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Effect of Common Oxidative Water Treatments on Acanthamoeba Internalized Legionella

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EFFECT OF COMMON OXIDATIVE WATER TREATMENTS ON *ACANTHAMOEBA* INTERNALIZED *LEGIONELLA*

By

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Abstract

Effect of common oxidative water treatments on *Acanthamoeba* internalized *Legionella*

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Legionella pneumophila can cause pneumonic and non-pneumonic disease in humans. Infections occur from aerosolized contaminated water. This bacterium is an opportunistic intracellular pathogen able to infect both protozoans, such as *Acanthamoeba polyphaga*, and human macrophages. Both *L. pneumophila* and *A. polyphaga* resist commonly used water treatments, such as chlorination, but *L. pneumophila* has displayed greater resistance in the presence of *A. polyphaga*. Therefore, there is concern that *L. pneumophila* could become established in plumbing systems after water treatment, leading to infections. The objective of this study was to show the effect of chlorine and chlorine dioxide exposures on the survival of *A. polyphaga* internalized *L. pneumophila*. Gentamicin was used to kill extracellular *L. pneumophila* and samples were exposed to the oxidants, then the reactions were quenched and incubated at 30°C. The concentration of *L. pneumophila* was determined by culture analysis following lysis of *Acanthamoeba* on days 0, 7, and 14. Chlorine achieved ~1 log reduction at a concentration of 56.7 mg.min/L and ~2 log reduction at 376.3 mg.min/L. Chlorine dioxide achieved ~1 log reduction at a concentration of 74.21 mg.min/L and ~2 log reduction at 249.4 mg.min/L. All but one ClO_2 concentration tested showed increasing log reduction throughout the 14-day monitoring period. This project addresses a concern of water treatment facilities and

public health officials regarding the survival of intracellular *Legionella*. The results of this study show the need for greater understanding of other microorganisms' impact on *Legionella* control and will be useful to water treatment in determining oxidant levels needed for ensuring that potable water does not pose a delayed threat to the public.

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Dedications

This thesis is dedicated to my parents Bonnie and Tom Park. Your support and encouragement have truly made this possible.

Table of Contents

List of Tables

List of Figures

Chapter 1: Introduction

Legionella

Legionella is a ubiquitous microorganism that is capable of causing disease in people that spend time in or around engineered environments that potentially create aerosols. A few examples of such aerosol sources are showers, water fountains, air conditioners, and hot tubs. The Centers for Disease Control and Prevention (CDC) has identified *Legionella* as number two on their "Top 10 Causes - Outbreaks in Public Water Systems" list, only behind *Giardia duodenalis* for number of outbreaks (CDC, 2015). *Legionella* is a gram negative rod-shaped bacterium that is frequently found in aquatic environments with a tendency to take residence in biofilms that develop in both natural and engineered water systems (Uzel, Hames, & Ebrary, 2010).

As an infectious organism, *Legionella* is able to cause two different diseases that fall under the term legionellosis. These present in two forms: the non-pneumonic form, known as Pontiac Fever and the pneumonic form, called Legionnaires' disease. Pontiac Fever generally has milder symptoms than Legionnaires' disease, with primary differences in that pneumonia only develops in Legionnaires' cases and Pontiac Fever usually does not require medical intervention. Legionnaires' disease often presents with symptoms of shortness of breath, cough, fever, headaches, and muscle aches (Buchrieser *et al*., 2013). Occasionally Legionnaires' disease will include diarrhea, nausea, and confusion (WHO, 2016). Legionnaires' disease is fatal in 1 out of every 10 treated patients and the death rate if no treatment is administered is 70 to 80 percent (Correia *et al*., 2016). Over 6,000 cases were reported to the CDC in 2015; this is likely an underestimation due to legionellosis being underdiagnosed (CDC, 2017).

Although anyone can contract legionellosis, there is an increased danger in those with risk factors including chronic lung disease, over 50 years of age, smoking history, systemic malignancy, diabetes, renal failure, immune system disorders, hepatic failure, and travel with an overnight stay (CDC, 2017). This is of great concern in relation to nosocomial infections and the population at large. Many people with the risk factors for legionellosis are found in healthcare facilities, and there have been enough occurrences of hospital acquired legionellosis that the disease has become a major concern in healthcare settings (Agarwal, Abell, & File, 2017). According to the World Health Organization (WHO, 2016), *Legionella* is also of concern due to the current aging populations of many countries; as such, Legionnaires' disease has become a prominent health threat.

Legionnaires' disease was discovered in 1976 after an American Legion convention held in Philadelphia, Pennsylvania. The disease received its name due to this outbreak affecting members of the American Legion, which is made up of older individuals. An epidemiological investigation found that 182 became ill and 29 died in relation to this outbreak, and *Legionella* was discovered to be the causative agent. The investigation later led to the identification of the cause of Pontiac Fever, which had been seen many times, but the causative agent had remained a mystery previously. There have been many outbreaks since this incident with similar results (CDC, 2017).

Notably, the species *Legionella pneumophila* is the most common cause of legionellosis infections. Together, all other species of *Legionella* make up an estimated 5% of legionellosis cases. The source of these infections can come from water, soil, or in only one recorded case person-to-person (Correia, *et al*., 2016). *L. pneumophila* has been described as a facultative parasite. This is due to *L. pneumophila* being capable of existing in a free-living or an

intracellular state. The intracellular state occurs in response to being enveloped by a predatory protozoan. It is able to resist such predation; in fact, it is protected within the phagocytic cell, even being able to replicate (Uzel, *et al*., 2010).

Interestingly, it appears that *L. pneumophila* becomes more virulent after being exposed to predatory amoebas such as *Acanthamoeba.* It is believed that this greater virulence is due to this exposure to phagocytosis causing *L. pneumophila* to be more adept to enter the intracellular state. This may also be connected to the similarities between the phagocytosis performed by predatory protozoans and the macrophages of the human immune system (Molmeret, Horn, Wagner, Santic, & Abu Kwaik, 2005).

For water providers, it is important to detect the presence of *L. pneumophila* in water samples, and accurate detection is vital in deciding if disinfection is effective. Detection can be performed through several methods, but the most common method is culturing samples on Buffered Charcoal Yeast Extract (BCYE) agar due to its usefulness in detecting viable cells (Conza, Casati, & Gaia 2013). This method measures colony forming units (CFU). When performing spread plating, a sample is spread across the agar surface and an individual cell will grow and divide to form a single visible colony. This allows one to quantify the number of cells in the sample through counting colonies and calculating the number of cells present in the sample. One potentially important factor for quantification is that intracellular *Legionella* may appear as only one colony on an agar plate even if there are numerous bacteria present within an infected cell. This can result in inaccurate estimations of cells. Therefore, it is important that water providers can be reassured that intracellular *Legionella* have all been released before quantifying the number of bacteria present (Conza, *et al*., 2013).

Ecology

Water sources and treated water commonly have a variety of microbial life in them. Many of these organisms are capable of creating biofilms. A biofilm is a matrix of extracellular material that is produced by several different species of bacteria. These biofilms protect the organism from chemical threats, physical removal, predation, and many other environmental dangers (López, Vlamakis, & Kolter, 2010). Biofilms can become occupied by a diverse group of microscopic organisms, including *Legionella*, and become complex microbial communities. Some organisms take advantage of the protection biofilms provide while predators, such as *Acanthamoeba*, have developed the ability to take advantage of these communities as rich feeding grounds. These communities can be composed of organisms that are harmless or have varying levels of virulence, and this has made many of these organisms of concern to the water industry (López *et al*., 2010).

Chemical Water Treatments

Legionella continues to show that it is an impressively resilient microbe due to its ability to resist the commonly used oxidative water treatments chlorine and chlorine dioxide. In water distribution, such chemicals are used effectively with an initial high dose to deactivate pathogenic organisms and residual concentrations being present while the water is being transported to customers. The residual is the amount of chemical left after it has reacted with organic material in the water, and this remaining free portion of the chemical is what is available to prevent organisms from recovering. Treatment is often measured in CT value (i.e., concentration minimum and contact time), which is used in water treatment to express exposure of the contaminants in the water to the treatment chemical as it relates to time, and represents

inactivation credits in the water treatment industry. Inactivation credits are used to indicate if treatment would have been effective in damaging dangerous microorganisms, such as *Cryptosporidium*, to a point that they are no longer a health threat to customers (EPA, 2016b). The CT value refers to the time integrated concentration of the oxidant, $CT = Concentration_{minimum} \times contact\ time$ (Rush, 2002). Concentration is usually measured in mg/L and contact time is measured in minutes.

A challenge for water providers is using a high enough dose of these chemicals without being so high that it negatively impacts the taste or safety of the water (CDC, 2008). The Environmental Protection Agency (EPA) requires that regular sampling be performed throughout the water system and at no point in the water system can the residual drop below 0.1 mg/L, but not exceed 4 mg/L, as free chlorine (Cl_2) (EPA, 2016a). When disinfectant chemicals are applied in too high of a dose, disinfectant byproducts (DBP) can be produced at health threatening levels. These DBPs include trihalomethanes (THM), haloacetic acids (HAA), chlorite, and bromate, among others, and are created when organic materials located in the source water react with applied disinfectant chemicals (EPA, 2017). It is important to note that a water provider's responsibility ends once the water reaches any secondary system, such as water softeners or water purification systems in households, hotels, or hospitals. *Legionella's* resistance to disinfection is an area of continued interest in public health and water treatment as these treatments are often the standard for removing harmful microorganisms, such as coliform bacteria (King, Shotts, Wooley, & Porter, 1988). Paths of entry for health threatening microorganisms into water systems are still poorly understood and will likely become an area of interest for water treatment as well as public health.

Chlorine

When using chlorine, water providers must be cautious in the amount being used. This is because when chlorine comes into contact with organic material or naturally occurring chemicals in source waters, it can form chemicals that can have negative health effects. Two of these are THMs and HAAs, both of which are regulated by the EPA. THMs are believed to be carcinogenic, and chronic exposure has been associated with increased risk of several different cancers, including bladder and colon (Rivera-Núñez *et al*., 2012). HAAs have not been shown to be carcinogenic, but there is evidence of them being genotoxic and cytotoxic (Zhang *et al*., 2010). Genotoxic substances cause damage or mutation to DNA and cytotoxic substances are harmful to entire cells (Silva *et al*., 2015).

Chlorine is the most commonly used water disinfectant employed in water treatment around the world. The addition of chlorine to drinking water is usually done as sodium hypochlorite (liquid), chlorine gas, or calcium hypochlorite (solid). Sodium hypochlorite is often preferred in the treatment of plumbing and other buildings that have potable water (Rosenblatt & McCoy, 2014). This chemical has been used as a principal disinfectant since 1908 when it came into use in Jersey City, New Jersey. Since then, it has been effective in dramatically reducing outbreaks of waterborne disease within the United States and many other countries globally (Calomoris & Christman, 1998). The CDC recommends an initial dose high enough to leave a residual concentration of 2 mg/L after 30 minutes of contact and 0.2 mg/L after 24 hours (CDC, 2014). The World Health Organization proposes a 5 mg/L initial and a 0.5mg/L residual to be present throughout the distribution system (WHO, n.d. a). The requirements from these agencies are most commonly based on CT for common bacteria, viruses, and protozoa, but do not include *Legionella*. There is currently very limited information available with regards to disinfection of

Legionella, which can also thrive when internalized in other organisms, such as amoebas. Cooper and Hanlon's (2010) research has indicated that *Legionella* that have formed a biofilm are resistant to levels of chlorine that are 0.5 mg/L or even higher after 1 hour of exposure. In a previous study by Cooper and colleagues (2008) it was shown that one facility's water system was repeatedly colonized by one strain of *Legionella* after multiple 1 hour exposures at 50 mg/L during a 2.5-year period. This indicates that there is a need to ensure *Legionella* is not able to recover after the initial treatment. This is especially true when areas of water systems can have low chlorine residuals due to low flow rate, dead ends, or premises that use water purification systems (EPA, 2002). Ensuring the initial treatments' effectiveness could help prevent the formation of a biofilm in areas of low residual.

Chlorine has been tested repeatedly for its effectiveness against *Legionella* under many different conditions. Kuchta *et al.* (1983) tested chlorine residuals between 0.1 and 0.5 mg/L and found that chlorine was most effective with higher temperatures and lower pH for up to 60 minutes with a CT of up to 9 min.mg/L (Table 1). Their experiments also indicated that while a concentration of 0.1 mg/L of chlorine allowed *Legionella* to survive for a long period of time, a concentration of 0.5 mg/L was capable of showing a 2 log reduction in bacteria. This study, like most studies, reports only oxidant dose, which assumes that the concentration remains constant throughout the reaction, and this is not an accurate depiction. In water systems, the kinetics of oxidant decay differ depending on the reactive organic and inorganic substances in the source water (Rush, 2002).

Jacangelo (2002) used higher concentrations of chlorine from 1.0 to 4.0 mg/L on several different emerging pathogens and found that an exposure time of more than ten times greater was

needed to get a similar reduction in *Legionella* (Table 1). Similar pH and temperatures were used in the experiment, but trends were not as apparent.

With a different focus, Cooper and Hanlon (2010) investigated the effects of chlorine on *Legionella* in a planktonic state (i.e., single cellular and suspended cells) and a biofilm associated state of 3, 28, and 56 days old cultures (Table 1). The samples were exposed to an initial concentration of 50 mg/L chlorine and continued levels between 0.2 and 0.5 mg/L for 28 days. The results indicated that the planktonic bacteria were unable to recover during the 28- and 56-day period while the biofilm associated bacteria were able to recover from the treatment.

An experiment of chlorine's impact on biofilms used copper and stainless-steel coupons with *L. pneumophila* biofilms. The authors concluded that a one-hour contact time of 50 mg/L chlorine allowed *L. pneumophila* to grow following the exposure, having approximately 106 CFU present on the coupons. The *Legionella* was also capable of surviving with free chlorine levels maintained at 0.5 mg/L (Cooper & Hanlon, 2009).

Chlorine dioxide

Chlorine dioxide (ClO_2) was used for water treatment as early as 1940 in Europe and has been used by many water systems in the United States for water disinfection, usually in small facilities. As a water-soluble gas, ClO_2 is typically generated on site of the intended treatment and has a recommended maximum concentration of 0.08 mg/L in drinking water. It has been indicated that 0.1–0.5 mg/L at the tap is sufficient to control *Legionella* in most situations, but in some water systems, the level of contamination and age of colonization can lead to a need for higher doses (HSE, 2014). A minimum level for ClO_2 is not readily available. ClO_2 has also shown to be highly effective at permeating biofilms compared to chlorine.

ClO_2 use avoids many of the byproducts generated by chlorine, but it also produces harmful byproducts. Of particular concern is chlorite, a contaminant regulated by the EPA with a maximum level of 1 mg/L in drinking water. In order to avoid producing chlorite, water providers cannot use more than 1.4 mg/L of ClO_2. Chlorite can cause damage to red blood cells, inhibiting the body's ability to transport oxygen, and has been shown to cause delayed brain development in animal models (EPA, 2006).

Dupuy *et al*. (2011) compared chlorine dioxide, monochloramine, and chlorine as well as their effectiveness in treating *L. pneumophila,* multiple *Acanthamoeba* spp., and the two cultured together (Table 1). The doses used were 0.4 mg/L of ClO_2 and 2–3 mg/L of chlorine. Exposure was for a 1-hour period. Chlorine and ClO_2 were most efficient at reducing co-cultured *L. pneumophila* and free-living *L. pneumophila*, achieving a 99.9% bacterial reduction. ClO_2 was highly effective against some *Acanthamoeba* species, but not all species were tested. All sampling was performed immediately after exposure, but did not address potential recovery from these treatments.

Jacangelo (2002) also investigated the impact of ClO_2 on *Legionella* with varying temperatures and pH levels (Table 1). The ClO_2 dose was 1.0 mg/L during testing. The test showed a 99% reduction with 5°C at pH 6.0 and at pH 8.0. This reduction was also observed with 25°C at 6.0 min-mg/L and at pH 8.0.

Table 1. Literature review on the effects of chemical treatment on survival of *Legionella*.

Ox.	Dose (mg/L)	Residual (mg/L)	Time	CT (min.mg/L)	Effect	°C	pH	Reference
Cl_2	n/r	0.1 & 0.5	0-60 min	0.5-9	<u>99% reduction</u> 4°C: 6-9 min (pH 7.6), 21°C: 0.5min (pH 6), 1–6 min (pH 7), 4min (pH 7.6) 32°C: 3.2 (pH 7), <3(pH 7.6)	 4 21 32	 6.0 7.0 7.6	Kuchta *et al.* (1983)
Cl_2	1.0-4.0 mg/L	n/r	0-60 min	30-60	<u>99% reduction</u> 5°C: CT>50->320 (pH 6), 50-250 (pH 7), 250-1,000 (pH 8) 15°C: CT 100->320 (pH 6),60->320 (pH 7),25->710 (pH 8) 25°C: CT 40-500 (pH 6), 100-160 (pH 7),130-250 (pH 8)	 5 15 25	 6.0 7.0 8.0	Jacangelo *et al.* (2002)
Cl_2	50 mg/L	0.2 & 0.5	28 days	n/r	Planktonic negative at 28 days Biofilm viable after 56 days	36	n/r	Cooper & Hanlon (2010)
Cl_2	50 mg/L	0.5	60 min	n/r	*Legionella* persisted	n/r	n/r	Cooper & Hanlon (2009)
ClO_2 Cl_2	n/r	0.4, 2–3, & 0.8	1 hr	5	ClO_2=99.9% reduction Cl_2 =99.9% reduction	30 50	n/r	Dupuy *et al.* (2011)
ClO_2	n/r	1.0	n/r	1-2	99% reduction 5°C (pH 6-8) 99% reduction 25°C (pH 6-8)	5 25	6.0 8.0	Jacangelo *et al.* (2002)

n/r = not reported
Ox. = oxidant
CT = reported oxidant concentration versus time in mg.min/L

Acanthamoeba

Protozoans, in particular amoebas, commonly appear in biofilms both in natural and man-made aquatic environments. Among amoebas, the *Acanthamoeba* are of particular interest to water treatment. *Acanthamoeba* is a ubiquitous microorganism, having been found in soil, sea water, fresh water, brackish water, sewage, swimming pools, contact lens equipment, medicinal pools, dental treatment facilities, dialysis machines, heating systems, and air conditioning systems (CDC, 2016). There are more than 20 species of *Acanthamoeba,* eight of which have been shown to cause a disease called amoeboid keratitis. Of these, *Acanthamoeba polyphaga* and *Acanthamoeba castellani* are the most common causative agents of *Acanthamoeba* keratitis (Maycock, & Jayaswal, 2016). *Acanthamoeba* also causes Granulomatous Amebic Encephalitis, which is a serious infection of the central nervous system, typically occurring in those with compromised immune systems (CDC, 2010). *Acanthamoeba* keratitis is an uncommon disease, though more common than Granulomatous Amebic Encephalitis, that is caused by the infection of the cornea by *Acanthamoeba.* The disease presents with blurred vision, eye pain, light sensitivity, eye redness, and excessive tearing (Maycock, & Jayaswal, 2016). If left untreated, this condition can lead to vision loss or even blindness. Anyone can develop *Acanthamoeba* keratitis, but it is most common in people who wear contact lenses (CDC, 2010).

A. polyphaga is a predatory amoeba that is commonly found in engineered water systems, feeding on cyanobacteria, bacteria, fungi, and other amoebas (CDC, 2011). This protozoan exists in one of two forms, the trophozoite form and the cyst form. As a trophozoite, *A. polyphaga* is highly mobile, is able to feed, and is considered infectious. In the cyst form, the amoeba becomes inert and produces a two-layered cyst wall. Encystment is usually a reaction to environmental stressors such as chemical exposure or predation. The cyst wall is primarily

composed of cellulose, allowing the cell to survive harsh environments (Lemgruber, Lupetti, De Souza, Vommaro, & da Rocha-Azevedo, 2010). The versatility of this organism has been shown to allow it to survive common water treatments and is a primary reason why this organism is of concern to both health and water officials.

A. polyphagia's resistance to oxidants has been studied under differing circumstances by several investigators. Coulon *et al*. (2010) investigated chlorine's effectiveness on several strains of *A. polyphaga*. A 2,500 ppm Cl_2 residual showed to be completely effective but extending exposure time was necessary. One strain survived this concentration for an exposure time of 30 minutes. Their results indicate that chlorine is not effective in treating *Acanthamoeba* cysts at concentrations of 2 to 5 ppm, which are commonly employed in the treatment of drinking water.

As mentioned previously, *L. pneumophila* is capable of existing in an intracellular state. To be affected by water disinfectants, *Legionella* needs to be exposed directly to the chemical and when in an intracellular state, the bacteria are protected from exposure. Once an oxidant lyses the amoeba, intracellular *Legionella* can be exposed to the oxidant, but until such time the bacteria will likely remain unaffected (Figure 1). A study from the University of Poitiers, France, tested the effectiveness of Cl_2 and ClO_2on *L. pneumophila* grown in co-culture with *A. polyphaga*. The results indicate that being in co-culture protects *L. pneumophila* to a point from Cl_2 and ClO_2 (Dupuy *et al*., 2011). This study did not address *L. pneumophila*'s ability to recover after a given time.

These studies suggest that in the presence of *A. polyphaga*, *L. pneumophila* is able to recover from treatment given time, but none of these studies have thoroughly addressed the

exposure times needed for complete deactivation or the time to recovery. Recovery can be defined as the "increase in numbers of culturable cells" in a bacterial sample (Bolster, Bromley, & Jones, 2005). The results from the present research project will expand on these published studies and will expand on the knowledge of water treatment to reduce exposure to *Legionella*.

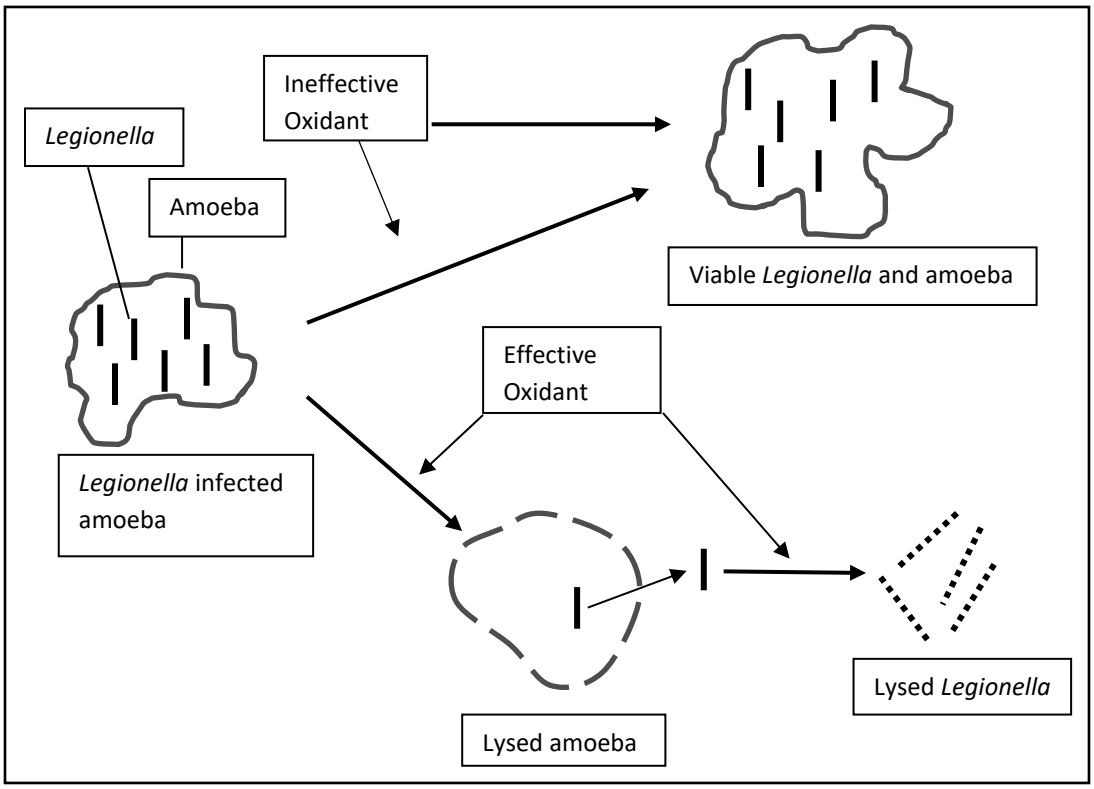

Figure 1. Diagram representing internalized *Legionella* survival after oxidative treatment.

Objectives

The objectives of this study were to:

(1) Compare the effectiveness of chlorine and chlorine dioxide on *A. polyphaga* internalized *L. pneumophila* given time to recover as is possible in some water plumbing systems.

(2) Determine the CT of the oxidants chlorine and chlorine dioxide necessary to effectively reduce the number of *A. polyphaga* internalized *L. pneumophila* and prevent potential recovery after water treatment.

Research questions

1. What are the most effective CTs for chlorine and chlorine dioxide to reduce the survival of internalized *L. pneumophila* after treatment?
2. What is the difference in the effectiveness of chlorine and chlorine dioxide in preventing *Legionella* from recovering after treatment at different CTs?

Chapter 2: Research Design and Methods

All experiments were performed at the Southern Nevada Water Authority's (SNWA) River Mountain campus in the Water Quality Biology Laboratory. The SNWA provided access to maintained cultures of *L. pneumophila* and *A. polyphaga* for all experiments, as well as provided all safety equipment and materials needed for experimentation. Both of the organisms used in this study are considered to be infectious; therefore, Biosafety Level 2 precautions were followed when working with either organism, particularly methods with the potential of producing aerosols, as recommended by the CDC (Department of Health and Human Services, CDC, & NIH, 2009). All experiments were performed in a biosafety cabinet and researchers wore gloves and a laboratory coat whenever working with samples. *Legionella pneumophila* strain 33152 (American Type Culture Collection, Manassas, VA) and *Acanthamoeba polyphaga* strain 30461 (ATCC) were used in this study. The experiments were performed in autoclaved raw water sourced from Lake Mead, the water source for much of the Las Vegas valley, and were filter-sterilized to remove particulate matter that could impact oxidant decay. This sterile lake water (SLW) acted as a representative water sample for the experiment.

Growth Curve

Initial testing was required to establish a growth curve for *L. pneumophila,* in order to identify the growth phase of the bacteria during the experiment. This was performed by culturing *L. pneumophila* for isolation on BCYE agar plates (BD Diagnostics, Durham, NC) and incubating for 7 days at 35°C. In triplicate, three isolated colonies were inoculated into a 15 mL centrifuge tube containing 10 mL of *Legionella* Enrichment Broth (Sigma-Aldrich, St. Louis, MO), along with one that was not inoculated with *Legionella* to serve as a blank, and incubated at 35°C for 7 days. During the growth in the broth, absorbance (Abs) was measured daily on

days 0, 1, 2, 3, and 7 (based on laboratory access to the researcher) on a DR 5000 UV-Vis Laboratory Spectrophotometer at a wavelength of 570 nm (Hach Company, Loveland, CO). The results were used to create a graphical representation of absorbance over time. This allowed the determination of when the *L. pneumophila* was in post-exponential phase as this was the preferred growth state for infecting *A. polyphaga*. Previous research has shown that post-exponential growth is connected to increased virulence in *L. pneumophila* (Molmeret, et al., 2004).

Gentamicin Controls

Pre-testing was performed to ensure that gentamicin treatment was effective against free *L. pneumophila,* but does not impact *A. polyphaga* viability. This was performed by: 1) exposing a 10 mL sample of 5×10^5 cells/mL of *Acanthamoeba* alone in SLW to 200, 100, 50, and 0 µg/mL of gentamicin, and 2) the same cell concentration of *Legionella* alone to 100 µg/mL of gentamicin for 90 minutes. Samples were prepared and exposed to gentamicin in 10 mL SLW, and after 90 min. of exposure time the samples were washed twice. The samples were (ten-fold) serially diluted to 10^{-5}. Notably, the viability for *A. polyphaga* and *L. pneumophila* needed to be tested differently*;* these were performed as indicated below.

For *A. polyphaga,* in triplicate, 0.1mL of each dilution was transferred to Corning 24 well cell culture multiwell plates (Sigma-Aldrich) that contained 0.9mL of peptone, yeast extract, yeast nucleic acid, folic acid, and hemin (PYNFH) (Sigma-Aldrich, St. Louis, MO) media to improve amoeba growth. Therefore, each sample was diluted by another factor of ten at this point. Each row of six wells was an individual sample starting with a dilution of 10^{-3} up to 10^{-6} (Figure 2). Multiwell plates were incubated at 30°C for 7 days; then each well was microscopically examined under an inverted microscope to detect the presence of viable amoeba,

based on microscopically observable cellular activity, in each well. The results were entered into the EPA's Most Probable Number Calculator to estimate the number of viable cells present in the original sample; this method is regularly used by SNWA staff for monitoring of multiple species of amoeba from environmental samples.

For estimating *L. pneumophila,* in triplicate, 0.1 ml of each sample dilution was spread plated on BCYE agar plates, incubated at 34°C for seven days, and then colonies were counted and CFU per sample was calculated (Figure 2).

Oxidant Exposure

For each oxidant, residuals were measured at several points throughout the 1-hour exposure time that was used. At the measurement points, the residual of each chemical was measured to calculate the CTs of each oxidant finding the time integrated concentration of each. This was done using the formulas: $CT = \int [Cl_2] dt$ and $CT = \int [ClO_2] dt$. Residuals for chlorine and chlorine dioxide were measured by the *N*,*N*′-diethyl-*p*-phenylenediamine (DPD) method with a Hach DR/890 Portable Colorimeter (Hach Company). For chlorine, 10 mL of sample acted as a blank in the sample cell, 10 mL of sample had a free chlorine DPD powder pillow added, swirled for 20 seconds, and inserted into the cell holder to be read. For chlorine dioxide, 10 mL of sample acted as a blank in the sample cell, 4 drops of glycine (Hach Company) were added to 10 mL of sample, then a free chlorine DPD powder pillow was added, swirled for 20 seconds, and inserted into the cell holder to be read. This was used to establish the decay of the oxidant during testing to calculate the resulting CT (Figure 3) (APHA, 2005).

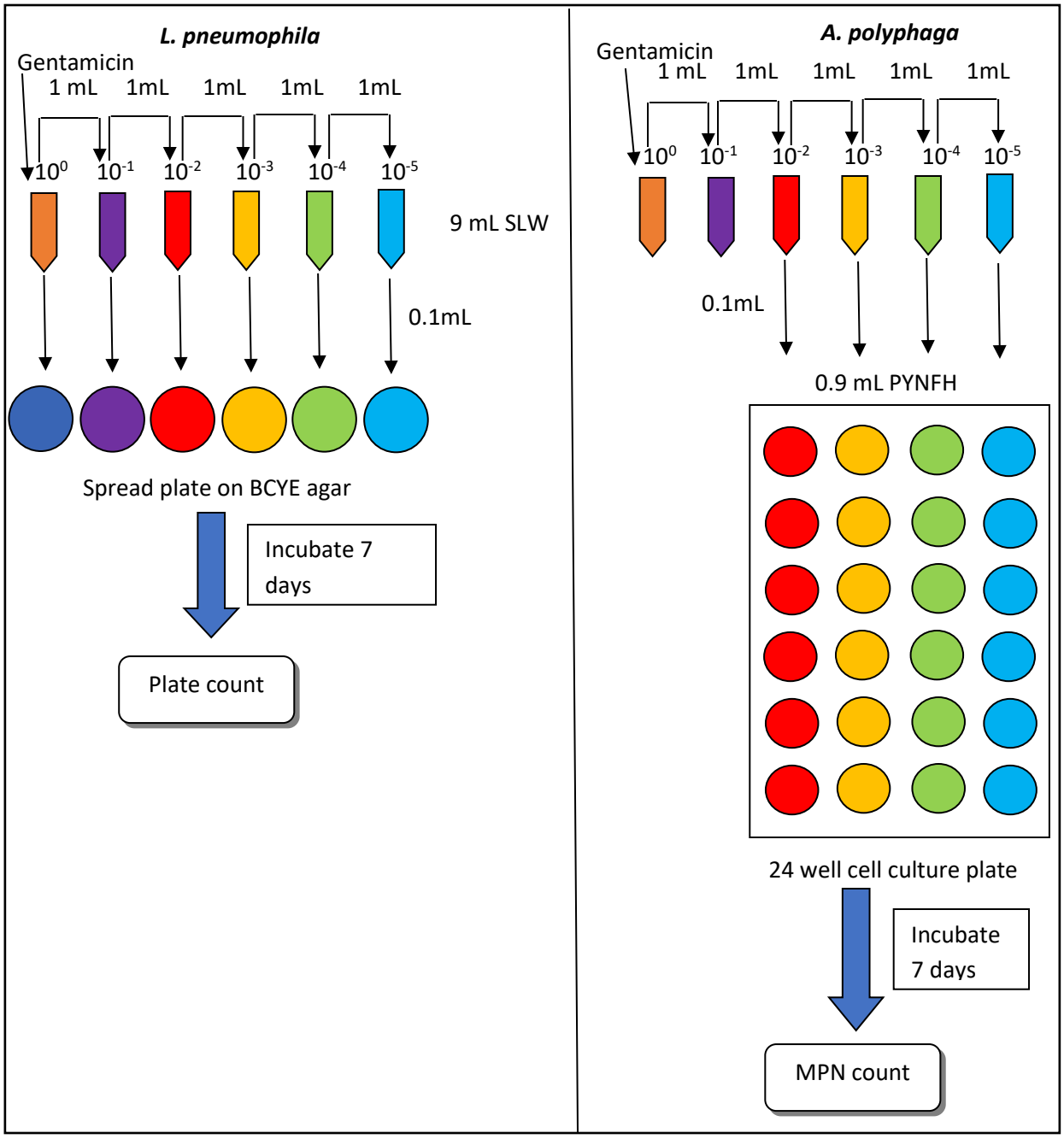

Figure 2. Flow chart of gentamicin effectiveness on free *L. pneumophila* and impact on *A. polyphaga* (MPN=Most Probable number, SLW=Sterile Lake Water).

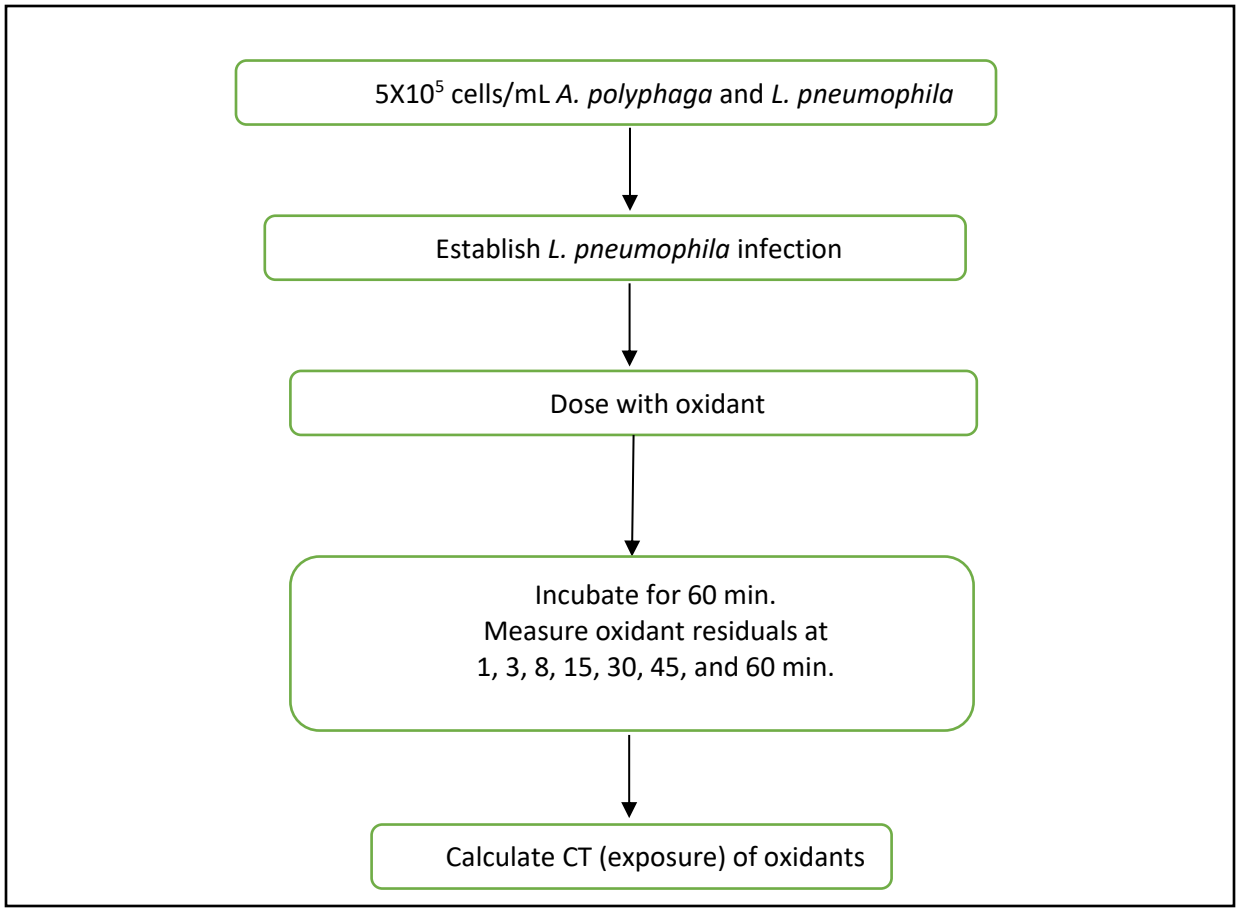

Figure 3. Flow chart illustrating determination of exposure times.

Testing preparation and infection

For testing, a fresh working culture of *A. polyphaga* was prepared by vigorously hand shaking the stock culture 100 times and firmly tapping every 20 shakes, to release the amoeba from the surface. The stock samples were maintained in PYNFH liquid media (Sigma-Aldrich) and stored at 30°C. A two mL aliquot of the working culture was transferred into a fresh tissue culture flask with 23mL of PYNFH media and incubated at 30°C for 4 days. PYNFH is commonly used in the growth and cultivation of many types of amoeba samples. Samples of *L.*

pneumophila (in post-exponential phase) were prepared using the procedure described in the growth curve section, but only incubated for 4 days. The experiment was performed with 80 mL samples under different CTs to have a variety of CTs to compare. Replicates were run when possible along with positive and negative controls.

The positive controls were untreated infected *A. polyphaga* in SLW and the negative controls were 5×10^5 cells/mL *A. polyphaga* in SLW. The *A. polyphaga* stock was shaken 100 times (firmly tapping the flask every 20 shakes), transferred to a 15 mL centrifuge tube, washed twice in SLW (centrifuging for 15 min at 600×g), and resuspended in SLW. The *A. polyphaga* sample was counted with a hemocytometer and inoculated into 20 ml of SLW in tissue culture flasks at a concentration needed for ~5×10^5 cells/mL in the final sample volume of 80 mL. This was then incubated at 30°C for 2 hours, to allow *A. polyphaga* to settle to and establish on the bottom of the flask.

During this time, the *L. pneumophila* culture was prepared by centrifugation at 5000×g for 10 min, washing twice, and then resuspending in SLW. The *L. pneumophila* sample was stained with 10µL per 10 mL of sample of CellTrace CFSE Cell Proliferation Kit (Thermo Fisher Scientific, Waltham, MA), prepared by following the manufacturer's instructions by combining the Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) lyophilized powder with 13 µL Dimethyl sulfoxide (DMSO). This produced a 5 mM concentration that was incubated for 20 min., and then washed with SLW. This stain was chosen due to it being effective for cell enumeration, is not toxic to cells, and made the detection of *Legionella*, both free and internal, more accurate. The cell culture stock was then counted with a hemocytometer under fluorescent microscopy (excitation/emission (nm): 492/517) and prepared to be ~5×10^5 cells/mL in the final sample volume of 80 mL. To inoculate the settled *A. polyphaga* culture, *L. pneumophila* was

transferred to the flask and gently agitated. The culture was incubated for 24 hours at 30°C to establish the infection (Figure 4). Infection was confirmed through fluorescence microscopy by observation of green fluorescing vacuoles within the *Acanthamoeba*.

Immediately before oxidant testing, the flasks with samples were dosed with 100 µg/mL of gentamicin (Thermo Fisher Scientific), and incubated at 30°C for 90 minutes, in order to kill and reduce the impact of extracellular *Legionella* on the experimental results (Moffat, & Tompkins, 1992). Gentamicin is an antibiotic that has been shown to kill only extracellular *Legionella* and does not penetrate the *A. polyphaga* cell membrane (Gao, Harb, & Abu Kwaik, 1997). The infected cultures were decanted into centrifuge vials, each sample flask was rinsed gently 3 times with 5 mL SLW and transferred into the vials. Samples were washed twice with SLW (centrifuging at 600×g for 15 min.), and resuspended in 30 mL SLW. Samples were returned to their original tissue culture flask, and then brought up to 80 mL with SLW for exposure to oxidative treatments (Figure 4).

Infection rate was determined in order to choose the approximate best time to begin exposure testing. *A. polyphaga* and *L. pneumophila* were prepared with *A. polyphaga* being infected, as described, and concentrations were adjusted to 5×10^5 cells/mL of both organisms in a final volume of 80 mL of SLW. Gentamicin was not dosed. The samples had a cell scraper applied in a side to side motion from back to front 3 times covering the entire surface of the tissue culture flask at 1, 2, 4, 6, 24, and 48 hours. Then 10 µL was taken from the sample and viewed on a hemocytometer for counting. This was counted by switching between fluorescence microscopy, excitation/emission (nm): 492/517, and light microscopy counting all *Acanthamoeba* and

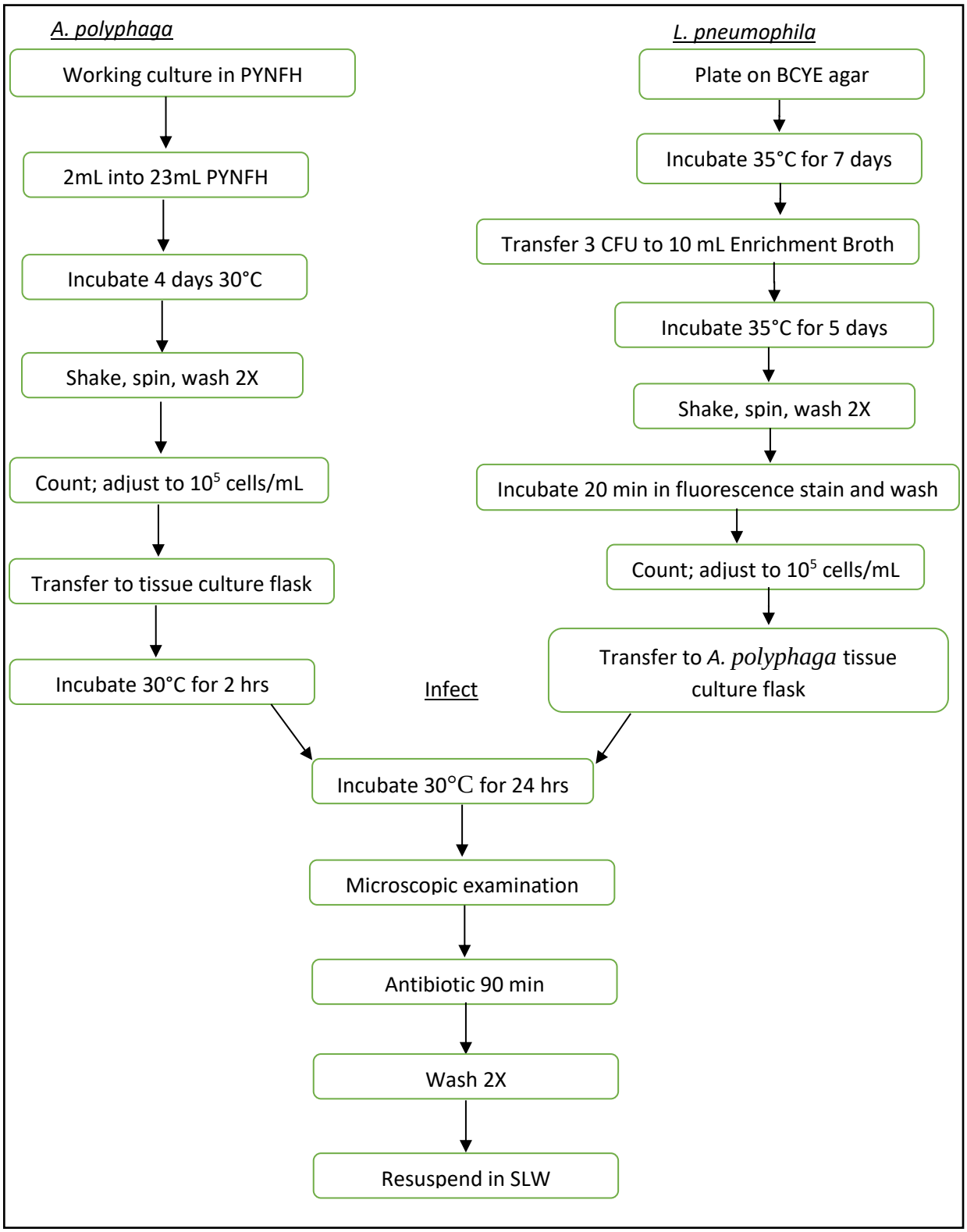

Figure 4. Experimental diagram for *A. polyphaga* and *L. pneumophila* preparation.

infected *Acanthamoeba*. Infected amoeba were considered to be those with fluorescent vacuoles. These results were then entered into an infection rate formula applied as $\frac{\#\ of\ Infected\ amoeba}{Total\ Amoeba} \times 100\%$ (CDC, 2012).

Experimental Design

The study involved a series of steps. First, infection of the amoeba was established in several tissue culture flasks over the 24-hour period as previously described and then treated with gentamicin to remove extracellular *Legionella* before washing twice. Second, these co-infected cultures were treated with several concentrations (i.e., doses) of oxidant (Table 2). All of these doses, with the exception of Cl_2 and ClO_2 at 30 mg/L, were performed in duplicate or triplicate (i.e., 2-3 trials). Following oxidant treatment, these samples were then incubated at 30°C, diluted to 10^{-3}, and each of these dilutions was spread-plated in triplicate on days 0, 7, and 14. After incubating for 7 days at 35°C these plates were analyzed individually, and the concentration in CFU/mL was calculated. All the day 0 culture results for a given oxidant dose were averaged (n=9), and the standard error around the mean was calculated. The same was done for the day 7 and day 14 results. Culture results from the positive control (no oxidant dosed on co-infected culture) were used to adjust for expected variance of *Legionella* concentrations. Log reduction was calculated taking the $\log_{10}$ of the average of a given oxidant dose and using the positive control as the basis for untreated samples. The standard deviation around the mean was determined (see Data analysis section). This was performed for day 0, 7, and 14.

Table 2. Experimental design.

Oxidant	Dose mg/L	# of Trials	Dilutions (plated in triplicate)	Plates at day 0	Plates at day 7	Plates at day 14	Total number of plates
Cl_2	3	3	4	36	36	36	108
	5	3	4	36	36	36	108
	7	2	4	24	24	24	72
	20	2	4	24	24	24	72
	30	1	4	12	12	12	36
ClO_2	7	3	4	36	36	36	108
	12	2	4	24	24	24	72
	20	2	4	24	24	24	72
	30	1	4	12	12	12	36
						Total # of plates	684

Oxidative treatment

Prior to the experiment, pilot-testing was performed with sodium thiosulfate to ensure it did not affect internalized *Legionella*. In triplicate, 10 mL samples that had been infected and treated with gentamicin, as previously discussed, were dosed with 0.8 mL of 1,000mg/L sodium thiosulfate (Fisher Scientific) then incubated at 30°C. These were serially diluted, spread plated on days 0, 7, and 14, and counted after incubating for 7 days. A positive control was included following the same procedures excluding dosing of sodium thiosulfate and a negative control was included with SLW and *Acanthamoeba* only.

The experiment was performed with the two oxidative treatment chemicals at multiple exposures in order to obtain a usable CT (Figure 5). These treatment chemicals were chlorine dioxide (ClO_2) and chlorine (Cl_2). As described previously, oxidant CTs were obtained based on oxidant residuals at different reaction times throughout the study. When possible, trials were run

in triplicate or duplicate, as described in experimental design. Initially, for Cl_2, one sample was exposed to 2 mg/L, three samples were exposed to 3 mg/L, three samples to 5 mg/L, and two to 7mg/L (Table 2). Due to not achieving even a 1 log reduction (i.e., low clearance) from these samples, the following weeks samples were exposed with two to 12 mg/L, two to 20 mg/L, and one to 30 mg/L Cl_2. For ClO_2, three samples were exposed to 7 mg/L, two samples were exposed to 12 mg/L, two samples were exposed to 20 mg/L, and one sample was exposed to 30mg/L (Table 2).

The volume of each remaining sample of each exposure was reduced to 20 mL (to be consistent for all samples), and sterile sodium thiosulfate (1,000 mg/L) was used to quench all reactions at 60 min., using 35 µL for every 3 mg/L of oxidant residual remaining; this stopped any further oxidant reactions. Samples were kept and incubated at 30°C (Figure 5).

Culturability/cell count

Determination of culturability was performed by spread-plating on days 0, 7, and 14. To prepare for plating, the amoeba were released from the tissue culture flask by applying a cell scraper in a side to side motion from the back of the flask to the front three times. A 4.5ml aliquot was transferred immediately to a 15mL centrifuge tube and then centrifuging at 5,000×g for 8 minutes followed by 1 minute of vortexing on high three times; this method lysed the *Acanthamoeba* releasing any internalized *Legionella* (Figure 5) (Dietersdorfer, Cervero-Aragó, Sommer, Kirschner, & Walochnik, 2016; Alleron, Merlet, Lacombe, & Frère, 2008). The lysed solutions were diluted to 10^{-4} and spread plated on BCYE agar. These were incubated at 34°C for 7 days, followed by colony counting to estimate the number of viable *Legionella* cells (CDC,

2017). Recovery was shown by the number of *Legionella* CFU approaching the concentrations seen in the untreated positive control (Bolster *et al*., 2005).

Legionella culturability was used to measure the effectiveness of each oxidant and indicated the change in viability that occurred over time with each sample. To address differences that may have occurred in the samples when prepared on different days the results were converted to percent survival by dividing the samples growth results by the positive controls growth results $\left(\frac{Average\ Sample\ CFU/mL}{Positive\ Control\ \ CFU/mL} \times 100\%\right)$. Using the percent survival of the exposures allows for reasonable caparisons between oxidant tests, days, and normal cell viability loss.

Data analysis

Data analysis was based on the *Legionella* culture results with samples plated on BCYE agar in triplicate and enumerated as CFU/mL. Percent survival for each CT was determined by calculating the mean of the replicate culture results of each trial, dividing this by the mean of the positive control culture results, and multiplying by 100. The standard deviation was calculated based on the percent survival of each trial, and used for comparing the CFU results from the different CTs each day. Log reduction of *L. pneumophila* from each oxidants' CTs were calculated in order to quantitatively demonstrate how each oxidant would behave in a real-world application. The log reduction (LR) was found using the formula $LR = \log_{10} \frac{CFU\ untreated}{CFU\ treated}$ with the standard deviation (SD) determined using the formula $SD = \left[\left(\frac{SD\ ^2 untreated}{n_{untreated}}\right) + \left(\frac{SD\ ^2 treated}{n_{treated}}\right)\right]$ (Zelver, Hamilton, Goeres, & Heersink, 2001). This approach was used to normalize the data between each testing day and take into account normal cell death and growth.

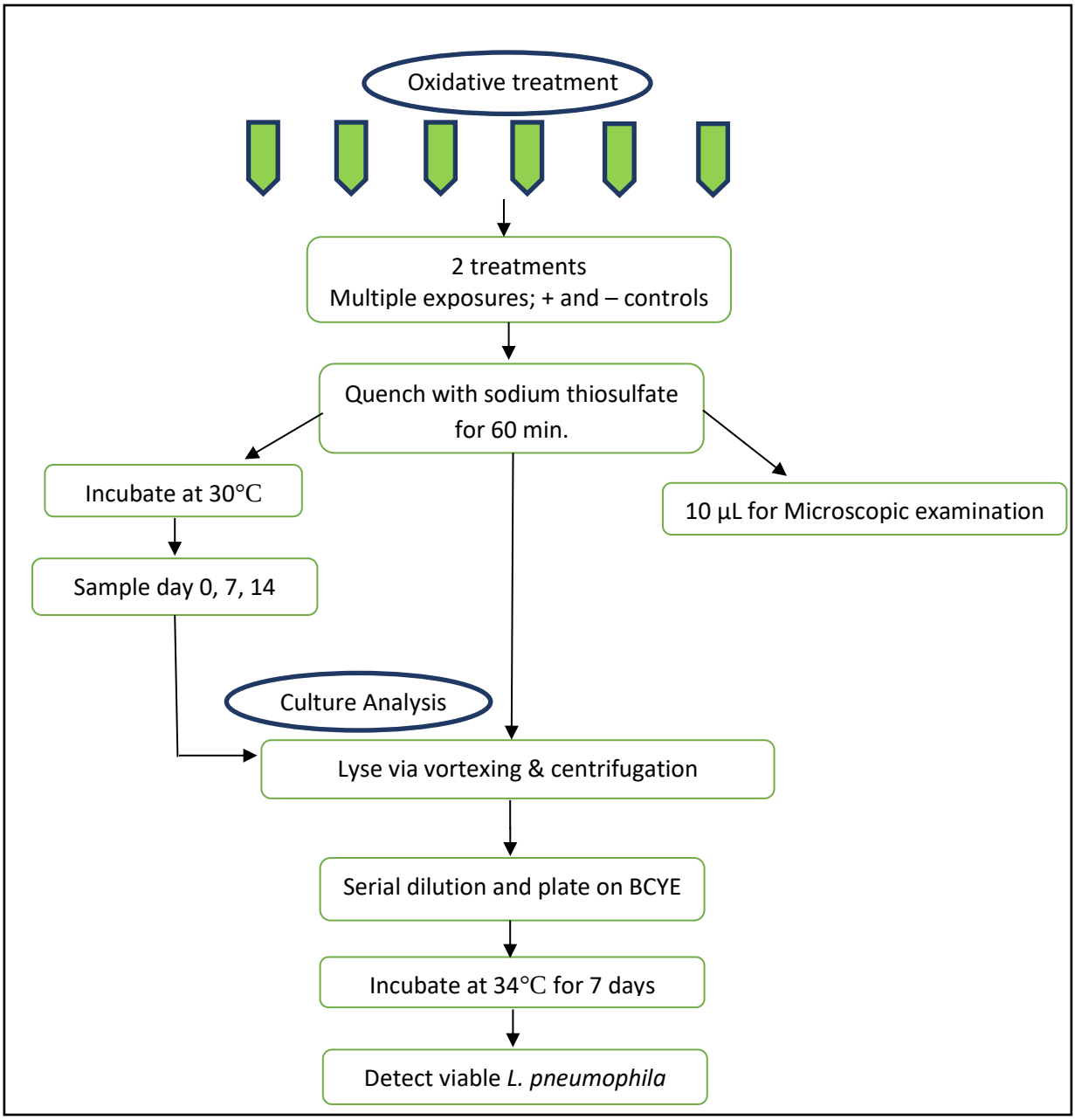

Figure 5. Flow chart illustrating the oxidation and culturability procedures.

Oxidant kinetics were calculated by finding time versus the natural log of the oxidant residual divided by the concentration of oxidant dosed $\left(\ln\frac{A}{A_0}\right)$, to ensure that our oxidant decay rates were representative.

Chapter 3: Results

Growth Curve

Spectrophotometric measurements of triplicate cultures were made to establish a growth curve between days 0 and 7 (Figure 6). These were averaged and used to determine the best day for infection. The growth curve results indicated that post-exponential growth, which is more infectious, would occur between day 4 and 7; with limited access to the laboratory in mind day 5 was chosen for infecting the amoeba (Molmeret, et al., 2004).

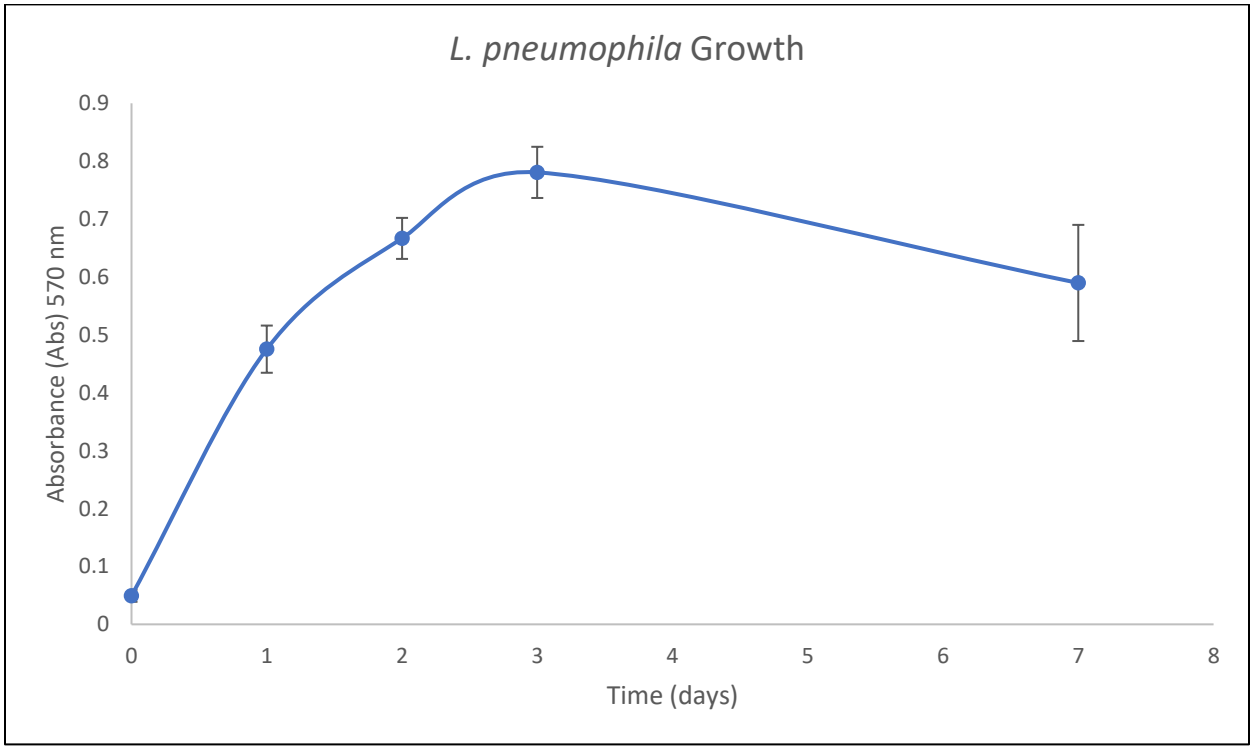

Figure 6. Growth curve of *L. pneumophila* in *Legionella* enrichment broth (n=3; error bars = standard error).

Controls

After exposure of *Acanthamoeba* for 90 min. to gentamicin and incubating for 7 days in PYNFH media, the colonies were enumerated and the concentration was calculated using the most probable number (MPN) method. The results of this test were used as an indication that gentamicin does not meaningfully impact the survival or growth of *A. polyphaga* until 200 µg/mL was dosed (Table 3 and Figure 7). A concentration of 100 µg/mL was chosen as the preferred concentration for removal of extracellular *Legionella* and was then tested on free *Legionella*.

Table 3. *A. polyphaga* ATCC #30461 viability after 90 min. gentamicin exposure

Gentamicin Dose	MPN/mL	Mean (MPN/mL) (n=3)
0 µg/mL	8.18×10^4	9.58×10^4
	1.47×10^5	
	5.87×10^4	
50 µg/mL	8.18×10^4	1.03×10^5
	1.47×10^5	
	8.19×10^4	
100 µg/mL	7.36×10^4	1.03×10^5
	5.76×10^4	
	1.78×10^5	
200 µg/ml	4.24×10^4	4.88×10^4
	4.24×10^4	
	6.15×10^4	

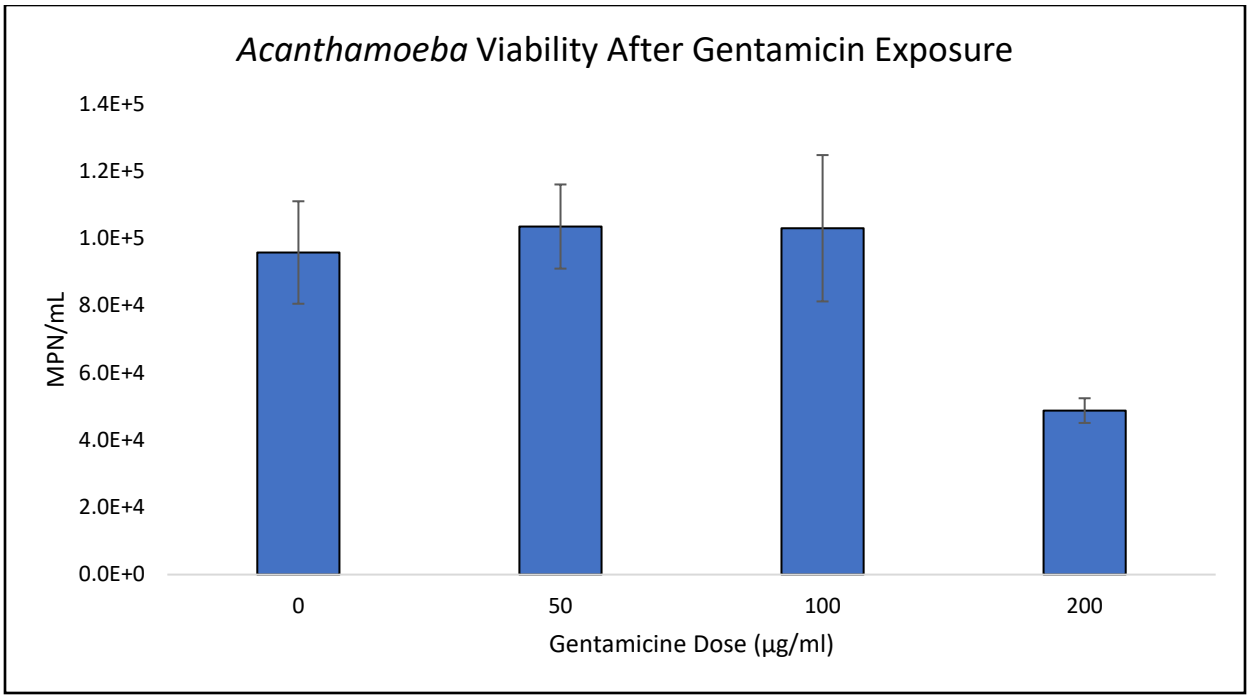

Figure 7. *A. polyphaga* ATCC #30461 viability after 90 min. gentamicin exposure (MPN = most probable number; n=3; error bars=standard deviation).

When free *Legionella* were exposed to 100 µg/mL dose of gentamicin only 3% of the cells were viable compared to the positive control that had no gentamicin exposure. The results from *Legionella* exposure to the gentamicin control further supported the choice of 100 µg/mL for eliminating extracellular *Legionella* (Table 4 and Figure 8).

Table 4. *L. pneumophila* ATCC# 33152 viability after 90 min. gentamicin exposure.

Gentamicin	CFU/mL	Mean (CFU/mL) (n=3)
0 µg/mL	1.85×10^3	1.91×10^3
	2.02×10^3	
	1.86×10^3	
100 µg/mL	8×10^1	6.3×10^1
	5×10^1	
	6×10^1	

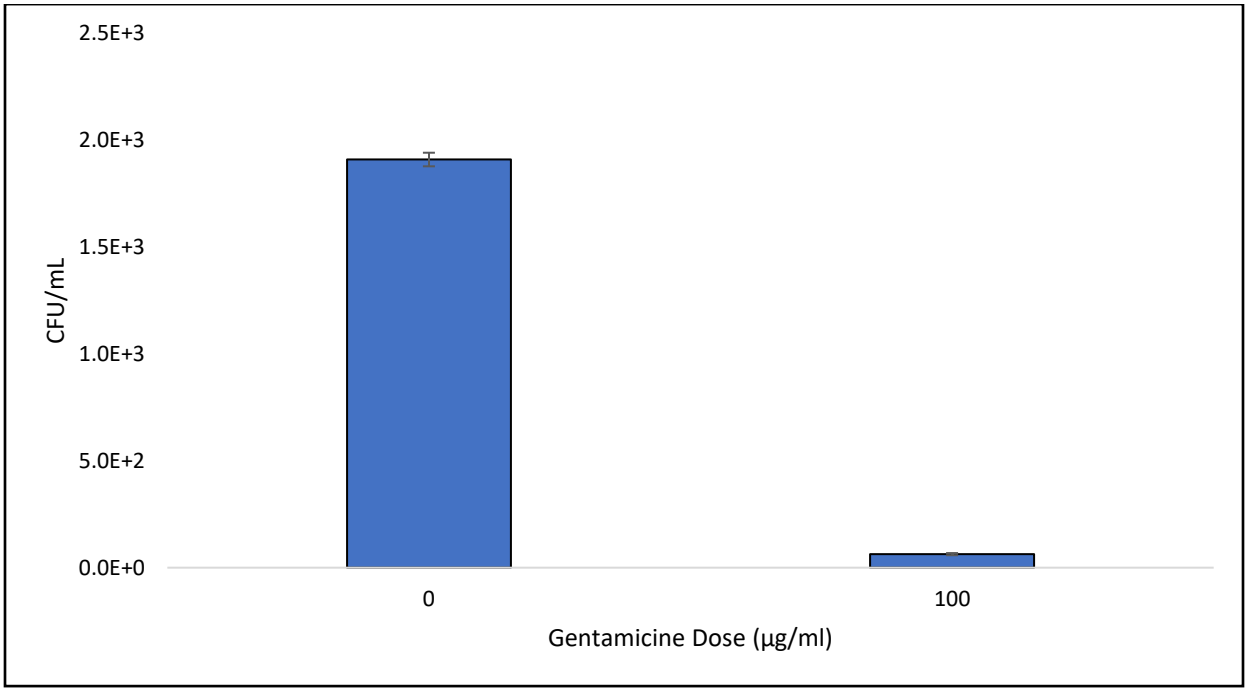

Figure 8. Mean *L. pneumophila* ATCC# 33152 viability after 90 min. gentamicin exposure (n=3; error bars= standard deviation).

After *Legionella* was exposed to sodium thiosulfate, there was some impact on viability, but the average difference between each plating day and the positive control was less than 5% (Table 5 and Figure 9). Thus, this impact was considered to be minimal and it was decided that the use of sodium thiosulfate had a negligible effect on *Legionella* survival within this experiment.

Table 5. Impact of sodium thiosulfate on *L. pneumophila* ATCC 33152 viability.

	Day 0	**Day 7**	**Day 14**
	CFU/mL		
L. pneumophila	110	120	120
	80	110	80
	100	100	80
Average (n=3)	97	110	100
Positive control	120	90	120
Negative control	No Growth		

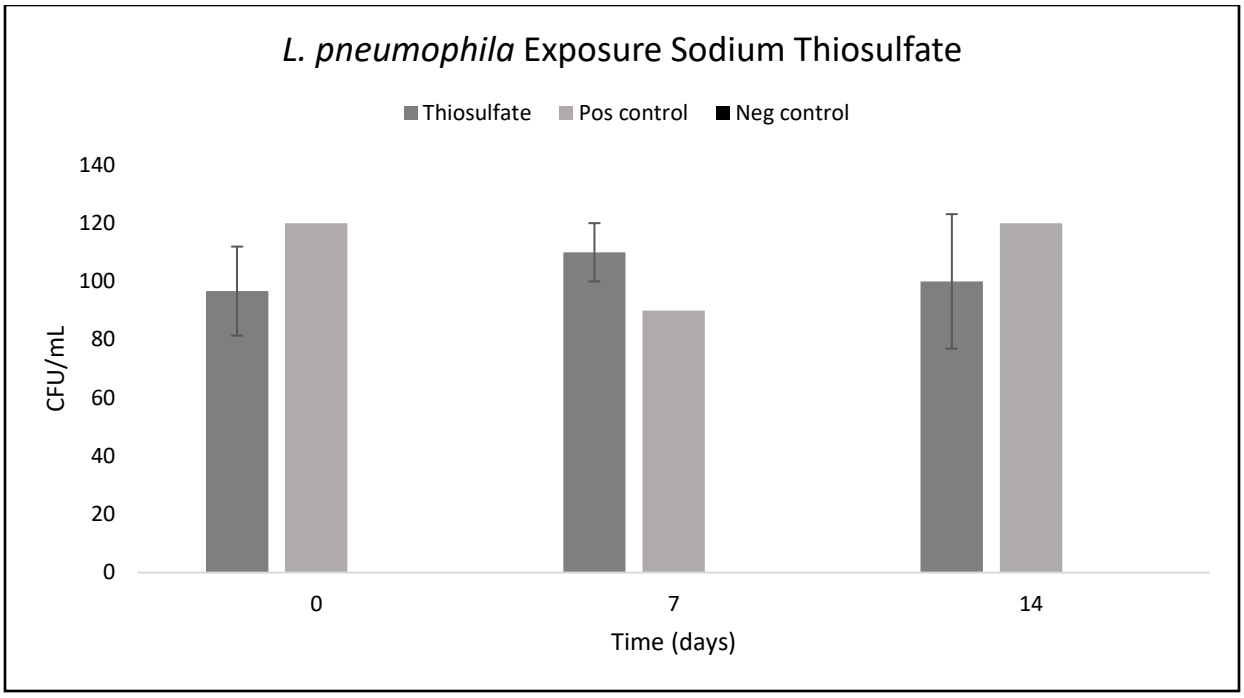

Figure 9. Mean survival of *L. pneumophila* exposure to 200 mg/L sodium thiosulfate (n=1 for pos. controls; no growth for neg. controls; error bars=standard deviation).

Infection Rate

The CellTrace CFSE Cell Proliferation Kit used to determine the infection rate by switching between fluorescent and light microscopy was an effective way to determine if amoeba cells were infected (Figure 10). By counting the sample on a hemocytometer multiple times, we observed that the greatest infection rate occurred between 4, 6, and 24 hrs. after *Acanthamoeba* samples were inoculated with *Legionella*, with an infection rate of 52%, 56%, and 50%, respectively (Figure 11). An incubation time of 24 hrs. after inoculation was chosen for experiments.

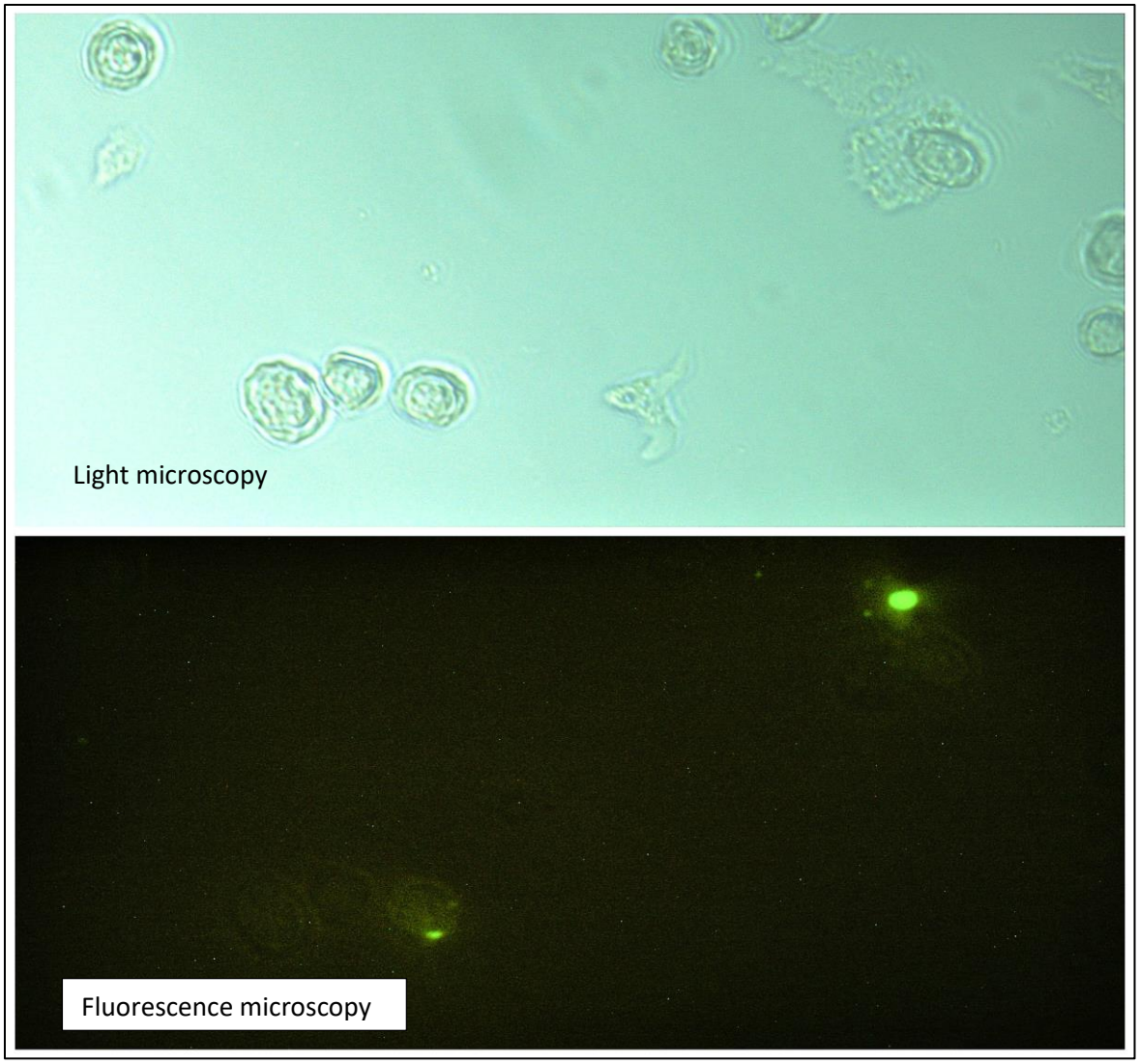

Figure 10. Photomicrographs of *A. polyphaga* sample infected with fluorescent *L. pneumophila*.

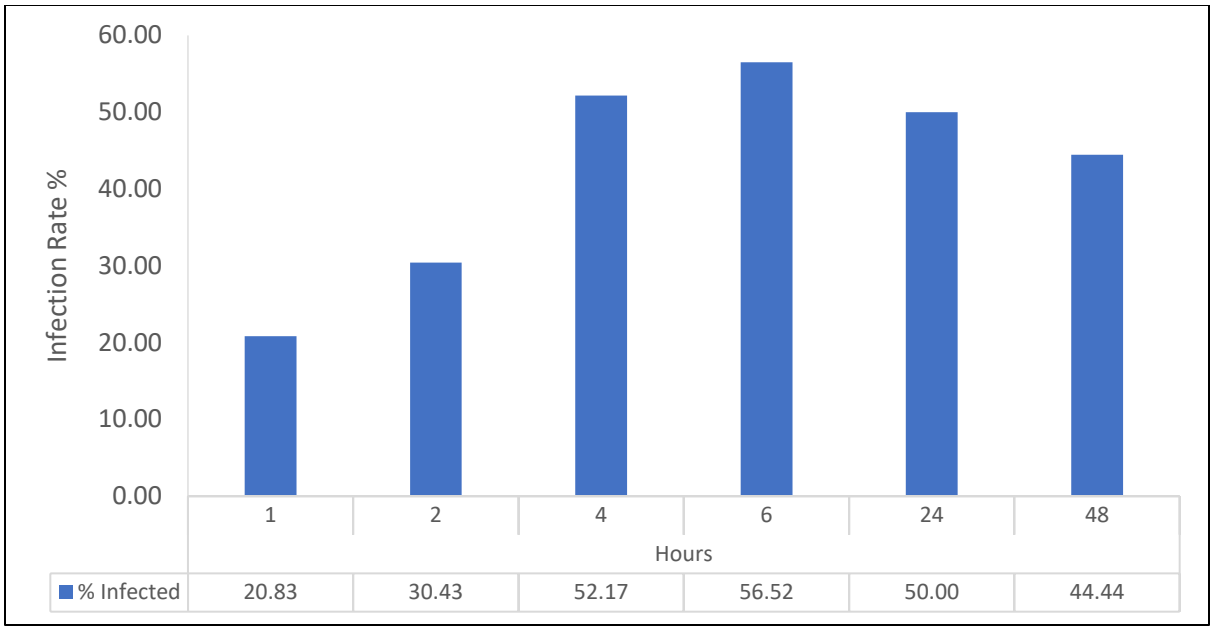

Figure 11. *L. pneumophila* infection rate of *Acanthamoeba*. Number of infected *Acanthamoeba* to not infected expressed as a percent.

Oxidative Decay

Chlorine and chlorine dioxide were measured throughout the exposure time to calculate the final CT and the decay kinetics for each dose (Figures 12, 13, 14, and 15). For Cl_2, the CT after 60 min. of exposure were: 3 mg/L= 5.6 mg.min/L, 5 mg/L= 29.5 mg.min/L, 7 mg/L= 56.7 mg.min/L, 20 mg/L= 376.3 mg.min/L, and 30 mg/L= 718 mg.min/L (Figure 12). Also, 12 mg/L of Cl_2 was tested and found to produce a CT of 99 mg.min/L, but it produced abnormal growth results that greatly exceeded the positive control; therefore, this data point was omitted. The kinetics of the decay of the chlorine samples all showed R^2 values greater than 0.95 indicating that this decay was reasonably represented by the measurements (Figure 13).

As for chlorine dioxide, the CT after 60 min exposure were: 7 mg/L=98.9 mg.min/L, 12 mg/L=249.4 mg.min/L, 20 mg/L=532.1 mg.min/L, 30 mg/L=997.7 mg.min/L (Figure 14). The kinetics of the decay of the chlorine dioxide samples all showed R^2 values greater than 0.95 indicating that this decay was reasonably represented by the measurements (Figure 15). A notable difference between the chlorine and chlorine dioxide was that free chlorine depleted at a somewhat steady rate while chlorine dioxide depleted quickly during the first 3 minutes, but the decay slowed greatly after this maintaining a relatively stable residual. This difference between the oxidants showed chlorine dioxide to have notably higher CT compared to chlorine at the same dose.

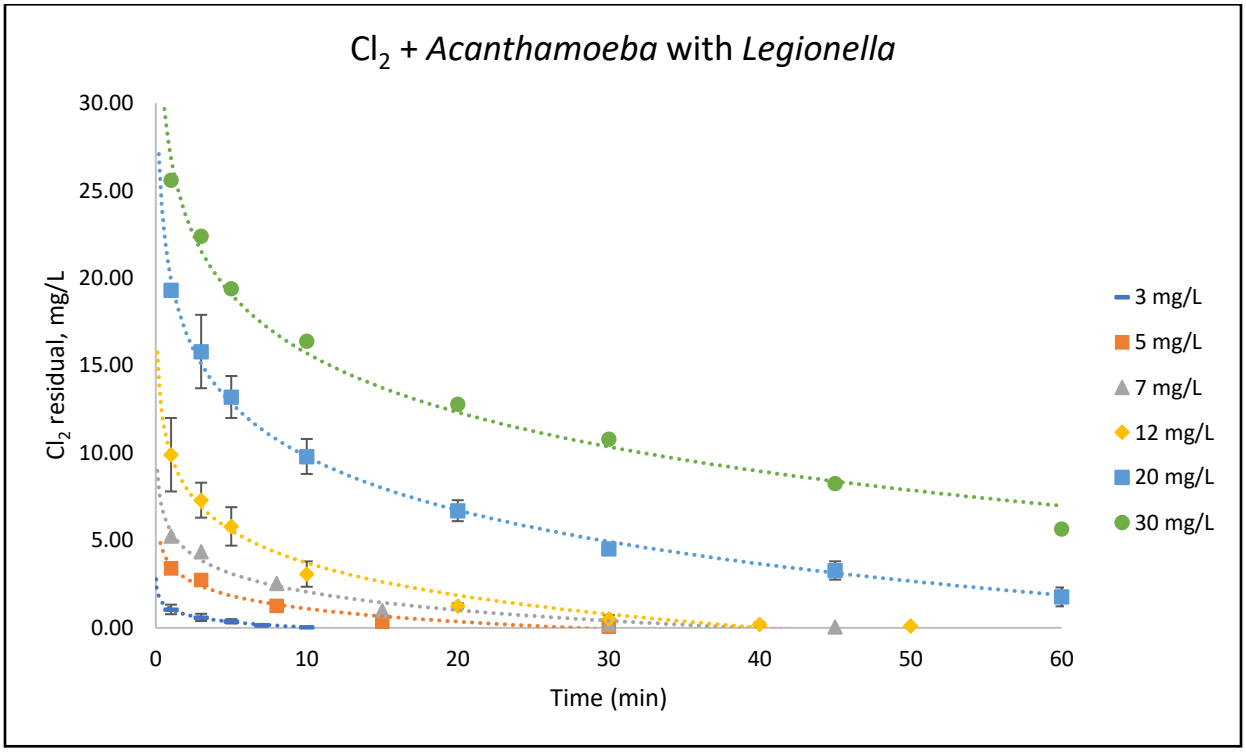

Figure 12. Chlorine decay during *Legionella* infected *A. polyphaga* exposure (n=3 for 3 mg/L and 5 mg/L; n=2 for 7 mg/L, 12 mg/L, and 20 mg/L; n=1 for 30 mg/L; error bars=standard deviation).

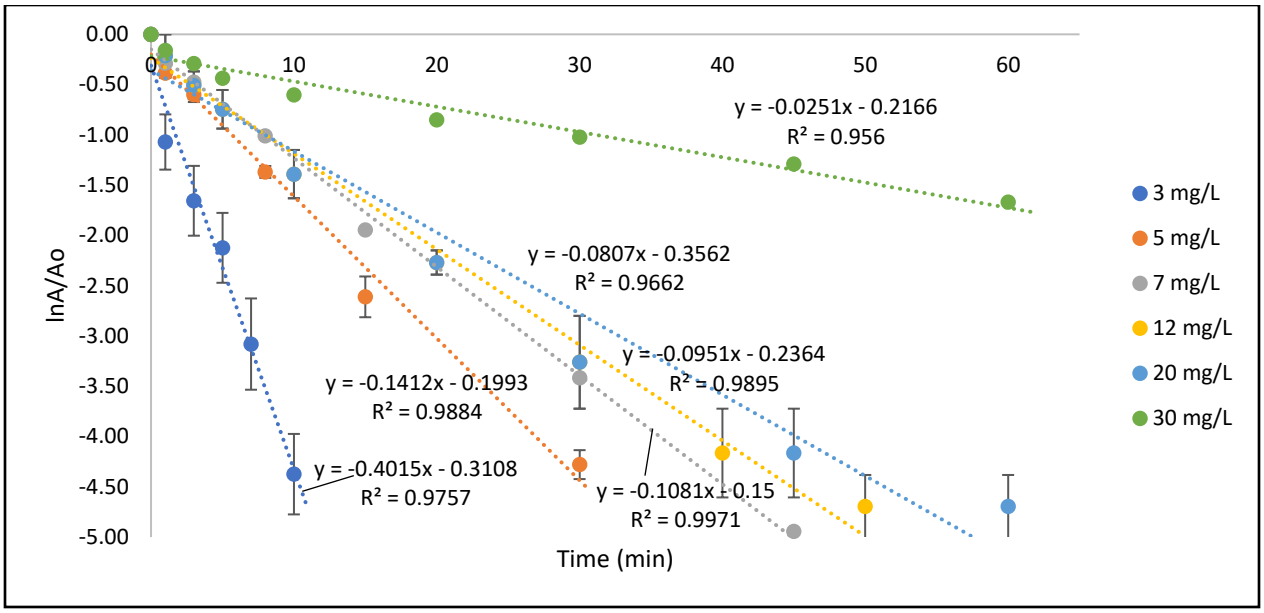

Figure 13. Chlorine decay kinetics during *Legionella* infected *A. polyphaga* exposure (n=3 for 3 mg/L and 5 mg/L; n=2 for 7 mg/L, 12 mg/L, and 20 mg/L; n=1 for 30 mg/L; error bars=standard deviation).

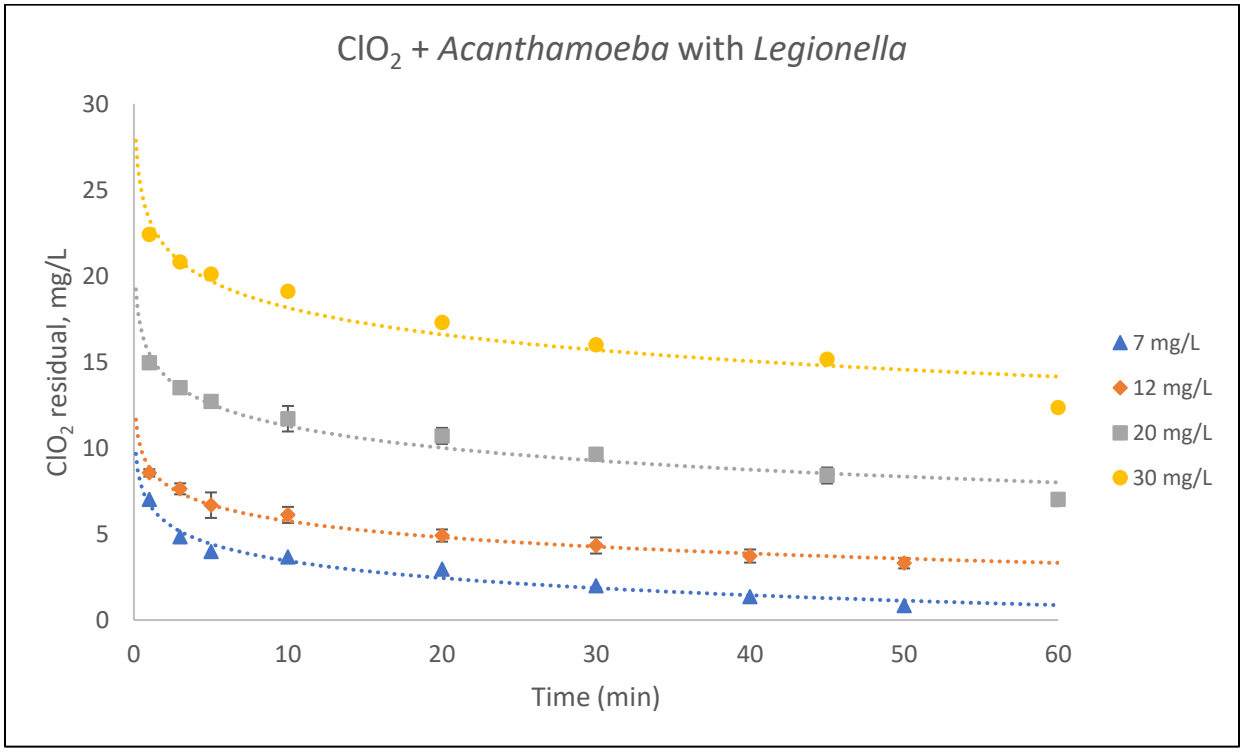

Figure 14. Chlorine dioxide decay during *Legionella* infected *A. polyphaga* exposure (n=3 for 7 mg/L n=3; n=2 for 12 mg/L and 20 mg/L; n=1 for 30 mg/L; error bars=standard deviation).

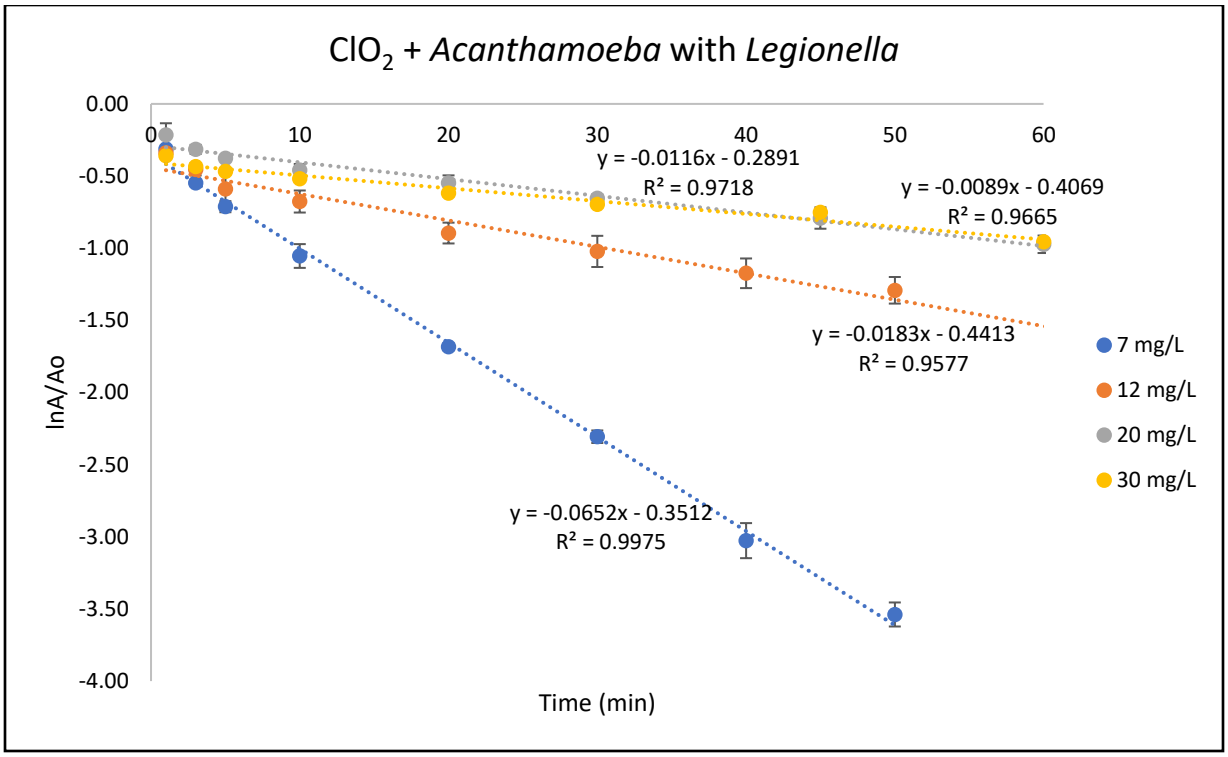

Figure 15. Chlorine dioxide decay kinetics during *Legionella* infected *A. polyphaga* exposure (n=3 for 7 mg/L; n=2 for 12 mg/L and 20 mg/L; n=1 for 30 mg/L; error bars=standard deviation).

Culturability

Chlorine generally produced decreasing concentrations of viable *Legionella* cells with increasing CT exposures. It was also observed that there was a decrease in *Legionella* CFU for CTs 5.6 and 29.5 mg.min/L over the recovery period and an increase in *Legionella* CFU for all other CTs as time increased (Table 6). When comparing percent survival of *L. pneumophila* after exposure to chlorine, it was notable that even with increasing CT the CFU generally increased over the 14-day period with all CTs (Figure 16). However, the percent survival of samples exposed to 5.6, 29.5, and 56 mg.min/L became relatively similar by day 14, showing 28, 27 and 30% survival, respectively.

Table 6. Mean CFU/mL of *L. pneumophila* with *A. polyphaga* following chlorine exposure.

CT	Dose	n	**CFU/mL** ± standard error		
			Day 0	**Day 7**	**Day 14**
5.6 mg.min/L	**3 mg/L**	3	$1.37\times 10^{2}\pm 0.58$	$1.56\times 10^{2}\pm 2.11$	$1.20\times 10^{2}\pm 2.58$
29.5 mg.min/L	**5 mg/L**	3	$1.30\times 10^{2}\pm 1.19$	$1.78\times 10^{2}\pm 0.36$	$1.14\times 10^{2}\pm 1.75$
56.7 mg.min/L	**7 mg/L**	2	$9.6\times 10^{1}\pm 0.11$	$1.21\times 10^{2}\pm 1.58$	$1.26\times 10^{2}\pm 1.07$
Positive Control		1	8.60×10^{2}	5.07×10^{2}	4.20×10^{2}
376.3 mg.min/L	**20 mg/L**	2	$4.95\times 10^{1}\pm 0.06$	$1.07\times 10^{2}\pm 3.05$	$1.26\times 10^{2}\pm 3.19$
718 mg.min/L	**30 mg/L**	1	6×10^{0}	3×10^{0}	1.27×10^{1}
Positive Control		1	3.87×10^{3}	1.48×10^{3}	1.59×10^{3}

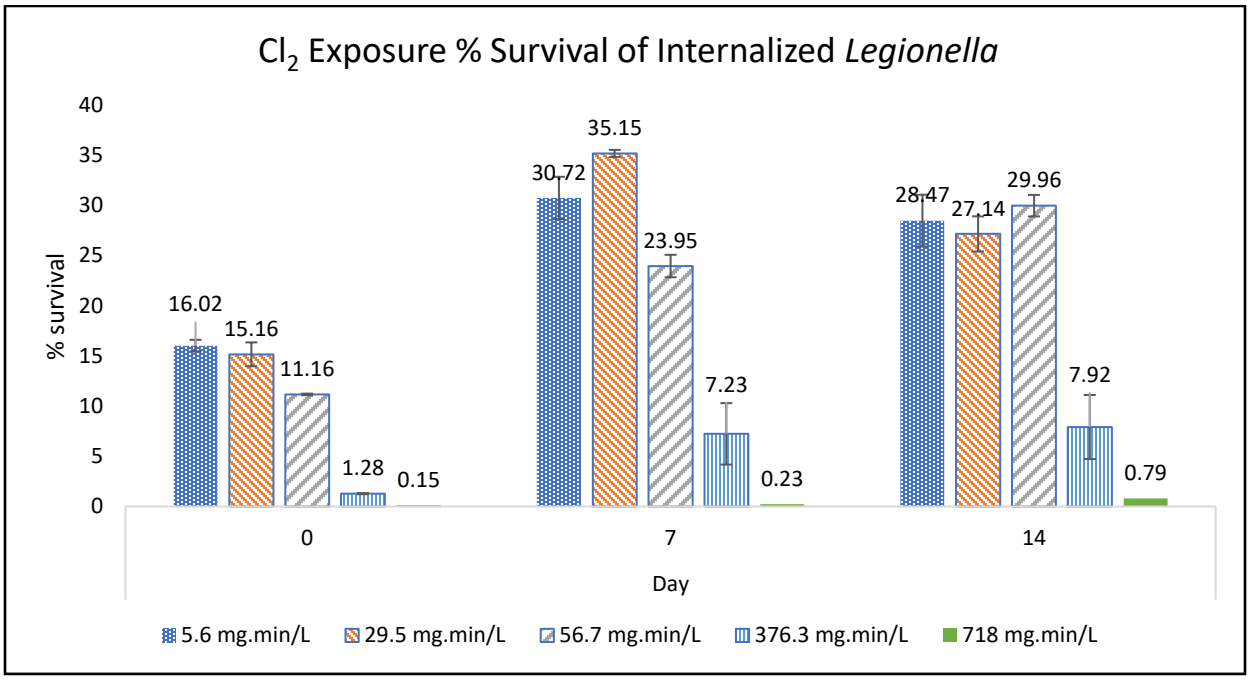

Figure 16. Survival of Cl_2 exposed *Acanthamoeba* internalized *Legionella* (n=3 for 5.6 mg.min/L and 29.5mg.min/L; n=2 for 56.7 mg.min/L and 376.3 mg.min/L; n=1 for 718 mg.min/L; error bars=standard error).

ClO_2 proved to be effective immediately and was able to limit recovery of *Legionella* over time. There is some indication that there was growth of *L. pneumophila* over time at 249.4 mg.min/L, noticeably increasing over the 14-days, but the other CTs of 74.21, 532.1, and 997.7 mg.min/L indicated a reduction in the number of viable cells as time increased (Table 7). When comparing percent survival of the different CTs, 249.4 mg.min/L produced an increase of *Legionella* from 0.94 to over 3.62% while 532.1 and 997.7 mg.min/L decreased from 0.51 and 0.60% to 0.29 and 0.06%, respectively (Figure 17).

Table 7. Mean CFU/mL of *L. pneumophila* with *A. polyphaga* following chlorine dioxide exposure.

CT	**Dose**	**n**	**CFU/mL** ± standard error		
			Day 0	**Day 7**	**Day 14**
74.21 mg.min/L	**7 mg/L**	3	$7.27\times 10^{2}\pm 23.6$	$5.40\times 10^{2}\pm 28.7$	$1.33\times 10^{2}\pm 5.19$
Positive Control		1	5.50×10^{3}	5.70×10^{3}	5.80×10^{3}
249.4 mg.min/L	**12 mg/L**	2	$1.42\times 10^{1}\pm 3.93$	$1.92\times 10^{1}\pm 1.13$	$2.1\times 10^{1}\pm 4.43$
532.1 mg.min/L	**20 mg/L**	2	$8\times 10^{0}\pm 2.48$	$1\times 10^{0}\pm 0.83$	$2\times 10^{0}\pm 0.58$
997.7 mg.min/L	**30 mg/L**	1	9×10^{0}	1×10^{0}	$<1\times 10^{0}$
Positive Control		1	1.51×10^{3}	9.67×10^{2}	5.80×10^{2}

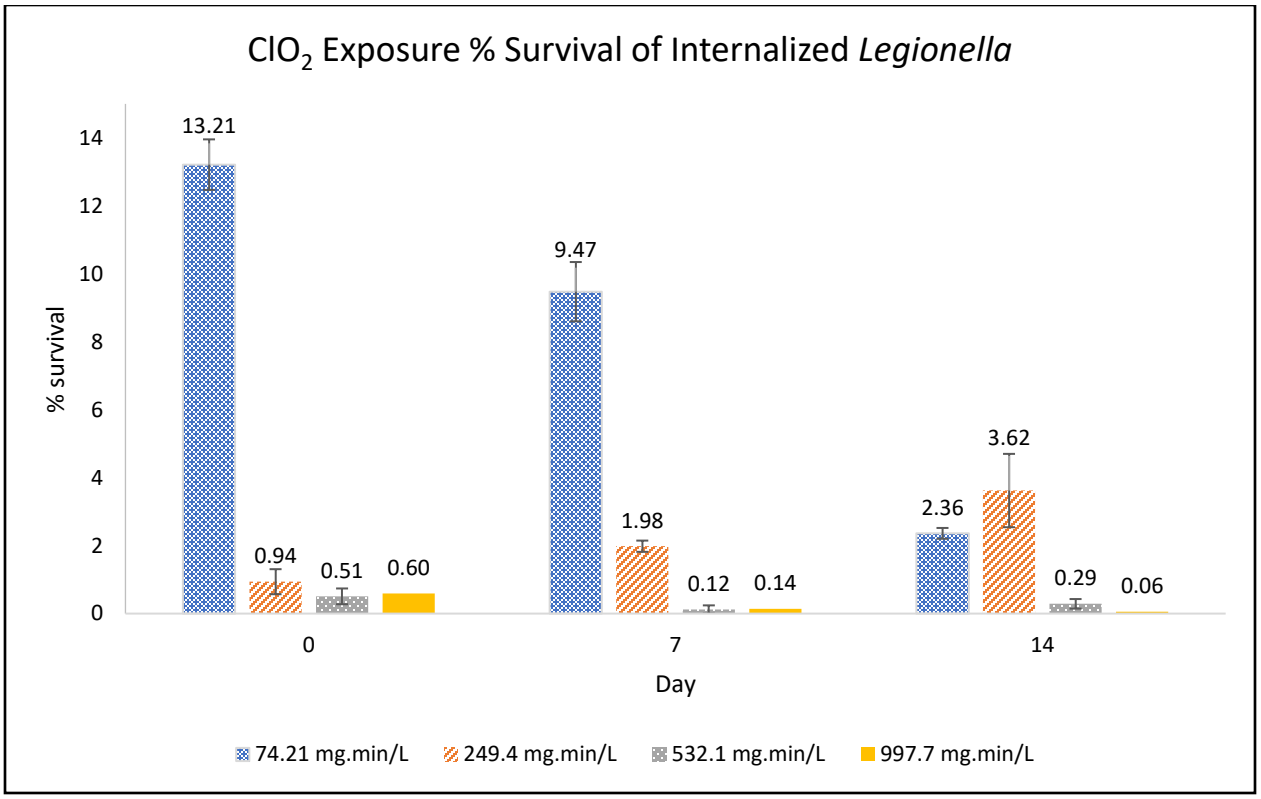

Figure 17. Survival of ClO_2 exposed *Acanthamoeba* internalized *Legionella* (n=3 for 74.21 mg.min/L; n=2 for 249.4 mg.min/L and 532.1 mg.min/L; n=1 for 718 mg.min/; error bars = standard error).

The chlorine CTs of 5.6 mg.min/L, 29.5 mg.min/L, and 56.7 mg.min/L produced an initial log reduction of 0.79, 0.82, and 0.95, respectively (Figure 18 and Table 8), showing an increase in *Legionella* Log reduction as CT increased. The CT of 56.7 mg.min/L produced an initial log reduction of 0.95, achieving a nearly 1 log reduction. At a CT of 376.3 mg.min/L, Cl_2 produced a log reduction approaching 2. The highest CT of Cl_2, 718 mg.min/L, produced an initial log reduction of 2.83 coming the closest of either oxidant to achieving an initial 3 log reduction. The significant difference between the Cl_2 CTs (Figure 18) can be used to answer research question one (what are the most effective CTs for chlorine and chlorine dioxide to reduce the survival of internalized *L. pneumophila* after treatment) for Cl_2. Though much higher

than may be reasonable for water treatment, 718 mg.min/L was the most effective Cl_2 CT. However, all Cl_2 CTs showed some *Legionella* recovery through the study time period (Figure 18 and Table 8).

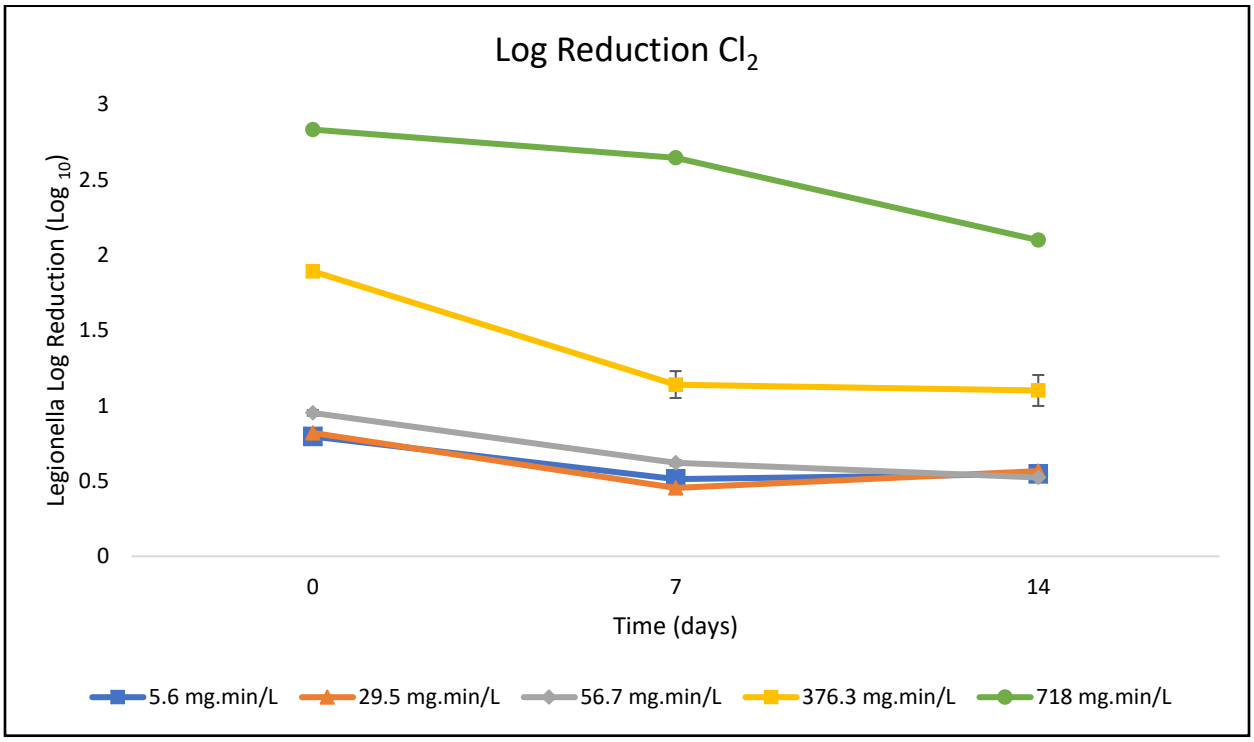

Figure 18. Log reduction of *Acanthamoeba* internalized *L. pneumophila* after exposure to Cl_2 in sterilized lake water. Normalized using positive control to account for normal cell death affecting results (n=3 for 5.6 mg.min/L and 29.5mg.min/L; n=2 for 56.7 mg.min/L and 376.3 mg.min/L; n=1 for 718 mg.min/L; error bars=standard deviation).

The ClO_2 CT 74.21 mg.min/L produced an initial log reduction of 0.88, achieving a nearly 1 log reduction (Figure 19 and Table 8). The CT 249.4 mg.min/L produced an initial log reduction of 2.03, achieving just above a 2-log reduction. The ClO_2 CT of 532.1 mg.min/L produced an initial log reduction of 2.29 (Figure 19 and Table 8). Finally, the ClO_2 CT 997.7 mg.min/L produced an initial log reduction of 2.22. The data showed a difference between all

CTs, with the exception of 532.1 and 997.7 mg.min/L. These results can be used to answer research question one (what are the most effective CTs for chlorine and chlorine dioxide to reduce the survival of internalized *L. pneumophila* after treatment) for ClO_2. Although, likely a much higher concentration than may be reasonable for water treatment, 532.1 mg.min/L was the most effective ClO_2 CT initially. However, by the end of the recovery period 997.7 mg.min/L achieved a log reduction of 3.24, the highest seen in any of the trials for either oxidant (Figure 19 and Table 8).

Table 8. Log reduction of *A. polyphaga* internalized *L. pneumophila* after chlorine and chlorine dioxide treatments.

CT	**Dose**	**n**	**Log Reduction (± standard error)**		
			Day 0	**Day 7**	**Day 14**
Cl_2					
5.6 mg.min/L	**3 mg/L**	3	0.79±0.022	0.51±0.021	0.55±0.026
29.5 mg.min/L	**5 mg/L**	3	0.82±0.026	0.45±0.014	0.57±0.020
56.7 mg.min/L	**7 mg/L**	2	0.95±0.018	0.62±0.017	0.52±0.014
376.3 mg.min/L	**20 mg/L**	2	1.89±0.028	1.14±0.089	1.10±0.103
718 mg.min/L	**30 mg/L**	1	2.83±0.193	2.65±0.073	2.10±0.086
ClO_2					
74.21 mg.min/L	**7 mg/L**	3	0.88±0.018	1.02±0.042	1.64±0.043
249.4 mg.min/L	**12 mg/L**	2	2.03±0.077	1.70±0.019	1.44±0.055
532.1 mg.min/L	**20 mg/L**	2	2.29±0.092	2.92±0.097	2.54±0.087
997.7 mg.min/L	**30 mg/L**	1	2.22±0.113	2.86±0.101	3.24±0.018

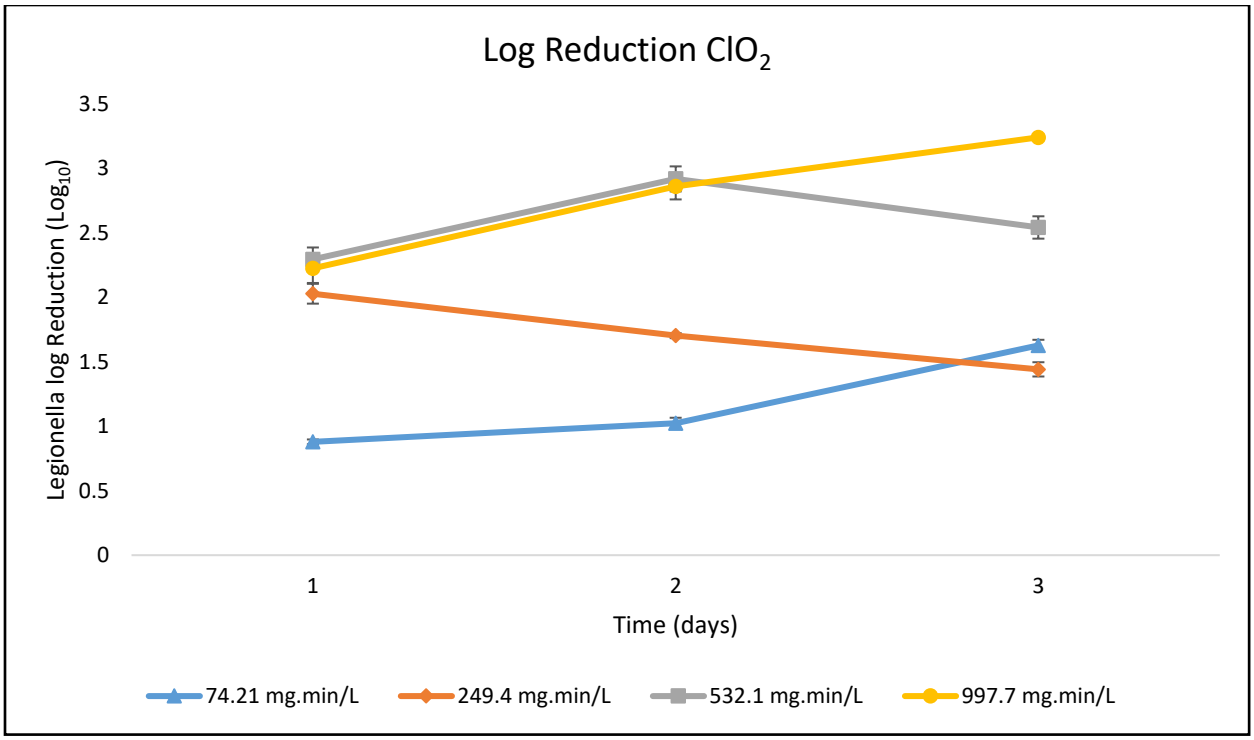

Figure 19. Log reduction of *Acanthamoeba* internalized *L. pneumophila* after exposure to ClO_2 in sterilized lake water. Normalized using positive control to account for normal cell death affecting results (n=3 for 74.21 mg.min/L; (n=2 for 249.4 mg.min/L and 532.1 mg.min/L; n=1 for 718 mg.min/L; error bars=standard deviation).

The lowest three Cl_2 CTs did not achieve even a 1 log reduction, and 56.7 mg.min/L produced a decreasing log reduction from 0.95 to 0.52 as recovery time increased (Figure 18 and Table 8). There appears to be little difference between these three CTs, taking into account how similar these trend lines were in Figure 18, showing that increasing CT maintains greater clearance of *Legionella*. The Cl_2 CT of 376.3 mg.min/L produced a decreasing log reduction of 1.89 and 1.10 as recovery time increased (Figure 18 and Table 8). Thus, this CT achieved nearly a 2-log reduction while maintaining at least a 1 log reduction over time. The highest CT of Cl_2, 718 mg.min/L, produced a decreasing log reduction of 2.83 and 2.10 with increasing recovery time (Figure 18 and Table 8), maintaining a 2-log reduction during the recovery time. With these results, part of research question two (what is the difference in the effectiveness of chlorine

and chlorine dioxide in preventing *Legionella* from recovering after treatment at different CTs) can be addressed in seeing that all Cl_2 CTs allowed recovery of *Legionella* (Figure 18).

For ClO_2 CTs, 74.21 mg.min/L produced an increasing log reduction from 0.88 to 1.64 as recovery time increased (Figure 19 and Table 8). The ClO_2 exposure of 249.4 mg.min/L produced a decreasing log reduction from 2.03 to 1.44 with increasing recovery time. In comparing these two CTs (Figure 19) the trend lines cross between day 7 and 14, with 74.21 mg.min/L showing a continued reduction and 249.4 mg.min/L having continued recovery. The ClO_2 exposure of 532.1 mg.min/L produced an increasing log reduction between 2.29 and 2.54 over the recovery time. This was an unusual growth pattern, as there was an initial loss of *Legionella*, but recovery was seen between 7 and 14-days (Figure 19). Finally, the ClO_2 exposure of 997.7 mg.min/L produced an increasing log reduction from 2.22 to 3.24 with increasing recovery time (Figure 19 and Table 8), showing a continued reduction of *Legionella* resulting in over a 3 log reduction. These results show that there is a difference between all of these CTs, except day 0 and 7 for 532.1 and 997.7 mg.min/L, but over the entire period the difference was notable. In comparing the results on Figure 19, there was a noticeable difference in the CT needed to reduce *Legionella's* ability to recover over time, but the trends were unclear. Even so, it is apparent that ClO_2 generally produced an increasing log reduction over the recovery period. These results can be used to address research question two (what is the difference in the effectiveness of chlorine and chlorine dioxide in preventing *Legionella* from recovering after treatment at different CTs). ClO_2 is the more effective oxidant for preventing *Legionella* recovery.

Chapter 4: Discussion

To our knowledge, this is the first study to compare water treatment oxidants and their impact given a recovery period after exposure to *Acanthamoeba* internalized *Legionella*. For the study, it was decided to perform culture analysis on days 0, 7, and 14, to simulate what can occur after internalized *Legionella* sit for a period of time in a building's plumbing system. It is not uncommon for buildings to have additional treatments that can remove oxidants (e.g., water purification systems) nor is it uncommon for water to sit for extended periods in these plumbing systems (Ling, Whitaker, LeChevallier, & Liu, 2018). Previous studies have looked only at the immediate impact of the oxidative treatment, with most focusing on either one oxidant, *Legionella* alone, or *Acanthamoeba* alone. These have been used in the development of some guidance for the treatment and prevention of *Legionella* in water systems, but have yet to fully address the continued appearance of *Legionella* in plumbing systems or how to protect the public from this danger (EPA, 2016c). With the increasing occurrence of Legionnaires' disease and the complex nature of *Legionella's* ecological niche, improving scientific understanding of *Legionella* becomes highly relevant to public health and water treatment (CDC, 2018).

The increased resistance of *L. pneumophila* to chlorine after infecting *A. polyphaga* was evident when comparing our results to those of Kuchta *et al.* (1983) and Jacangelo *et al.* (2002), both focused on free *Legionella*. These researchers reported a 2-log reduction with CTs of 0.5-9 mg.min/L, (Kuchta *et al.,* 1983) and 30-60 mg.min/L, (Jacangelo *et al.,* 2002). In contrast, our results indicate that a CT of between 376.3 mg.min/L and 718 mg.min/L would be necessary to achieve log reductions of 1.89 and 2.83, respectively on day 0. Also, by looking at the time after exposure, our study showed that 376.3 mg.min/L maintained only a 1.1 log reduction and 718 mg.min/L a 2.1 log reduction. Kuchta *et al.* (1983) and Jacangelo *et al.* (2002) did not report

effects of the chlorine given time. These results indicate that both the ecology and recovery time in relation to *Legionella* needs further investigation.

Dupuy *et al*. (2011) found a 1 to over a 2-log inactivation of *Legionella* grown in co-culture with different species of *Acanthamoeba* using CTs of only 5 mg.min/L. This originally appears to conflict with our results as we required much higher CTs to achieve similar results, but these researchers used rather different conditions. Some of the differences were: the temperatures used were 30-50°C (similar to hot water systems but allowing for faster reactions with oxidants), external *Legionella* was not removed, CT was determined based on only 4 sample points, and instead of a representative water sample for the medium they used phosphate buffer solution (decreasing reactions between the media and the oxidant). Our study focused on only internal *Legionella* (by use of gentamicin) and simulated more common conditions by using room temperature (~20°C) and sterilized lake water. Even so, Dupuy *et al*. showed that ClO_2 was more effective than Cl_2 in reducing *Legionella* co-cultured with *Acanthamoeba.* This finding is in agreement with our results showing that, in general, ClO_2 exposure had lower percent survival and greater log reduction of *Legionella* then Cl_2 exposure of similar or greater CT.

Our first research question was, "What are the most effective CTs for chlorine and chlorine dioxide to reduce the survival of internalized *L. pneumophila* after treatment?" Within water treatment, successful control of most infectious microorganisms is often based in log reduction, as seen in recommendations set forth by government organizations (e.g., EPA) and was our basis for measuring effectiveness (Alleron, *et al*., 2008). Depending on a facility's needs or goals for *Legionella* control, different CTs can be applied under different situations (e.g., a 1, 2, or 3 log reduction). To achieve approximately a 1 log initial reduction, Cl_2 required

a CT of 56.7 mg.min/L and ClO_2 required 74.21 mg.min/L, showing log reductions of 0.95 and 0.88, respectively. After the 14-day period, Cl_2 CTs showed recovery while ClO_2 presented increased log reduction. When comparing the change in percent survival of *Legionella* over the 14-days, the Cl_2 CT of 56.7 mg.min/L showed a 0.78 fold increase and the ClO_2 CT of 74.21 mg.min/L showed a 0.82 fold decrease, indicating that ClO_2 allowed continued reduction of *Legionella* while Cl_2 produced *Legionella* recovery. For an approximately 2 log reduction, Cl_2 required a CT of 376.3 mg.min/L and ClO_2 required 249.4 mg.min/L, achieving log reductions of 1.89 and 2.03, respectively. After the 14-day period, both the Cl_2 CT and ClO_2 CT allowed *Legionella* recovery, as shown by a decreasing log reduction. When comparing the percent survival over the 14-days, the Cl_2 CT of 376.3 mg.min/L showed a 5.18 fold increase and the ClO_2 of 249.4 mg.min/L showed a 1.92 fold increase, indicating that ClO_2 allowed less *Legionella* recovery than Cl_2. No CT achieved a 3-log reduction initially but the Cl_2 CT of 718 mg.min/L achieved an initial log reduction of 2.83. Interestingly, the ClO_2 CTs 532.1 mg.min/L and 997.7 mg.min/L achieved initial log reductions of 2.29 and 2.22, respectively. With the CT of 997.7 mg.min/L, the highest log reduction of 3.24 was observed on day 14, but 532.1 mg.min/L showed a log reduction decrease between day 7 and day 14. However, 532.1 mg.min/L did not reach the same reduction seen on day 0. It is evident that ClO_2 generally requires lower CTs than chlorine to achieve similar or higher log reductions than that seen with Cl_2. ClO_2 also maintains a higher log reduction by the end of 14-days.

Our second research question was, "what is the difference in effectiveness of chlorine and chlorine dioxide in preventing *Legionella* from recovering after treatment at different CTs?" In all, as seen in comparing log reduction results, ClO_2 exposure appeared to be the most effective at reducing recovery of *L. pneumophila* when compared to Cl_2. This may be due to the

difference in the reaction mechanisms of the oxidants. Cl_2 performs oxidative substitution and addition while ClO_2 reacts through free radical electrophilic abstraction (Baribeau, *et al*., 2002). Another possible explanation for the continued loss seen after ClO_2 exposure but not Cl_2 exposure is that ClO_2 produces over 40 disinfection byproducts (WHO, n.d. b) and at least one of the decay products, chlorite, is also an effective biocide (Gagnon et al., 2005). Chlorite was not measured as it was beyond the scope of this project but may have persisted and inhibited *Legionella* recovery. Over all, the comparison of these groups shows that ClO_2 is the better choice in reducing the recovery of *L. pneumophila* after treatment but the few unusual results indicate that more research is needed.

It is important to address the unusual reduction of viable *Legionella* that occurred with the ClO_2 CT 532.1 mg.min/L between day 0 and 7, with initial *Legionella* loss seen and regrowth evident between day 7 and 14, shown by a log reduction of 2.29, 2.92, and 2.54 for day 0, 7, and 14, respectively. This may be due to the morphological changes in *A. polyphaga* after being exposed to ClO_2 described by Mogoa, *et al*. (2011), as they reported cells becoming highly vacuolated and cytoplasm remained rather dense. Thus, the amoeba present may have been initially less active due to such structural changes, being unable to take up *Legionella* until day 7 and recovering between day 7 and 14, allowing *Legionella* to reproduce within these now functional amoebae. This may be supported by the similar initial reduction seen with the ClO_2 CT 997.7 exposure between day 0 and 7, but these amoebae were not able to recover as indicated by a continued drop in viable *Legionella*. These changes may also indicate that ClO_2 is more effective against *Acanthamoeba* than Cl_2, which is in agreement with the findings of Dawson and Brown (1987).

Another important result to note is the increasing clearance *of Legionella* seen in the ClO_2 CT 74.21, 532.1, and 997.7 mg.min/L while 249.4 mg.min/L showed recovery. A possible explanation for this is that the shock from exposure to the higher CT of 249.4 mg.min/L caused a greater number of amoeba cells to go into the cyst state sooner than that seen in the lower 74.21 mg.min/L CT. By responding sooner to the oxidant exposure, more of the *Acanthamoeba* may have survived this treatment period and thus maintained this route for *Legionella* to amplify. Higher CTs 532.1 and 997.7 mg.min/L may have been able to overcome the protective cyst membrane by destroying or penetrating the cellulose layer and allowing it to damage the amoeba cell. In contrast, the 74.21 mg.min/L CT may have been able to have a greater impact on a greater number of amoebas before they responded by entering a cyst state, thus killing or damaging more amoeba. This in turn, may have more effectively reduced the availability of this route of *Legionella* amplification. Unfortunately, without having measured *Acanthamoeba,* for enumeration of trophozoites and cysts, as well during the sampling days, it is difficult to fully address the amoeba's response. While microscopic examination of the samples dose with 249.4 mg.min/L ClO_2 CT did appear to have more amoeba cysts present on day 0 and more amoeba cells in general on the 7^{th} day in comparison to the 74.21 mg.min/L CT sample, this was not quantified.

This experimental study did not fully represent how these organisms would act in an actual plumbing environment. It is also worth noting that different CTs are applied to control different organisms. All these organisms can respond differently but this study was designed to only address *A. polyphaga* internalized *L. pneumophila*. In all, these data can be used for future recommendations, but remain only one part of what is considered in water treatment application and should be used in conjunction with other scientific results.

This study did have some limitations. First, a small sample size can limit our ability to see variation in the results. Second, a small sample volume was used. Due to limited space, flask size, and restrictions in the growth of the microorganisms, we were limited to 80 mL for each sample. This could result in limitations on the microbial growth as nutrients are depleted during the 14-day incubation period. Third, there was limited biodiversity of the sample. Although this study was intended to focus on the interaction of these two organisms, the biological communities in natural and man-made water systems can be far more diverse and would likely change the activity and interactions of *L. pneumophila* and *A. polyphaga*. Biodiversity should be the focus of future studies. Fourth, this study did not take into account viable but not culturable cells (VBNC). Due to limited sample volume, it was decided to focus on the current gold standard for *Legionella* enumeration, plating on BCYE agar, instead of the methods for detecting VBNC cells, such as flow cytometry, which can require a relatively large sample volume (CDC, 2018). VBNC cells could be a useful area of future study. Fifth, the limited number of sample days was limited. Due to time constraints, we were only able to sample once a week and this may have led to missing data points that could have been illuminating as to how *Legionella* reacts over time. Finally, only one source water was used. Part of the intent for this study was to address the concern of Legionnaires' disease in Southern Nevada and that was why only Lake Mead water was used, but adding other water sources would be enlightening for areas of future study.

Recommendations

For control of *Legionella*, it is important to consider the differences between the oxidative water treatment response of *Legionella* within *Acanthamoeba* and in the absence of *Acanthamoeba*. Previous studies focusing on extracellular *Legionella* found that a 2 log

reduction with Cl_2 was achieved with CTs of 0.5-9 mg.min/L, (Kuchta *et al*,. 1983) and 30-60 mg.min/L (Jacangelo *et al.,* 2002). To effectively address outbreaks of extracellular *Legionella* it would be best to achieve a Cl_2 CT of 30-60 mg.min/L. Based on our results for *Legionella* internalized by *Acanthamoeba,* a Cl_2 CT of 376.3 mg.min/L would be needed to achieve a 2-log reduction. ClO_2 would require a CT of 1-2 for a 2-log reduction of extracellular *Legionella* (Jacangelo *et al.*, 2002). Based on our results for *Legionella* internalized by *Acanthamoeba,* a ClO_2 CT of 249.4 mg.min/L would be needed to achieve a 2-log reduction.

Chapter 5: Conclusions

Water is a necessity in daily life, and providing safe water has been a cornerstone of public health since public health's inception (Stewart, 2017). While the water that reaches the public is made safe for consumption through treatment, it is not sterile. This water can contain complex biological communities that can be made up of infectious and noninfectious microbes that interact with one another. Although many efforts are in place to prevent infectious organisms from reaching the public, these microbes can still reach buildings and even become established in plumbing systems (Mara & Horan, 2003). These complex biological communities make understanding what treatments are most useful for different microbes difficult, but necessary for public health, particularly for microorganisms with complex life cycles, such as *L. pneumophila*. The increasing incidence rate of Legionnaires' disease shows the need to address how this bacterium is reaching the public, especially when considering it can cause one of the few preventable types of pneumonia.

The first objective of this study was to compare the effectiveness of Cl_2 and ClO_2 on *A. polyphaga* internalized *L. pneumophila* given time to recover as is possible in some water systems. The second objective was to determine the CT of the oxidants Cl_2 and ClO_2 necessary to effectively reduce the number of *A. polyphaga* internalized *L. pneumophila* and prevent potential recovery after water treatment. In all CTs of both chemicals tested in this study, initial reduction of *L. pneumophila* was seen, but only the ClO_2 CT values of 74.21 mg.min/L and 997.7 mg.min/L showed a continuing reduction throughout the entire 14-day period, with all of the other exposures showing some recovery during the recovery time period. However, none showed complete clearance of *Legionella.*

The results of this study indicate that ClO_2 is more effective at long term control of *L. pneumophila* when internalized by *Acanthamoeba* and that Cl_2 may be limited in its ability to prevent intracellular *Legionella* from reaching the public. This is of particular concern when building water systems employ their own additional treatment(s) beyond that used by water providers, which can remove or reduce the residual from the original treatment oxidants. Our results also indicate that even with the high CT required to reduce intracellular *Legionella*, it might not be possible for treatment facilities to completely remove this bacterium, as none showed complete clearance. Therefore, it would be prudent for building systems to ensure maintained levels of treatment oxidants within their systems as well. Future efforts in controlling this pathogen will need to be treated as a community effort with all stakeholders taking part in *Legionella* prevention (ASHRAE, 2015). In order to properly address its potential impact on *Legionella* management, it may be useful to know if amoeba, such as *Acanthamoeba*, are present when performing *Legionella* prevention and outbreak investigations.

Future research should focus on the interactions of *Legionella* with a variety of amoeba species and how these affect this bacterium's resistance and ability to recover from oxidative treatments. This is an important area of study because previous research has shown that *Legionella* is unlikely to be found by itself in natural or artificial water systems (López *et al*., 2010). Additional studies should also be done relating to other water treatment methods on intracellular *Legionella* as there are a variety of techniques currently employed that may be more or less effective. Our study showed that these oxidants are effective at reducing the amount of viable intracellular *Legionella* initially, but some oxidants are more effective at reducing its ability to recover. These results can be applied to further investigations of how microbial

diversity impacts our ability to prevent *L. pneumophila* exposure, may be used by water treatment officials for future regulation in *Legionella* control, and could be used by building managers in deciding the best approach to remediate outbreaks.

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