Legionella pneumophila in Domestic Hot Water Systems: Evaluation of Detection Methods and Environmental Factors Affecting Survival

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Abstract

Legionella is the causative agent of Legionnaires' disease which hospitalizes 8,000 to 18,000 people in the United States each year. The disease in transmitted through inhalation or aspiration of water containing the bacterium and can be acquired within the home. Studies have found that 0-37% of domestic water heaters contain *Legionella*, making household hot water systems a potential route of exposure.

The objective of this research was to evaluate different methods for testing environmental samples for *Legionella pneumophila* and to analyze potable water conditions that affect survival of free living *Legionella pneumophila* in hot water tanks. Three heat pretreatment methods (50ºC for 30 minutes, 55ºC for 15 minutes, and 60ºC for 3 minutes) were not effective at recovering *Legionella* in this study. There was no statistically significant difference between the three acid pretreatment methods that were tested (pH 2.0 with a neutralizing solution, pH 2.2, and the CDC method). Six media (BCYE, DGVP, PCV, GPCV, CCVC, and GPVA) exhibited similar *Legionella* recovery, except for when high levels of non-*Legionella* organisms were present, in which case BCYE demonstrated lower recovery. When disinfectant was present, if sodium thiosulfate was not added before the disinfectant, *Legionella* recovery was lower. However, this result was not statistically significant for free chlorine until after 5 minutes. *Pseudomonas aeruginosa* (up to 67.5 cfu/ml) and pyocyanin (up to 9 mg/l) did not have an effect on *Legionella* recovery under the tested conditions.

Environmental factors affecting survival of free living *Legionella pneumophila* in hot water tanks were also studied. After one day exposure in small-scale simulated water heaters at 55ºC, viable *Legionella* could not be recovered. At 44ºC, *Legionellae* were recovered after one day but only at very low levels after eight days. Between 23 and 37ºC, *Legionella* could survive longer

than eight days. Copper (Cu^{2+}) concentrations above 2160 ppb were found to be toxic to *Legionella*, but iron (Fe^{3+}) between 1 and 2160 ppb did not affect survival. Above pH 11 survival was greatly reduced. No effect was observed between pH 5-10. When glass fiber filters were added to the reactors and they were seeded with tap water and sediment slurry, *Legionellae* were retained in 7 of 16 reactors for 327 days.

The results of this work will assist in optimal identification of *Legionella* via microbial analysis of potable water samples, thereby assisting in prevention and diagnosis of factors contributing to Legionnaires' disease, especially in settings with high-risk patients (e.g. hospitals). Water systems studying *Legionella* amplification in domestic hot water systems can use simulated or real distribution system sampling to reproduce and study factors that prevent or reduce *Legionella* growth and persistence.

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Attribution

A brief description of the contributions of each co-author is included here.

Dr. Marc A. Edwards, Charles Lunsford Professor of Civil and Environmental Engineering, Virginia Tech. Dr. Edwards is the primary Advisor and Committee Chair. He provided the research direction for all work contained in this thesis, contributed to the conclusions of this work, and edited the manuscript.

Dr. Joseph O. Falkinham, III, Professor of Biological Sciences, Virginia Tech. Dr. Falkinham provided laboratory support and assisted with microbiological analyses. In addition, he contributed to the preparation of a paper resulting from this work which will be submitted for publication.

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CHAPTER 1

LEGIONELLA IN POTABLE WATER DISTRIBUTION SYSTEMS

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ABSTRACT

Legionella, the causative agent of Legionnaires' Disease, affects thousands of people each year in the United States. This critical review emphasizes several aspects of *Legionella* relative to potable water treatment and premise plumbing including the microbiology of the organism, laboratory methods for its detection, environmental factors that affect survival, and colonization within 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 that had been saved from earlier pneumonia-associated deaths, and further analysis led to identification of several *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 population, which is very similar to the rate of 10 cases per million population detected in Europe (WHO 2005, CDC 2007). Due to 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, whereas the

Occupational Safety and Health Administration (OSHA) estimates over 25,000 cases occur each year (OSHA). These estimates equate to 28 – 86 cases each year per million population, and are slightly higher than the 20 cases per million population estimated for Europe (WHO 2005). 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%. For individuals who are not immuno-suppressed the death rate is 10-15% and total estimated deaths per year in the U.S. is about 4,000 (OSHA). The direct healthcare cost of nosocomial (i.e., acquired in hospital) legionellosis in America is estimated to be over $34 million (McCoy 2005).

1.2 MICROBIOLOGY

1.2.1 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 make cell staining difficult (Garrity 2005, WHO). *Legionellae* are urease-negative, catalase-positive, heterotrophic, aerobic, chemoorganotrophic, and transitionally motile (Garrity 2005). When motile, they have one or more straight or curved polar or lateral flagella. *Legionellae* utilize amino acids for energy and carbon, do not oxidize or ferment carbohydrates, and require L-cysteine-HCl and iron salts for growth amongst other nutrients. There are presently 48 identified species of *Legionella*, of which 20 have been associated with Legionnaires' disease and/or Pontiac fever (Fields *et al* 2002). Several species are divided into serogroups. Most human infections are associated with *L. pneumophila*, especially serogroups 1, 4, and 6 (OSHA).

1.2.2 Life Cycle

Legionella has a two phase lifecycle, and is infectious only 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). During this phase *Legionellae* search for a host to infect. Once the bacteria

find a host, they enter it through coiling phagocytosis and reside within the phagosome (McCoy 2005). *Legionellae* inhibit phagosome-lysosome fusion, thereby avoiding bacterial lysis. Living freely 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). *Legionellae* multiply within the host and overpopulate the phagosome, completely overwhelming it in as little as 48 hours (McCoy 2005). The bacteria then return to the infectious phase and the protist undergoes necrosis, bursting and releasing *Legionellae* into the environment to find another host. Without a host, growth of *Legionella* has only been observed on 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). Some of the *Legionella* hosts are also known to cause infection. *Acanthamoebae* can infect contact lenses 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).

1.3 LEGIONELLOSIS

1.3.1 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). At the beginning 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. Nervous system symptoms including disorientation or confusion are also present in about 25% of cases.

Legionnaires' disease is transmitted through inhalation or aspiration of water containing *Legionella* (OSHA). Recently, more emphasis has been placed on aspiration as the primary mode of transmission (EPA 1999, Yu 1993). There is no evidence of person-to-person transmission or of human infection from animals, hence human infection is not considered to be part of the *Legionella* life cycle (EPA 2001). Instead, human infection can be viewed as a "dead-end" for *Legionella* because once they enter the human body they are not re-released into the environment in high numbers. Human exposure to water containing *Legionella*, or aerosols derived from this water, is considered the primary source of infection (EPA 1999). Water sources that have been implicated in Legionnaires' disease infections include cooling towers, domestic hot water systems, humidifiers, spas, and dental water lines (OSHA). Although *Legionella* has been detected in ice machines, this source has never been implicated as a source of infection (Stout *et al* 1985b).

Legionella infects the macrophages of a human body similar to the way it infects protozoa. 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 and protozoa. These differences are in the requirement for actin polymerization, the role of host cell protein synthesis, and the induction of apoptosis (Fields *et al* 2002).

The general population is resistant to *Legionella* infection, as evidenced by the low attack rates of Legionnaires' disease despite widespread prevalence of the bacteria (EPA 2001). However, certain risk factors can increase a person's chances of contracting the disease. Some of these risk factors are due to increased exposure, such as patients who require ventilators. Other factors are due to increased susceptibility, such as men over the age of 50, heavy smokers, heavy drinkers, cancer patients, and bone-marrow and organ transplant patients (AWT 2003, EPA 2001). Legionnaires' disease rarely occurs in HIV patients, 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, however people without risk factors can certainly acquire the disease.

The risk factors for Legionnaires' disease often result in high rates of nosocomial infections, because hospitalized patients are very susceptible. In a study of nosocomial Legionnaires' disease in England and Wales, it was found that 8% of cases were hospital-acquired (Joseph *et al* 1994). The death rate for nosocomial infections is much higher than the average and may be up to 50% (NIH 2007). Legionnaires' disease is also often acquired during travel, perhaps due to exposure through hotel water or whirlpool spas (EPA 1999). It is difficult to detect travel-related outbreaks 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 and the patient must have positive laboratory results through culture or antigen testing. It is impossible to differentiate Legionnaires' disease from other pneumonias using a chest x-ray, thus follow-up laboratory testing is necessary (OSHA). However, follow-up testing is often not completed unless the patient exhibits risk factors for Legionnaires' disease. Treatment 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. Several *Legionella* species also produce β-lactamase to inactivate penicillin (McCoy 2005). It has been shown that proper diagnosis and early treatment is very important in reducing death rates (Heath *et al* 1996). There is no vaccine available for Legionnaires' disease (EPA 1999).

1.3.2 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). Unlike Legionnaires' disease, the incubation

period is one to three days and recovery occurs within 2 to 5 days without antibiotics. The attack rate of Pontiac fever is much higher than Legionnaires' disease; approximately 90 percent of people who are exposed will contract the illness.

1.3.3 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 most similar to that in guinea pigs, thus guinea pigs are often used in experimental studies. Berendt *et al.* determined that the median lethal dose of aerosolized *L. pneumophila* serogroup 1 is 1.4×10^5 cells, and intake of as little as 5 colony forming units into the lungs will infect 100% of guinea pigs (Berendt 1980).

1.4 LABORATORY METHODS

1.4.1 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. If there is chlorine in the water, it should be neutralized with 0.5 ml of 0.1N sodium thiosulfate for each liter of disinfectant (CDC 2005b). However, the CDC does not specify timing for the sodium thiosulfate addition. An alternative is to use sample bottles containing sodium thiosulfate pellets (McCoy 2005).

Since *Legionella* often grows within biofilms, swab samples should be collected from faucets and showerheads, preferably with the aerator or showerhead removed. Swabs should be submerged in 3-5 ml of water collected from the site to prevent drying during transportation (CDC 2005b). Stout *et al.* recommends that samples should be stored at 2-8ºC both before and after processing, unless it is a hot water sample which will be processed within 24 hours (Stout 1998b). OSHA, however, 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).

1.4.2 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 1-1). The purpose of the charcoal is to decompose exogenous hydrogen peroxide to which *Legionella* is sensitive to (Hoffman *et al* 1983). Casein acid hydrolysate contains sodium chloride, which was in the first culture medium, was later found to inhibit the growth of virulent *Legionella pneumophila* (Catrenich and Johnson 1989). Thus, this protein source was later replaced with yeast extract. The ACES buffer and α-ketoglutarate increase the recovery of *Legionella* on the medium (Edelstein 1981). Adding 1.0% bovine serum albumin to the media (ABCYE) has been shown to enhance the growth of *Legionella micdadei* and *Legionella bozemanii* (Morrill *et al* 1990). Other media have been developed to culture *Legionella*, 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). However, BCYEα is considered to be the standard medium for *Legionella* isolation. The net improvement in *Legionella* detection limits attributable to improvements on the original Mueller-Hinton media has not been established.

Table 1-1: Improvements 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

If a sample contains low levels of *Legionella* it may be necessary to concentrate the sample. 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 free the bacteria from the filter (CDC 2005b). This method can be used to concentrate *Legionella* (and other bacteria) by a factor of up to 100, however if samples larger than 1 L are collected a greater concentration is possible. In centrifugation, the water sample is centrifuged and the supernatant decanted. Centrifugation has been examined at 1000g for 10 minutes, 3800g for 30 minutes, and 8150g for 15 minutes (Ta *et al* 1995, Boulanger and Edelstein 1995). In each of these cases filtration has been demonstrably more effective at concentrating *Legionella* than centrifugation. However, with either 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 BCYE and 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 media and streaked perpendicularly with an inoculating loop (ACHD 1997). Culture media should be incubated between 35 and 37ºC in a humid atmosphere and colonies will appear after 3 to 5 days. Although the CDC recommends incubation with 2.5% CO_2, *L. gormanii* is the only *Legionella* species that has been shown to grow better in this condition (CDC 2005b, Garrity 2005).

To improve the detection of *Legionella* in environmental and clinical samples, it is sometimes desirable to add antibiotics to the media. These compounds serve to inhibit non-*Legionella* organisms which may compete with *Legionella* and reduce detection limits. Anisomycin inhibits yeasts, cycloheximide inhibits fungi, cefamandole and vancomycin inhibit staphylococci, and glycine helps to inhibit environmental flora, but not respiratory flora (Stout 1998b, Harrison and Taylor 1988). It should also be noted that 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 (Tables 1-2, 1-3).

Table 1-2: 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 1-3: 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

In addition to selective media, there are heat and acid pretreatment methods which can also inhibit non-*Legionella* organisms. *Legionella* is relatively acid and heat resistant compared to other bacteria, so controlled exposure to heat and acid allow survival of *Legionella* while inactivating many competitors. Heat pretreatment of collected environmental samples occurs via exposure to a 50ºC water bath for 30 minutes followed by rapid cooling. This method 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 55ºC water bath for 15 minutes or a 60ºC water bath for 3 minutes (WHO, Wilkinson 1987, Edelstein *et al* 1982b). There is also a heat enrichment method designed to improve recovery of *Legionella* from samples that initially appear to be negative.

By incubating samples at 35ºC and culturing every 2 weeks for up to 6 weeks, *Legionella* can multiply within protozoa and reach a detectable level (CDC 2005b). This method is useful for recovering *Legionella* from samples that initially appear to be negative, however the initial concentration of *Legionella* in the sample cannot be determined. Since this process can take up to 6 weeks, the concentration of *Legionella* in the water system may have changed during this time.

There are several methods for acid pretreatment (Table 1-4). 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. Still 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). It has been shown that these acid pretreatments have a negative effect on *L. wadsworthii*, but does not appear to have a statistically significant effect on other *Legionella* species (Calderon and Dufour 1984).

Table 1-4: 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 HCl	Not specified	1:1	15; 30 if needed	CDC 2005b	

When processing samples, 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. General guidance has been written to help laboratories decide which method(s) to use on a given sample (Table 1-5).

Table 1-5: 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

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 are round and convex with younger colonies having a 'ground-glass' appearance (CDC 2005b, Harrison and Taylor 1988). The edges are typically pink or purple however younger colonies may appear to have 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 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).

1.4.3 Molecular Methods

The standard culture method is an expensive, time-consuming process and can require over a week to produce results. Molecular methods produce results much faster, typically after one or two days (McCoy 2005). Polymerase chain reaction (PCR), can be used to determine the presence or absence of *Legionella*, however it cannot differentiate between viable and non-viable bacteria. The possibility also exists for PCR inhibition in which the presence of compounds such as rust or some divalent cations (calcium, magnesium, silver, or copper) can cause false negative results (Miskowski, Maiwald *et al* 1994). However, an inhibitor control can be used to determine if PCR inhibition is a concern for the sample. In a study of 260 samples from hot water systems and cooling towers in France, Joly *et al.* determined that PCR inhibition prevented analysis of up to 2.7% of samples (Joly *et al* 2006). Additionally, PCR is species-specific and many laboratories are not able to identify species other than *L. pneumophila* (Miskowski). Quantitative real-time PCR can be used to enumerate *Legionella* in a sample, however the results of this test are in genome units (GU) per liter instead of colony forming units (cfu) per liter used in the culture method (Joly *et al* 2006). GU/l only has a weak correlation with cfu/l, thus it is difficult to compare the two methods (Wellinghausen *et al* 2001). Samples containing less than approximately 250 cfu/l are not measurable by the quantitative real-time PCR method and cutoff values can differ greatly between laboratories (Joly *et al* 2006). Villari *et al.* proposed a method in which samples are analyzed using PCR and, if negative, concentrated using filtration and then tested using culture methods (Villari *et al* 1998). This method has the advantage of fast detection if *Legionella* concentrations are high. 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).

1.4.4 Air Detection Methods

The two most successful methods for detecting airborne *Legionella* involve capture of cells via gas impingement to a liquid and or gas impingement on a solid media 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 study comparing 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). Due to the high 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. Two Anderson samplers should be used and run simultaneously, one containing BCYE media and the other containing a selective *Legionella* medium (CDC 2005b). Air sampling is not a recommended method for measuring potential exposure because there is a high probability of false-negative results (DHS 2007).

1.5 ENVIRONMENTAL FACTORS

1.5.1 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, and 25 to 68% of dental-unit waters are colonized with *Legionella* (Table 1-6).

Table 1-6: *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	

Hot water tanks are especially prone to *Legionella* colonization. Although the recommended temperature to control *Legionella* is 60ºC, scalding can occur within six seconds at this temperature (SCBPS 2005, CPSC). Thus, the United States Consumer Product Safety Commission recommends 49ºC to control scalding. But this relatively low temperature allows *Legionella* to colonize these tanks (CPSC). The Canadian Standing Committee on Building and Plumbing Services seeks to address this issue by recommending that water heaters be set at 60ºC and that measures be taken, such as installing a temperature regulator, to ensure the temperature supplied to distal sites does not exceed 49ºC (SCBPS 2005). In addition, the factory setting for water heater thermostats in Canada is 60ºC (Stanwick *et al* 1981). Even when water heater thermostats are set at 60ºC, electric water heaters can still become colonized with *Legionella* due to the position of the heating elements. That is, in electric water heaters, the lower heating element is positioned 15-20 cm above the bottom of the tank (Joly 1985). This creates a temperature gradient where the bottom of the tank can be much cooler than the bulk water. The bottom of the tank can also collect sediment, which serves as a nutritional source for *Legionella* 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). This is believed to be due to the position of the heating element at the very bottom of the tank in gas water heaters, which prevents temperature stratification.

In order to link occurrence of Legionnaires' disease with a water source, the same serogroup and subtype of *Legionella* that is found in the patient's sputum must be found in the water (OSHA). This has been done in several studies, which is considered strong proof that the household water supply can be the 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). A study in the United Kingdom found that 12% of *Legionella* cases were due to drinking water (VROM 2004).

1.5.2 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). In two surveys in Italy, copper levels above 50 ppb were correlated with lower *Legionella* colonization (Borella *et al* 2005, Borella *et al* 2004). One survey noted that six times less *Legionellae* were present in water samples containing greater than 50 ppb copper. The effect of iron on *Legionella* is less clearly defined. Some studies have found 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). However, it is known that *Legionellae* need iron to grow in culture media, and virulence is significantly reduced in iron-limited conditions (McCoy 2005, James *et al* 1995). States *et al.* found that very high levels of iron (>50 ppm) had a toxic effect (States *et al* 1985). The samples were collected from hot water tanks with pH between 7.14 and 7.76, thus pH was not a factor. Zinc concentrations both above 200 ppb and below 100 ppb have been correlated with lower *Legionella* levels, indicating that the optimal amount of zinc for *Legionella* growth may be between 100 and 200 ppb (Borella *et al* 2005, Borella *et al* 2004).

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). Although a study of Italian hotels demonstrated that magnesium has a negative correlation with *Legionella*, a study of Pittsburgh water supplies indicated that there was no correlation (Borella *et al* 2005, Stout *et al* 1992a). Conflicting results have been found regarding the association of calcium and *Legionella*, with two studies showing positive, one negative, and another no correlation (Zacheus and Martikainen 1994, Marrie *et al* 1994, Stout *et al* 1992a, Borella *et al* 2005). While one study found there was a positive correlation between nitrate and *Legionella*, another found a negative correlation between nitrate + nitrite and *Legionella* (Zacheus and Martikainen 1994, Marrie *et al* 1994). Negative correlations have also been found with sodium, barium, chloride, and hardness, and positive correlations with potassium, phosphate, sulphate,

and TOC (Zacheus and Martikainen 1994, Marrie *et al* 1994, States *et al* 1985, Ortiz-Roque and Hazen 1987).

The pH range that is 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). *Legionellae* are believed to survive in dissolved oxygen concentrations between 0.3 and 9.6 ppm, however in one study naturally occurring *L. pneumophila* grew between 6.0 to 6.7 mg/l and not 1.7 to 2.2 mg/l (Wadowsky *et al* 1985). *Legionellae* are well-adapted to surviving in low-nutrient conditions. At lower temperatures (5 and 24ºC), it was found that *L. pneumophila* could survive in tap water for up to 299 days with the maximum survival rate (80%) occurring at 5ºC in sterilized tap water (Hsu *et al* 1984). It has also been 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).

At lower temperatures (4 to 20ºC) *Legionella* can survive at up to 3% sodium chloride, while at higher temperatures (30 and 37ºC) 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 study demonstrated that while *L. pneumophila* was noncultivable in hot spring water with salt concentrations almost the same as seawater, the bacterium maintained metabolic activity (Ohno *et al* 2003).

Since amino acids serve as the carbon source for *Legionella*, 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 had similar results, except instead of phyenylalanine or tyrosine they found that glutamic acid was required (George *et al* 1980, Tesh and Miller 1981). Tesh *et al.* also 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, however they are present only at low concentrations, if at all, in potable water (Chinn and Barrett 2000, Chellam and Xu 2004). Amino acids can also be destroyed by exerting a chlorine demand on the system (Hureiki *et al* 1994).

1.5.3 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* (Wadowsky and Yee 1983, Toze *et al* 1990). Some bacteria are also inhibitory 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).

In addition to growth on culture media, *Legionella* growth in water can be influenced by other organisms. Stout *et al*. found that environmental bacteria and sediment both improved the survival of *Legionella pneumophila* in environmental samples (Stout *et al* 1985a). However, it is 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 protist hosts (Declerck *et al* 2005). In a study in Finnish apartments, it was found that microbial biomass, thermophilic bacteria at 50ºC, and fungi at 20ºC were all positively correlated with *Legionella* (Zacheus and Martikainen 1994). Algae, *Pseudomonas* and flavobacteria are noted by OSHA for their ability to supply essential nutrients for *Legionella* growth, and *P. aeruginosa* has been shown to support the replication of *L. pneumophila* in *Naegleria lovaniensis* and *Acanthamoeba castellanii* (OSHA, Declerck *et al* 2005). In contrast, 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).

1.5.4 Physical Factors

Legionellae can survive in a wide range of temperatures. In a survey of lakes and rivers, Fliermans *et al.* found *Legionella* at temperatures between 5.7 and 63ºC (Fliermans *et al* 1981). 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 60ºC (Flannery *et al* 2006, Arnow and Weil 1984, Borella *et al* 2005). Older 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). Larger hospitals have an increased prevalence of *Legionella*, as well as buildings greater than 10 stories (Marrie *et al* 1994, Flannery *et al* 2006).

It has been found that copper pipes limit *Legionella* colonization, but iron pipes promote it (van der Kooij *et al* 2005, 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, but also by testing the water in actual hotel water systems and correlating it to the nearest pipe material. Rubber and silicone within the distribution system also can support *Legionella* growth, and perhaps *Legionella* can attach to its surface easier than other distribution system components (Schofield and Locci 1985, Schofield and Wright 1984). Stagnation and interruptions in water service increase the prevalence of *Legionella*, possibly due to lower disinfectant levels (Storey *et al* 2004, Flannery *et al* 2006). Turbulent conditions can cause large clusters of *Legionella* to become detached and move into the bulk water (Storey *et al* 2004).

1.5.5 Airborne Legionella

Since *Legionella* is an aquatic pathogen, and humidity is also an important factor in determining the persistence and suspension of aerosols, humidity is an important consideration relative to human exposure. Hambleton *et al.* found that *L. pneumophila* aerosols stayed in the air and survived better at 65% relative humidity relative to 30%, 55%, or 90% (Hambleton *et al* 1984). Alternatively, Dennis and Lee found that it 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 completed by Dennis *et al.*, a shower bath was studied in which the initial relative humidity was 55% and the relative humidity after 15 minutes was 100%. Before the shower was turned on and after 15 minutes, no *Legionella* were recovered from the air, however *Legionellae* were found between 0 and 15 minutes. This indicates that at a relative humidity between 55% and 100%, *Legionellae* were able to become airborne and stay airborne for a significant time period (Dennis *et al* 1984b). It is possible that at lower levels of humidity there is not enough water present for *Legionella* to survive in air and at higher levels of humidity droplets are much larger and the *Legionella* settle out faster and do not stay airborne.

Deloge-Arbarkan *et al*. found that the concentration of *Legionella* in the water was not correlated with their airborne abundance, which obviously provides a challenge relative to setting hazard guidelines because ultimately exposure depends on specifics of the local environmental and other factors such as 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. For *Mycobacterium*, another 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).

Similar factors may affect the concentration of *Legionella* within aerosols and might be important to human infection under some 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). This issue has never been researched.

1.6 PREVENTION OF LEGIONNAIRES' DISEASE

1.6.1 Risk Assessment

There are conflicting views about whether sampling for *Legionella* is worthwhile when no Legionnaires' disease cases have been reported. The Allegheny County Health Department in Pennsylvania recommends that hospitals should sample yearly if no transplants are performed and more often if they are (ACHD 1997). This is to detect *Legionella* in the water system before outbreaks occur. At least ten distal sites (water fountains, showers, faucets etc.) should be tested if there are less than 500 beds. If there are more than 500, two sites should be tested for every 100 beds. 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).

A level-one investigation consists of an overview of the water system, a walk-through 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 walkthrough where water samples are collected, initiation of an employee awareness program, and a review of worker absences. If more than one case of Legionnaires' disease has been diagnosed, a level-two investigation should be implemented. Otherwise, a level-one investigation is sufficient. If the outbreak is considered to be ongoing, control measures should be immediately implemented.

Several action levels have been published to assist hospitals and building managers when *Legionella* is detected in the water. OSHA recommends prompt cleaning and/or biocide

treatment when *Legionella* levels in cooling towers exceed 100 cfu/ml, domestic water exceeds 10 cfu/ml, or a humidifier exceeds 1 cfu/ml (OSHA). Immediate cleaning and/or biocide treatment and limitation of employee exposure is recommended at levels above 1000 cfu/ml for cooling towers, 100 cfu/ml for domestic water, or 10 cfu/ml for humidifiers. The biocide treatment recommended by OSHA is either continuous chlorination above 1 mg/l or the addition of bromine.

Pathcon Laboratories has also developed suggested action criteria with five different levels 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).

1.6.2 Disinfection

There are several methods for disinfecting 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 are the super heat and flush method, chlorination, chloramination, chlorine dioxide, bromine, and copper-silver ionization. The super heat and flush method involves heating hot water tanks to 70ºC and flushing for 30 minutes, ensuring the temperature at each outlet is at least 60ºC (ACHD 1997). Recolonization can occur within weeks to months (Lin *et al* 1998). However, if the hot water temperature is maintained at 60ºC this can be delayed. A 90% reduction of *Legionella* can be achieved at 45ºC for 2500 minutes, 50ºC for 380 minutes, 60ºC for less than 5 minutes, and 70ºC for less than 1 minute.

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). 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; one university reported 30 times more leaks during 3 years of continuous chlorination (Rutala and Weber 1997). Chlorine is also less effective when the pH of the water is above 8.0 (OSHA). Another disadvantage of continuous hyperchlorination is the formation of trihalomethanes, which are suspected carcinogens (EPA 2001). 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).

Chloramine 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). This may be because chloramine is more stable and penetrates biofilms better than free chlorine (Lin *et al* 2000). However, chloramines act slower than free chlorine, and thus require longer contact time (WHO).

Chlorine dioxide penetrates biofilms better than chlorine and is also a better oxidizer (WHO). Shock treatment of chlorine dioxide (50 – 80 mg/L for 1 hour) followed by continuous treatment (3 – 5 mg/L) greatly reduces 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 are not many studies that have been completed on this topic (Thomas *et al* 2004).

Bromine also has suspected advantages relative to chlorine in that it is less corrosive, is 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.

Copper-silver ionization entails releasing positively charged copper and silver ions into the system. These positive ions bond with the negatively charged cell wall, altering cell wall permeability, denaturing proteins, and leading to cell death (ACHD 1997). Recolonization takes about 2 months after the treatment 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). Copper-silver ionization may not be an effective long-term disinfection method. 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*, however it decomposes quickly and is more expensive (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 (Domingue *et al* 1988). Disinfection with ultraviolet light involves treating 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 88ºC 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.

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CHAPTER 2

COMPARATIVE PERFORMANCE OF *LEGIONELLA PNEUMOPHILA* DETECTION METHODS

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ABSTRACT

Recovery of *Legionella pneumophila* from potable water samples was systematically evaluated using published culture methods and pretreatments designed to improve detection in the presence of competing organisms. The heat pretreatment methods (50ºC for 30 minutes, 55ºC for 15 minutes, and 60ºC for 3 minutes) were not effective at recovering *Legionella* in this study using an oligotrophic synthetic potable water. Three acid pretreatment methods (pH 2.0 with a neutralizing solution, pH 2.2, and the CDC method) were equally effective in reducing competition from other organisms and improving detection of *Legionella*. No difference was seen in *Legionella* recovery on six media (BCYE, DGVP, PCV, GPCV, CCVC, and GPVA) except when high levels of non-*Legionella* organisms were present, in which case BCYE demonstrated much lower recovery due to overgrowth of background bacteria. When sodium thiosulfate was not added before the disinfectant, *Legionella* recovery was lower. However, this effect was not statistically significant for free chlorine until after 5 minutes. *Pseudomonas aeruginosa* (up to 67.5 cfu/ml) and pyocyanin (up to 9 mg/l) had no effect on *Legionella* recovery under the tested potable water conditions.

2.1 INTRODUCTION

Legionella pneumophila is an aquatic opportunistic pathogen that causes Legionnaires' Disease. It has been estimated that Legionnaires' Disease causes 8,000-18,000 cases of hospitalization in the United States every year, of which 5-30% are lethal (CDC 2005a). *Legionella pneumophila* is transmitted through inhalation or aspiration of water containing the bacterium. Assessing risk

and diagnosing sources of infection requires effective detection of *Legionella* in environmental water samples via culture methods. The main challenges in quantifying *Legionella* in potable water samples, and therefore in identifying risks, include: 1) collecting samples that are representative of "worst case" exposure, 2) quenching of disinfectant, 3) overcoming competition from other microbes, and 4) identifying *Legionella* even at low levels.

2.1.1 Improving Detection by Reducing Competition from Other Organisms

The basic medium for growing *Legionella* is Buffered Charcoal Yeast Extract (BCYE) (Edelstein 1981). However, many non-*Legionella* organisms grow on BCYE as well, and overgrowth of these organisms can reduce detection limits for *Legionella*. Thus, there are several methods to inhibit non-*Legionella* organisms in samples. There are acid and heat pretreatment methods as well as selective media that are made by adding antibiotics to BCYE agar.

Heat Pretreatment. The main heat pretreatment method used for environmental samples is performed by placing the sample in a 50ºC hot water bath for 30 minutes and then submerging it in ice water before plating (Dennis *et al* 1984). Two other methods for heat pretreatment are submerging the sample in a 55ºC hot water bath for 15 minutes or a 60ºC hot water bath for 3 minutes (WHO, Edelstein *et al* 1982). This study will examine recovery of *Legionella* using three published heat pretreatment methods.

Acid Pretreatment. Acid pretreatment methods combine the sample with an acid-buffer solution in a specified ratio and allow the mixture to react for a specific amount of time before plating. The acid-buffer solution consists of hydrochloric acid and potassium chloride in a ratio which can range from 3.9:25 to 1:18 depending on which method is used (Ta *et al* 1995, CDC 2005b). The solution pH can range from 2.0 to 2.5, the acid to sample ratio can range from 1:1 to 1:9, and the reaction time can range from 3 to 30 minutes (Gorman *et al* 1985, Bollin *et al* 1985, Ta *et al* 1995, Bopp *et al* 1981, Rowbotham 1983). In addition, a neutralizing solution may be added to raise the solution to a neutral pH before plating (Gorman *et al* 1985). It is also believed that Cu^{+2} can be toxic to *Legionella*, and copper is often present in premise plumbing water at high levels

due to copper pipes. Thus, it was deemed of interest to test whether acidification of samples containing particulate copper might sometimes hinder recovery of *Legionella* due to the change in copper speciation at the lower pH.

Selective Culture Media. BCYE agar can be made more selective by adding antibiotics to the media. While the CDC suggests the use of PCV and GPCV media, Standard Methods calls for GPVA and CCVC (Table 2-1) (CDC 2005b, Clescerl *et al* 1999). Also, Stout *et al.* suggest the use of DGVP for environmental samples (Stout 1998). These five selective media as well as BCYE were compared for *Legionella* recovery.

Table 2-1: Antibiotic Additions to BCYE

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
PCV	CDC 2005b	100	80	5					
GPCV	Dennis *et al* 1984	100	80	5	0.3%				
DGVP	Vickers *et al* 1987	50		1	0.3%	10			
GPVA	CDC 1992	100		5	0.3%				80
CCVC	Bopp *et al* 1981		80	0.5			4	16	

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

2.1.2 Sample Handling

There are several published methods for recovering *Legionella* from environmental samples. However, there are currently no well-defined protocols for sample collection and treatment. *Legionella* is expected to be associated with sediment and thrive in stagnant parts of the hot water system with low chlorine residual and moderate temperatures such as that found in the bottom of water heaters (Stout *et al* 1985). Thus, detection of *Legionella* can be expected to be a strong function of the manner in which samples are collected as is known for detection of lead hazards in premise plumbing (Triantafyllidou *et al* 2007). Factors that are likely to be important include stagnation time in the system before sampling, flow rate at which the sample is be collected, wasting of water before collection, and the effect of mixing samples before plating. Each of these factors can be expected to impact *Legionella* recovery. Another issue of concern is

time of thiosulfate addition. While the United States Centers for Disease Control and Prevention (CDC) calls for the addition of 0.5 ml of 0.1 N sodium thiosulfate to each liter of sample, it does not identify the critical time period at which sodium thiosulfate should be added (CDC 2005b).

2.1.3 Potential Specific Inhibition

Several bacteria including *Aeromonas hydrophila, Aeromonas salmonicida, Flavobacterium meningosepticum,* and *Pseudomonas aeruginosa* are known to inhibit *Legionella* growth on culture media (Paszko-Kolva *et al* 1993). This effect has also been observed in our laboratory (Figure 2-1). Leoni *et al.* found that *Pseudomonas aeruginosa* has a negative correlation with *Legionella* in environmental samples, however this effect has not been reproduced in a laboratory setting (Leoni *et al* 2001). Under some conditions, such as iron limitation, *Pseudomonas aeruginosa* produces pyocyanin which can have lethal effects on human cells and gram-positive bacteria (O'Malley *et al* 2003).

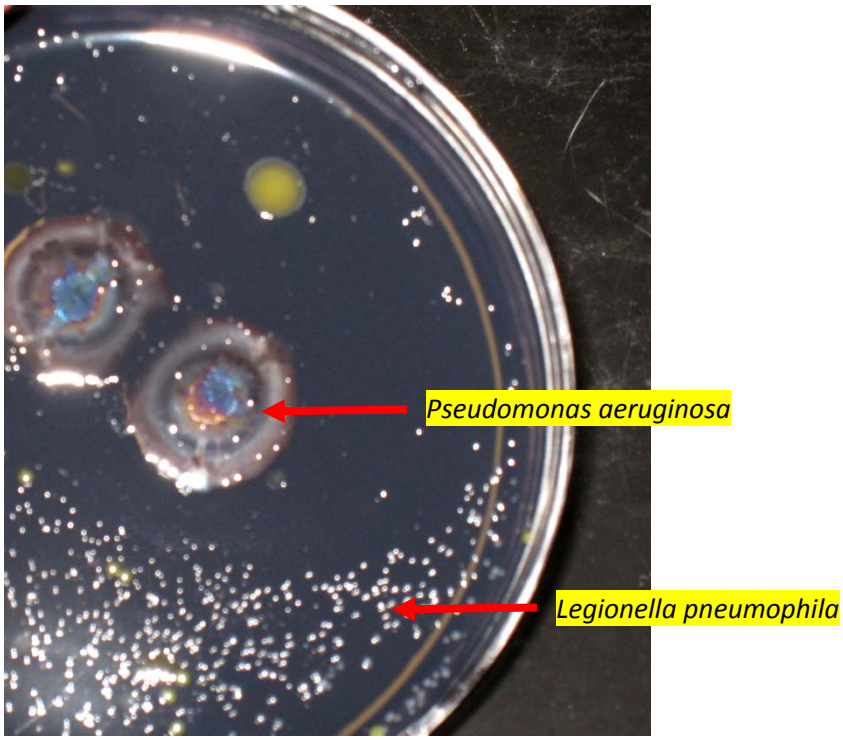

Figure 2-1: Inhibition of *Legionella pneumophila* by *Pseudomonas aeruginosa* on BCYE agar

The overall goal of this research is to systematically examine the impact of sodium thiosulfate addition time, methods of inhibiting non-*Legionella* organisms, and the effects (if any) of *Pseudomonas aeruginosa* and pyocyanin on *Legionella* recovery in synthesized potable water.

2.2 EXPERIMENTAL METHODS

A synthesized potable water in which viable *Legionella* could be maintained was used as a base solution (Table 2-2) and ozonated organic matter (humic acid that was ozonated until there was 80% color destruction) was added as a carbon source. H_2SO_4 and KOH were used to adjust the pH.

Table 2-2: Base Water Components

Chemical	Concentration (ppm)
Ozonated Natural Organic Matter as Total Organic Carbon	0.18
Na_2HPO_4	0.01
KNO_3	9.655
$NaHCO_3$	170.52
$MgSO_4$	39.3
$Al_2(SO_4)_3$	0.732
$NaSiO_2$	26.296
$CaSO_4$	25.25
$CaCl_2$	20.54
$CuCl_2$ (as Cu^{2+})	0.01
$FeCl_3$ (as Fe^{3+})	0.01
Chelated Iron ($C_{10}H_{12}FeN_2NaO_8$ as Fe)	0.005
pH	7

2.2.1 Heat Pretreatment

Three heat pretreatment methods were compared by first creating three 100-ml synthetic water solutions and inoculating them with *Legionella pneumophila*. The *Legionella* inoculating solution was created by adding medium-grown *Legionella pneumophila* (ATCC 33152) to sterile deionized water. Testing of this solution via serial dilutions on BCYE agar (0.1 mL spread with

a glass rod) determined the concentration of viable *Legionella* added (Edelstein 1981). The plates were incubated at 37ºC for 4 days and *Legionella* colonies were counted.

The three heat pretreatment methods were performed by placing 2 ml into a 50ºC hot water bath for 30 minutes, 55ºC for 15 minutes, or 60ºC for 3 minutes (Dennis *et al* 1984, WHO, Edelstein *et al* 1982). After the designated time the sample was placed in ice. A control was also used for each solution which was not subjected to heat pretreatment. 0.1 ml of the sample was spread onto 3 BCYE and 3 DGVP agar plates with a sterile glass rod. The plates were incubated at 37ºC for 4 days and examined for possible *Legionella* colonies with a 20x stereo microscope. Possible *Legionella* colonies were streaked onto BCYE and blood agar and incubated at 37ºC for 48 hours. If the colony grew on BCYE but not on blood agar, it was considered a presumptive *Legionella* colony.

2.2.2 Acid Pretreatment

Three acid pretreatment methods were compared and tested for *Legionella* at 3, 5, 15, and 30 minutes. The acid-buffer solutions tested were pH 2.2, pH 2.0 with a neutralizing solution, and the CDC method. The pH 2.0 and 2.2 solutions were created by mixing 25 ml of a 0.2 M KCl solution and 3.9 ml of a 0.2 M HCl solution and adjusting to the proper pH with 0.1 M KOH (Bopp *et al* 1981, Gorman *et al* 1985). The neutralizing solution was a 0.01 M KOH solution at pH 12.0. The solution for the CDC method was created by mixing 18 parts 0.2 M KCl with 1 part 0.2 M HCl (CDC 2005b). All acid pretreatment solutions were autoclaved to sterilize before use. Three 100-ml synthetic water solutions were created and inoculated with *L. pneumophila* as previously described. Each of these solutions was then subjected to the three acid pretreatment methods. 2 ml of the synthetic water solution was added to 2 ml of the acid-buffer solution and allowed to react for the specified amount of time. If the neutralizing solution was used, 2 ml of the neutralizing solution was added. The mixture was then spread onto 3 BCYE plates to test for *Legionella*. Incubation conditions and presumptive identification of *Legionella* were completed as previously described. The effect of copper on acid pretreatment was also studied. A 10 ppm solution of copper particulate solids collected from a water distribution system producing “blue water” was added in a 1:1 ratio to a synthetic water solution containing *Legionella* to create a 5

ppm copper solution. This solution was then tested for *Legionella* by plating onto DGVP and reacting with pH 2.0 buffer solution for 5 minutes and plating on BCYE.

2.2.3 Selective Culture Media

Five antibiotic combinations were added to BCYE agar to make the media more selective (Table 2-1). These selective media were compared by preparing three 100-ml synthetic water solutions and inoculating with *L. pneumophila* as previously described. 0.1 ml of each solution was then spread with a sterile glass rod onto 3 of each selective agar. Incubation and presumptive identification for *Legionella* was completed as previously described.

2.2.4 Sample Handling

The effect of the time of sodium thiosulfate addition on *Legionella* concentration was tested by first preparing and inoculating three 100-ml synthetic water solutions as previously described. 0.5 ml of 20 mg/l free chlorine or chloramine was added to 5 ml of each solution and 0.5 ml of 0.001 N sodium thiosulfate was added at a specified time before or after the disinfectant. These times were: before the disinfectant, 5 seconds after, 1.5 minutes after, and 5 minutes after. This method was used to simulate a sample collection in which *Legionella* growing in a biofilm with no chlorine was suddenly mixed with chlorinated or chloraminated bulk water and some time elapsed before sodium thiosulfate was added to the sample to neutralize the disinfectant. The condition where sodium thiosulfate was present in the bottle before collecting the sample is henceforth referred to as the sample in which sodium thiosulfate was added at time 0. A control for each synthetic water was also performed in which deionized water was added instead of disinfectant. 2 ml of each solution was reacted with 2 ml of pH 2.2 acid-buffer solution for 5 minutes. 0.1 ml of the acid pretreated solution was spread onto 3 BCYE plates and 0.1 ml of the non-acid pretreated solution was spread onto 3 DGVP agar plates with a sterile glass rod. The plates were incubated and examined for *Legionella* as previously described.

2.2.5 Potential Specific Inhibition

To test the potential inhibiting effect of *Pseudomonas aeruginosa* on *L. pneumophila*, six 100-ml synthetic water solutions were prepared and inoculated with *L. pneumophila* as previously described. *Pseudomonas aeruginosa* (ATCC 9027) was added to create a 67.5 cfu/l solution in two bottles and 67.5 cfu/ml in two bottles. *P. aeruginosa* was not inoculated into two bottles to serve as a control. The *Pseudomonas aeruginosa* inoculating solution was prepared in the same manner as with *Legionella*, however serial dilutions were spread onto cetrimide agar instead of BCYE. The cetrimide was incubated at 37ºC for 24 hours and *P. aeruginosa* colonies were counted. Each solution was tested for *Legionella* 30 minutes and 24 hours after *P. aeruginosa* was added to determine the effect that *P. aeruginosa* had on *L. pneumophila* in this aquatic system. The solutions were tested for *Legionella* in the same manner as the sodium thiosulfate tests.

To test the effect of pyocyanin on *Legionella*, an additional six 100-ml synthetic water solutions were prepared and inoculated with *L. pneumophila*. Pyocyanin was added to create a 0.041 mg/l solution in two bottles, 0.41 mg/l in two bottles, and 9 mg/l in one bottle. Pyocyanin was not added to the sixth bottle to serve as a control. After 24 hours the bottles were tested for *Legionella* in the same manner as the sodium thiosulfate tests.

2.3 RESULTS AND DISCUSSION

2.3.1 Heat Pretreatment

The listed heat pre-treatment methods all killed the *Legionella* in the synthesized tap water, with the exception of the sample was immersed in a 60ºC water bath for 3 minutes in which less than 40% of the *Legionella* were recovered relative to the control without heat pretreatment (Figure 2-2). No *Legionellae* were recovered after 15 minutes at 55ºC or 30 minutes at 50ºC. Several studies have used these heat pretreatment methods successfully while only slightly affecting the *Legionella* concentration (Jousimies-Somer *et al* 1993, Dennis *et al* 1984). These studies used colonies that were isolated from tap water and may have contained host organisms which protect

Legionella from environmental stresses. No amoebae could be identified from the samples utilized in this research. These results indicate that in some situations the heat pretreatment methods may not always effectively recover *Legionella* from environmental samples. Of the available methods, treatment with 60ºC for 3 minutes seems to be the least likely to cause problems with recovery.

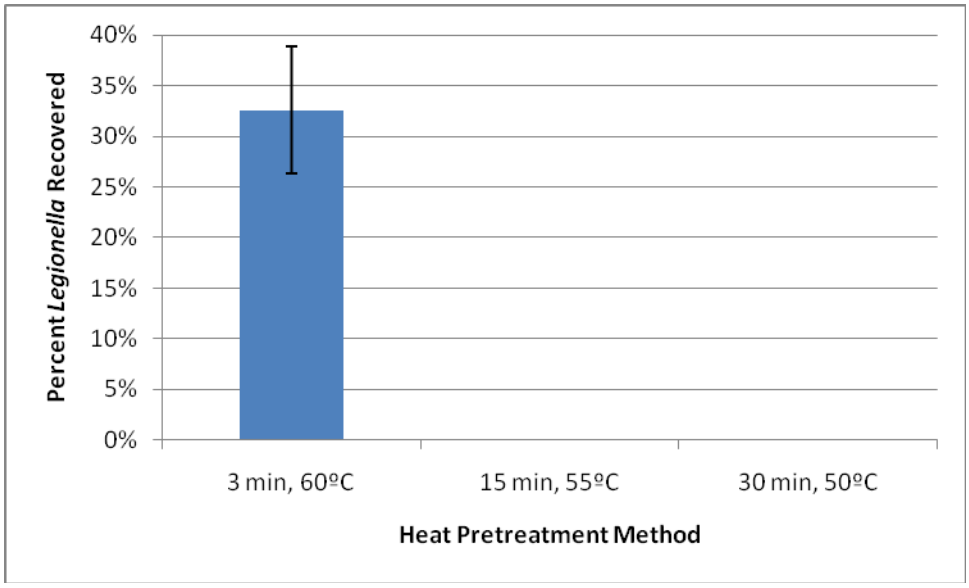

Figure 2-2: Comparison of percent *Legionella* recovered with different heat pretreatment methods. The error bars denote 95% confidence intervals.

2.3.2 Acid Pretreatment

All three acid pretreatment methods were equally effective in recovering *Legionella* (Figure 2-3). The 3 and 5 minute reaction times did not show statistically significant differences. After 15 minutes, all methods showed less *Legionella* recovery than at 3 or 5 minutes, however this was only statistically significant for pH 2.2. After 30 minutes of acid pretreatment, recovery of all methods was significantly impacted. These results are similar to those found by Ta *et al.* who compared the recovery at 3 and 15 minutes after treatment with pH 2.2 (Ta *et al* 1995). Ta found that acid pretreatment for 15 minutes reduced *Legionella* however not to a statistically significant level. If acidification was conducted in the presence of 5 ppm copper, *Legionella* survival was not different than without acidification (Figure 2-4).

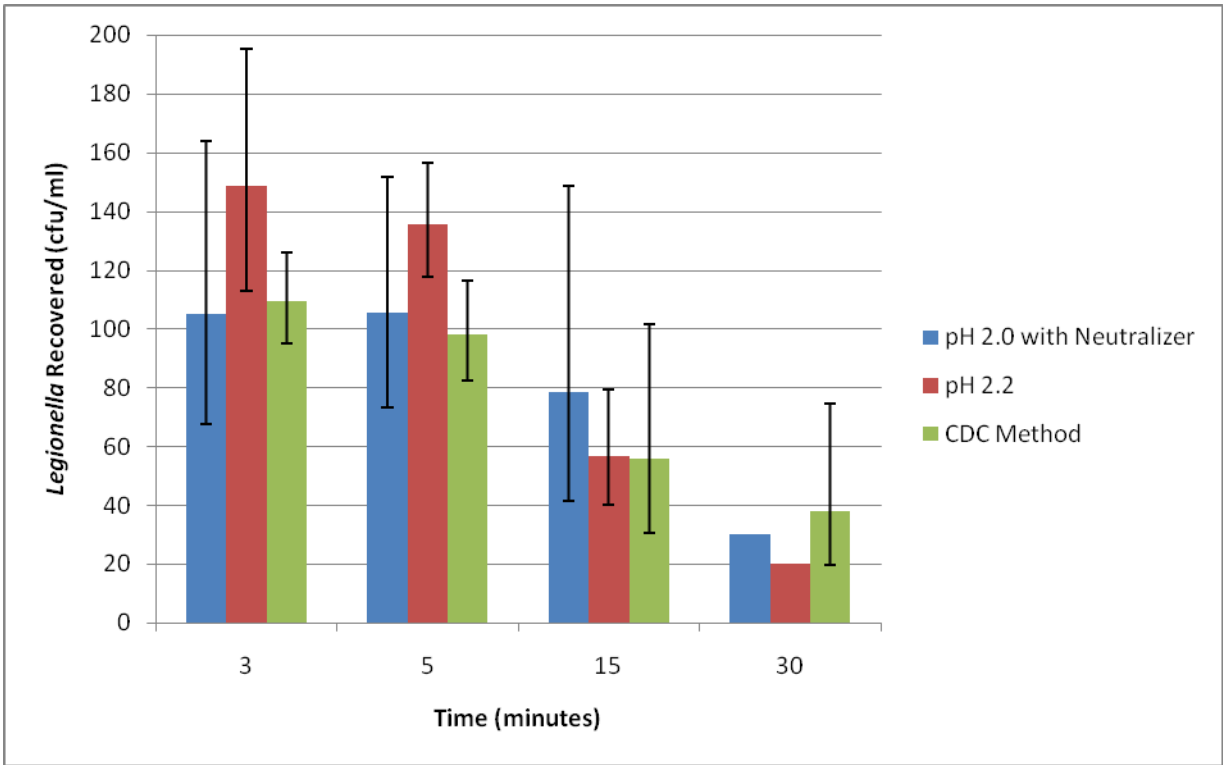

Figure 2-3: Comparison of *Legionella* recovered with different acid pretreatment methods over time. The error bars denote 95% confidence intervals.

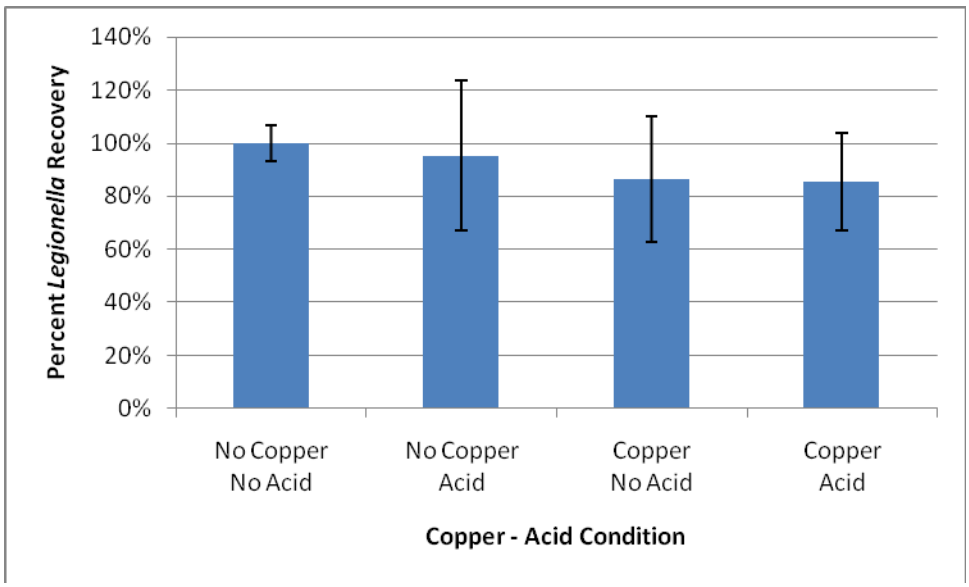

Figure 2-4: Comparison of *Legionella* recovery as percent of average amount recovered with no copper and no acid when subjected to 5 ppm copper and/or pH 2.0 acid for 5 minutes. The error bars denote 95% confidence intervals.

2.3.3 Selective Culture Media

There was no statistically significant difference in *Legionella* recovery between the selective media tested using a one-way ANOVA test ($p = 0.21$) (Figure 2-5). The selective media were also not significantly different than BCYE. This was probably due to the low levels of background bacteria present in these samples. When similar tests were performed on samples containing higher levels of background bacteria, detection limits on BCYE for *Legionella* were greatly reduced due to overgrowth of other bacteria (Figure 2-6).

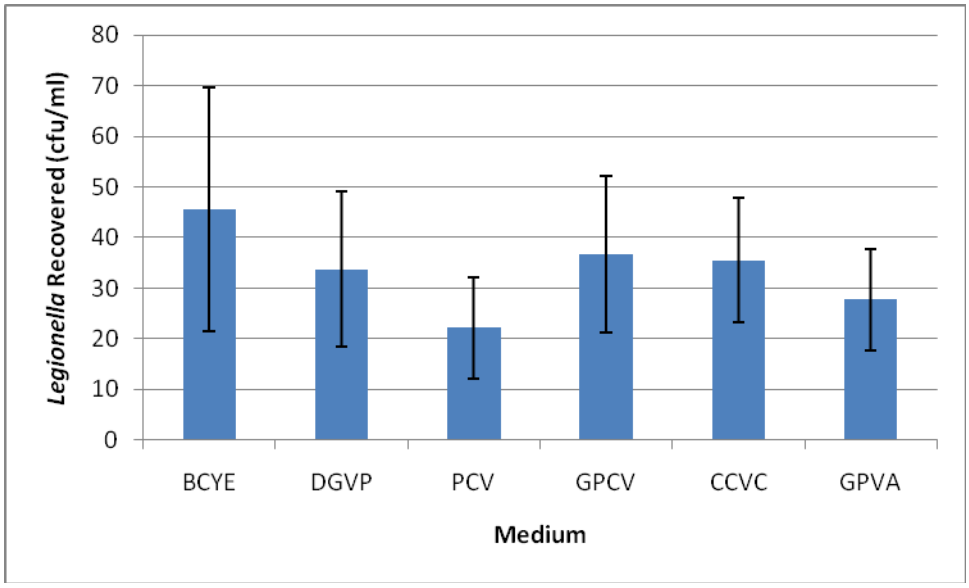

Figure 2-5: Comparison of *Legionella* recovered on selective media from samples containing low levels of background bacteria. The error bars denote 95% confidence intervals.

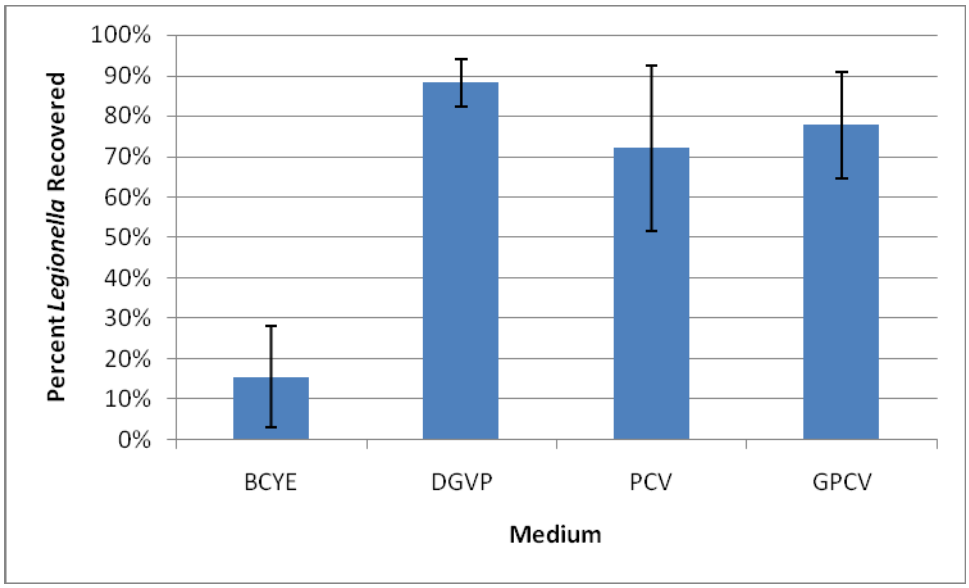

Figure 2-6: Comparison of percent *Legionella* recovered on selective media from samples containing high levels of background bacteria. The error bars denote 95% confidence intervals.

2.3.4 Sample Handling

When sodium thiosulfate was added before the disinfectant, it did not matter if disinfectant was present in the sample or not (Figure 2-7). In the case where sodium thiosulfate was added 5 seconds after the disinfectant, less *Legionella* was recovered than when no disinfectant was added. However, this difference was only statistically significant for chloramine using the student's t-test ($p = 0.007$). After 1.5 minutes the difference was statistically significant for both chloramine and free chlorine ($p = 0.03$ for chloramine, $p = 0.009$ for free chlorine). Decay rates were similar for free chlorine and chloramine. These results indicate that sodium thiosulfate should be present in sample bottles before water sample collection in order to immediately scavenge the disinfectant and to maximize *Legionella* recovery.

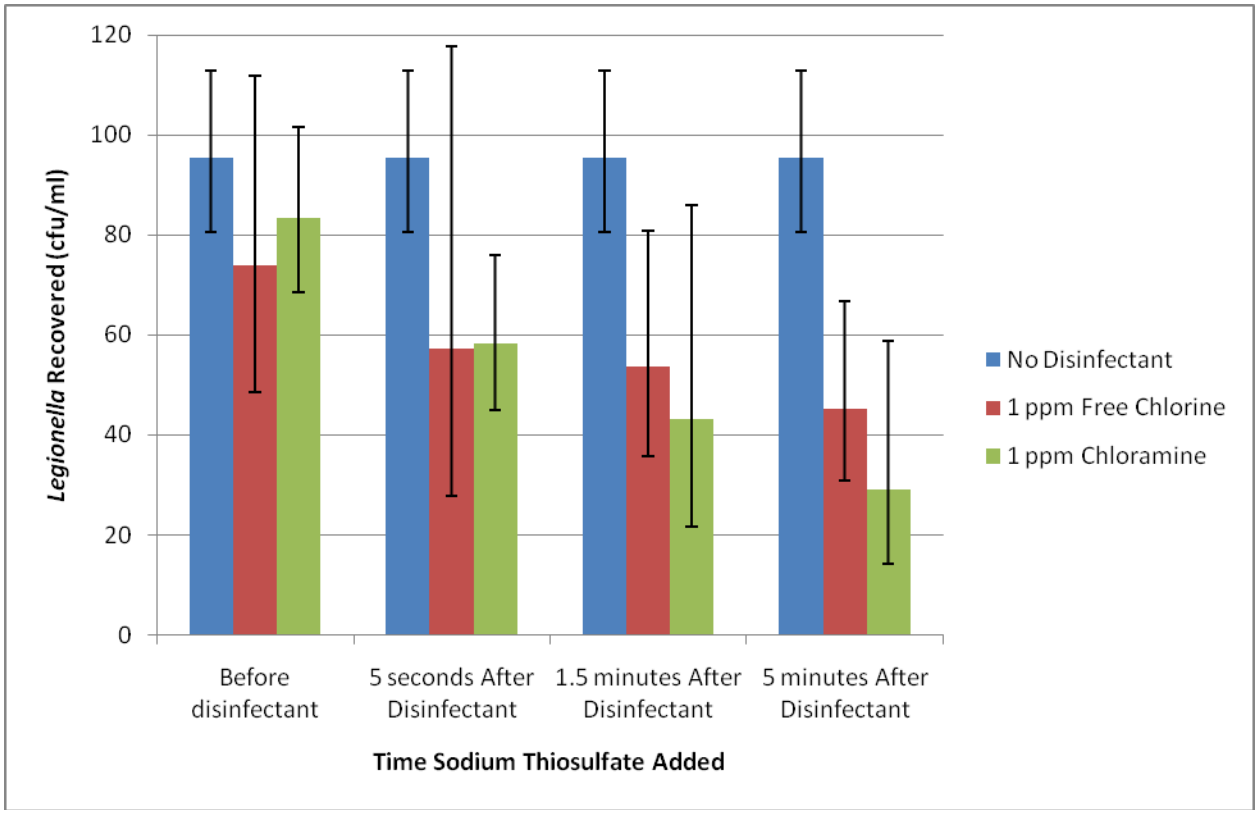

Figure 2-7: Comparison of *Legionella* recovered with time sodium thiosulfate added. The error bars denote 95% confidence intervals.

2.3.5 Potential Specific Inhibition

Pseudomonas aeruginosa concentration did not have an effect on *Legionella* in the synthetic water solution (Figure 2-8). This indicates that the inhibitory factor observed on solid media is either not produced in an aquatic environment, is too dilute in an aquatic environment, or is not produced in the conditions tested in this study. More studies should be performed to determine if there are *P. aeruginosa* virulence factors that can inhibit *Legionella* in aquatic systems and the environmental conditions under which these virulence factors may be expressed. Pyocyanin also had no effect on the *Legionella* concentration.

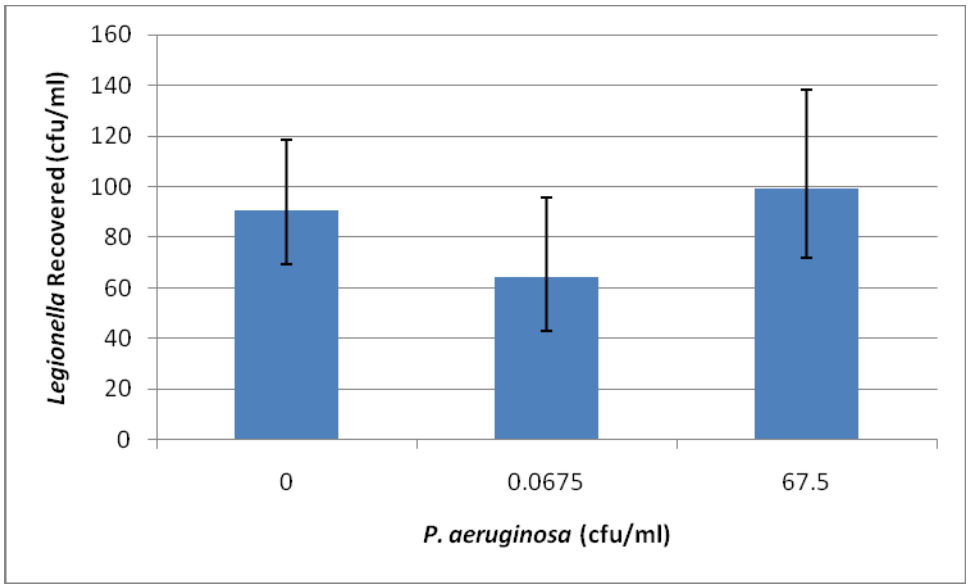

Figure 2-8: Comparison of *Legionella* recovered with *Pseudomonas aeruginosa* concentration. The error bars denote 95% confidence intervals.

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CHAPTER 3

ENVIRONMENTAL FACTORS AFFECTING THE SURVIVAL OF *LEGIONELLA PNEUMOPHILA* IN DOMESTIC HOT WATER SYSTEMS

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ABSTRACT

Environmental factors that affect the short-term survival of free living (without a host organism present) *Legionella pneumophila* in domestic water heaters were examined. After exposure in small-scale simulated water heaters using synthetic potable water above 55ºC, viable *Legionella* was not recovered after one day exposure. At 44ºC *Legionella* survived one day but were recovered only at very low levels after eight days. Between 23 and 37ºC, *Legionella* survived more than 8 days. Copper (Cu^{2+}) that can be present in domestic plumbing systems was highly toxic to *Legionella* at levels above 2.16 ppm. Iron (Fe^{3+}) between 1 and 2160 ppb did not affect *Legionella* survival. Survival was generally only a weak function of pH, but above pH 11 was much reduced. *Legionellae* were retained in the system for longer periods of time when extra surface area from glass fiber filters was present and if bottles were seeded with tap water and a sediment slurry. The filters presumably provided surface area for biofilm growth as well as environmental factors that enhanced survival in water heater environments.

3.1 INTRODUCTION

Legionella pneumophila is a waterborne pathogen estimated to cause between 8,000 - 25,000 cases of Legionnaires' Disease in the United States every year (CDC 2005, OSHA). Transmission is via inhalation or aspiration and potable water has been determined to be the cause of several cases of Legionnaires' disease (OSHA, Stout *et al* 1992b). Surveys have found that 0 to 37% of residential water systems contain detectable *Legionella*, and one study in Quebec determined that 37% of the electric water heaters contained *Legionella* (Stout *et al*

1992a, Arnow and Weil 1984, Alary and Joly 1991). Thus, it is of considerable interest to better understand physical and chemical factors that influence *Legionella pneumophila* survival in domestic water heaters.

Legionellae are known to multiply within protist hosts, including 14 species of amoebae, two species of ciliated protozoa, and one species of slime mold (Fields *et al* 2002). Without a host, growth has only been observed on laboratory media. With few exceptions, previous research on the subject has studied *Legionella* in either real tap water environments or synthetic growth media. While both approaches have advantages, when using tap water it is impossible to measure the exact composition of the water, which can vary markedly especially in premise plumbing systems. This variation also hinders comparison of results between different studies. The synthetic growth media that have been used to date contain nutrients at much higher levels than are encountered in potable water (Table 3-1). To complement prior research on this subject, this work utilized oligotrophic synthetic potable water, which was systematically modified to determine environmental factors that affect short term *Legionella* survival in water heaters. The work also attempted to determine whether *Legionella* could ever replicate in a significant way without an amoeba host, considering the confines of nutrient levels that might be encountered in potable water systems.

Table 3-1. Comparison of carbon, nitrogen, potassium, and phosphorus in Ristroph's chemically defined liquid medium and the synthetic water tested in this study

	Carbon (ppm)	Nitrogen (ppm)	Potassium (ppm)	Phosphorus (ppm)
Chemically Defined Liquid Medium (13)	8500	36.5	92	31
Range tested for synthetic water in this study	8 - 25	0.05 - 1.7	0.1 - 3.7	0 - 0.04

3.2 EXPERIMENTAL METHODS

Small-scale simulated water heaters were created using 118 ml square glass bottles to simulate the glass which is bonded to the inside of hot water tanks. Each bottle was filled with 100 ml of

synthetic water and inoculated with 100 µl of *Legionella pneumophila* inoculation water. The inoculation suspension was prepared by adding culture-grown *Legionella pneumophila* (ATCC 33152) to sterile deionized water. The inoculation water was tested for *Legionella* to determine the amount that was added to the synthetic water. The solution was tested by creating serial dilutions and spreading 0.1 ml of each dilution with a sterile glass rod onto BCYE agar (Edelstein 1981). The glass bottles were placed in a covered container and heated to a constant 37ºC. The synthetic base water was created by adding chemicals into deionized water to simulate potable water from the Potomac River in Potomac, Maryland (Table 3-2). The ozonated natural organic matter was prepared by ozonating humic acid until 80% color destruction was achieved. pH was altered using H_2SO_4 or KOH. Several modifications to this base water were studied (Table 3-3). Another modification studied utilized addition of amino acids at levels typical of the range found in potable water (Table 3-4) (Chinn and Barrett 2000, Chellam and Xu 2004). The amino acids added were based on those added by Ristroph *et al.* in a chemically defined liquid medium for *Legionella pneumophila* (Ristroph *et al* 1981).

Table 3-2. Base water components

Chemical	Concentration (ppm)
Ozonated Natural Organic Matter as Total Organic Carbon	0.18
Na_2HPO_4	0.01
KNO_3	9.655
$NaHCO_3$	170.52
$MgSO_4$	39.3
$Al_2(SO_4)_3$	0.732
$NaSiO_2$	26.296
$CaSO_4$	25.25
$CaCl_2$	20.54
$CuCl_2$ (as Cu^{2+})	0.01
$FeCl_3$ (as Fe^{3+})	0.01
Chelated Iron ($C_{10}H_{12}FeN_2NaO_8$ as Fe)	0.005
pH	7

Table 3-3. Modifications to base water

Modification	Concentrations Tested
Ozonated Natural Organic Matter as Total Organic Carbon	0, 45, 180, 720 ppb
Na_2HPO_4	0, 10, 200 ppb
KNO_3	0.322, 9.655 ppm
$NaHCO_3$	56.84, 170.52 ppm
$CuCl_2$ as Cu^{2+}	1, 10, 60, 360, 2160 ppb
$FeCl_3$ as Fe^{3+}	1, 10, 60, 360, 2160 ppb
pH	5, 6, 7, 8, 9, 10, 11
Temperature	23, 30, 37, 44, 55 ºC
Ammonia	0, 1.0 ppm
Chlorine	0, 0.25 , 2.5 ppm
Chloramine	0, 0.40, 4.0 ppm

Table 3-4. Concentration of amino acids in base water

Amino Acid	Concentration (ppb)
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-Lysine	10.30
L-Methionine	3.17
L-Phenylalanine	5.55
L-Proline	1.82
L-Serine	10.30
L-Threonine	5.23
L-Tryptophan	1.58
L-Tyrosine	6.34
L-Valine	7.60
L-Choline	0.32
L-Rhamnose	25.35

After one day, the water was tested for *Legionella.* Longer-term tests were also performed in which the initial water containing *Legionella* was replaced with synthetic water that did not contain *Legionella*. The bottle was filled again within one minute of emptying the bottle. This

water change was performed two times per week. This was to simulate the emptying and filling of a water heater that occurs within a house.

The effect of glass fiber filters and seeding with non-synthetic water was also studied. In these cases, a 1.75 inch by 1.75 inch square glass fiber filter was placed in the bottom of the bottle. The bottle was seeded with 50 ml of water collected from an unchlorinated domestic water heater supplied with well water from Blacksburg, Virginia and 30 µl of sediment slurry from a small outdoor pond on the Virginia Tech campus in Blacksburg, Virginia. The bottles were allowed to sit for one week before replacing it with synthetic water and inoculating with *Legionella*. One month after inoculation the glass fiber filter was removed.

100 ml samples were collected in sterile polypropylene sample bottles and stored at 4ºC for a maximum of 24 hours before processing. 0.1 ml of the sample was spread with a sterile glass rod on 2 DGVP plates (Vickers *et al* 1987). 2 ml of the sample was acid pretreated with 2 ml of pH 2.0 acid-buffer solution for 5 minutes (Bopp *et al* 1981). 0.1 ml of the acid pretreated sample was spread with a sterile glass rod on 2 BCYE plates (Edelstein 1981). The plates were incubated for 4 days in a humid atmosphere at 37ºC. Plates were then inspected with a 20x stereo microscope. Possible *Legionella* colonies were streaked onto BCYE and blood agar and incubated at 37ºC for 48 hours. If the colony grew on BCYE and not on blood agar, it was considered a presumptive *Legionella* colony.

3.3 RESULTS AND DISCUSSION

The concentration and persistence of *Legionella* varied between each reactor. At first this was interpreted as differences in *Legionella* amplification rates as a function of nutrient levels, but a longer term study demonstrated that the differences were due to highly variable washout rates between the different conditions. Using this experimental setup and considering cumulative *Legionella* recovery in the range of waters tested, there was no strong evidence that *Legionella* replication occurred. That is, in no case was the cumulative recovery of *Legionella* greater than the initial number of *Legionella* inoculated even after 327 days (Figure 3-1). Stout *et al*,, who found that although *Legionella* could survive as long as environmental bacteria were present, it

could not amplify unless sediment (and perhaps host organisms) were present as well (Stout *et al* 1985). This finding extends Stout's results to synthesized potable waters covering a wide range of nutrient levels.

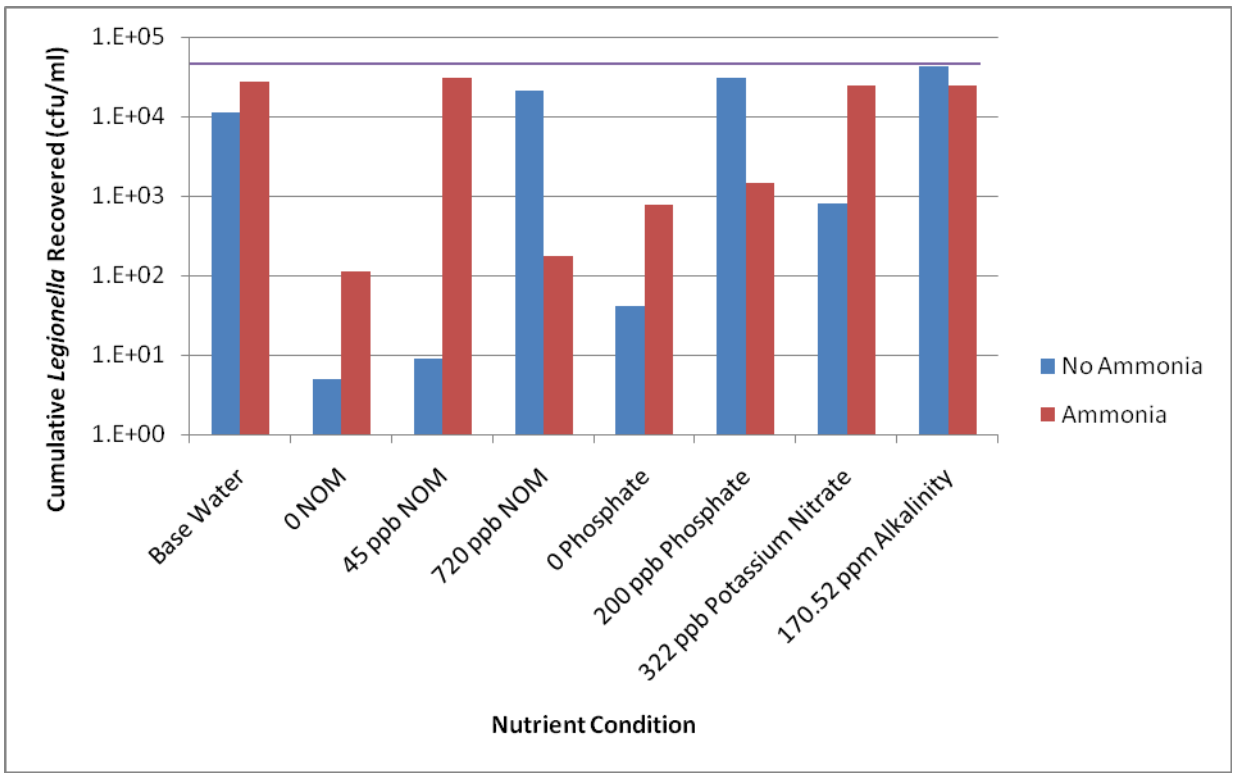

Figure 3-1. Cumulative amount of *Legionella* recovered after 327 days. The purple line represents the initial inoculation amount, which was 43905 cfu/ml

Although there was no evidence of *Legionella* amplification, there were marked differences amongst the reactors in terms of *Legionella* washout rates. The cumulative amount of *Legionella* recovered ranged from 0.01 to 95.98% after 327 days (Figure 3-1). It is uncertain why *Legionella* recovery was so variable. Amongst the likely factors contributing are variable rates of bacterial death, the presence of viable but non-culturable bacteria, or bacterial attachment to the walls of the reactor. For example, it is known that some nutrients such as phosphate can reverse surface charges and limit bacterial adhesion, and it is possible that similar factors were contributing in this study as well (Appenzeller *et al* 2002). Similar results were obtained between conditions when replicates were present.

When glass fiber filters were added and the bottles were seeded with non-synthetic water and 43,905 cfu/ml *Legionella pneumophila*, greater than 1000 cfu/ml *Legionella* could be recovered from 7 of 16 bottles after 7 months. The glass fiber filter provides additional surface area and may aid *Legionella* survival and retention by promoting attachment to a fixed biofilm. This result demonstrates that, even without a host organism present and detectable amplification of *Legionella* in water heaters, high concentrations of *Legionella* can persist in the effluent of such reactors for months after a single inoculation event.

One day after inoculation, percent *Legionella* survival was slightly lower at 37ºC than at 23 or 30ºC, but at 44ºC or higher survival was impaired (Figure 3-2). Almost no *Legionella* survived at 55ºC. After eight days and water replacement, the same trend was present but with lower levels of *Legionella*, however, nearly no *Legionella* had survived at 44ºC (Figure 3-3). This seems to indicate that if water containing *Legionella* enters a water heater, the *Legionella* can persist for very long periods of time if portions of the heater are maintained at temperatures at or below 37ºC. The *Legionella* in this study were more susceptible to heat than in laboratory studies which used tap water. These studies found a 90% reduction of *Legionella* after 2500 minutes (41.67 hours) at 45ºC and less than 5 minutes at 60ºC (Lin *et al* 1998).

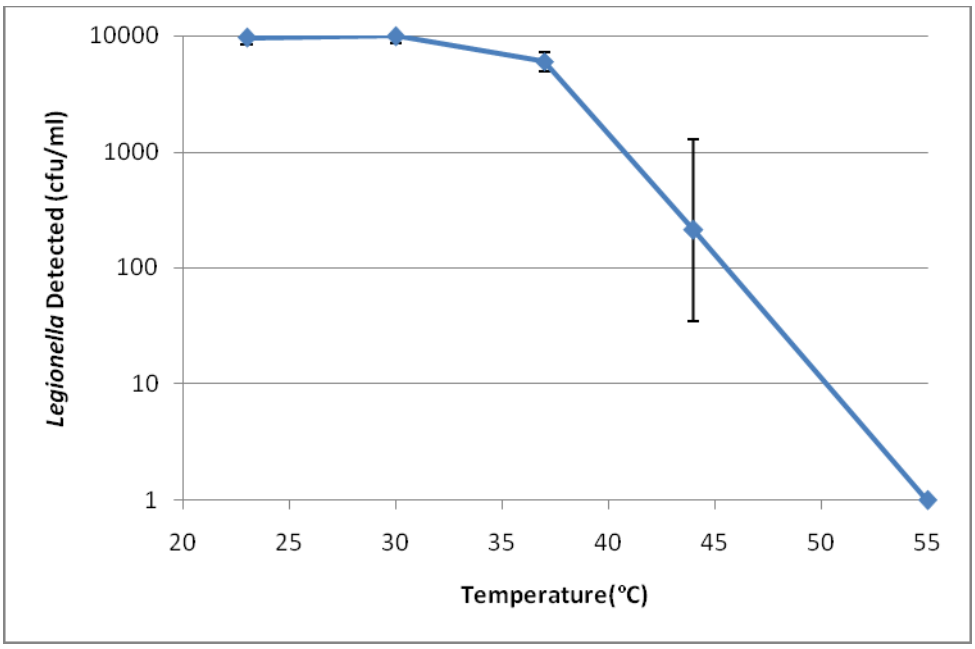

Figure 3-2. *Legionella* detected after one day at different temperatures. Initial inoculation amount was 10750 cfu/ml. The error bars denote 95% confidence intervals.

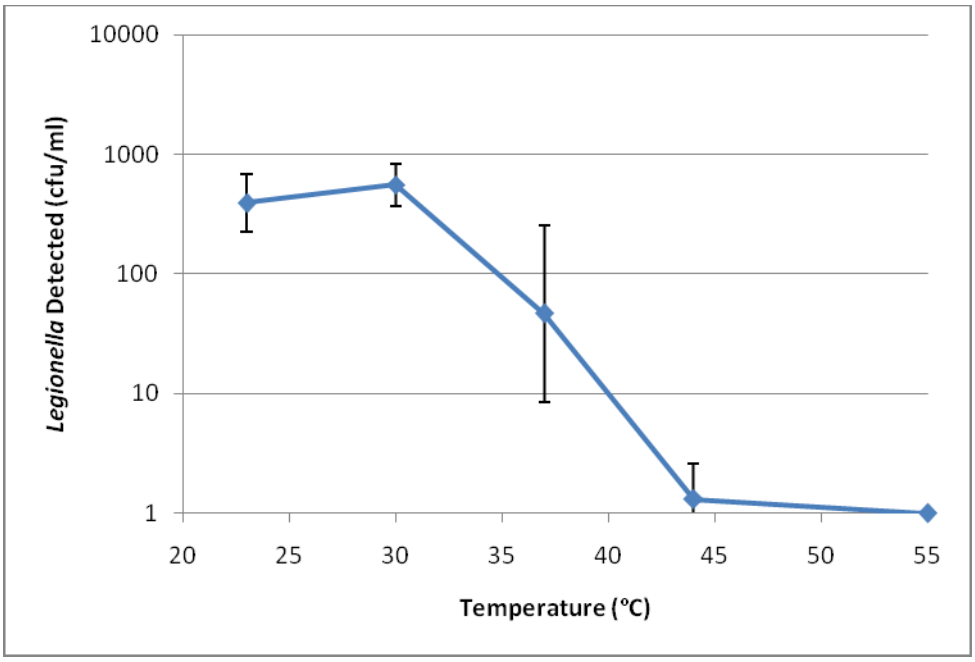

Figure 3-3. *Legionella* detected after eight days at different temperatures. The water was replaced after one day with synthetic water not containing *L. pneumophila*. Initial inoculation amount was 10750 cfu/ml. The error bars denote 95% confidence intervals.

The presence of 360 ppb copper (Cu^{2+}) slightly reduced *Legionella* survival and 2160 ppb significantly reduced survival (Figure 3-4). This indicates that Cu^{2+} has a toxic effect on *Legionella*. Copper toxicity has been noted in other studies and copper pipes have also been shown to have an inhibitory effect on *Legionella* colonization (Leoni *et al* 2005, van der Kooij 2005). These earlier studies used statistical tests relating encountered *Legionella* to concentration present during monitoring, whereas the current study is a head to head test in which the only variable is the level of Cu^{+2} dosed.

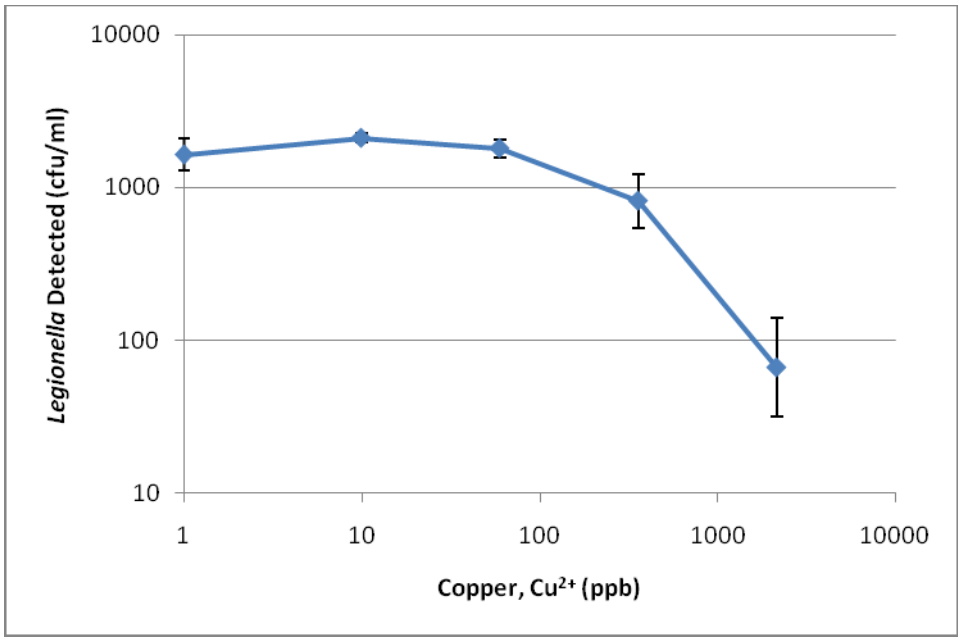

Figure 3-4. *Legionella* detected after one day at different copper levels. Initial inoculation amount was 1915 cfu/ml. The error bars denote 95% confidence intervals.

Iron (Fe^{3+}) did not alter *Legionella* survival at levels between 1 and 2160 ppb (Figure 3-5). While Borella *et al.* found that lower levels of iron inhibit *Legionella*, Zacheus and Martikainen found iron to be negatively correlated with *Legionella* occurrence (Borella *et al* 2004, Zacheus and Martikainen 1994). It is possible that the negative correlation was due to uncontrolled variables other than iron, that the inhibitory effect varies from water to water, or that Fe^{+2} may be the active inhibitory agent versus Fe^{+3} used in this study.

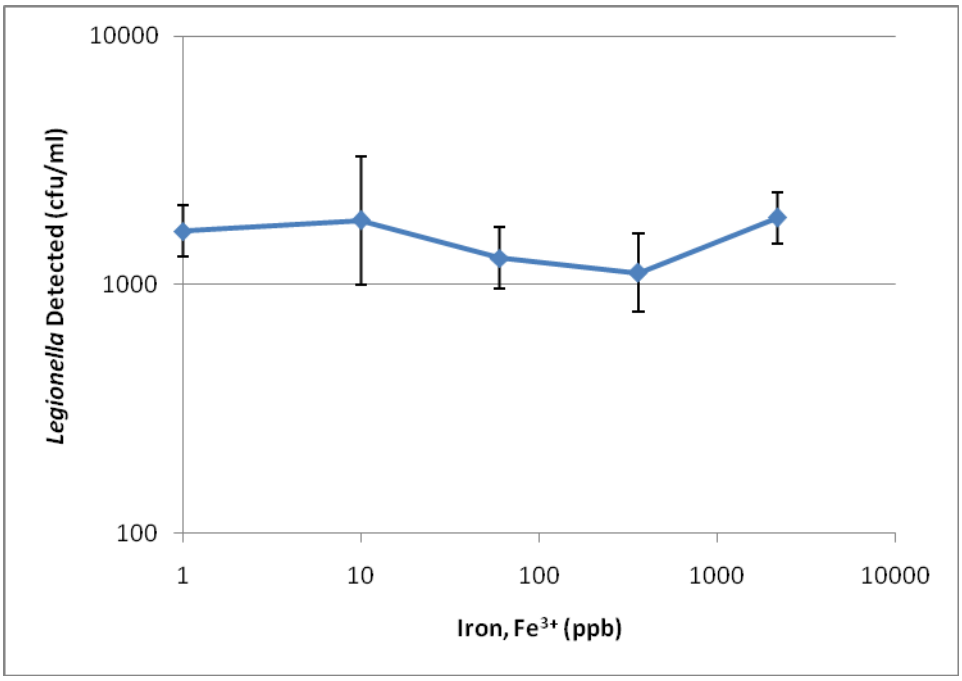

Figure 3-5. *Legionella* detected after one day at different iron levels. Initial inoculation amount was 1915 cfu/ml. The error bars denote 95% confidence intervals.

Legionella survival was much lower at pH 11 than at pH levels between 5 and 10 (Figure 3-6). Wadowsky *et al.* found that *L. pneumophila* could multiply between pH 5.5 and 9.2 (Wadowsky *et al* 1985). This result indicates that *Legionella* can survive in a greater pH range than it can multiply, and that pH ranges encountered in potable water are unlikely to influence *Legionella* survival.

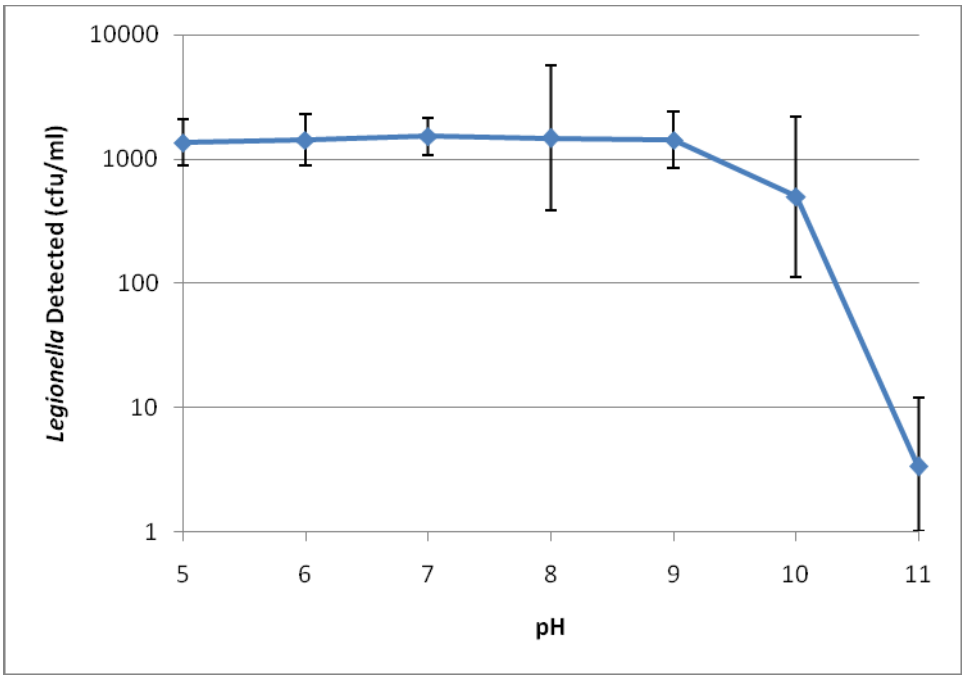

Figure 3-6. *Legionella* detected after one day at different pH levels. Initial inoculation amount was 1680 cfu/ml. The error bars denote 95% confidence intervals.

No definitive link between the presence of amino acids and *Legionella* survival could be established. However, at the end of the time period during which amino acids were added less *Legionellae* were recovered from the reactors (Figure 3-7). Additionally, the amount recovered continued to decrease even after amino acids were removed from the system. This may be due to the natural death of the bacteria or the amino acids may have somehow increased the susceptibility to environmental stresses.

It is very significant that *Legionella* persisted in the reactors and could still be recovered after 327 days, even with water changes twice per week during which 100% of the water was poured out and new synthetic water not containing *Legionella* was used for replacement. The 100% water change was used to simulate the worst-case scenario in which all the water was drained

from the hot water tank. This indicates that *Legionella* remained in the bottles, possibly by first becoming attached to the glass surface, and then detaching slowly.

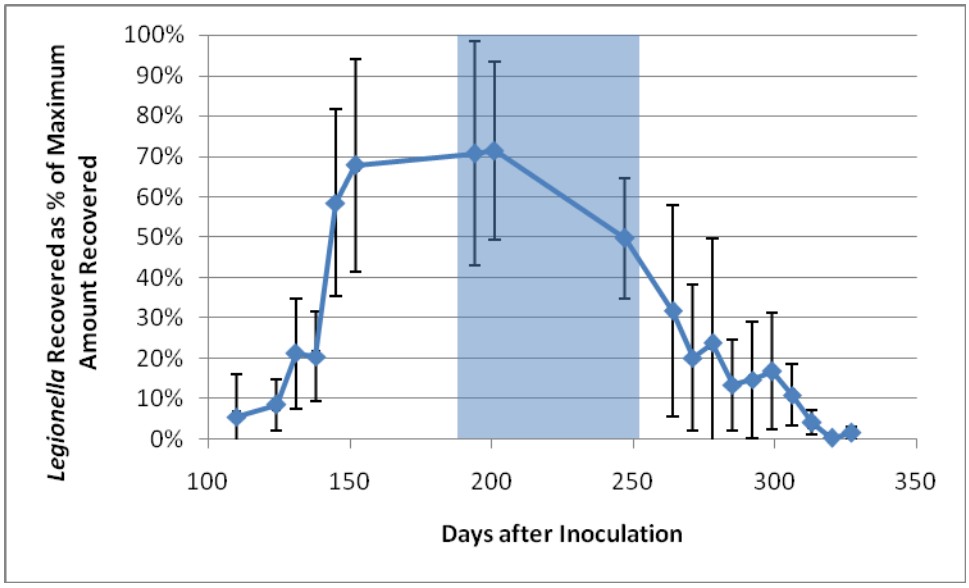

Figure 3-7. Long-term *Legionella* survival. The shaded portion represents the time in which amino acids were added. The error bars denote 95% confidence intervals.

No *Legionella* was recovered from reactors containing chlorine (0.25 and 2.5 ppm) or chloramine (0.40 and 4.0 ppm), indicating that both disinfectants are useful for eliminating free-living *Legionella*. However, *Legionella* are known to survive within a protist host with up to 50 mg/l free chlorine present for a period of 18 hours (Kilvington and Price 1990). The level of ozonated organic matter, Na_2HPO_4, KNO_3, $NaHCO_3$, and ammonia had no effect on *Legionella* survival for the range of water conditions tested.

This work confirms prior research that suggests amplification of *Legionella* in potable water systems is unlikely without a thriving protist host, even at levels of nutrients that would be considered very high in potable water systems. Similar studies should be performed with *Legionella* in the presence of a host organism, such as an amoeba, which is necessary for *Legionella* replication and provides protection from environmental stresses (Fields *et al* 2002, Kilvington and Price 1990). Nutritional demands of the host should be established so that water utilities can better anticipate the range of chemistries under which *Legionella* amplification might occur.

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CHAPTER 4

CONCLUSIONS

- Heat pretreatment may not be an effective method for optimal recovery of *Legionella* from some tap water samples
- The three tested acid pretreatment methods were effective in recovering *Legionella* at 3 and 5 minutes
- Acidification did not affect *Legionella* recovery when copper was present
- There was not a significant difference between the five selective culture media tested
- If sodium thiosulfate was not present in bottles before sampling, *Legionella* recovery was lower in the presence of chlorine
- *Pseudomonas aeruginosa* (up to 67.5 cfu/ml) and pyocyanin (up to 9 mg/l) did not have an effect on *Legionella* recovery under the tested conditions
- *Legionella* persisted up to 327 days when glass fiber filters were added to the reactors and they were seeded with tap water and a sediment slurry
- At 55ºC, *Legionella* could not be recovered from the reactors after 1 day
- At 44ºC, *Legionellae* were recovered after 1 day but only at low levels after 8 days
- Between 23 and 37 ºC, *Legionellae* survived longer than 8 days
- Copper (Cu^{2+}) concentrations above 2160 ppb were toxic to *Legionella*
- Iron (Fe^{3+}) concentrations between 1 and 2160 ppb did not affect *Legionella* survival
- *Legionella* survival was reduced at pH 11
- No culturable *Legionella* were recovered in the presence of 0.25 or 2.5 ppm free chlorine or 0.40 or 4.0 ppm chloramine
- No short-term effect of varying natural organic matter, phosphate, potassium nitrate, or alkalinity on *Legionella* survival could be detected