11 (1.69 x 10^3 gene copies/mL and 1.46 x 10^3 amoeba/mL). *L. pneumophila* was inoculated (6.78 x 10^6 gene copies/mL and 6.7 x 10^4 CFU/mL from count data) on 3/21/11. Regular 80% water changes were initiated on 3/31/11 (Day 0 of experiment, after 13 days of stagnation) with only weekly water changes. Due to some

amplification observed under these conditions, *L. pneumophila* was reinoculated into the SGWH at an initial concentration of 1.27 x 10^5 gene copies/mL on 4/29/11 to investigate the repeatability of the previous iteration.

2.4 RESULTS

After analyzing the relationship between organic matter substrates and general growth of bacteria as measured by 16S rRNA gene analysis and HPC, amplification of specific pathogens are discussed separately.

Heterotrophic Plate Count Correlations and 16S rRNA gene analysis

There were often strong correlations between TOC (0-15,000 µg/L) and either 16S rRNA or HPC during time periods of the regular 80% water changes (Average R^2 values of 0.971 and 0.889 for 16S rRNA and HPC analysis respectively, Experiment A1). At points of the experiment when significantly longer stagnation times or re-inoculation was imposed to improve potential pathogen recovery, the correlations for 16S rRNA and HPCs experienced a decrease to R^2 values of 0.626 and 0.645, respectively (Table 12, Experiment A2). Finally, in examining data with overall stagnation periods of 1 week, correlations with added TOC are still weaker (Experiment A3). Thus, with less frequent stagnation periods improved correlations were maintained between overall bacterial levels and added TOC, as would be expected intuitively.

Table 12: Overall Experimental Correlation Summary: TOC (0-15,000 µg/L) vs. 16S rRNA, HPC

Summary of Experiments	Experiment Title	Dates Performed	Average R^2 value 16S rRNA qPCR	Times Sampled (n) 16S rRNA qPCR	Average R^2 value HPC	Times Sampled (n) HPC
Preliminary Experiments	N/A	12/1/09-4/15/10	N/A	N/A	0.952	11
Routine Water Changes (3 times per week)	A1	4/28/10-8/3/10	0.971	3	0.889	9
Routine Water Changes (3 times per week) Following Stagnation	A2	10/13/10-3/7/11	0.626	3	0.645	3*
Water Changes with 1 Week	A3	3/31/11-4/18/11	0.168	3	0.295	3

Stagnation						

***Additional samples were collected but failed routine QA/QC criteria**

In examination of this same data in greater detail, during routine water changes (3 times per week), there were often very strong correlations between added organic carbon and either 16S rRNA or HPCs was observed (Table 13, Figure 3). For example, both 16S rRNA and HPCs were positively correlated with TOC on 7/6/10 with R^2 values of 0.962 and 0.941, respectively. HPC and qPCR quantification of bacteria were also strongly correlated at this point of the experiment (R^2 value 0.818). Thus, data was expected based on previous findings from work in water distribution systems (LeChevalier et al., 1991), TOC (and AOC) encourage heterotrophic and overall bacterial regrowth potential in simulated water heaters.

Table 13: R^2 values Representing Strong Positive Correlations with TOC (Experiment A1). P values indicate significance under 95% confidence. *N/A denotes Not Applicable

Sample Date	HPC R^2 value	16S rRNA R^2 value
6/7/10	0.941 (P=6.5E-05)	N/A
6/14/10	0.925 (P=1.0E-04)	N/A
6/23/10	0.865 (P=9.0E-04)	0.981 (P=1.7E-05)
7/6/10	0.941 (P=0.002)	0.962 (P=6.7E-05)
7/14/10	0.939 (P=7.1E-05)	0.971 (P=5.2E-05)
7/19/10	0.993 (P=1.2E-06)	N/A

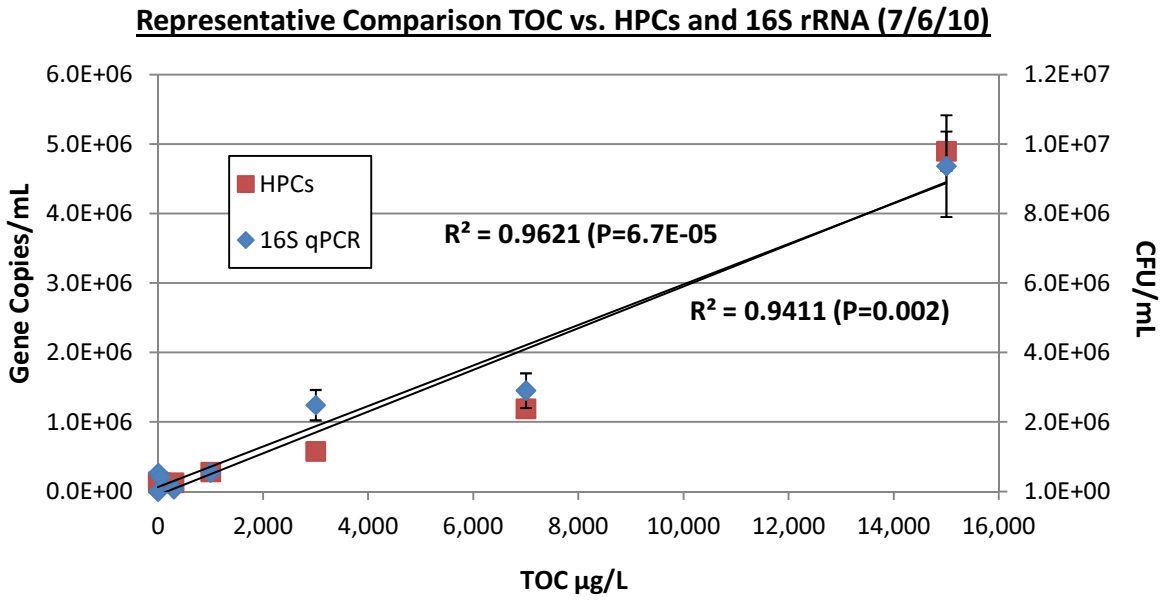

Figure 3: Plot of HPC and 16S rRNA genes vs. TOC. Errors bars denote 95% confidence intervals (Experiment A1).

However, strong correlations were significant only at the higher levels of added TOC. At lower levels of added TOC (<300 µg/L), R^2 values on 7/6/10 were as low as 0.164 and 0.050 for 16S rRNA and HPCs, respectively. During this time period, SGWH were being run under conditions of three water changes per week without stagnation for at least two months after the initial inoculum. In general, throughout the experiment correlations between 16S rRNA gene analysis and HPC at the lower levels of added TOC consistently yielded low correlations (R^2 =0.004-0.580, Figure 4). The low correlations at low added TOC are not unexpected, given that even ultrapure water is known to support high levels of bacteria unless the water is continually re-circulated and disinfected (Kulakov 2002; McAlister, 2001). But, in this regard, the simulated water heaters differed from cold water distribution systems for which only 50 µg/L AOC is cited as a threshold for much higher regrowth of bacteria (LeChevalier et al., 1991), although under more frequent water changes than tested herein the thresholds for significant correlations might be expected to change.

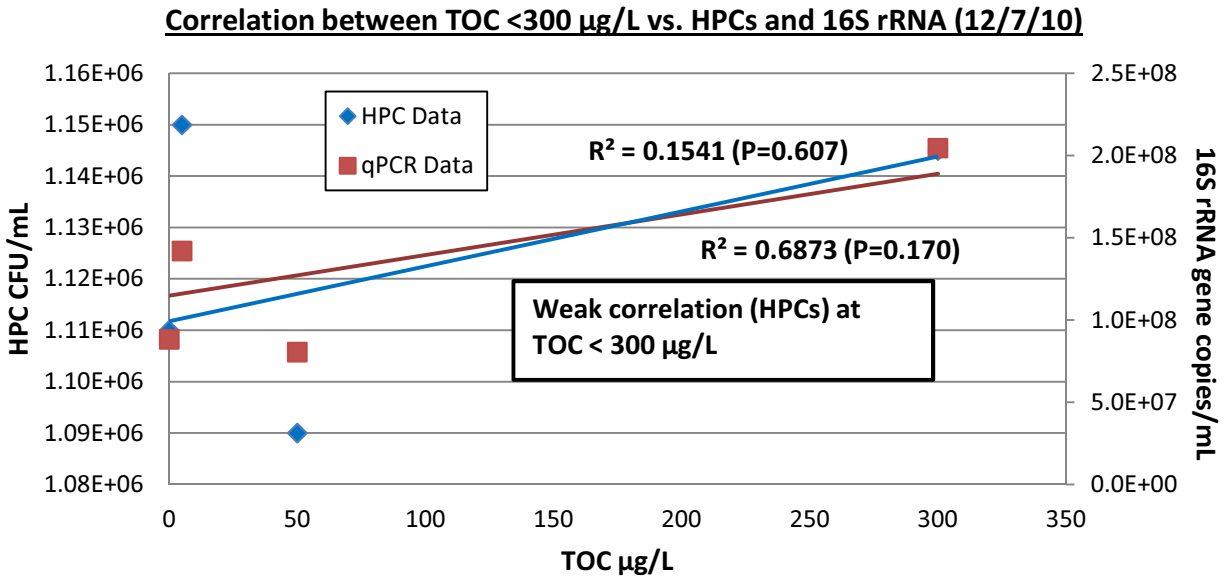

Figure 4: Weak or No Correlation for HPC or 16S rRNA at low TOC values (12/7/10, Experiment A2).

Correlations were commonly observed at higher levels of added TOC (> 1,000 µg/L) for both 16S rRNA data and HPCs with R^2 values of 0.995 and 0.869, respectively (Figure 5). Correlations at this level of TOC ranged from R^2=0.702-0.998.

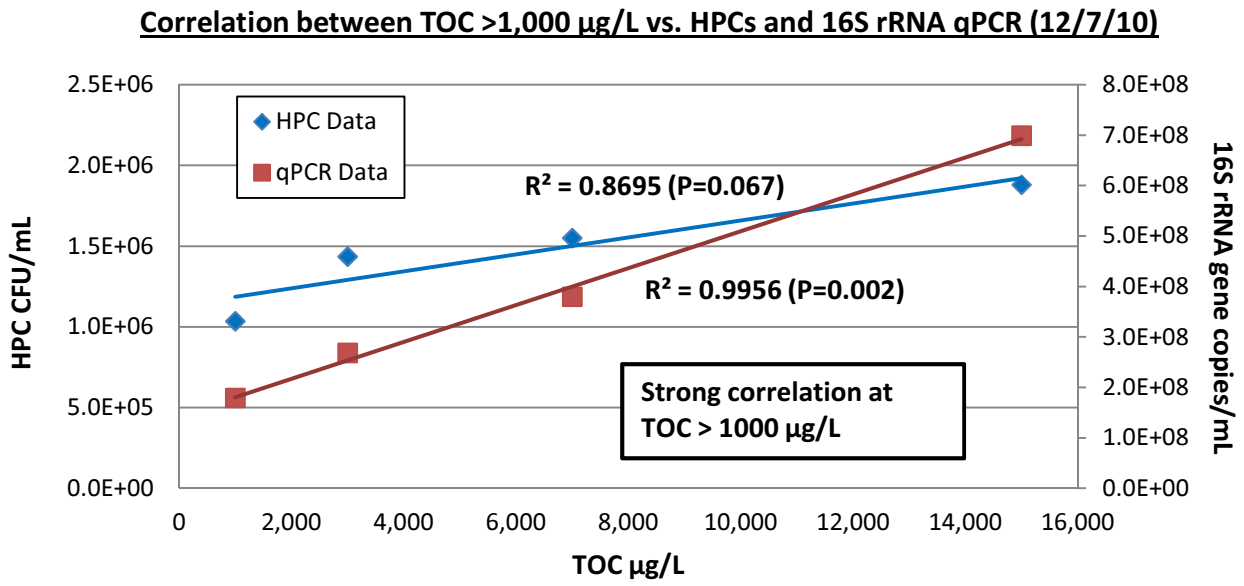

Figure 5: Representative Strong Correlation of HPC and 16S rRNA at TOC concentrations >1,000 µg/L (12/7/10, Experiment A2).

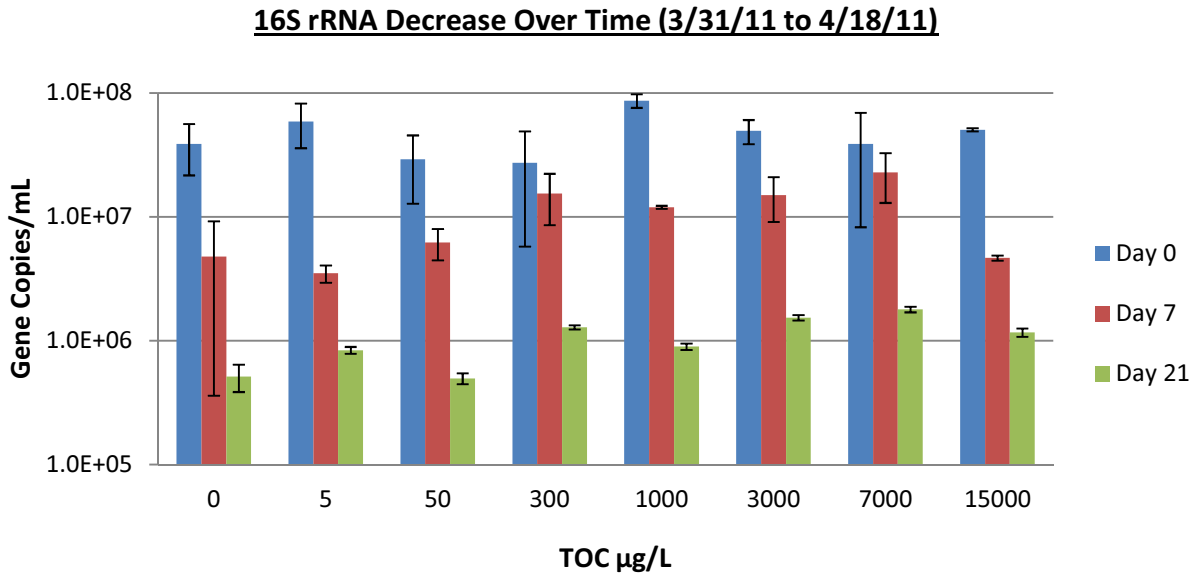

Figure 6: 16S rRNA gene analysis from 3/31/11 to 4/18/11 (Day 0 to Day 21) after stagnation (Experiment A3); initial quantification revealed no correlation between TOC (or AOC) and the total microbial biomass. Subsequent

analysis revealed a weak positive correlations for TOC concentrations >1,000 µg/L. Error bars denote the 95% confidence interval.

M. avium

M. avium demonstrated a positive correlation (R^2 value of 0.949, P=4.2E-05) with TOC (0-15,000 µg/L) based upon initial qPCR measurements (Figure 7)**.** Thus, it appears that *M. avium* growth is also positively correlated with the other heterotrophic organisms and general bacterial biomass, and that *M. avium* was not outcompeted at higher levels of added TOC as might be predicted (Torvinen et al., 2007).

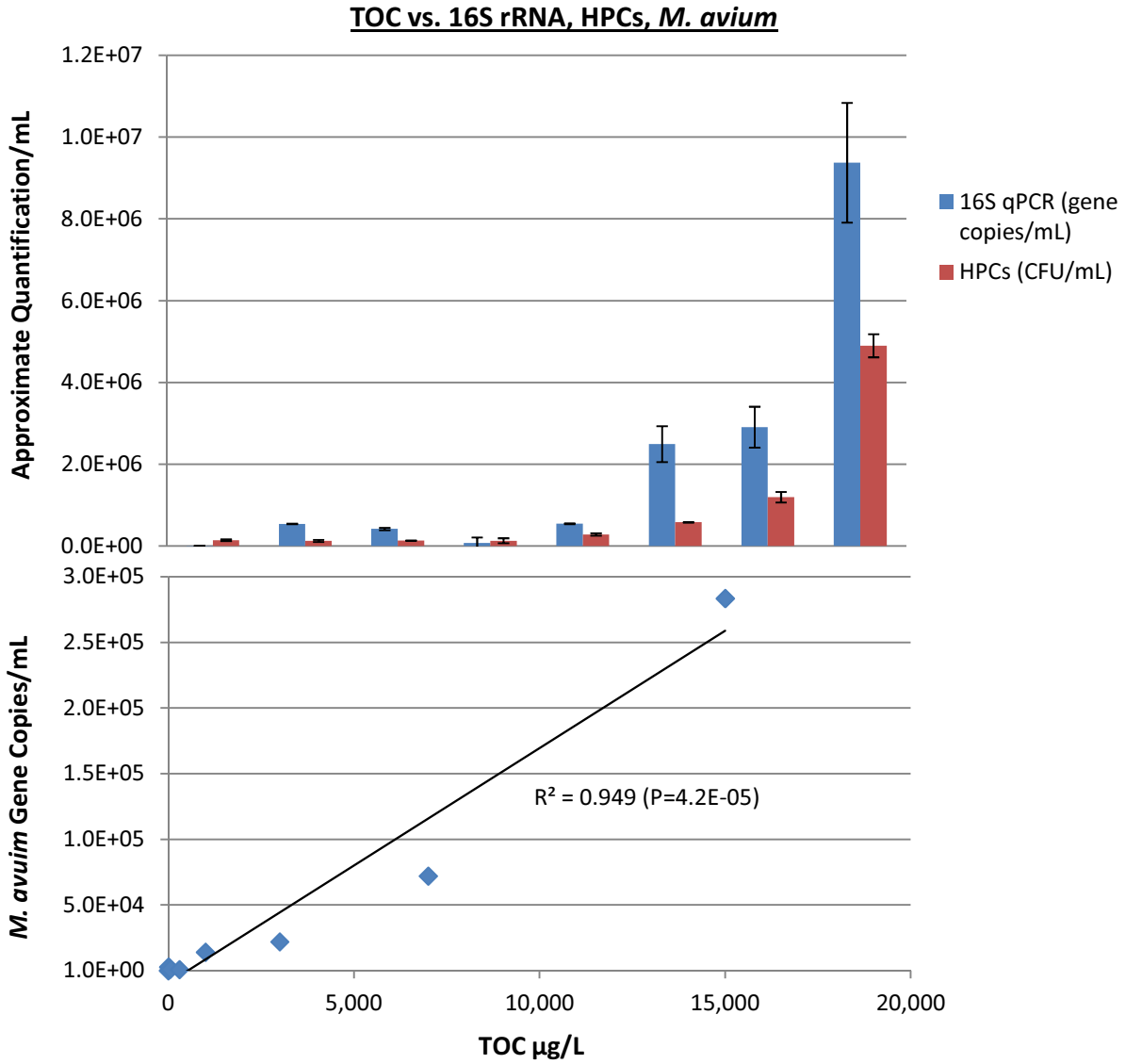

Figure 7: *M. avium* quantifications from culturing and qPCR initially strongly correlated with added TOC (Experiment A1).

As the experiment progressed, changes were made to the source water and initial stagnation periods. *M. avium* in the SGWH effluent revealed significant correlations at lower levels of TOC but inconsistently over time (Data 10/13/10 to 3/7/11; Table 14, Experiment A2). If higher levels of added TOC were included, correlations disappeared because *M. avium* abundance diminished at higher TOC concentrations. This data is consistent with the expectation that *M. avium* would be outcompeted by other heterotrophic bacteria. Overall, there was not a consistent overall correlation of *M. avium* with low (5-1,000 µg/L) or high (>1,000 µg/L) TOC ranges (Table 14, Figure 8) throughout the study.

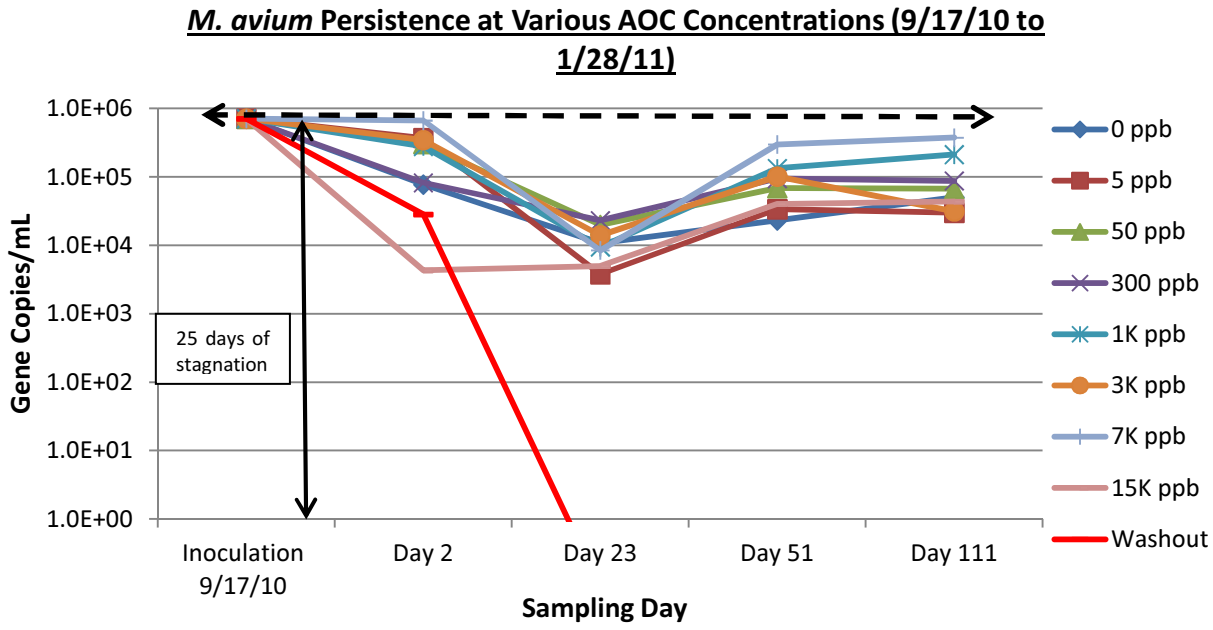

Figure 8: *M. avium* was present in SGWH effluent at levels above washout (Experiment A2).

Table 14: Correlation of TOC vs. *M. avium* (Experiment A2)

	M. avium **(R^2 value) qPCR Low Range TOC (0-1,000 µg/L)**	***M. avium*** **(R^2 value) Culture Data Low Range TOC (0-1,000µg/L)**
10/13/2010 (Day 2)	0.007 (P=0.890)	0.007 (P=0.890)
11/3/2010 (Day 23)	0.003 (P=0.929)	0.004 (P=0.929)
12/7/2010 (Day 51)	0.819 (P=0.034)	0.818 (P=0.035)
1/28/2011 (Day 111)	0.974 (P= 0.001)	N/A

L. pneumophila

At no point during the experiment was *L. pneumophila* ever correlated, either positively or negatively, with increasing TOC. *Legionella was* measured from all SGWH with qPCR a total of 10 times, and the R^2 vs. added TOC ranged from -0.567 to 0.231 with a median R^2 of 0.174. Culturing of *L. pneumophila* was also attempted 9 times from each set of SGWH; in no instances were viable cells detected. Clearly, despite the numerous modifications made in the experiment expressly intended to induce amplification of culturable *L. pneumophila*, AOC was never observed to be a driving factor in *L. pneumophila* proliferation.

The qPCR data was scrutinized using mass balances and a washout model to determine whether any significant amplification or persistence occurred in any of the SGWH at any point of the experiments. In a typical experiment, the concentrations (> 1.0 x 10^5 gene copies/mL) of *L. pneumophila* initially detected decreased by several orders of magnitude, as DNA was washed out from the SGWH via the 80% water changes (Figure 9, Experiment A2). A washout model that assumes 20% of the original DNA remains in the SGWH after each water change is plotted for comparison. However, it is acknowledged that the washout model does not account for the capture of DNA in biofilms and later release to water. Nonetheless, there was little evidence of *L. pneumophila* amplification during most trials of the experiment.

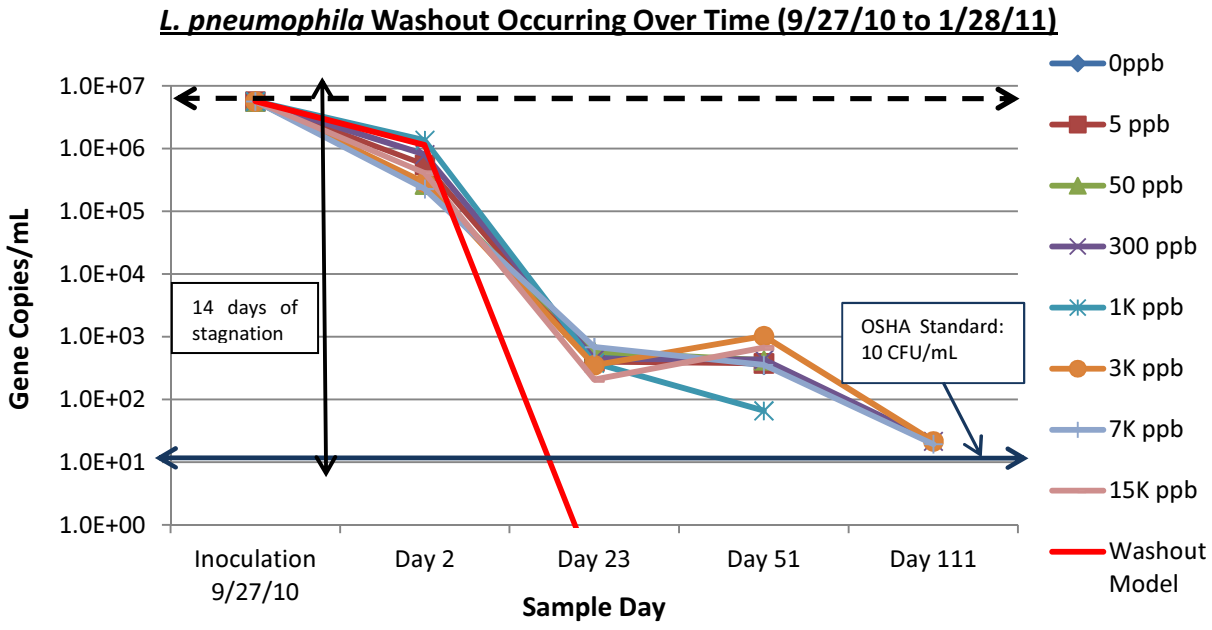

Figure 9: *L. pneumophila* did not amplify in the SGWH conditions as evidenced by qPCR (Experiment A2). The dotted line denotes the initial inoculum concentration added to the SGWH. The washout model assumes that 20% of original inoculum value remains after a single water change and that all cells are in the planktonic state.

The exception was a period following inoculation (10 days stagnation) after changing conditions to include greater surface area from 1 mm glass beads, addition of amino acids, reduced pH, and reduced dissolved oxygen levels (Experiment A3). Before beginning formal water changes, a stagnation period of approximately 2 weeks was imposed. Subsequently, 80% water changes were performed only on a weekly basis. Sampling from this experiment revealed significant initial increases in *L. pneumophila* concentration (Figure 10) for a two week period (> 95% confidence); interestingly, no culturable cells were detected. Following the first two weeks of sampling, there was a rapid decrease in the abundance of *L. pneumophila* that closely followed washout predictions (Figure 10, Figure 11). In examining the SGWH with significant *L. pneumophila* amplification, there was no correspondence with initial TOC (or AOC), as samples with the most significant amplification contained moderate levels of added TOC (1,000 and 7,000 µg/L). During this same time period and as discussed previously, no correlations were obtained for 16S rRNA and HPC values with R^2 values of 0.053 and 0.001 respectively.

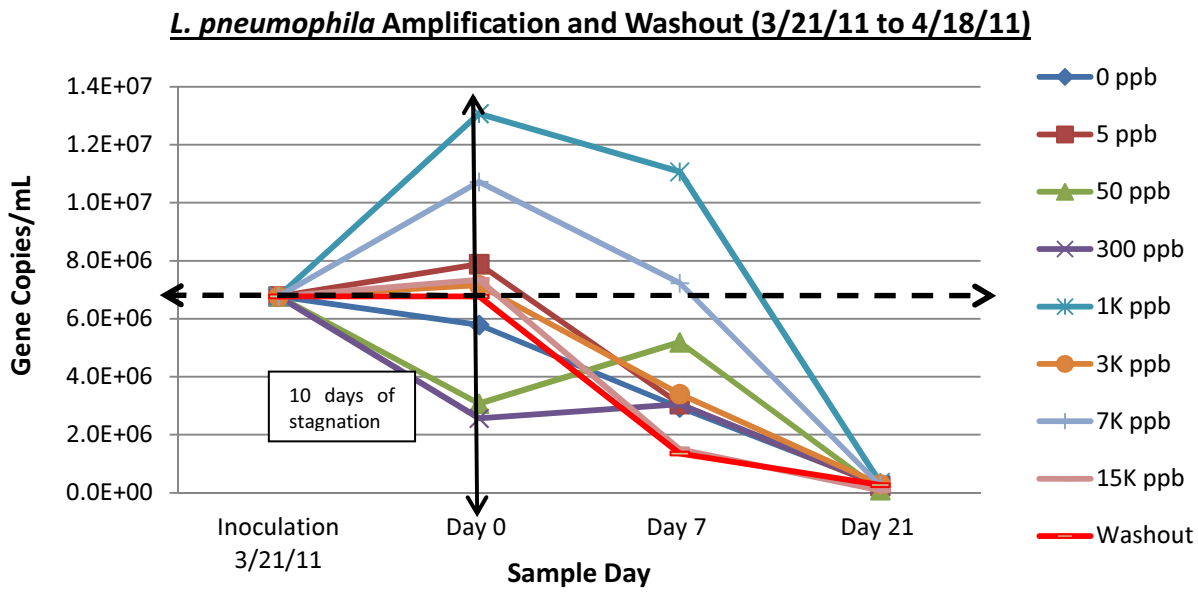

Figure 10: *L. pneumophila* was reinoculated into all SGWH on 3/21/11 (Experiment A3). The dotted line denotes the initial inoculum concentration added to the SGWH. Initial qPCR measurements indicate short term amplification in the first two weeks of sampling (See 1K µg/L and 7K µg/L lines).

Although the exact factors that led to the amplification of *L. pneumophila* were not identified, it is speculated that a biodegradable growth source from dead cellular biomass may have played a role. This

theory stems from the comparison of 16S rRNA gene analysis results to those from *L. pneumophila* (Figure 11). The greatest amount of *Legionella* amplification occurred from a SGWH sample with 1K added TOC, for which *L. pneumophila* increased 6.0 x 10^6 gene copies/mL compared to the inoculum value. This particular sample also possessed the greatest initial concentration of quantified 16S rRNA with a value of 8.6 x 10^7 gene copies/mL (Experiment A3). Considering the SGWH had been stagnant for at least a two week period prior to beginning formal 80% water changes, it is possible that the high concentrations of 16S rRNA was present as dead cellular biomass. A recent study by Temmerman et al., 2006 found that *L. pneumophila* increased 1.57 ± 0.32 log units through necrotrophic growth. The quantifiable values attained for amplification in this experiment were about 0.5 log units given the calculated inoculation value.

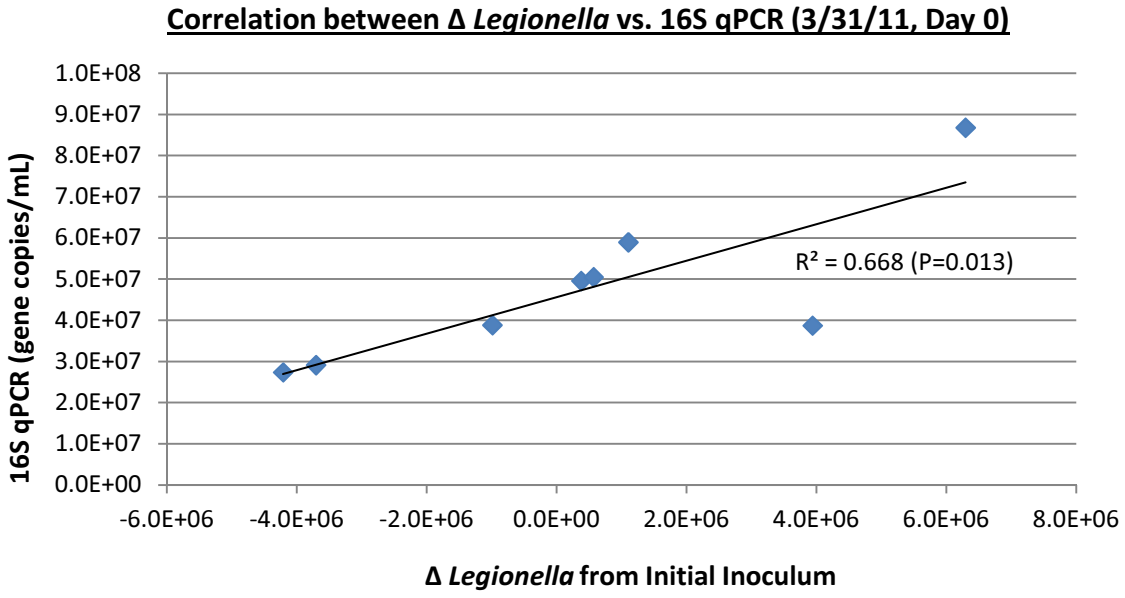

Figure 11: Correlation of Δ *Legionella* (inoculum value ± quantified *L. pneumophila*) vs. 16S rRNA gene analysis data from 3/31/11 (Day 0, Experiment A3). Positive correlation observed possibly due to significant stagnation and necrotrophic growth potentials. *Legionella* amplification from this experiment did not exceed 0.5 log units. During this same study period, R^2 values analyzing TOC vs. 16S rRNA and HPCs were very weak (average R^2 values of 0.168 and 0.295 respectively).

Table 15: Comparison of *L. pneumophila* qPCR data and 16S rRNA gene analysis on 3/31/11 (Experiment A3)

Sample Name	*Legionella* qPCR Quantification Day 0	16S rRNA gene analysis Quantification Day 0	% of *Legionella* from 16S rRNA Quantification
0 µg/L	5.79E+06	3.88E+07	14.9
5 µg/L	7.88E+06	5.90E+07	13.4
50 µg/L	3.08E+06	2.91E+07	10.6
300 µg/L	2.57E+06	2.73E+07	9.4
1K µg/L	1.31E+07	8.68E+07	15.1
3K µg/L	7.16E+06	4.96E+07	14.4
7K µg/L	1.07E+07	3.87E+07	27.7
15K µg/L	7.35E+06	5.05E+07	14.6

Given that there was only transient success inducing *Legionella* amplification as discussed above, the experiment was repeated (Experiment A4). As before, amplification of *Legionella* above background inoculum densities was evident in the 300, 3K, and 7K µg/L condition (> 95% confidence), followed by washout in later weeks of testing (5/16/11, Day 7) following implementation of regular water changes. Positive culturing results for *Legionella* were also obtained during this experiment A4 following sample pretreatment of 50 °C for 30 minutes. Results indicated colony formation from 0 and 7,000 µg/L samples with average results of 30 and 175 CFU/mL respectively. Correlations between the increase in *Legionella* vs. 16S rRNA gene analysis (from 5/9/11, Day 0) produced only an R^2 value of 0.256, and there were no apparent overall correlations between TOC (or AOC) values and overall *L. pneumophila* concentration (-0.081 and -0.562).

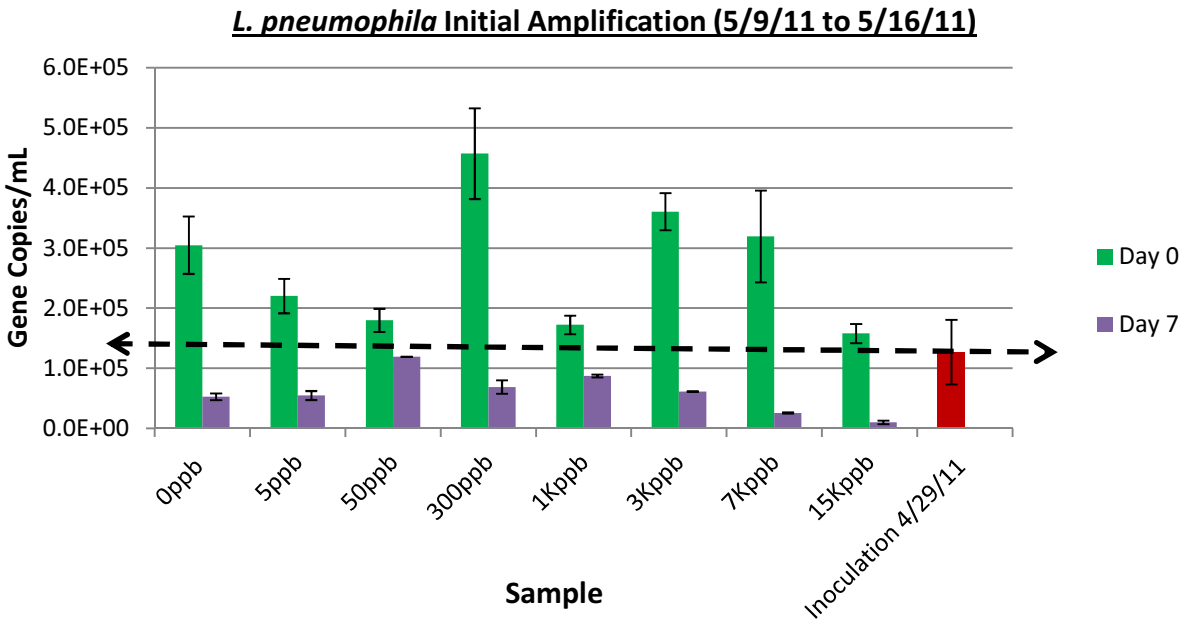

Figure 12: *L. pneumophila* initial growth after inoculation on 4/29/11 from qPCR analysis (Experiment A4). Initial analysis reveals significant amplification in pathogenic bacteria concentrations in the 300, 3K, and 7K SGWH samples above the 95% confidence interval on the inoculum. There is no apparent correlation between TOC (or AOC) and *L. pneumophila* concentrations. Error bars represent the 95% confidence interval. The dotted line denotes the initial inoculum concentration added to the SGWH.

Overall, the specific factors that would cause large scale *L. pneumophila* amplification in simulated water heaters remain elusive. There was clearly a trend towards slight amplification of *L. pneumophila* during stagnation events exceeding 10 days with added amino acids and micronutrients. Given that there are a variety of factors (e.g. temperature, pH, predator-prey relationships, necrotrophic growth, and microbial inhibition) potentially involved in amplification, the lack of clear correlations between added organic matter and *L. pneumophila* concentrations is not unreasonable. Indeed as *L. pneumophila* cannot directly use AOC constituents present in fulvic acids as a carbon source, any relationship between the two would be expected to be indirect. This concept is further illustrated by AOC correlation analysis between AOC and *L. pneumophila* for which consistent correlations are not observed (Table 16).

Table 16: Correlations of AOC vs. *L. pneumophila* (1K-15K samples, Experiments A3, A4)

Sample Date	*L. pneumophila*
3/31/2011 (Day 0, A3)	0.235 (P=0.514)
4/7/2011 (Day 7, A3)	-0.434 (P=0.340)
4/18/2011 (Day 21, A3)	-0.938 (P=0.031)
5/9/2011 (Day 0, A4)	0.138 (P=0.627)

5/19/2011 (Day 7, A4)	-0.873 (P=0.340)

A. polyphaga and *H. vermiformis*

A. polyphaga was a model protozoan inoculated and analyzed throughout the investigation. There was no consistent correlation between low range TOC values (0-1,000 µg/L) with this protozoan over time (Figure 13, Experiment A2). While there was never an increase in concentration above the inoculated value, there was evidence of persistence above washout predictions. Although significant detection was obtained via qPCR, microscopy revealed only encysted cells at the start of the experiment: during inoculation (9/17/10) and Day 2 (10/13/10). Overall, during the study, there was no definitive evidence of protozoan regrowth. There were a few times when *A. polyphaga* was correlated with lower levels of added organic matter (Table 17) but significant correlations were not observed when the entire range of added TOC was considered.

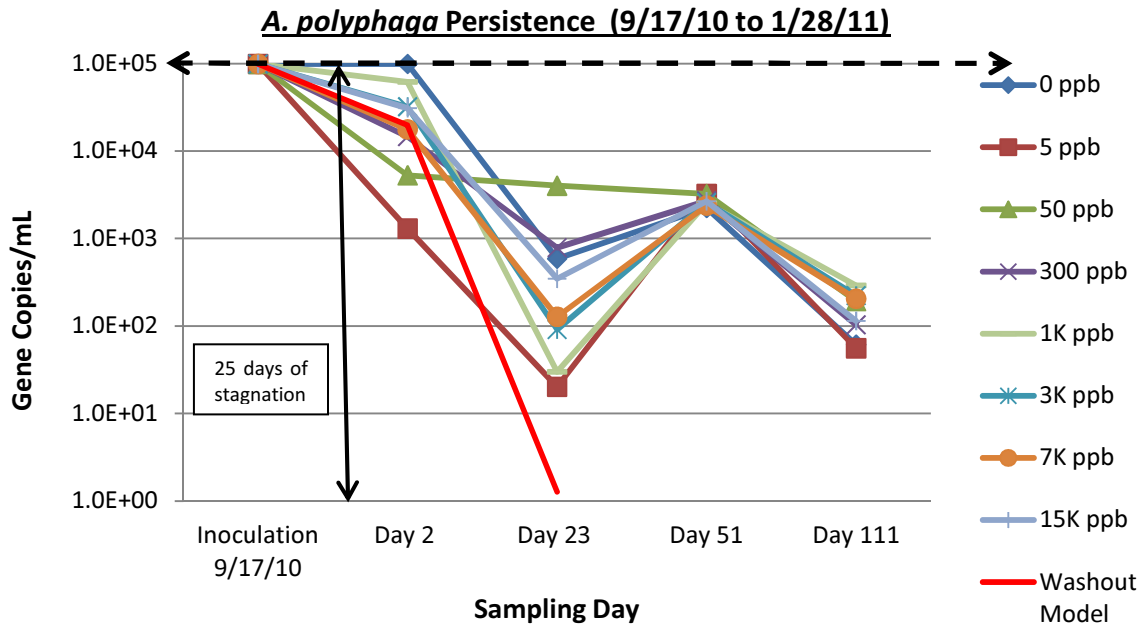

Figure 13: *A. polyphaga* persistence above washout from qPCR quantification (Experiment A2).

Table 17: Correlation of TOC vs. *A. polyphaga* qPCR Results (Experiment A2).

	***A. polyphaga* (R^2 value) Low Range TOC (0-1,000 µg/L)**
10/13/2010 (Day 2)	0.991 (P= 0.004)
11/3/2010 (Day 23)	-0.125 (P=0.559)
12/7/2010 (Day 51)	-0.118 (P=0.569)
1/28/2011 (Day 111)	0.683 (P=0.084)

H. vermiformis had colonized the SGWH at low levels and in the latter experiments in which GAC water was incorporated; it was inoculated along with *Legionella* to promote potential amplification. *H. vermiformis* amplified markedly, with a peak concentration about 1 log unit higher than inoculated levels about two weeks after inoculation in the condition with 0 mg/L added TOC (Experiment A3). If anything, the higher levels of added TOC tended to be amongst the SGWH with the lower levels of *H. vermiformis* amplification. At no point was *H. vermiformis* correlated with added organic matter (linearized plots of qPCR vs. added TOC resulted in R^2 values of -0.175, -0.462, -0.368 for sample dates 3/31/11 (Day 0), 4/4/11 (Day 7), and 4/18/11 (Day 21) respectively). During another part of the experiment R^2 values ranged from -0.991 to 0.018 (Table 18). Thus, transient correlations were apparent during the time period of this investigation.

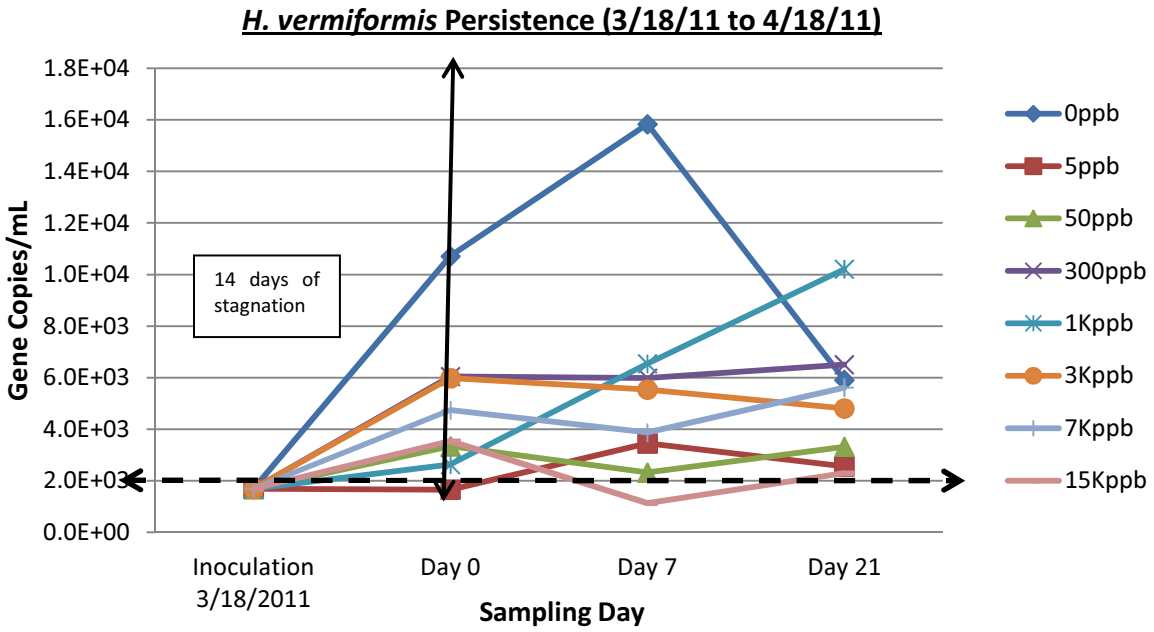

Figure 14: *H. vermiformis* persistence (from qPCR) in all SGWH samples over a period from 3/18/11 to 4/18/11 (Experiment A3). There is no correlation between the concentration of this organism and initial TOC (or AOC) concentrations within SGWH.

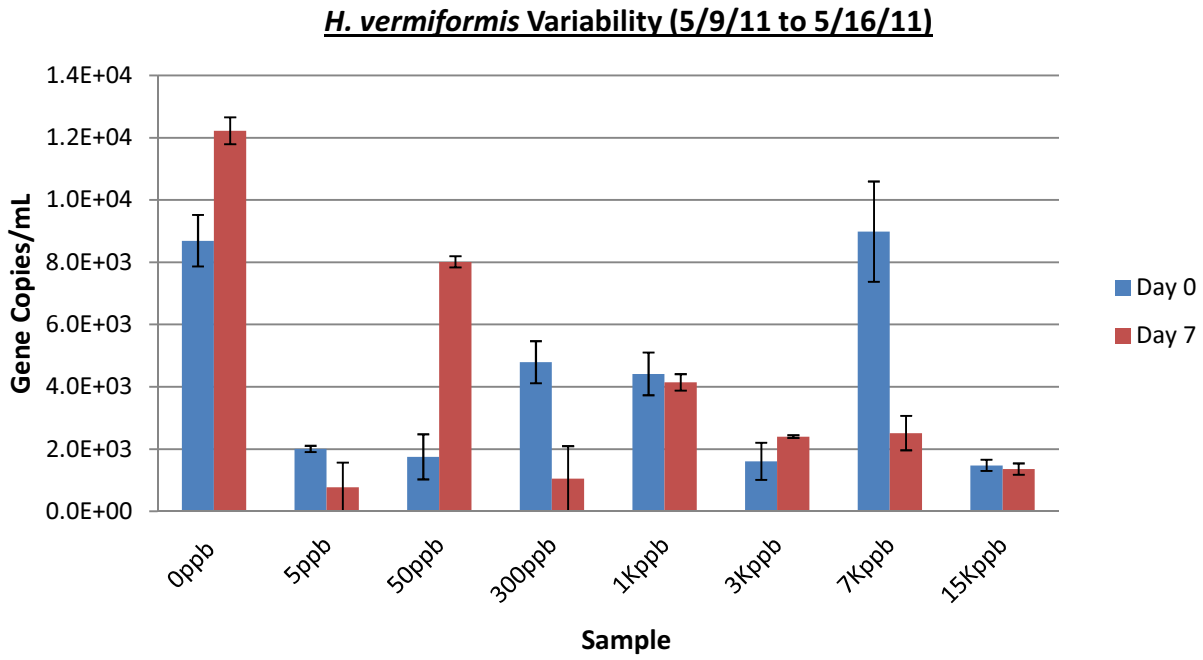

Figure 15: *H. vermiformis* variability in SGWH series (Experiment A4). No discernable correlation between TOC (or AOC) and *H. vermiformis* growth patterns. Data does not appear to have correlations to initial *L. pneumophila* amplification in Figure 12. Error bars represent the 95% confidence interval.

Table 18: Correlations of AOC vs. *H. vermiformis* (1K-15K samples)

Sample Date	*H. vermiformis*
3/31/2011 (Day 0, A3)	0.018 (P=0.862)
4/7/2011 (Day 7, A3)	-0.991 (P=0.004)
4/18/2011 (Day 21, A3)	-0.627 (P=0.207)
5/9/2011 (Day 0, A4)	-0.011 (P=0.894)
5/16/2011 (Day 7, A4)	-0.693 (P=0.167)

2.5 DISCUSSION & KEY FINDINGS

In situations with regular water changes (3 changes per week), the concentration of total bacteria in water from simulated water heaters (as measured by qPCR and HPC) were often strongly correlated with increasing organic matter concentrations. A general observation was that for infrequent flow regimes (i.e. weekly water changes and longer) and organic matter source, when water changes became less frequent the strength of the correlation between pathogens examined weakened. Moreover, significant correlations were not observed at lower levels of added organic matter (< 300 µg/L). In retrospect neither of these trends is unexpected, given serious problems experienced with rampant microbial re-growth observed for ultrapure water applications (US Filter, 2001; Kayser et al., 1975). These systems are most representative of situations of long stagnation, decreased disinfectant concentrations, and when organic matter and other nutrients are obviously at their practically achievable minimums. Furthermore, given that *L. pneumophila* cannot directly utilize ozonated fulvic acids as a carbon source, any relationship with added TOC (or AOC) would be expected to be indirect.

Results from these studies provide some potential insights to situations that may be encountered typical domestic water heaters. On the one hand, more frequent flow events in a typical building could be expected to strengthen the correlation between organic matter in water and bacterial concentration. However, these events would also tend to increase the concentration of secondary disinfectants in the system which would tend to decrease the total concentrations of bacteria (Zhang et al., 2009). One key finding from this study is that the typical threshold used to indicate problems with regrowth in potable water systems (i.e., HPC counts < 500 CFU/mL) cannot be consistently achieved in domestic warm water distribution systems through control of TOC alone. Even after examining conditions with no added TOC,

HPC counts regularly exceeded 500 CFU/mL in this work and in prior work with ultra-pure water systems (US Filter, 2001; Kayser et al., 1975).

M. avium was sometimes strongly correlated with added organic matter levels, confirming the results of Norton et al., 2004 obtained under continuous flow conditions using the same baseline water tested from this investigation. However, consistent correlations were not observed consistently, perhaps due to the infrequent flow events tested, or because in some circumstances with higher TOC *M. avium* is outcompeted by faster growing heterotrophic bacteria. For pathogens such as *Legionella*, *A. polyphaga* and *H. vermiformis*, no correlations between TOC (or AOC) were observed at any point of the experiment. Indeed, for *Legionella* and *A. polyphaga* there was limited evidence of amplification and when it was observed it was not consistently at either high or low added TOC. The specific factors that cause massive amplification of *Legionella* under representative conditions in potable water remain elusive at this time. Amongst the potential factors that might induce amplification include heat shock treatment, unknown trace micronutrients, variations in overall SGWH temperature, absence of microorganisms that might inhibit the *Legionella* amplification (but not *Mycobacterium*), and other complex predator-prey relationships given the apparent necessity of a Protozoan host. A review of the literature, however, revealed that *Legionella* amplification has never been convincingly obtained in completely synthesized potable water under representative oligotrophic conditions (i.e., lower organic carbon, representative phosphate, and nitrogen levels). This represents a critical step for the identification of key factors influencing *L. pneumophila* growth in representative drinking water conditions from consumer plumbing systems.

REFERENCES

APHA, Method 9215. In Standard Methods for the Examination of Water and Wastewater, 20 ed.; Clesceri, L. S.; A.E., G.; Eaton, A. D., Eds. American Water Works Association and Water Environment Federation: Washington, D.C., 1998.

Camper, A. 2004. Involvement of humic substances in regrowth. Int. J. Food Microbiol, 92:355-364.

Camper, A., Brastrup, K., Sandvig, et al., (2003). Effect of distribution system materials on bacterial regrowth. J. AWWA. **95**(7):107-121.

Clark, R.A., and Clarke, D.R. (2004). Microbiologically Influenced Corrosion in Hydronic Heating Systems. Journal of Failure Analysis and Prevention. 4:4 38-42

Edwards, M., Falkinham, J., Pruden, A., Lieberman, R. (2009). Relationship between Biodegradable Organic Matter and Pathogen Concentrations in Premise Plumbing. Final AWWARF Research Proposal RFP: 4251.

Hoffman, P. S, H. Friedman, M Bendinelli (2008). *Legionella pneumophila*: Pathogenesis and Immunity. New York: Springer Science & Business Media, LLC

Nazanian, E.J., et al.,(2008)." Design and implementation of a protocol for the detection of *Legionella* in clinical and environmental samples." Diagnostic Microbiology and Infectious Disease, **62**: 125-132

Kayser, W.V., K.C. Hickman, W.W. Bond, et al., (1975). "Bacteriological Evaluation of an Ultra-Pure Water-Distilling System." Applied Microbiology, **30**(4): 704-706

Kool, J. L., J. C. Carpenter, et al., (1999a). "Effect of mono disinfection of municipal drinking water on risk of nosocomial Legionnaires' disease." Lancet **353**(9149): 272-277.

Kuiper MW, et al. (2006) "Quantitative detection of the free-living Amoeba *Hartmannella Vermiformis* in surface water by using real-time PCR." Applied and Environmental Microbiology, **72**(9): 5750-5756.

Kulakov LA, MB McAlister, KL Ogden, MJ Larkin and JF O'Hanlon.(2002) "Analysis of bacteria contaminating ultrapure water in industrial systems." Applied and Environmental Microbiology, **68**: 1548– 1555

LeChevalier, M.W,; Schulz, W., Lee, R.G. (1991). Bacterial nutrients in drinking water. Applied and Environmental Microbiology, **57** 857.

Mathys, W., Stanke, J., Harmuth, M., Junge-Mathys, E. 2008. Occurrence of *Legionella* in hot water systems of single-family residences in suburbs of two German cities with special reference to solar and district heating. International Journal of Hygiene and Environmental Health, 211:179-185.

McAlister MB, LA Kulkaov, MJ Larkin and KL Ogden.(2001) "Analysis of bacterial contamination in different sections of a high-purity water system."Ultrapure Water, **18**: 18–26

Moore, M.R., Pryor, M., Fields, B., Lucas, C., Phelan, M., and Besser, R.E. Centers for Disease Control and Prevention (CDC). 2006. Introduction of monochloramine into a municipal water system: impact on colonization of buildings by *Legionella* spp. Appl. Environ. Microbiol, **72**(1):378-383.

Norton, C.D., LeChevallier, M.W. and Falkinham, J.O. III.(2004). Survival of *Mycobacterium avium* in a model distribution system. Water Res, **38**:1457-1466.

Oliver, J.D. (2009). "Recent findings on the viable but nonculturable state in pathogenic bacteria." FEMS Microbiology Reviews. **34**: 415-425

Riviere, D. et al., (2006) "Development of a real-time PCR assay for quantification of *Acanthamoeba* trophozoites and cysts." Journal of microbiological methods, **64**: 78-83

Servais, P., Billen, G., and Hascoet, M.C. 1987. Determination of the biodegradable fraction of dissolved organic matter in waters. Water Res, **21**:445-450.

Smith, C., D. Nedwell, et al., (2006) "Evaluation of quantitative polymerase chain reaction-based approaches for determining gene copy and gene transcript numbers in environmental samples" Environmental Microbiology, **8**(5): 804-815

Steinert, M., Emody, L., Amann, R., and Hacker, J. (1997) "Resuscitation of viable but nonculturable *Legionella* pneu-mophila Philadelphia JR32 by *Acanthamoeba* castellanii". Applied and Environmental Microbiology, **63**: 2047–2053.

Strickhouser, A. 2006. *Legionella pneumophila* in domestic hot water systems: evaluation of detection methods and environmental factors affecting survival. Master's thesis: Virginia Tech.

Temmerman, R., H. Vervaeren et al., (2006) "Necrotrophic Growth of *L. pneumophila*" Applied and Environmental Microbiology, **72 (6):** 4323-4328.

Torvinen, E., M. Lehtola, et al., (2007) " Survival of Mycobacterium avium in Drinking Water Biofilms as Affected by Water Flow Velocity, Availability of Phosphorus, and Temperature." Applied and Environmental Microbiology, **73**:6201-6207.

U.S. Filter (2001). "Preventing Microbial Contamination in Analytical Grade Water with Regular Sanitation"

Valster RM, Wullings BA, and van der Kooij D. (2010) Detection of Protozoan Hosts for *Legionella pneumophila* in Engineered Water Systems by Using a Biofilm Batch Test. Applied and Environmental Microbiology, **76**(21): 7144-7153.

van der Kooij, D. 2002 Assimilable organic carbon (AOC) in treated water: determination and significance. In: Encyclopeida of Environmental Microbiology, Bitton, G. (Ed.), John Wiley & Sons, Hoboken, NJ, USA pp. 312-327.

van der Kooij, D. (1992). “Assimilable organic carbon as indicator of bacterial regrowth”. Journal of the American Water Works Association, **84**:57.

Weinrich, L. A., E. Giraldo, et al., (2009). ”Development and Application of a Bioluminescence-Based Test for Assimilable Organic Carbon in Reclaimed Waters.” Applied and Environmental Microbiology, **75**(23): 7385-7390

Wilton, S. and Cousins, D. (1992)” Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube”. Genome Research, **1**:269-273

CHAPTER 2: APPENDIX A

qPCR Inhibition Studies

After conducting QA/QC on q-PCR detection in these investigations, it was discovered that qPCR inhibition was sometimes preventing detection of *L. pneumophila* in undiluted samples. In general, use of a 1:5 dilution was found to eliminate inhibition in 16S rRNA gene analysis (Figure 16), and further dilution did not produce improved recovery of spiked DNA but decreased the detection limit.

Supplemental experiments were directed at identifying the specific factors present in the SGWH that may have contributed to inhibition in the SGWH. For the analysis waters containing a range of candidate inhibitors were spiked with *L. pneumophila* template DNA and recovery was quantified (Figure 17). The red line in Figure 17 represents 100% detection of *Legionella* and the green bars represent the percentage of *L. pneumophila* actually recovered. Inhibition can result in the presence of high levels of dissolved iron, magnesium, copper, aluminum, and NOM. On the other hand, a concentrate of particulates from the GAC filter (200 mL filtered and re-suspended) meant to simulate high levels of background biomass did not cause problems with inhibition. In general, ICP-MS results from SGWH with plumbing deficiencies, and therefore higher levels of iron and magnesium (Chapter 3), contained a maximum of 10 mg/L of magnesium and 3 mg/L of iron in the effluent, and iron and magnesium were not present at elevated concentrations for in any of the samples discussed in Chapter 2. Hence, while it is clear that inhibition poses considerable problems for qPCR of *L. pneumophila* in this particular potable water, and elevated iron, aluminum, and NOM can induce inhibition, the specific constituents causing inhibition could not be identified with certainty (Chapter 3).

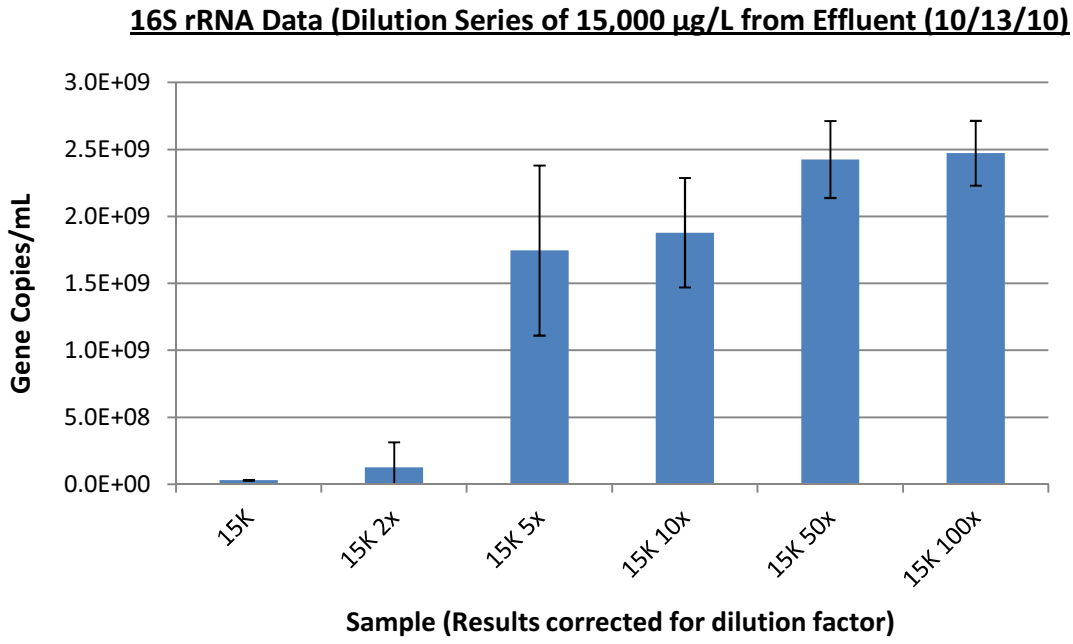

Figure 16: Gene copies in the original sample quantified after 0, 2X, 5X, 10X, 50X and 100X dilutions (Experiment A2). At 0 dilution inhibition eliminated recovery of at least 98% of the *Legionella* DNA, whereas 5X and higher dilutions consistent recovery using 95% confidence intervals. Similar results were obtained for spike and recovery of DNA.

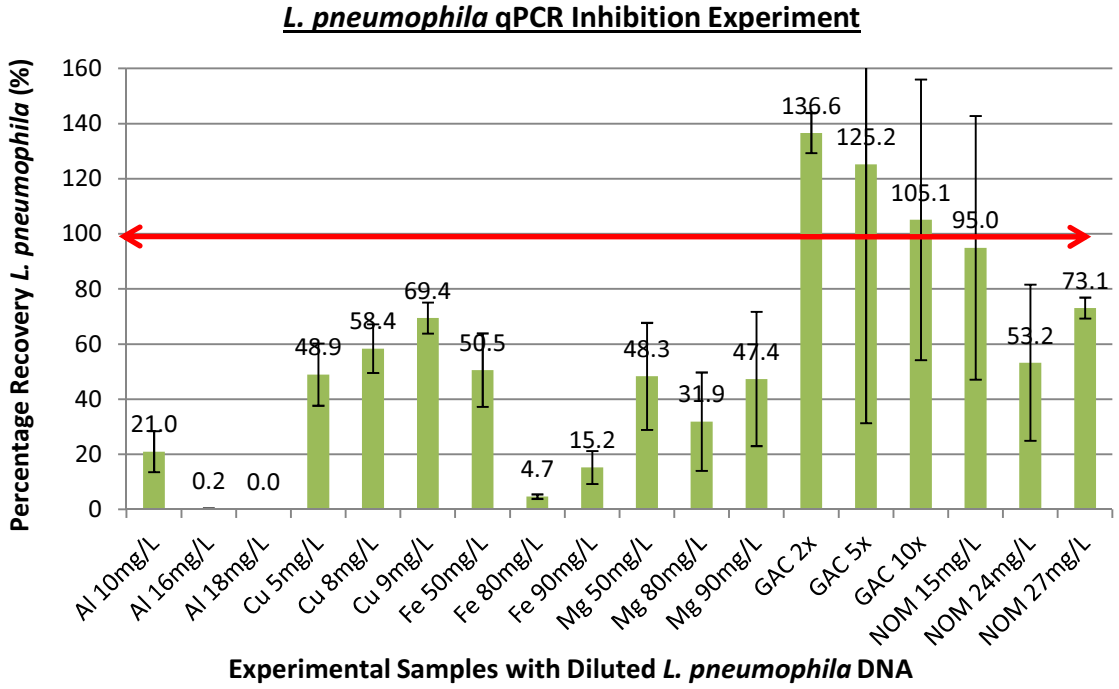

Figure 17: Percentage recovery of *L. pneumophila* in the presence of constituents with potential inhibitory effects (Constituent concentrations listed in (Table 19). If no inhibition had occurred recovery is expected to be 100%. In samples with GAC particulate, recovery is believed to be above 100% due to presence of a small amount of *Legionella* DNA present within the source water.

Table 19: Stock solution concentrations utilized in *L. pneumophila* inhibition experiment

Initial Stock Solution Concentration	Principal Constituent	2x Dilution Concentration	5x Dilution Concentration	10x Dilution Concentration
20mg/L	as Al	10mg/L	16mg/L	18mg/L
100mg/L	as Mg	50mg/L	80mg/L	90mg/L
100mg/L	as Fe	50mg/L	80mg/L	90mg/L
10mg/L	as Cu	5mg/L	8mg/L	9mg/L
30mg/L	NOM Stock	15mg/L	24mg/L	27mg/L

Table 20: Stock amino acids added to baseline water source (Experiment A3)

Amino Acid	Concentration µg/L (µg/L)
L-Arginine	5.55
L-Aspartic Acid	8.08
L-Cystine	2.53
L-Cysteine	6.34
Glycine	2.85
L-Glutamic Acid	9.51
L-Histidine	2.38
L-Isoleucine	7.45
L-Leucine	10.14
L-Lysing	10.3
L-Methionine	3.17
L-Phenylalanine	5.55
L-Proline	1.82
L-Serine	10.3
L-Threonine	5.23
L-Tryptophan	1.58
L-Tyrosine	6.34
L-Valine	7.6
L-Rhamnose	25.35

CHAPTER 2: APPENDIX B

***L. pneumophila* Culturing Studies**

To understand why *Legionella pneumophila* recovery from culturing methods proved inadequate within this study, an experiment was devised to measure *L. pneumophila* survival in SGWH water conditions (Lead by Dr. Falkinham and Myra Williams). The study utilized the following conditions:

- A suspension of *Legionella pneumophila* was prepared in buffered-saline-gelatin (BSG) and adjusted to equal the turbidity of a No. 1 McFarland standard;
- The number of colony-forming units (CFU)/mL suspension was 2.0 x 10^8/mL;
    - suspension was then serially diluted in 10-fold increments in either BSG or simulated SGWH water
- 0.1 mL of the resulting dilution series was spread on BCYE plates in triplicate. The plates were prepared at the same time and in the same day to avoid variation.

Results from this investigation reveal at least an order of magnitude difference in comparison of BSG dilutions with dilutions made from the Norton et al., 2004 tap water (Table 21). Thus, the recipe of Norton et al., tap water, as constituted in this research, rapidly acted to render inoculated *L. pneumophila* non-viable. The suspension of *Legionella* cells in a buffered media solution leads to a time dependent loss in culturability (although cells may possibly be in a viable form).

Table 21*: L. pneumophila* suspension with BSG and Norton et al., 2004 Experiment

Suspension Dilution	BSG-dilution (CFU/mL)			Norton et al., 2004-dilution (CFU/mL)		
1,000x	TMTC	TMTC	TMTC	TMTC	TMTC	TMTC
10,000x	TMTC	TMTC	TMTC	245	170	175
100,000x	226	208	132	21	33	31
1,000,000x	26	23	28	3	4	10

***TMTC indicates that >300 colony forming units were present on the plate and not statistically viable counts**

CHAPTER 2: APPENDIX C

Biofilm Investigations

Biofilm swabs were taken from a 30 cm^2 area on the glass wall of representative SGWH, extracted, and analyzed utilizing qPCR (Experiment A1). Results of this study indicated that *L. pneumophila* in SGWH biofilms was not statistically different from zero utilizing a 95% confidence interval (Figure 18); sample analysis also revealed similar findings for *A. polyphaga*. Data from 16S qPCR was conducive to what was expected as there was a positive correlation (R^2 value= 0.879) in examining added TOC vs. 16S rRNA genes quantified. *M. avium* results from this analysis suggested the optimal TOC concentration for growth potential may be around 7,000µg/L; however, given a 95% confidence interval the quantification value is not significantly different from zero (Figure 18). However, inhibition in sample analysis may have significantly affected the quantified values as it had not been ascertained during this experimental analysis (See Chapter 2: Appendix A, Chapter 3: Appendix A). Due to the error and concerns over qPCR inhibition (± 100%), biofilms findings should be approached with caution.

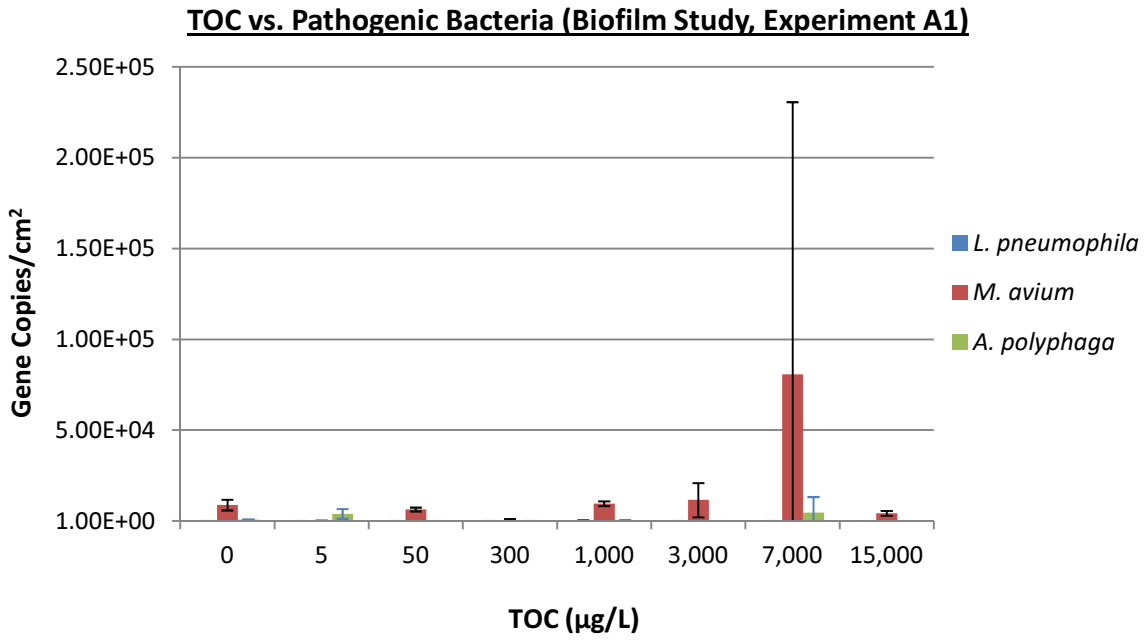

Figure 18: Biofilm data from the second experimental iteration. Results suggest samples of *L. pneumophila* and *A. polyphaga* are not statistically different from zero. This data set should be analyzed with caution as inhibition effects were not tested during this experiment (Experiment A1). Error bars delineate the 95% confidence interval.

CHAPTER 3: EFFECTS OF PREMISE PLUMBING DEFICIENCES ON WATER CHEMISTRY AND MICROBIAL GROWTH IN SIMULATED WATER HEATERS

Krista Williams Amy Pruden, Joseph Falkinham, and Marc Edwards

Virginia Tech, 418 Durham Hall; Blacksburg, VA 24060

ABSTRACT

Premise plumbing can markedly influence potable water chemistry by raising ambient pH, lowering dissolved oxygen concentrations, and by increasing Mg/Fe concentrations in water. Such factors may also encourage the growth of biofilms and the presence of other microorganisms. Potential relationships between assimilable organic carbon (AOC) generation mechanisms and pathogenic bacteria (*L. pneumophila*, *M. avium*, and *A. polyphaga*) were examined in conjunction with influential factors in premise plumbing including PEX pipe, iron sediment, magnesium anodes, nitrifying bacteria, and metallic iron. Although there is reasonable expectation that each of these factors could effectively increase overall AOC, premise plumbing conditions were not correlated with high pathogen concentrations with compared to simulated glass water heaters (SGWH) controls. Results from SGWH studies also imply that AOC can be generated within simulated systems containing iron, magnesium, and $Fe(OH)_3$ sediments above a 95% confidence intervals. However, these factors were not correlated with higher pathogen levels when compared to SGWH controls. An exception was *M. avium* at lower TOC conditions during transient time frames over the course of the study. ANOVA testing of mean concentrations on *M. avium* from low TOC deficient SGWH indicated significantly differences across conditions with a calculated p value of 0.010. *L. pneumophila and A. polyphaga* persisted at levels far above washout conditions under the same test conditions, including conditions with iron and manganese present, although no cells were found to be culturable.

3.1 INTRODUCTION

Premise plumbing refers to the portion of the potable water distribution system located within schools, hospitals, businesses, and private housing. Characteristics of plumbing systems such as surface area to volume ratio, stagnation periods, corrosive plumbing materials (e.g. copper, iron, magnesium), and low

disinfectant residuals vary markedly from the main water distribution system and even in comparison of consumer homes. Therefore, it is reasonable to expect that some of these factors may contribute to amplification and regrowth of pathogenic bacteria (Zhang et al., 2009). Even if organic substrates such as AOC and Biodegradable Dissolved Organic Carbon (BDOC) are controlled at the water treatment facility, certain characteristics of premise plumbing systems may undermine this potential nutrient limitation. Specifically, from the perspective of organic constituents, studies have suggested that cross linked polyethylene (PEX) pipes, nitrification, sacrificial magnesium anode rods, iron corrosion, and humic sorption to $Fe(OH)_3$ sediments can all potentially increase bioavailable organic carbon concentrations. Potential methods that generate AOC are discussed in the sections below.

Cross Linked Polyethylene (PEX) Pipes

Modern consumer plumbing systems typically consist of large proportions of PEX, PVC, HDPE, and copper materials. Rogers et al., 1994 demonstrated that high levels of TOC (>10 mg/L) can be leached from new PEX pipe which may have contributed to growth of *L. pneumophila* cells within those water systems. However, another study by van der Kooij et al., 2005 did not demonstrate an increased likelihood of *Legionella* occurrence in PEX plumbing systems. In addition, investigations have also revealed that low weight organic materials can permeate into potable water at levels up to 200 µg/L though PEX pipes under extreme circumstances (Skjevra et al., 2003; Durand et al., 2007; Heim et al., 2007). Still other studies have demonstrated that phosphate and other nutrients can leach from PEX. If potable water usage is lowered by prolonged stagnation as commonly occurs in premise plumbing, the impacts might be much more significant (Zhang et al., 2009).

H_2 Oxidizing Bacteria

In the process of corrosion from iron, aluminum, and magnesium, significant amounts of hydrogen gas may evolve. H_2 is capable of supporting autotrophic hydrogen oxidizing bacteria which, as autotrophs, effectively generate AOC from inorganic carbon on the surfaces of pipelines. Studies from Morton et al., 2005 have suggested that as much as 0.2 mg of organic carbon could be generated from autotrophic growth per mg of H_2 generated. The Morton study also implicated this process occurs in main distribution lines but the evolution was insignificant due to high flows and low overall surface area to volume ratios. Morton et al. predicted that impacts could be dramatically different in consumer premise plumbing systems. Even more extreme effects could potentially arise in typical hot water heater in which an aluminum or magnesium sacrificial anode rod prevents corrosion of the tank. An additional

study reported relatively high concentrations of microbial biomass present on corroding magnesium anode rods (Bagh et al., 2004), consistent with this hypothesis.

Humic Sorption to Iron Rust and Sediments

Humic acids are ubiquitous naturally occurring organic compounds, which can be subject to oxidation and polymerization reactions in water treatment plants and in nature (Edwards et al., 1993; Camper et al., 2004). Recent investigations have revealed that sorption of humic substances to iron oxides may increase overall biodegradability of the organic material (Butterfield et al., 2002a, 2002b). Extrapolating this result to premise plumbing systems, humic substances sorbed to rusts on galvanized iron pipe, iron sediment trapped in a whole house filter, or sediments at the bottom of a hot water heater tank, may increase effective concentration of AOC. Camper et al., 2004 suggested that these increases in overall sorped organic material may support relatively high rates of microbial growth.

Nitrification

Nitrification can occur utilizing naturally occurring ammonia or added ammonia from chloraminated systems as an electron donor. Autotrophic nitrifiers utilize a two step process in which new biomass is generated from ammonia oxidation (Zhang et al., 2009). Zhang et al., 2009 observed an upper bound of 87 µg of organic carbon created per mg NH_3 consumed, and this could be increased if iron or lead was available to reform ammonia from nitrate via corrosion reactions. Moreover, nitrification may be more prevalent in premise plumbing than in the main distribution system due to higher temperature, greater stagnation periods, and low disinfectant residual concentrations.

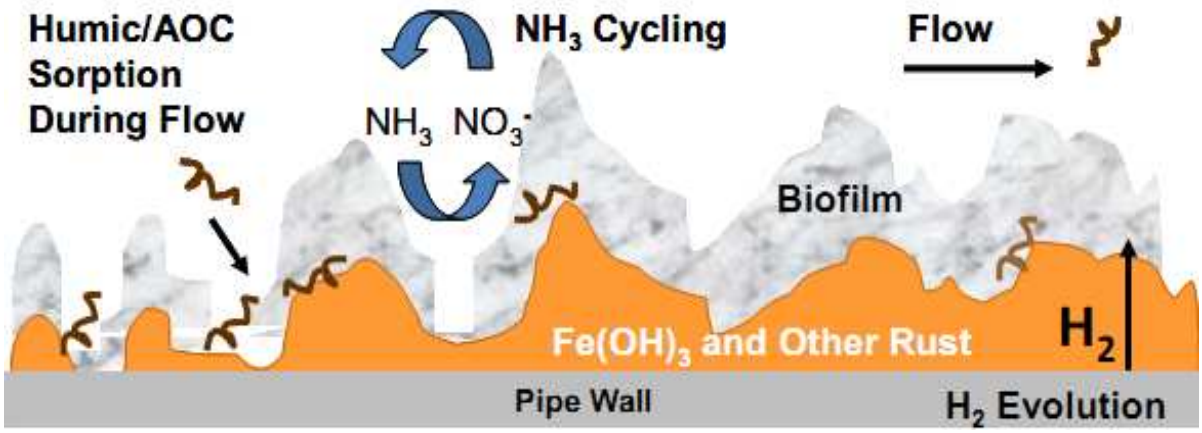

Figure 19: Schematic of potential AOC generation mechanisms occurring in consumer plumbing systems including: PEX pipes, nitrification processes, H_2 evolution from corroding materials, and humic sorption to iron sediments. Figure adapted and modified from Edwards et al., 2009.

AOC Generation Potential in Premise Plumbing

The mechanisms listed above can theoretically undermine any control the utility places on effluent AOC or BDOC leaving the treatment plant (Figure 20). As water enters consumer premise pluming, mechanisms such as PEX pipes, humic sorption, nitrification, and H_2 oxidizing bacteria can all act to increase the overall concentration of AOC.

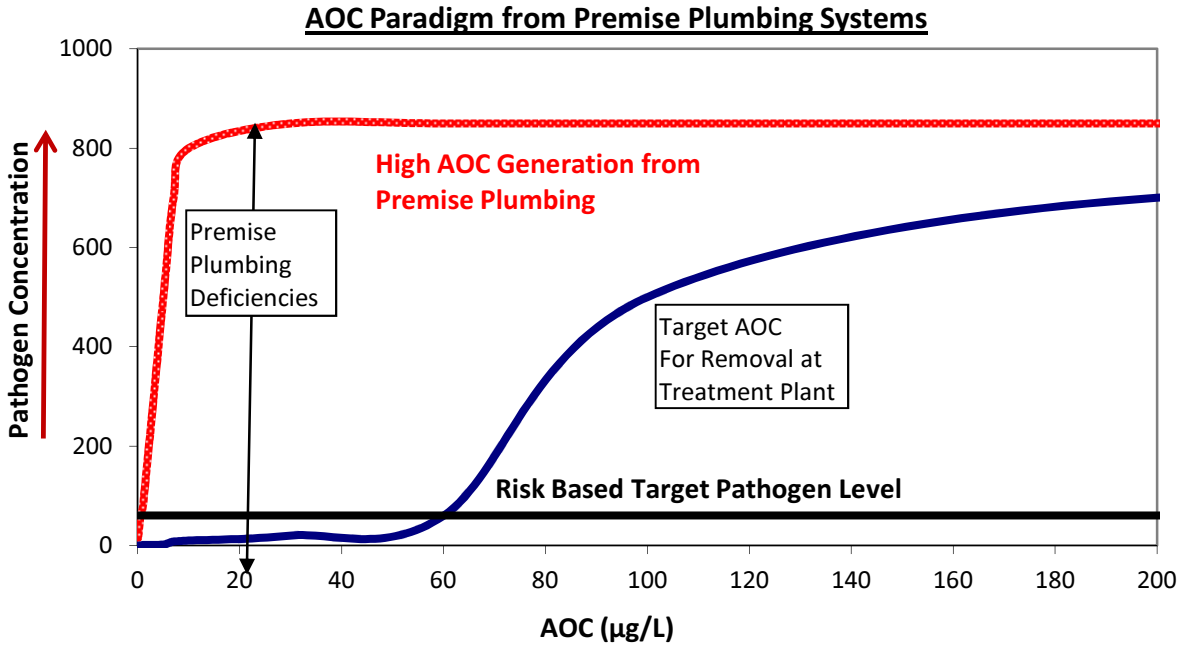

Figure 20: Potential realities of AOC generation in premise plumbing systems. Utility control is potentially undermined by reactions occurring within consumer systems. These reactions have the potential to produce considerable amounts of AOC and increase overall concentrations of pathogenic bacteria. Figure adapted and modified from Edwards et al., 2009.

The goals of this research were to 1) examine the impacts of a range of premise plumbing deficiencies on chemistry of water in simulated water heaters that might influence microbial growth, and 2) determine the relationship between total and pathogenic bacterial growth in different SGWH to examine influential premise plumbing deficiencies.

3.2 PLUMBING DEFICIENCY SGWH SETUP

These experiments were conducted in parallel with those discussed in Chapter 2. Two levels of TOC were tested (5 and 1,000 µg/L) in order to gain insight into potential interactions between AOC and plumbing deficiencies on pathogen proliferation with realistic AOC concentrations. An array of conditions was imposed on the water heaters to simulate unique conditions found in premise plumbing. Each SGWH series was run in triplicate with a total of 36 SGWH (6 SGWH (3 at 1,000 µg/L; 3 at 5 µg/L) x 6 experimental conditions= 36). The following conditions were examined:

- **NB SGWH:** Nitrifying bacteria isolated from potable water were inoculated at the start of the experiment, along with 1 mg/L free NH_3-N provided during each water change to establish nitrification (NH_3-derived AOC).
- **Mg Anode SGWH:** a 1.4 cm Mg rod was mounted to the bottom of SGWH with a surface area to volume ratio of 3.33 cm^{-1} (H_2-derived AOC).
- **$Fe(OH)_3$ SGWH**: Freshly precipitated $Fe(OH)_3$ sediment layer of a 1 mm depth served as an Fe nutrient source and sorbed organic carbon reservoir.
- **IRON SGWH:** A crescent shaped cast iron coupon was mounted to the bottom of the iron SGWH with a surface area to volume ratio of 0.5 cm^{-1} (H_2, Fe nutrient, sorped organic carbon reservoir).
- **PEX SGWH:** 10 cm of a 3/4" diameter PEX pipe was taken and subsequently cut into 2 cm pieces and mounted to the bottom of the SGWH (PEX-derived AOC). The surface area to volume ratio was calculated to be 0.48 cm^{-1} and 4.20 cm^{-1} for the outer and inner pipe diameters respectively. Given an initial SGWH surface area of 1.24 cm^{-1}, the total surface area equates to 5.92 cm^{-1}.
- **COMB SGWH:** A Combination of factors present at the same time including: nitrifying bacteria (NB SGWH), Mg anode rods (Mg SGWH), and cast iron coupons (Iron SGWH).

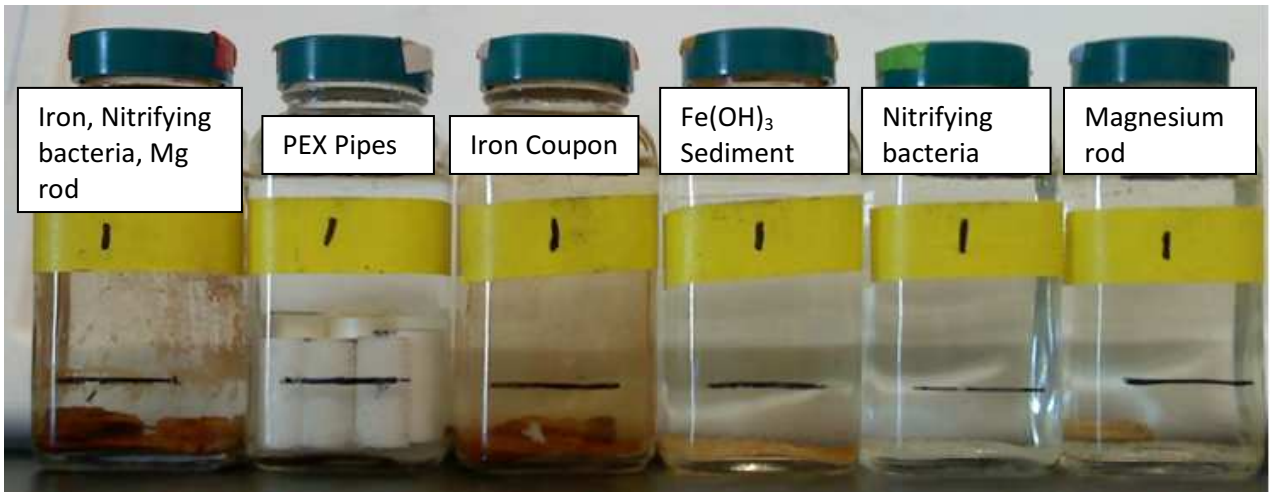

Figure 21: SGWH setup showing simulated premise plumbing deficiencies. SGWH were run utilizing 5 and 1,000 µg/L TOC water and compared to identical controls (5 and 1,000 µg/L) listed in Chapter 2.

Table 22: Abbreviated Names of SGWH with Plumbing Deficiencies

Iron L	Iron Coupon SGWH 5 µg/L TOC
Iron H	Iron Coupon SGWH 1,000 µg/L TOC
NB L	Nitrifying Bacteria SGWH 5 µg/L TOC
NB H	Nitrifying Bacteria SGWH 1,000 µg/L TOC
FE L	$Fe(OH)_3$ Sediments SGWH 5 µg/L TOC
FE H	$Fe(OH)_3$ Sediments SGWH 1,000 µg/L TOC
COMB L	Combination SGWH 5 µg/L TOC
COMB H	Combination SGWH 1,000 µg/L TOC
PEX L	Cross-Linked Polyethylene SGWH 5 µg/L TOC
PEX H	Cross-Linked Polyethylene SGWH 1,000 µg/L TOC
MG L	Magnesium Rod SGWH 5 µg/L TOC
MG H	Magnesium Rod SGWH 1,000 µg/L TOC

3.3 METHODS

On 3/18/10, 5 mL of dechlorinated Blacksburg, VA tap water was added to each SGWH and subsequently filled to 100 mL with 5 µg/L TOC of Norton et al., 2004 synthetic tap water. Additionally, inoculation of the nitrifying bacteria from positive Nitrifying Biological Activity reaction tests (N-BART) reaction tests was also performed at this time. On 3/29/10 (after 11 days of stagnation) 1 mL from a 30K SGWH (Chapter 2) and 1 mL from a 0 µg/L SGWH (Chapter 2) was inoculated into every SGWH within this phase. These steps were performed to introduce additional microbial flora to induce the establishment of biofilm. On 4/2/10, a 1mL aliquot of *L. pneumophila* was inoculated into each SGWH at

an approximate initial concentration of 2.03 x 10^5 gene copies/mL. On 4/9/10, a 500 µL aliquot of *A. polyphaga* was inoculated into each SGWH at an approximate concentration of 2,079 gene copies/mL. 5 µg/L TOC water was applied initially in all SGWH conditions. On 4/28/10, the 5 µg/L TOC water was elevated to 1,000 µg/L TOC in 18 of the 36 SGWH. Following this experimental change, 80% (80mL) water changes on a Monday, Wednesday, and Friday schedule proceeded on 4/28/10 (Day 0).

In later phases of the study, a series of adjustments were made to the baseline water and nutrients other than added AOC and BDOC in an attempt to improve the conduciveness for *L. pneumophila* amplification (see Chapter 2, Section 2.3 & Table 10 for listed modifications).

3.4 RESULTS

Effect of Plumbing Deficiencies on Dissolved Oxygen, pH, Mg and Iron in Simulated Water Heaters

The presence of premise plumbing deficiencies significantly impacted the chemistry of the water in the simulated water heaters relative to the control conditions. In several of the SGWH (i.e. MG, IRON, and COMB SGWH) corrosion tended to markedly increase the ambient pH and lower the overall dissolved oxygen (DO) concentration (Table 23, Table 24). This occurrence was likely due to corrosive reactions such as the following:

$$4Fe^{\circ} + 2O_2 + 4H_2O \rightarrow 4Fe^{+2} + 8OH^-$$

Table 23: Comparison of representative chemical conditions in effluent of SGWH 5 µg/L samples (Experiment B3). (Data collected from a condition with initial pH of 7.5 and initial DO of 4.0 mg/L. Baseline synthesized water from Norton et al., 2004.)

Parameters	Control Water 5 µg/L into SGWH	Magnesium (Mg) Coupon	Iron Coupon	$Fe(OH)_3$ (Fe)	PEX	Nitrifying (NB)	Combination (COMB)
DO (mg/L)	4.0	3.5	2.7	3.8	3.7	3.2	3.8
pH	7.5	9.7	8.1	8.5	7.5	6.7	9.8
Mg (mg/L)	1.7	10.7	1.7	1.7	1.7	1.7	10.4
Iron (mg/L)	0.042	0.042	3.4	2.7	0.042	0.042	3.4
Phosphorus (mg/L)	0.310	0.256	0.146	0.350	0.313	0.310	0.183
Effluent AOC (µg/L)	170	707	111	228	247	810	203

Table 24: Comparison of representative chemical conditions in effluent of SGWH 1,000 µg/L samples (Experiment B3). (Data collected from a condition with initial pH of 7.5 and initial DO of 4.0 mg/L. Baseline synthesized water from Norton et al., 2004.)

Parameters	Control Water 1,000 µg/L into SGWH	Magnesium (Mg) Metal	Iron Metal	$Fe(OH)_3$ (Fe)	PEX	Nitrifying (NB)	Combination (COMB)
DO (mg/L)	4.0	3.5	2.6	3.9	3.6	3.2	3.7
pH	7.5	9.9	8.2	8.3	7.4	6.9	9.8
Mg (mg/L)	1.7	10.7	1.7	1.7	1.7	1.7	10.4
Iron (mg/L)	0.042	0.042	3.4	2.7	0.042	0.042	3.4
Phosphorus (mg/L)	0.310	0.256	0.146	0.350	0.313	0.310	0.183
Effluent AOC (µg/L)	385	340	219	630	273	277	413

The DO within SGWH with simulated deficiencies all decreased by at least 0.2 mg/L relative to control conditions (Table 23, Table 24). IRON SGWH displayed the greatest difference in DO where an oxygen decrease of over 1 mg/L was noted for the both the "high" and "low" conditions as compared to the control. Given that the COMB SGWH contain both iron and magnesium coupons, it was somewhat surprising a greater change in DO was not detected as all SGWH operate under sealed conditions. Relative to the controls, an increase in overall pH (between 0.6 and 2.4 pH units) was detected in SGWH with a chance for corrosion processes as expected based on typical overall reactions (e.g. IRON, COMB, and MG SGWH). These reactions also elevated the iron (+3.4 mg/L) and magnesium (+10.7 mg/L) in the effluent relative to the control conditions.

Clearly, corrosive processes (e.g., MG, COMB, and IRON SGWH) reduced ambient DO levels from SGWH effluent samples, but nitrifying bacteria also exert an oxygen demand and reduce overall pH as a result of nitrification reactions. Evidence of nitrification was verified due to a loss of overall ammonia (from 2.30 to 0.11 mg N/L) and increases in nitrite (from approximately 0.01 to 1.47 mg N/L). The remaining ammonia is likely to have been converted completely to nitrate as part of the oxidation process.

All of these chemical changes may alter the ability of pathogenic and heterotrophic cells to proliferate. Ambient pH commonly affects microbial growth and population dynamics, and shift from 2-3 pH units

may affect the growth of many heterotrophic organisms (Maier et al., 2009). The optimal reported pH level given for supporting *L. pneumophila* growth has been reported to be 5.0 to 8.5 (Wadosky et al., 1985).

Possible Generation of AOC

It was expected that the different plumbing conditions might lead to generation of AOC in the simulated water heater and provide additional sources of organic carbon in SGWH. This hypothesis was confirmed in preliminary experimental measurements with require further QA/QC analysis. From the data obtained the NB L, MG L, and $Fe(OH)_3$ H SGWH, significantly more AOC was present in the effluent of the SGWH than in the 1,000 µg/L influent TOC (see all "High Conditions" in Figure 22). In the 5 µg/L influent TOC water, all simulated deficiencies produced higher AOC values from SGWH effluent compared to the influent source water, with the exception of the iron metal condition (see all "Low" Conditions in Figure 22). Further research on AOC generation is necessary, but the increased AOC was significant at > 95% confidence as compared to the control values ($P < 0.05$). Many other conditions had equal or lower AOC values in the SGWH effluent than in the influent. AOC may have still been generated in SGWH yielding a lower effluent than influent AOC concentration given that AOC uptake by microbes during long stagnation events may have exceeded the AOC generated.

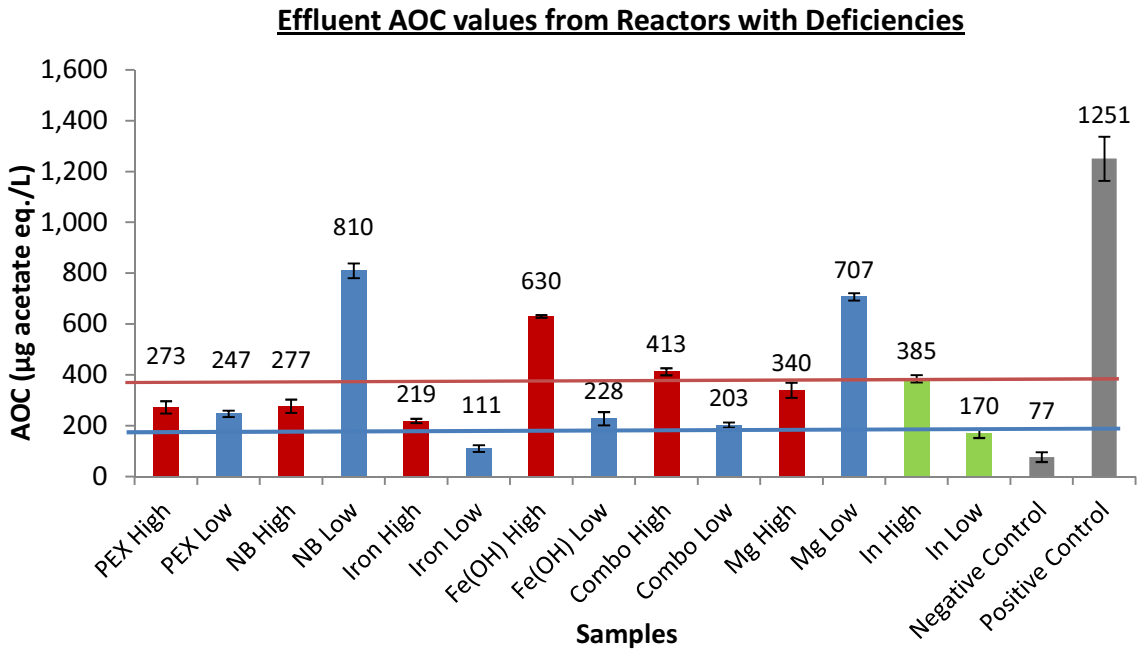

Figure 22: AOC concentrations from effluent SGWH samples (Experiment B3). The green bars with influent AOC levels are compared to the red and blue bars for analysis. The 1,000µg/L influent TOC water was found to have an AOC concentration of 385 µg/L and the 5µg/L influent water was found to have an AOC concentration of 170 µg/L. Given that several samples had greater effluent AOC concentration (above 95% confidence), the primary hypothesis of this research that AOC can be generated internally in water heaters is validated *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

Heterotrophic Plate Count and 16S rRNA Gene Analysis

It was anticipated that the differences in chemistry would translate to marked differences in general bacterial levels in the effluent of SGWH with various deficiencies. However, using single factor analysis of variance (ANOVA) tests of 16S rRNA gene analysis and HPC results indicated that the mean value from each SGWH was not significantly different from each other or the control 5 µg/L condition (representative data in Table 25, p value = 0.999). A similar result was obtained for every other condition including 1,000 µg/L and added TOC using 16S rRNA and the analogous data for HPCs. In other words, despite the marked differences in chemistry and AOC in each SGWH, these changes did not translate to higher levels of total bacteria. More detailed analysis attempting to consider a paired analysis at each sampling date is under consideration, as this may realize improved statistical power in discerning trends because the trend cited in Table 4 may be overwhelmed by the high initial value in the first sample (e.g., see Figure 23).

Table 25: Example 16S rRNA gene analysis (gene copies/mL) ANOVA table examining differences between sample means. Additional data tables are provided in Appendix: Chapter 3.

Date	Control	PEX L	NB L	COMB L	FE L	IRON L	MG L
3/31/11	5.90E+07	4.74E+07	3.94E+07	6.55E+07	3.99E+07	2.45E+07	6.20E+07
4/4/11	3.50E+06	5.00E+06	8.96E+06	4.91E+06	7.55E+06	7.77E+06	1.82E+06
4/18/11	8.37E+05	5.75E+06	2.01E+07	8.21E+06	6.66E+06	2.30E+06	2.85E+06
5/16/11	2.02E+06	7.94E+06	3.00E+06	3.43E+06	7.91E+06	1.60E+06	8.08E+05
Mean	**1.63E+07**	**1.65E+07**	**1.78E+07**	**2.05E+07**	**1.55E+07**	**9.05E+06**	**1.69E+07**
Variance	8.09E+14	4.25E+14	2.56E+14	9.04E+14	2.65E+14	1.14E+14	9.05E+14

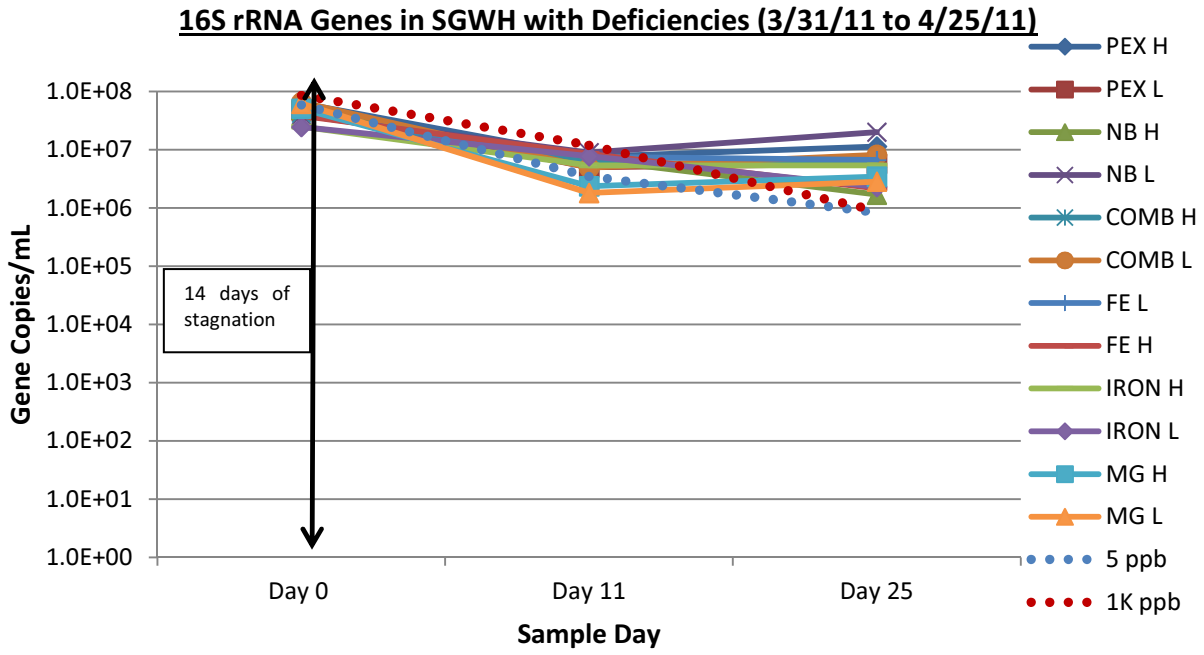

Figure 23: 16S rRNA gene analysis observed over time (3/31/11 (Day 0) to 4/25/11 (Day 25), Experiment B3). ANOVA analysis revealed no statistical differences between deficient conditions and control values. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

If anything, there was a trend to slightly lower HPC in the conditions with the influential plumbing factors versus the control conditions. On the one hand, this might suggest a shift to organisms that were not culturable, but the combined data for 16S rRNA and HPC indicate that the plumbing deficiencies had relatively little impact on the effluent bacterial levels, despite the potential for higher AOC.

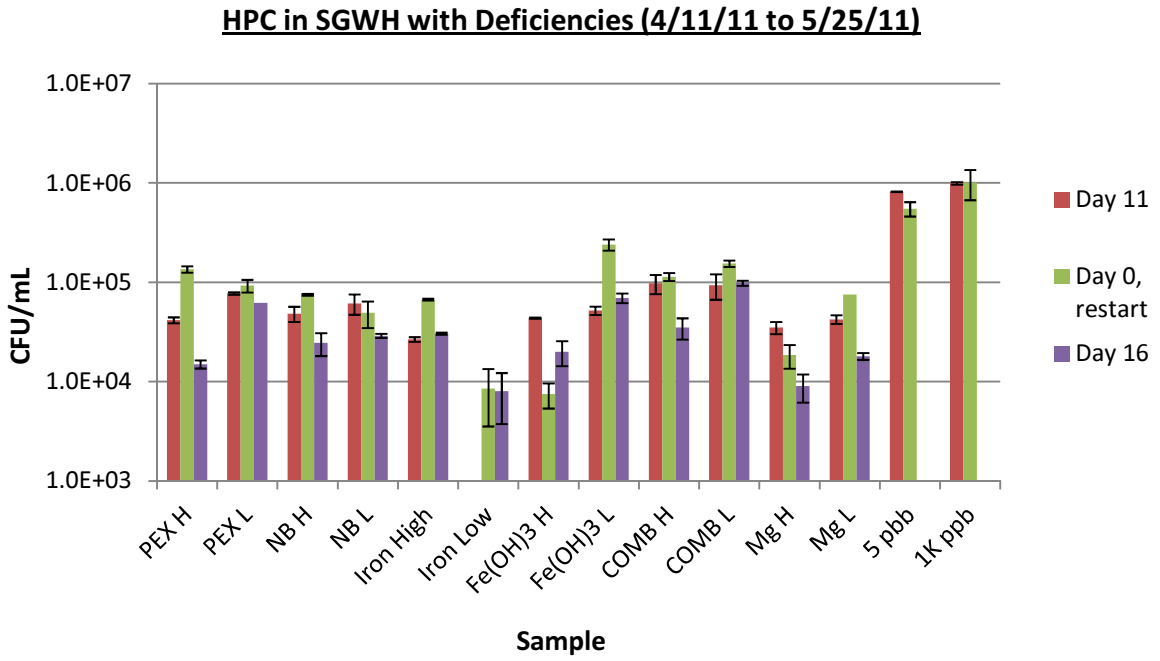

Figure 24: HPC data over time (4/11/11 (Day 11) to 5/25/11 (Day 16), Experiments B3 & B4). Error bars denote 95% confidence intervals. HPC increases obtained from the 5/9/11 (Day 0) sampling date may account from significant stagnation. ANOVA analysis revealed no statistical differences between mean values from deficient and control conditions (Chapter 2).

M. avium

Effluent *M. avium* concentrations as determined by qPCR tended to be slightly higher in the control conditions versus the conditions with influential plumbing factors (Figure 25) and significant stagnation. Subsequent ANOVA testing revealed that mean values were significantly lower at 95% confidence ($p = 0.01$). However, results obtained using two tailed t-tests assuming unequal variance show no statistical difference ($p > 0.05$). In utilizing ANOVA to examining deficient conditions with 1,000 µg/L added TOC p-values were also >0.05 and indicated no statistical difference (see Appendix: Chapter 3 for raw data sets).

Given these results, it appears that even under relatively constant environmental conditions in the simulated water heaters, the factors influencing persistence of *M. avium* were complex and not a direct function of AOC or influential plumbing factors.

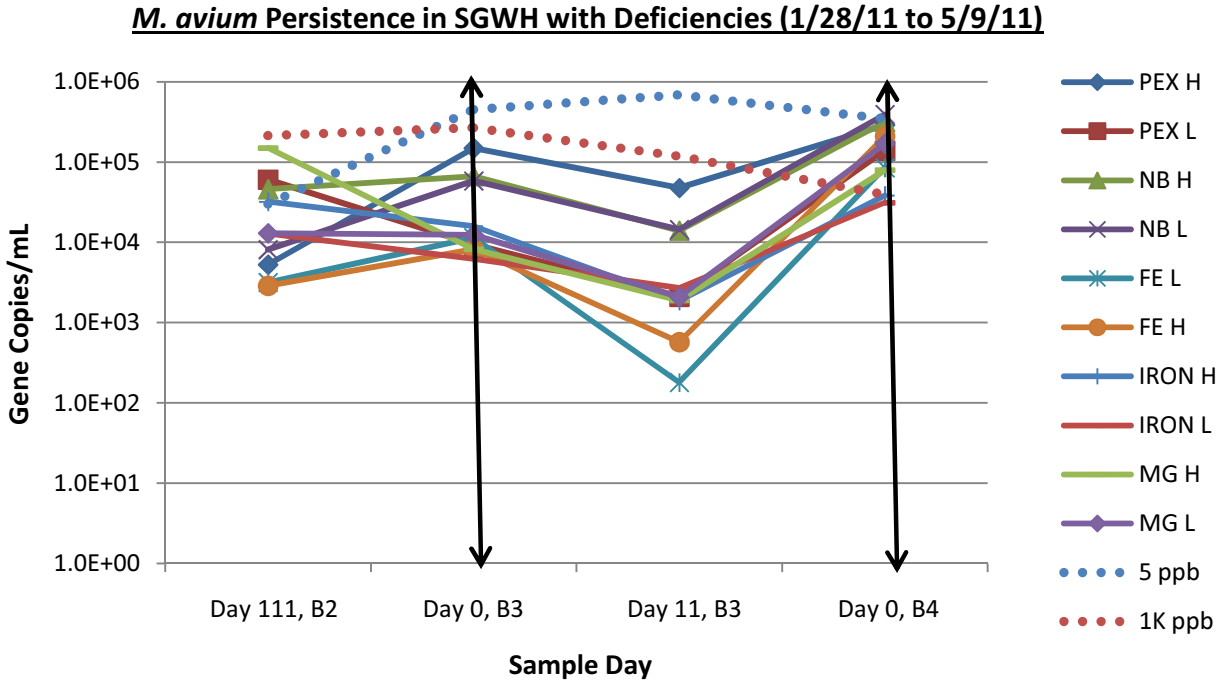

Figure 25: *M. avium* persistence in deficient SGWH conditions (1/28/11 to 5/9/11, Experiments B2, B3, and B4). Control conditions were generally had higher quantification than SGWH with deficiencies. Vertical lines represent instances when SGWH were restarted but with no additional *M. avium*. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

Under conditions with regular water changes (3 times per week) and no stagnation, the results obtained showed only slight variations from the 5 and 1,000 µg/L control conditions. The range of quantified effluent *M. avium* values showed significant variation over the study period (approximately 1 x 10^3 to 1 x 10^5 gene copies/mL under regular water changes; Figure 26). There was no statistical difference between identical conditions at 5 and 1,000 µg/L added AOC.

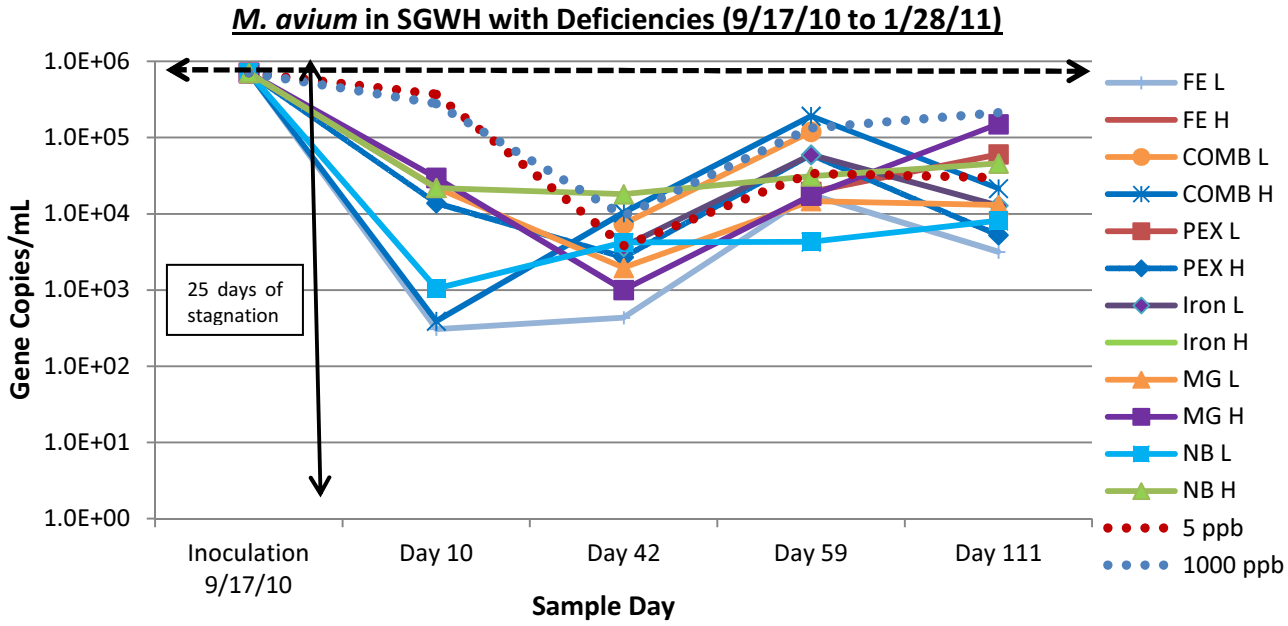

Figure 26: *M. avium* persistence (Experiment B2). The dotted line denotes the initial inoculum concentration added to the SGWH. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

L. pneumophila

In analyzing periods in which regular water changes were occurring (3 times per week) over the duration of the study, no significant differences were observed between conditions using ANOVA (p=0.999). However, clear trends did emerge near later phases of the experiment (Figure 27). For example, after 4 months of continual water changes samples taken on 1/28/11 (Day 111) from the MG H, COMB H, and IRON L SGWH were significantly different from control values with 95% confidence and demonstrated a high propensity for *L. pneumophila* to persist far above washout conditions. Interestingly, the condition with a Magnesium anode with the most persistent *L. pneumophila* was at a pH deemed far above the optimum levels for *L. pneumophila* (Wadosky et al., 1985). At no point is the study were culturable *Legionella* isolated on plating media and also confirmed to be *L. pneumophila* colonies (data not shown, n =15), suggesting that the *Legionella* detected in Figure 27 were in the VBNC state. SGWH yielding the most persistence above washout were noted to be were those that potentially sorb additional organic material (IRON, COMB) and/or produce H_2 gas as a result of corrosive processes (COMB, IRON, MG).

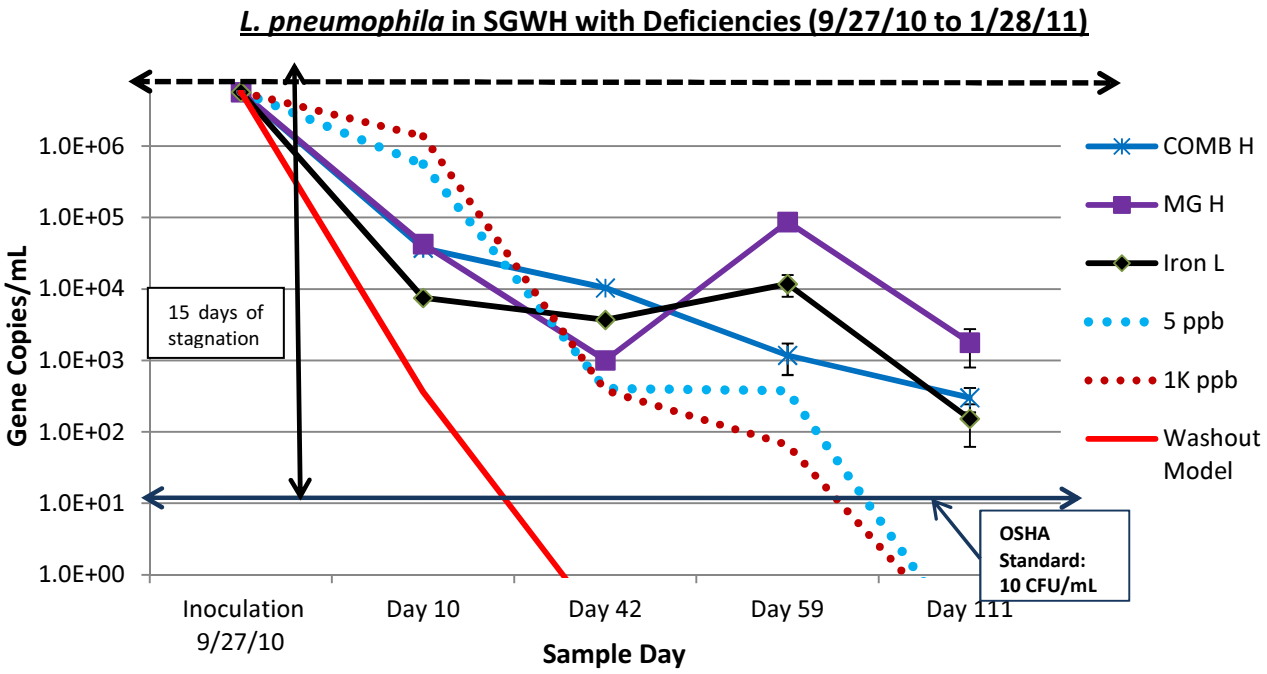

Figure 27: Persistence of *L. pneumophila* in SGWH above washout conditions and comparisons from the 5 µg/L and 1K µg/L of added TOC control conditions (Experiment B2). The dotted line denotes the initial inoculum concentration. The washout model assumes that 20% of original inoculum remains after a single water change and that all cells are in the planktonic state; bacterial retention in biofilm matrices is not considered and could account for some of the levels detected above the washout model. Error bars denote 95% confidence intervals. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

After several modifications and extensions of stagnation periods, slight amplification of *L. pneumophila* became apparent in the NB H and PEX H effluent SGWH. However, the observed amplifications were not greater than the 95% confidence interval relative to the initial inoculum concentration. Subsequent monitoring revealed significant declines in overall *L. pneumophila* concentrations over time. Overall, the results were comparable to those obtained before modifications (Figure 27) with marked decline observed in all SGWH over the study period. ANOVA revealed no statistical differences between control and deficient SGWH with a reported p value of 0.999 for the low and high TOC SGWH.

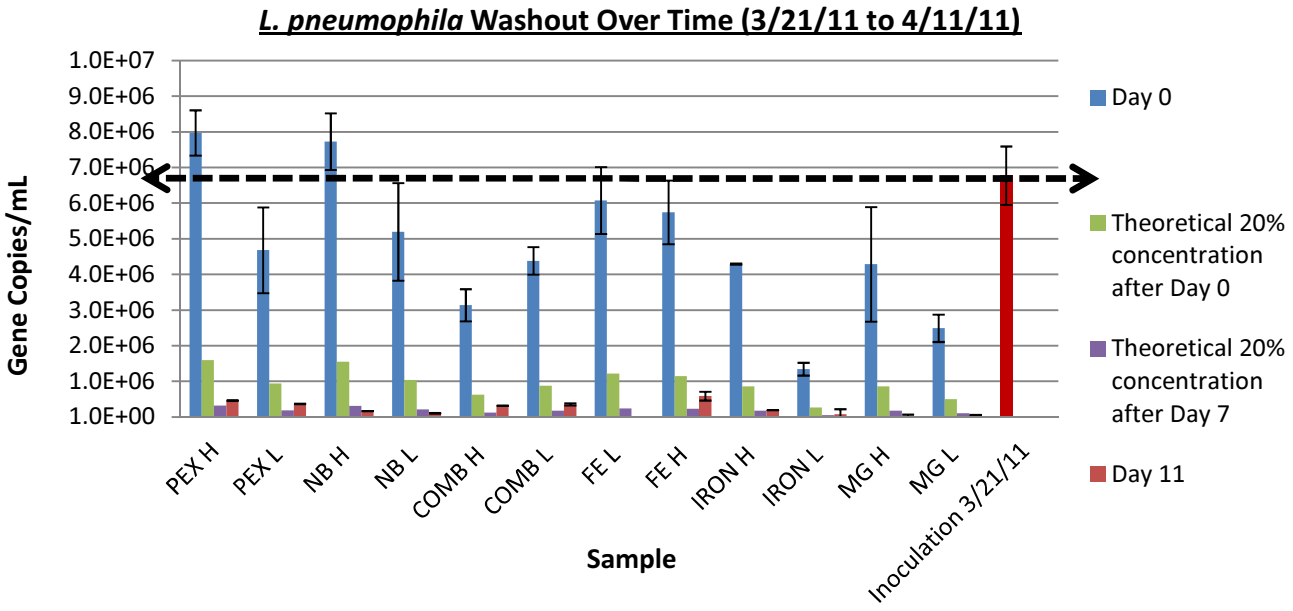

Figure 28: Comparison of the initial and final *L. pneumophila* with corresponding washout estimates (Experiment B3). Initial growth from PEX H and NB H is not above the 95% confidence interval of the inoculum; thus, amplification from these data points is inconclusive. The dotted line denotes the initial inoculum concentration added to the SGWH. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

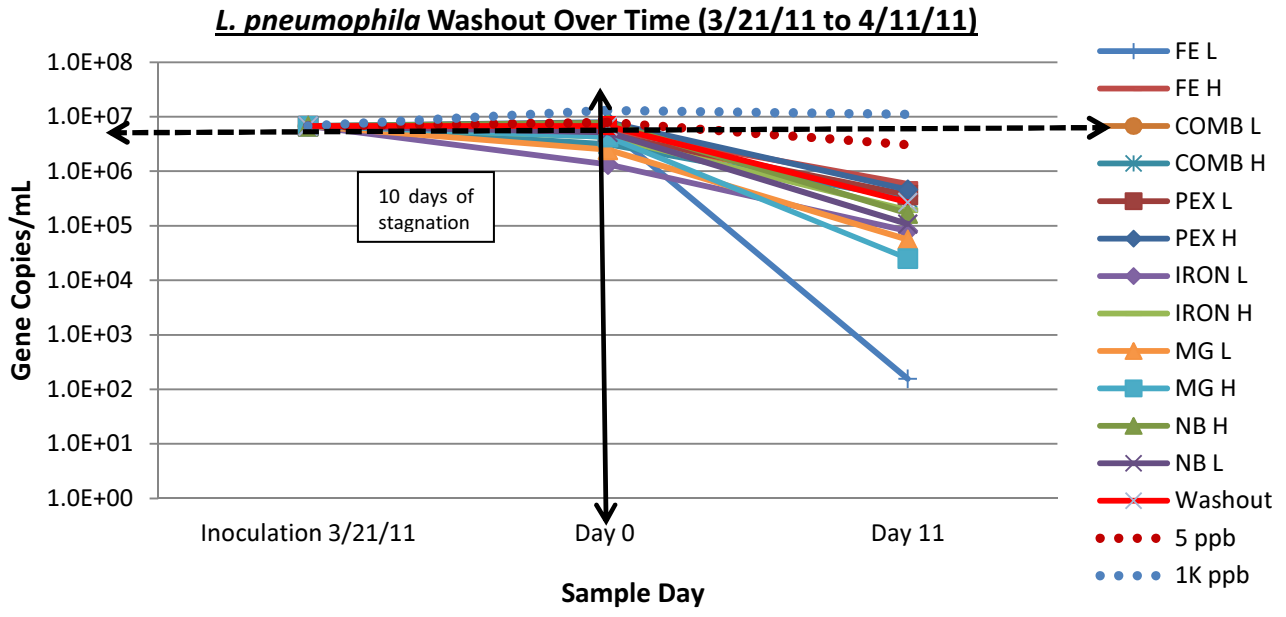

Figure 29: Alternative presentation of Figure 28 (Experiment B3). *L. pneumophila* persistence compared to washout and control conditions. The washout model assumes that 20% of original inoculum value remains after a single water change and that all cells are in the planktonic state; bacterial retention in biofilm matrices is not

considered and could account for some of the levels detected above the washout model. The dotted line denotes the initial inoculum concentration added to the SGWH. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

Surprisingly amplification of pathogens in SGWH with deficiencies was not noted corresponding to the second round of modifications to the water and stagnation conditions, as had been observed in the control SGWH (Figure 29). It is unclear why none of the conditions with plumbing deficiencies yielded at least the same level of amplification as in the control condition. Moreover, enhanced survival of *L. pneumophila* was not observed in these SGWH through the latter phases of the experiment.

A. polyphaga and *H. vermiformis*

A. polyphaga was analyzed in order to ascertain if there was a relationship between the persistence observed earlier from the COMB, IRON, and MG SGWH from Figure 28 (Experiment B3) during regular water changes. Results indicated no significantly greater persistence of *Acanthamoeba* as compared to the control conditions of 1K µg/L and 5 µg/L of added TOC. ANOVA confirms this hypothesis as p-values of 0.310 and 0.176 were obtained for the high and low TOC SGWH, respectively. Thus, conditions representing the array of plumbing factors did not enhance overall survival of this organism compared to controls. In addition to the qPCR data below, no counts were observed from microscopy after 11/20/10 (Day 42), even though *A. polyphaga* DNA was detected throughout this time period at levels above washout (Figure 30).

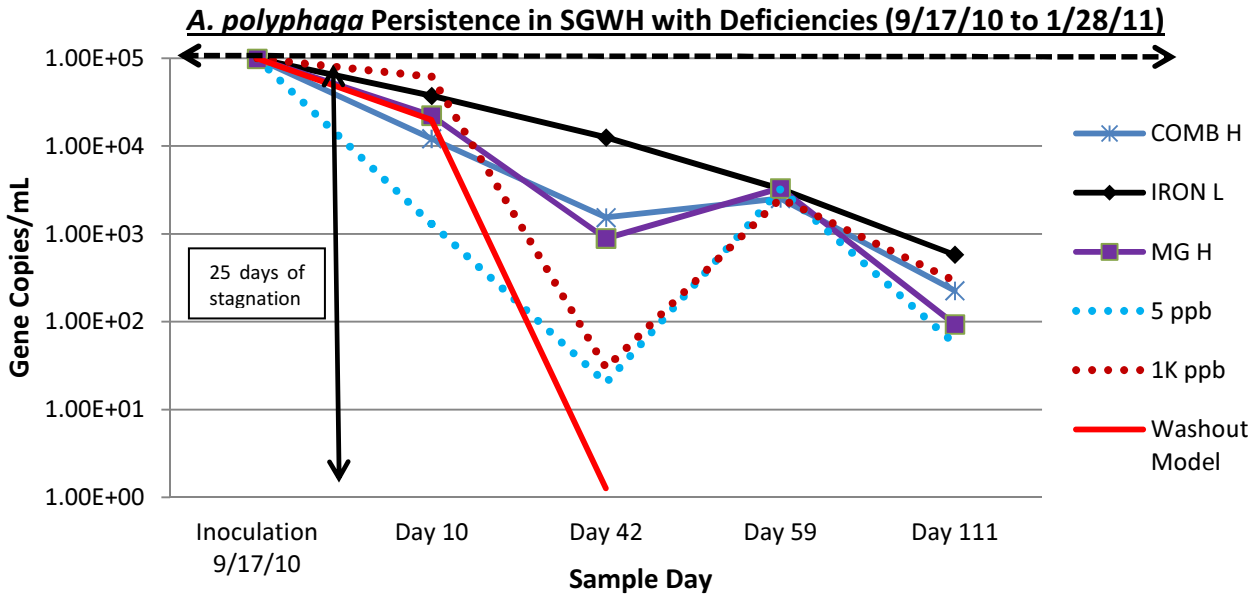

Figure 30: *A. polyphaga* persistence in SGWH (Experiment B2). The dotted line denotes the initial inoculum concentration added to the SGWH. The washout model assumes that 20% of original inoculum value remains after a single water change and that all cells are in the planktonic state; bacterial retention in biofilm matrices is not considered and could account for some of the levels detected above the washout model. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

H. vermiformis was discovered to have naturally colonized the SGWH, presumably due to GAC filtered Blacksburg, VA water added to the SGWH. In later study phases where additional *H. vermiformis* was inoculated, amplification above 95% confidence intervals occurred in MG L, IRON L, Fe L, NB L, and PEX H (Figure 31). *H. vermiformis* appeared to be better adapted to surviving in the simulated water heaters tested in this research than was *A. polyphaga,* in which no amplification was evident over time. The FE H sample also remains a point of considerable interest, as significant *L. pneumophila* amplification was observed 4/11/11 (Day 21) with a concomitant decrease in *H. vermiformis* on the same sampling date. It seems plausible that *L. pneumophila* was being actively phagocytized by active *Hartmannella* cells, resulting in amplification and eventual necrosis of *Hartmannella* cells. Given the limited number of sampling periods investigated with this organism and continual influent *Hartmannella* cells from the GAC filter, ANOVA analysis was not performed on data collected from this organism.

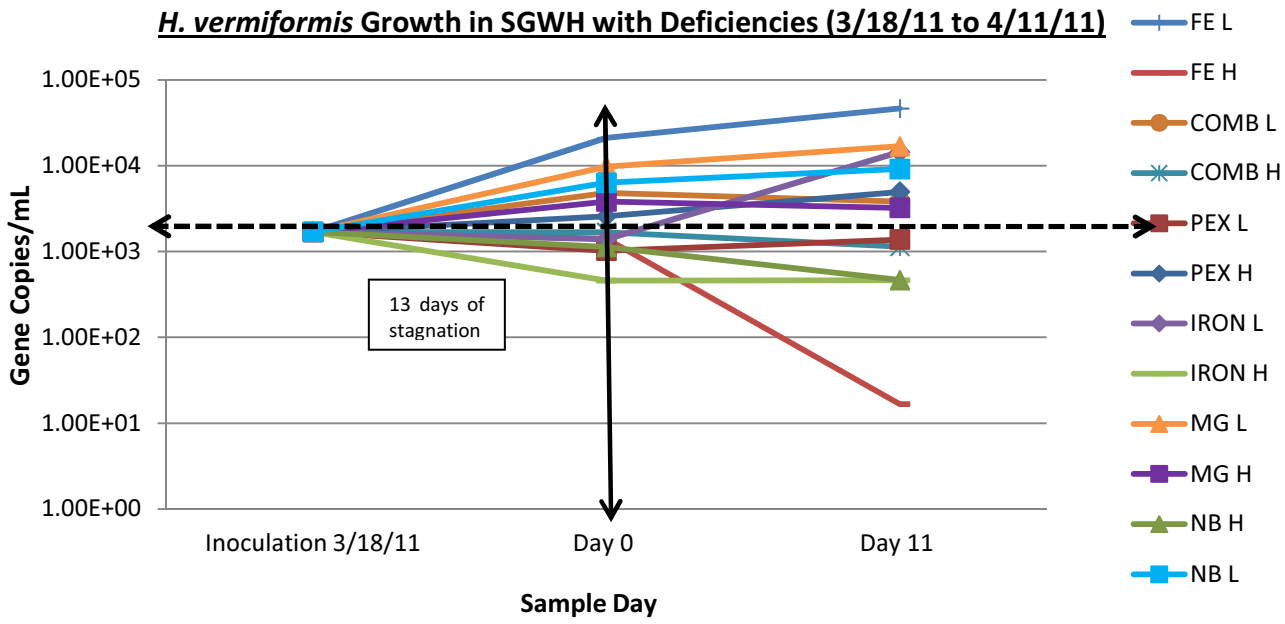

Figure 31: *H. vermiformis* persistence and growth in SGWH conditions (Experiment B3). The FE H sample showed significant concentration decreases as *L. pneumophila* showed a corresponding increase in overall concentration. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

3.5 DISCUSSION & KEY FINDINGS

Although the tested array of influential plumbing factors exerted a measureable impact on pH, dissolved oxygen, iron and magnesium concentrations, and some evidence was obtained for AOC generation in the SGWH, these conditions had surprisingly little impact on levels of either heterotrophic or pathogenic bacteria. These findings generally support the results from Chapter 2, which indicates that AOC is not a fundamental driver of pathogen abundance.

However, there were notable exceptions at certain times during this investigation. During periods of regular water changes, *L. pneumophila* persisted at levels above washout models in Mg H SGWH. Compared to the analogous 5 µg/L control condition (Chapter 2), *L. pneumophila* quantification from these SGWH showed statistical difference above 95% confidence. Thus these findings may suggest that sacrificial magnesium anode rods may enhance *Legionella* persistence within consumer hot water heater systems. The only statistically supported finding with respect to *M. avium* was from the low TOC SGWH under stagnation in which one tailed t-tests revealed the COMB L, $Fe(OH)_3$ L, IRON L, and MG L had p values < 0.05 and differ from the 5 µg/L control.

At first glance these results are considered to be contrary to expectations, but on the other hand, it is clear that factors that trigger *L. pneumophila* amplification could not be reproduced herein, either with or without plumbing factors initially thought to be influential (and previously considered "worst" case conditions). Amplification of pathogenic bacteria is dependent on a multitude of biotic and abiotic factors, and is clearly not a simple function of both AOC levels and tested deficiencies (including Mg anodes, nitrifying bacteria, PEX pipes, and iron coupons) in potable water systems.

3.6 FUTURE WORK

If future progress is to be made, better characterization of nutrient levels for source waters with high levels of protozoans and *Legionella* is essential. Field work should also better characterize the types of premise plumbing systems, corrosivity, ambient water temperatures, stagnation periods, acclimation time, biofilm diversity, and total disinfectant concentrations to better understand the factors that trigger amplification. It is also possible that larger scale studies using more complex water heaters will be important, due to the wider array of microbial niches that occur throughout the volume of such SGWH.

REFERENCES

APHA, Method 9215. In Standard Methods for the Examination of Water and Wastewater, 20 ed.; Clesceri, L. S.; A.E., G.; Eaton, A. D., Eds. American Water Works Association and Water Environment Federation: Washington, D.C., 1998.

Bagh, L.K., Albrechtsen, H.J., Arvin, E., and Ovesen, K. 2004. Distribution of bacteria in a domestic hot water system in a Danish apartment building. Water Res. **38**:225-235.

Butterfield, P.W., Camper, A.K., Biederman, J.A., and Bargmeyer, A.M., 2002. Minimizing biofilm in the presence of iron oxides and humic substances. Water Res. **36**:3893–3910.

Butterfield P.W., Camper, A.K., Ellis, B.D. and Jones, W.L., 2002. Chlorination of model distribution system biofilm: implications for growth and organic carbon removal. Water Res. **36**:4391–4405.

Camper, A. 2004. Involvement of humic substances in regrowth. Int. J. Food Microbiol. **92**:355-364.

Durand, M.,L. and Dietrich, A.M. (2007). Contributions of silane cross-linked PEX pipe to chemical/solvent odours in drinking water. Wat. Sci. Tech. 55:153-160.

Edwards, M., Falkinham, J., Pruden, A., Lieberman, R. (2009). Relationship between Biodegradable Organic Matter and Pathogen Concentrations in Premise Plumbing. Final AWWARF Research Proposal RFP: 4251.

Edwards, M., and M. M. Benjamin. 1992. Effect of Preozonation on Coagulant-NOM Interactions. J. Am. Water Works Assn. **84**(8):63-72.

Heim, T.H. and Dietrich, A.M (2007). Sensory aspects and water quality impacts of chlorinated and chloraminated drinking water in contact with HDPE and cPVC pipe. Water Res. **41**:757-764.

Hoffman, P.S, H. Friedman, M Bendinelli (2008). *Legionella pneumophila*: Pathogenesis and Immunity. New York: Springer Science & Business Media, LLC

Kulakov LA, MB McAlister, KL Ogden, MJ Larkin and JF O'Hanlon.(2002)Analysis of bacteria contaminating ultrapure water in industrial systems. Applied and Environmental Microbiology. **68**: 1548– 1555

McAlister MB, LA Kulkaov, MJ Larkin and KL Ogden.(2001) "Analysis of bacterial contamination in different sections of a high-purity water system."Ultrapure Water, **18**: 18–26

Skjevrak, I., Due, A., Gjerstad, K.O., and Herikstad, H. 2003. Volatile organic components migrating from plastic pipes (HDPE, PEX and PVC) into drinking water. Water Res. 37:1912-1920.

Morton, S. C., Zhang, Y., and M. Edwards. 2005. Implications of nutrient release from iron metal for microbial regrowth in water distribution systems. Water Res. **39**(13): 2883-2892.

Oliver, J.D. (2009). "Recent findings on the viable but nonculturable state in pathogenic bacteria." FEMS Microbiology Reviews. **34**: 415-425

Rogers, J., A. B. Dowsett, et al. 1994. Influence of temperature and plumbing material selection on biofilm formation and growth of *Legionella pneumophila* in a model potable water system containing complex microbial flora. Applied and Environmental Microbiology **60**: 1585-1592.

Skjevrak, I., Due, A., Gjerstad, K.O., and Herikstad, H. 2003. Volatile organic components migrating from plastic pipes (HDPE, PEX and PVC) into drinking water. Water Res. **37**:1912-1920.

Steinert, M., Emody, L., Amann, R., and Hacker, J. (1997) "Resuscitation of viable but nonculturable *Legionella* pneu-mophila Philadelphia JR32 by *Acanthamoeba* castellanii".Applied and Environmental Microbiology, **63**: 2047–2053.

Valster RM, Wullings BA, and van der Kooij D. (2010) Detection of Protozoan Hosts for *Legionella pneumophila* in Engineered Water Systems by Using a Biofilm Batch Test. Applied and Environmental Microbiology, **76**(21): 7144-7153.

van der Kooij, D. 2002 Assimilable organic carbon (AOC) in treated water: determination and significance. In: Encyclopeida of Environmental Microbiology, Bitton, G. (Ed.), John Wiley & Sons, Hoboken, NJ, USA pp. 312-327.

van der Kooij, D. (1992). "Assimilable organic carbon as indicator of bacterial regrowth". Journal of the American Water Works Association **84**:57.

van der Kooij D, Veenendaal HR, Scheffer WJ. 2005. Biofilm formation and multiplication of *Legionella* in a model warm water system with pipes of copper, stainless steel and cross-linked polyethylene. Water Res. **39**:2789-98.

Wilton, S. and Cousins, D. (1992)" Detection and identification of multiple mycobacterial pathogens by DNA amplification in a single tube". Genome Research. **1**:269-273

Zhang, Y., M. Edwards, A. Pinto, N. Love, A. Camper, M. Rahman, H. Baribeau. 2009b. Effect of Nitrification on Corrosion in the Distribution System. AWWARF Final Report.

CHAPTER 3: APPENDIX A

Inhibition studies from deficient SGWH Samples

As discussed from Chapter 2, qPCR inhibition posed a significant problem in the overall recovery of *L. pneumophila*. As performed previously, 1:10 sample dilutions were made and compared with a control sample containing no known qPCR inhibitors. As shown in Figure 32 samples from deficient SGWH, a 1:10 dilution was sufficient in recovering all sample DNA with 95% confidence. This experiment is illustrative in that a 1:10 dilution from SGWH samples containing corrosive materials will be sufficient in ensuring 100% sample recovery of *L. pneumophila*.

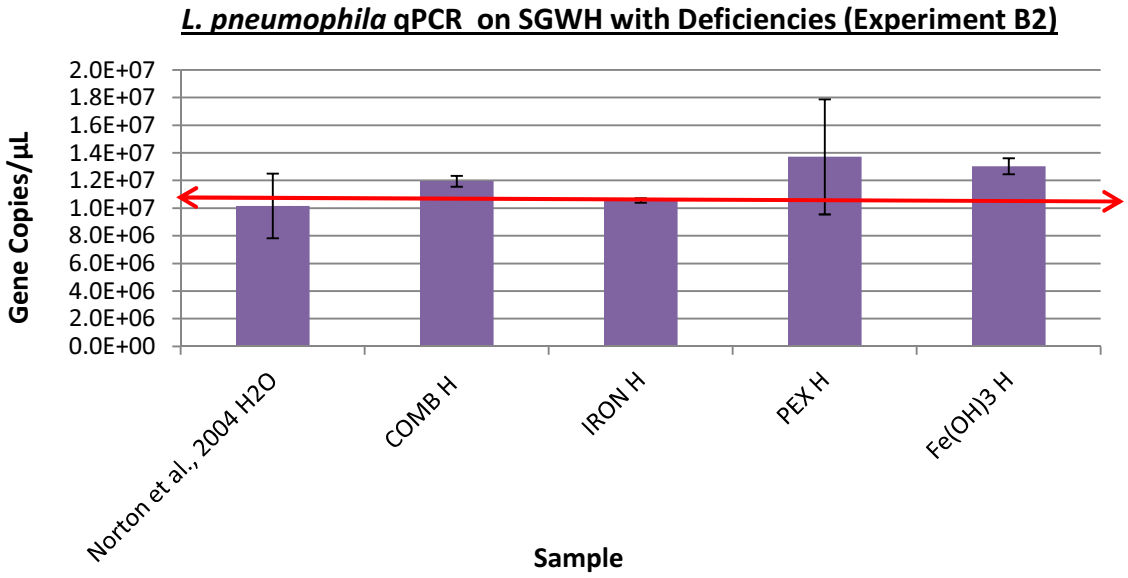

Figure 32: Relative inhibition from deficient water conditions with 1:10 dilutions (Experiment B2). Each effluent SGWH sample is spiked with a known amount of template *L. pneumophila* DNA. All samples are compared to the analogous inhibitor free reaction (red line) in which no inhibition factors are present. Error bars delineate the 95% confidence interval. *Note: High (H) has 1,000 µg/L TOC, Low (L) has 5 µg/L TOC in the ingoing water.

CHAPTER 3: APPENDIX B

Variability in Effluent SGWH Samples

Analysis of individual SGWH effluent was performed to examine for variability between replicate samples (Figure 33). During experiment B2 it was observed that the Mg H SGWH was considerably persistent as compared to the 1,000 µg/L control sample apparent by days 59 and 111 (Figure 27). Thus, effluent samples from each Mg H SGWH were analyzed. Findings indicate a significant difference between replicate samples with 95% confidence intervals. This experiment demonstrates that utilizing identical influent conditions may be insufficient in producing consistent regrowth potential across replicates.

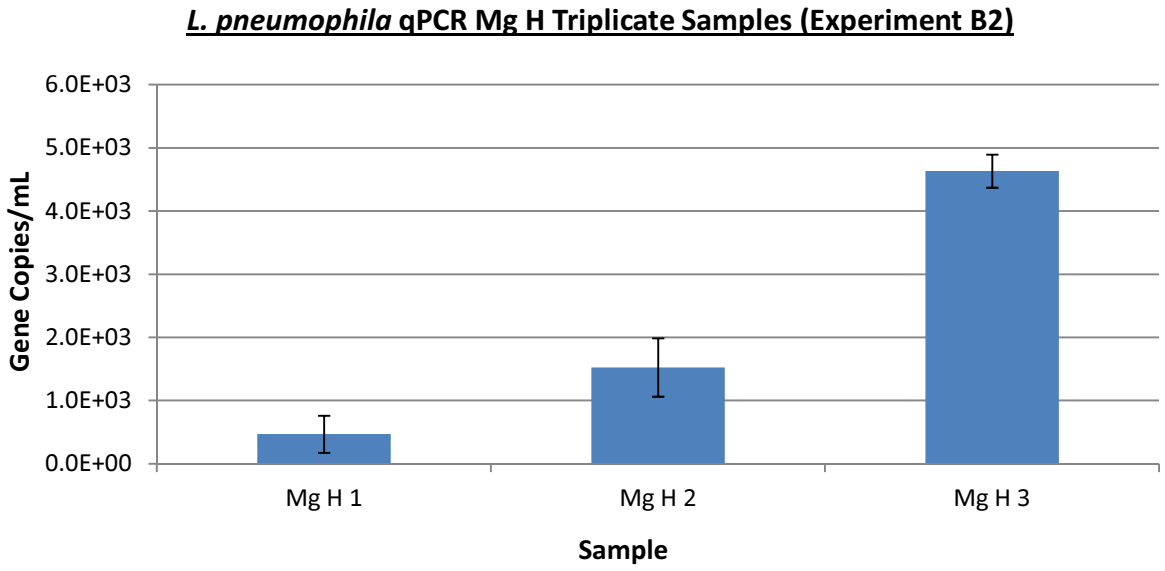

Figure 33: Differences in Mg H SGWH replicate samples with 95% confidence intervals (Experiment B2).

CHAPTER 3: APPENDIX C

ANOVA Testing Data Sets/Raw Data

ANOVA: 16S rRNA Low Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
5 µg/L	4	65313292	16328323	8.09E+14
PEX L	4	66081450	16520363	4.25E+14
NB L	4	71381500	17845375	2.56E+14
COMB L	4	82092333	20523083	9.04E+14
FE L	4	62010597	15502649	2.65E+14
IRON L	4	36211167	9052792	1.14E+14
MG L	4	67436220	16859055	9.05E+14

ANOVA: 16S rRNA High Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1,000 µg/L	4	1.14E+08	28397000	1.55E+15
PEX H	4	87756500	21939125	7.52E+14
NB H	4	57008447	14252112	5.25E+14
COMB H	4	58867500	14716875	3.49E+14
FE H	4	50416500	12604125	3.04E+14
IRON H	4	43101250	10775313	9.17E+13
MG H	4	58351833	14587958	5.85E+14

ANOVA: HPC Low Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
5 µg/L	4	5.37E+08	1.34E+08	7.13E+16
PEX L	4	416666.7	104166.7	9.14E+09
NB L	4	253333.3	63333.33	1.22E+09
COMB L	4	179666.7	44916.67	3.8E+08
FE L	4	428500	107125	7.91E+09
IRON L	4	388833.3	97208.33	2.08E+09
MG L	4	283500	70875	4.63E+09

ANOVA: HPC High Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1,000 µg/L	4	5.37E+08	1.34E+08	7.13E+16
PEX H	4	338833.3	84708.33	3.76E+08
NB H	4	251333.3	62833.33	1.23E+09
COMB H	3	72000	24000	7.44E+08
FE H	4	221666.7	55416.67	8.46E+08
IRON H	4	418166.7	104541.7	1.21E+09
MG H	4	211833.3	52958.33	2.98E+09

ANOVA: *L. pneumophila* Low 1 Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
5 µg/L	5	6247031	1249406	6.22E+12
Fe L	5	5693062	1138612	6.47E+12
COMB L	5	5719793	1143959	6.46E+12
PEX L	4	5691110	1422777	8.09E+12
Iron L	5	5713112	1142622	6.46E+12
Mg L	4	5695774	1423943	8.09E+12
NB L	5	5696694	1139339	6.47E+12

ANOVA: *L. pneumophila* Low 2 Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
5 µg/L	3	11166933	3722311	1.5E+13
PEX L	3	5188917	1729639	6.55E+12
NB L	3	5694950	1898317	8.17E+12
COMB L	3	4862617	1620872	5.73E+12
FE L	3	6163274	2054425	1.21E+13
IRON L	3	1455089	485029.7	5.53E+11
MG L	3	2715017	905005.6	1.89E+12

ANOVA: *L. pneumophila* High 1 Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*

1K µg/L	5	7060948	1412190	6.07E+12
Fe H	5	5691158	1138232	6.47E+12
COMB H	5	5739082	1147816	6.45E+12
PEX H	5	5695561	1139112	6.47E+12
Iron H	4	5711767	1427942	8.07E+12
Mg H	5	5822433	1164487	6.4E+12
NB H	5	5815394	1163079	6.41E+12

ANOVA: *L. pneumophila* High 2 Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1K µg/L	3	24316861	8105620	4.82E+13
PEX H	3	8723658	2907886	1.92E+13
NB H	3	8211883	2737294	1.87E+13
COMB H	3	3541967	1180656	2.89E+12
FE H	3	6541200	2180400	9.56E+12
IRON H	3	4521748	1507249	5.81E+12
MG H	3	4389070	1463023	5.97E+12

ANOVA: *M. avium* Low Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
5 µg/L	4	1509393	377348.3	7.37E+10
PEX L	4	210286.1	52571.51	3.94E+09
NB L	4	472957.5	118239.4	3.38E+10
COMB L	4	143493.8	35873.46	3.74E+09
FE L	4	99596.27	24899.07	1.62E+09
IRON L	4	53049.33	13262.33	1.62E+08
MG L	4	197210.2	49302.54	6.47E+09

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	3.97E+11	6	6.62E+10	3.750351	0.010784	2.572712
Within Groups	3.7E+11	21	1.76E+10			

Total	7.67E+11	27	

ANOVA: *M. avium* High Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1K µg/L	4	637465	159366.3	1.02E+10
PEX H	4	491192.7	122798.2	1.6E+10
NB H	4	442032.5	110508.1	1.91E+10
COMB H	4	112339.5	28084.87	1.46E+09
FE H	4	221640.4	55410.1	1.06E+10
IRON H	4	88169.83	22042.46	2.7E+08
MG H	4	239696	59924	4.86E+09

ANOVA: *A. polyphaga* Low Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
5 µg/L	4	4601.333	1150.333	2267599
Fe L	4	5993.603	1498.401	3189499
COMB L	4	5259.272	1314.818	2605041
PEX L	3	17909.28	5969.759	68482740
Iron L	4	53837.2	13459.3	2.82E+08
Mg L	4	15912.4	3978.1	27817871
NB L	4	18680.23	4670.057	26865681

ANOVA: *A. polyphaga* High Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
1K µg/L	4	64430.3	16107.57	9.2E+08
Fe H	4	5207.737	1301.934	2404260
COMB H	4	16476.08	4119.021	29696412
PEX H	4	32053.34	8013.336	1.98E+08
Iron H	2	88498.33	44249.17	1.74E+09
Mg H	4	26569.29	6642.323	1.1E+08
NB H	4	22780.3	5695.075	69615511

16S rRNA
Stagnation

5	PEX L	NB L	COMB L	FE L	IRON L	Mg L
5.90E+07	4.74E+07	3.94E+07	6.55E+07	3.99E+07	2.45E+07	6.20E+07
3.50E+06	5.00E+06	8.96E+06	4.91E+06	7.55E+06	7.77E+06	1.82E+06
8.37E+05	5.75E+06	2.01E+07	8.21E+06	6.66E+06	2.30E+06	2.85E+06
2.02E+06	7.94E+06	3.00E+06	3.43E+06	7.91E+06	1.60E+06	8.08E+05

16S rRNA
Stagnation

1K µg/L	PEX H	NB H	COMB H	FE H	IRON H	Mg H
8.68E+07	6.29E+07	4.84E+07	4.27E+07	3.83E+07	2.51E+07	5.08E+07
1.19E+07	7.59E+06	6.88E+06	5.96E+06	8.55E+06	5.43E+06	2.36E+06
8.96E+05	1.14E+07	1.70E+06	5.61E+06	2.18E+06	5.42E+06	3.45E+06
1.40E+07	5.86E+06	7.94E+04	4.58E+06	1.36E+06	7.17E+06	1.71E+06

HPC Stagnation

5 µg/L	PEX L	NB L	COMB L	FE L	IRON L	Mg L
5.35E+08	2.25E+05	1.06E+05	5.55E+04	6.70E+04	1.43E+05	1.65E+05
8.15E+05	4.17E+04	4.83E+04	2.67E+04	5.20E+04	9.73E+04	3.50E+04
4.63E+05	1.50E+04	2.45E+04	3.05E+04	6.95E+04	3.50E+04	9.00E+03
5.50E+05	1.35E+05	7.50E+04	6.70E+04	2.40E+05	1.14E+05	7.50E+04

HPC Stagnation

1K µg/L	PEX H	NB H	COMB H	FE H	IRON H	Mg H
5.35E+08	1.07E+05	1.12E+05	5.55E+04	8.30E+04	7.25E+04	1.33E+05
9.90E+05	7.73E+04	6.13E+04		4.37E+04	9.37E+04	4.23E+04
4.37E+05	6.20E+04	2.90E+04	8.00E+03	2.00E+04	9.80E+04	1.80E+04
1.01E+06	9.25E+04	4.95E+04	8.50E+03	7.50E+04	1.54E+05	1.85E+04

L. pneumophila
Stagnation

5 µg/L	PEX L	NB L	COMB L	FE L	IRON L	Mg L
7.88E+06	4.68E+06	5.19E+06	4.38E+06	6.08E+06	1.34E+06	2.49E+06
3.06E+06	3.68E+05	1.08E+05	3.54E+05	1.57E+02	8.08E+04	5.59E+04
2.20E+05	1.38E+05	3.92E+05	1.27E+05	8.48E+04	3.13E+04	1.70E+05

L. pneumophila
Stagnation

1K µg/L	PEX H	NB H	COMB H	FE H	IRON H	Mg H
1.31E+07	7.97E+06	7.73E+06	3.14E+06	5.74E+06	4.29E+06	4.28E+06
1.11E+07	4.62E+05	1.69E+05	3.21E+05	5.88E+05	1.94E+05	2.51E+04
1.72E+05	2.89E+05	3.15E+05	8.39E+04	2.10E+05	3.83E+04	7.93E+04

L. pneumophila
No stagnation

5 µg/L	FE L	COMB L	PEX L	IRON L	Mg L	NB L
5.69E+06	5.69E+06	5.69E+06	5.69E+06	5.69E+06	5.69E+06	5.69E+06
5.56E+05	3.31E+02	2.17E+04	6.39E+02	7.50E+03	2.55E+03	1.91E+03
4.03E+02	4.34E+02	7.38E+03		3.70E+03	1.96E+03	4.20E+03
3.78E+02	2.28E+03	7.03E+02	4.16E+02	1.18E+04	1.27E+03	5.81E+02
0.00E+00	1.81E+01	0.00E+00	5.50E+01	1.53E+02		0.00E+00

L. pneumophila
No stagnation

1K µg/L	FE H	COMB H	PEX H	IRON H	Mg H	NB H
5.69E+06	5.69E+06	5.69E+06	5.69E+06	5.69E+06	5.69E+06	5.69E+06
1.37E+06	4.90E+02	3.73E+04	2.26E+03	1.05E+04	4.27E+04	1.07E+05
3.82E+02	8.72E+01	1.03E+04	2.71E+03	1.11E+04	1.00E+03	1.81E+04
6.63E+01	5.81E+02	1.18E+03	6.00E+02		8.70E+04	2.79E+02
0.00E+00	0.00E+00	3.01E+02	0.00E+00	1.29E+02	1.77E+03	1.55E+01

M. avium
Stagnation

5 µg/L	PEX L	NB L	COMB L	FE L	IRON L	Mg L
2.99E+04	6.04E+04	8.13E+03	0.00E+00	3.17E+03	1.29E+04	1.30E+04
4.51E+05	9.39E+03	5.82E+04	1.48E+04	1.15E+04	6.21E+03	1.24E+04
6.84E+05	2.10E+03	1.45E+04	1.72E+03	1.80E+02	2.71E+03	2.08E+03
3.45E+05	1.38E+05	3.92E+05	1.27E+05	8.48E+04	3.13E+04	1.70E+05

M. avium
Stagnation

1K µg/L	PEX H	NB H	COMB H	FE H	IRON H	Mg H
212366.67	5.27E+03	4.59E+04	2.15E+04	2.88E+03	3.21E+04	1.50E+05
2.67E+05	1.49E+05	6.68E+04	6.27E+03	**8.17E+03**	1.59E+04	8.24E+03
1.18E+05	4.76E+04	1.40E+04	6.90E+02	5.73E+02	1.87E+03	1.90E+03
3.94E+04	2.89E+05	3.15E+05	8.39E+04	2.10E+05	3.83E+04	7.93E+04

M. avium **No stagnation**

5 µg/L	FE L	NB L	COMB L	PEX L	Mg L	IRON L
3.71E+05	3.07E+02	1.05E+03	0.00E+00	0.00E+00	2.21E+04	
3.80E+03	4.34E+02	4.20E+03	7.38E+03		1.96E+03	3.70E+03
3.37E+04	1.79E+04	4.29E+03	1.20E+05	1.94E+04	1.47E+04	5.95E+04
2.99E+04	3.17E+03	8.13E+03	0.00E+00	6.04E+04	1.30E+04	1.29E+04
2.45E+04	5.30E+02	1.67E+04	1.10E+04	1.58E+04	2.95E+03	8.86E+03

M. avium **No stagnation**

1K µg/L	FE H	NB H	COMB H	PEX H	Mg H	IRON H
2.80E+05	3.07E+02	2.19E+04	3.90E+02	0.00E+00	2.21E+04	
9.49E+03	4.34E+02	1.81E+04	1.03E+04		1.96E+03	1.11E+04
1.33E+05	1.79E+04	3.11E+04	1.91E+05	1.94E+04	1.47E+04	
2.12E+05	3.17E+03	4.59E+04	2.15E+04	6.04E+04	1.30E+04	3.21E+04
2.51E+04	5.30E+02	1.67E+04	8.15E+03	1.58E+04	2.95E+03	1.15E+04

A. polyphaga
No stagnation

5 µg/L	FE L	COMB L	PEX L	IRON L	Mg L	NB L
1.30E+03	5.61E+01	7.51E+01	1.55E+04	3.75E+04	1.17E+04	1.19E+04
2.02E+01	3.70E+03	1.79E+03		1.25E+04	1.19E+03	4.65E+03
3.23E+03	2.20E+03	3.39E+03	2.23E+03	3.26E+03	2.89E+03	2.07E+03
5.58E+01	3.47E+01	0.00E+00	2.24E+02	5.81E+02	1.26E+02	4.94E+01

A. polyphaga
No stagnation

1K µg/L	FE H	COMB H	PEX H	IRON H	Mg H	NB H
6.16E+04	9.85E+02	1.22E+04	2.91E+04	7.37E+04	2.23E+04	1.81E+04
2.99E+01	6.21E+02	1.54E+03	2.21E+02	1.48E+04	8.92E+02	2.03E+03
2.53E+03	3.55E+03	2.54E+03	2.54E+03		3.31E+03	2.53E+03
2.92E+02	4.72E+01	2.26E+02	2.23E+02		9.35E+01	1.05E+02

CHAPTER 3: APPENDIX D

Table 26: Experimental Summaries

Start Date	Experiment Title	Phase	Water/Experiment	Outcome
3/18/2010	B1	SGWH with Deficiencies (see Chapt 3)	• Norton et al., 2004 synthetic tap • initial addition 10mL from Blacksburg hot water heater • 37°C SGWH temperature • 3 water changes per week	**No amplification** or culturable *Legionella*. Identified issues with qPCR inhibition.
4/21/2010	A1	SGWH (Chap 2)	**SGWH Condition Change:** • Norton et al., 2004 synthetic tap, initial addition • 10mL from Blacksburg hot water heater • 37°C SGWH temperature • 3 water changes per week	**No amplification** of *Legionella*; issues with qPCR inhibition discovered during this phase of work; undetectable *Legionella* in biofilm.
9/27/2010	A2	SGWH (Chap 2)	**SGWH Condition Change:** following incubator increase to 50°C • 10% GAC filtered Tap • 90% WRF synthetic tap (Norton et al., 2004) • Temperature set to 32°C temperature following suggestion from Dr. Ashbolt (EPA). • 3 water changes per week	**No amplification** of *L. pneumophila* or *A. polyphaga*; *M. avium* had a correlation with low TOC range some of the time.
9/27/2010	B2	SGWH with Deficiencies (see Chap 3)	**SGWH Condition Change:** following incubator increase to 50°C • 10% GAC filtered Tap • 90% WRF synthetic tap (Norton et al., 2004) • Temperature set to 32°C temperature following suggestion from Dr. Ashbolt (EPA). • 3 water changes per week	**No amplification** of *L. pneumophila* or *A. polyphaga*; However, persistence beyond washout condition observed in Mg H, Iron L, and COMB H conditions.
3/21/2011	A3	SGWH (Chap 2)	**SGWH Condition Change:** • 10% GAC filtered Tap, 90% WRF synthetic tap (Norton et al., 2004), • Amino Acid addition Table 20 • pH adjustment to 7.5; DO adjustment to 4.0mg/L • Addition of Mg, Zn, and Fe • Inoculation with *H. vermiformis* • 1mm diameter glass beads • 32°C SGWH temperature • 1 water changes per week	**Slight amplification** of *L. pneumophila* from 1K and 7K samples with washout occurring in subsequent weeks; persistence and regrowth of *H. vermiformis* occurring in all conditions
3/21/2011	B3	SGWH with Deficiencies (see Chap 3)	**SGWH Condition Change:** • 10% GAC filtered Tap, 90% WRF synthetic tap (Norton et al., 2004), • Amino Acid addition Table 20 • pH adjustment to 7.5, DO adjustment to 4.0mg/L • Addition of Mg, Zn, and Fe • Inoculation with of *H. vermiformis* • 1mm diameter glass beads • 32°C SGWH temperature • 1 water change per week	**Slight amplification** of *L. pneumophila* from PEX H and NB H samples with washout occurring in subsequent weeks; persistence and regrowth of *H. vermiformis* in most SGWH conditions.
4/19/11	A4	SGWH (Chap 2)	**SGWH Condition Change:** • 10% GAC filtered Tap, 90% WRF synthetic tap (Norton et al., 2004), • Amino Acid addition Table 20 • pH adjustment to 7.5, DO adjustment to 4.0mg/L • Addition of Mg, Zn, and Fe • Inoculation with of *H. vermiformis* • 1mm diameter glass beads • 32°C SGWH temperature • 1 water change per week	**Slight amplification** of *L. pneumophila* from 300, 3K and 7K µg/L samples with washout occurring in subsequent weeks; persistence and some regrowth of *H. vermiformis.*

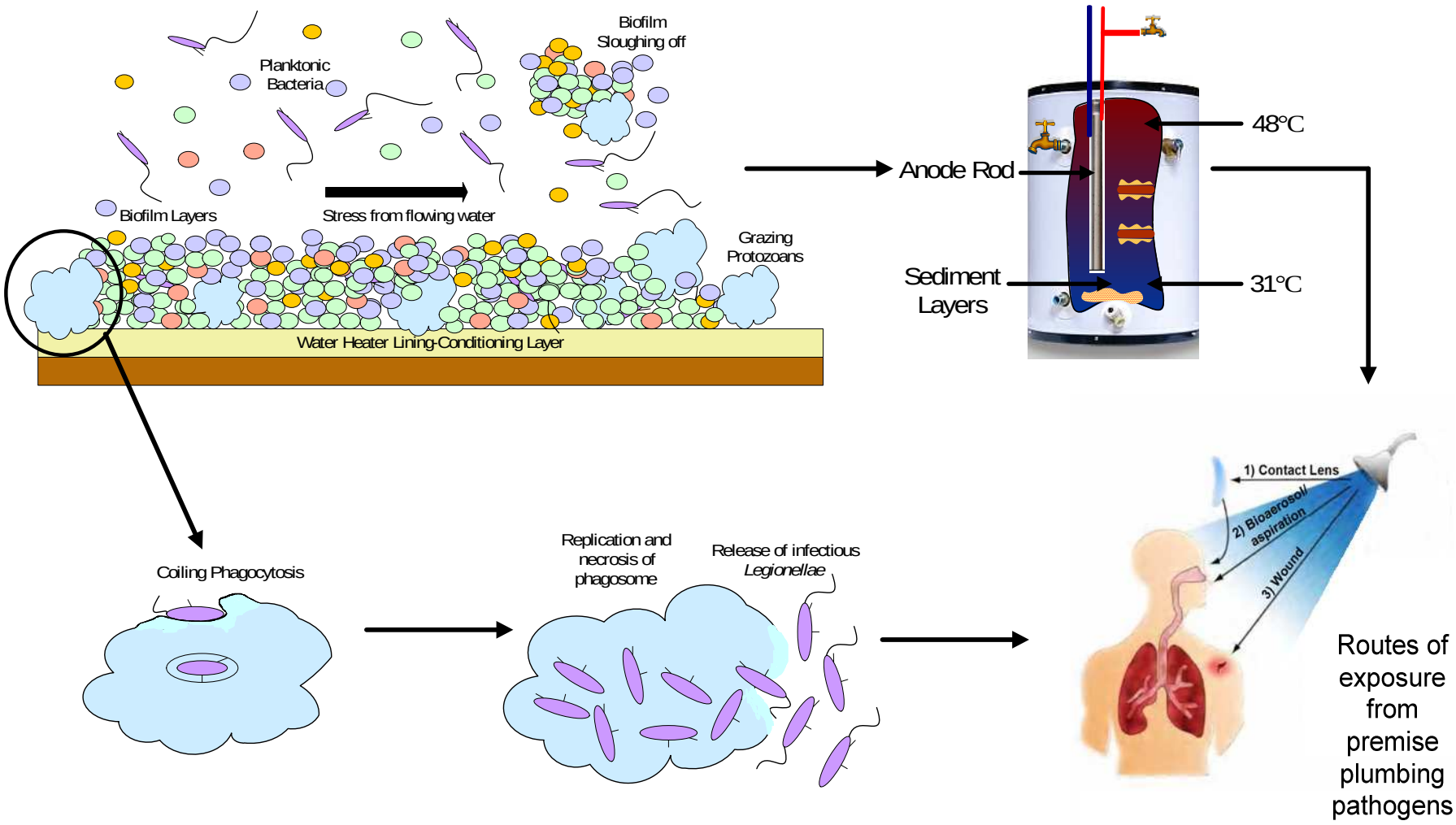

Figure 34: Life cycle schematic of *L. pneumophila* in a premise plumbling system. *L. pneumophila* can be phagocytized into a amoebae cell in pipe and water heater biofilms, proliferate, and ultimately cause necrosis of the host organism; free *Legionella* can then infect the lung cells of a consumer.