Microbial Ecology of *Acanthamoeba polyphaga* and Sulfate-Reducing Bacteria in Premise Plumbing

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ABSTRACT

Great advances have been made in the last 100 years in the effort to provide safe and reliable potable water. Unfortunately, organisms surviving the water treatment process still cause illnesses in the population. *Acanthamoebae* are ubiquitous in the environment and are resistant to commonly used disinfection methods. In addition to being pathogenic on their own, *Acanthamoeba* spp. are capable of acting as a host to pathogenic bacteria in potable water. The amoebae provide the bacteria with protection from chemical and physical means of disinfection. In this way many pathogens that would otherwise be killed in the water treatment process survive and are capable of infecting water customers.

Most likely due to experimental limitations discussed herein, the concentration of organic carbon in solution was not found to affect the number of *Acanthamoeba polyphaga* surviving within reactors designed to model residential water tanks. A copper ion concentration of 1.3 mg/L was determined to be an effective disinfectant against *A. polyphaga* trophozoites, while free chlorine at 10 mg/L and monochloramine at 50 mg/L were deemed effective against trophozoites.

Sulfate-reducing bacteria (SRB) are suspected to be causative agents in copper pitting corrosion. SRB have been found in tubercles covering pits in many homes experiencing pinhole leaks, but the mechanisms of the survival of these organisms in potable water systems are poorly understood. Nutrient studies conducted show that the absence of nitrogen in solution may encourage copper corrosion by SRB. In addition, a medium specifically designed to encourage SRB growth resulted in a large increase in copper corrosion as compared to the control water.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: THE IMPORTANCE OF *ACANTHAMOEBA SPP.* IN POTABLE WATER DISTRIBUTION SYSTEMS

Rachel Methvin and Marc Edwards

ABSTRACT: *Acanthamoeba* spp. are a genus of protozoan commonly found in premise plumbing. They are relatively resistant to extremes in pH and temperature and can survive for long periods of time in adverse conditions due to their ability to form protective cysts. Several species of *Acanthamoebae* are the causative agent of keratitis and granular amoebic encephalitis, and they can also serve as a host to a variety of pathogenic bacteria that pose a direct human health threat. *Acanthamoeba* spp. in their cyst form are highly resistant to all disinfectants currently used by water treatment systems. Future research is needed to better understand the prevalence of *Acanthamoebae* in drinking water systems and to develop mitigation strategies that can be used by drinking water providers to protect customers.

1.0 INTRODUCTION

In the developed world communities utilize complex water distribution systems to deliver hot and cold potable water to homes and other public buildings. The serious threat to human health arising from pathogens in source waters is omnipresent but has largely been mitigated through source water protection, disinfection, particulate removal, and utilization of a disinfectant residual. Water disinfection and treatment is appropriately recognized as one of the greatest engineering achievements of the 20th century.[1]

While it is necessary to remain vigilant in mitigating the hazard from source water pathogens, the focus on pathogen control has evolved to consideration of opportunistic pathogens including *Pseudomonas aeruginosa, Legionella pneumophila, Mycobacterium avium* and *Acanthamoebae*. These organisms pose unique challenges. For example, they are present in the treated water of most public water systems only at trace levels but can proliferate in the water distribution system under certain circumstances. Exposure to *Legionellae* and *Mycobacterium avium* in potable water occurs via respiration of airborne bio-aerosols, as opposed to consumption of water.

"Frank" pathogens are those that can cause disease in otherwise healthy individuals, while "opportunistic" pathogens only infect those who already have a compromised immune system due to a variety of risk factors including disease, smoking, being very young or elderly. In 2008, for the first time, the Centers for Disease Control (CDC) indicated that more waterborne disease outbreaks in the U.S. were attributed to opportunistic pathogens in the distribution system, rather than frank pathogens in the source water.[2] This is due to better understanding and documentation of waterborne disease outbreaks occurring from opportunistic pathogens, as well as continued improvements in control of waterborne disease from frank pathogens.

Potentially harmful bacteria find premise plumbing extremely conducive to growth and reproduction. Several characteristics of premise plumbing make this possible. The high surface area to volume ratio of pipes in a home compared to that in the distribution system provides a large area for biofilm growth. The materials used in premise plumbing are different than those used in distribution systems and can accelerate disinfectant decay or provide nutrients that spur growth. In addition, the extremes in

temperature, prevalence of dead ends (little used pipe sections), and infrequent flow in premise plumbing also give certain bacteria a chance to thrive.[3]

For pathogenic bacteria like *P. aeroginosa, Legionellae,* and *Mycobacteria*, the ability to survive ingestion by *Acanthamoebae* is an evolutionary precursor to surviving in humans and higher animals. Without the protection of *Acanthamoebae* and other protozoa, these pathogenic bacteria would be much more readily eradicated from plumbing systems. In fact, for a large number of endosymbiotic bacteria discussed in this review, the presence of *Acanthamoebae* or similar protozoa is necessary for their proliferation in drinking water.

Growth within *Acanthamoebae* may physically change the bacteria and make it more virulent. According to Cirillo et al., *L. pneumophila* grown within *Acanthamoebae* show dramatic changes in structure and appearance compared with bacteria that grow on agar. These changes may be due to the activation of a new protein. *L. pneumophila* with this protein enter human white blood cells more often than bacteria without it.[4] Thus, it is important that we study the conditions under which *Acanthamoebae* flourish and also examine current water treatment methods to see how they stack up against amoebae infection. Traditionally water treatment professionals have focused on disinfection at the water treatment facility for control of *Acanthamoebae* and other pathogens, and there has been less focus on regrowth within the distribution and premise plumbing. While disinfection plays an important role in pathogen control, physical methods of removal may also be important weapons against *Acanthamoebae* colonization.

The goal of this literature review is to critically review prior research on *Acanthamoebae,* with an emphasis on practical information relevant to its control and mitigation in the water industry.

1.1 MICROBIOLOGY OF *ACANTHAMOEBAE* IN THE ENVIRONMENT

Acanthamoeba species are commonly found in air, soil, and water. Their ubiquity is illustrated by the fact that 80% of the human population carries antibodies against *Acanthamoebae* infection.[5] This section explores the general microbiology of *Acanthamoebae*, the diseases for which it is a causative agent, as well as the possible bacterial endosymbionts that pose a danger to public health.

A. General Microbiology.

Lifecycle

The life cycle of *Acanthamoebae* consists of two stages: a trophozoite stage and a dormant, cyst stage. The size of the trophozoite ranges from 12-35 μm, while the cyst is slightly smaller (5-20 μm). The trophozoite stage is the active stage, in which the amoebae can replicate using binary fission. *Acanthamoebae* are capable of replicating both in the environment and within a host. *Acanthamoebae* are unicellular organisms with no defined shape. They use small extensions of the cell membrane, called acanthopodia, to facilitate movement. They feed through grazing on biofilms, and if suspended bacteria are the only available food source, a starvation response can result.[6] The amoebae feed on bacteria through phagocytosis and pinocytosis.

When the amoebae sense adverse environmental conditions, such as low nutrient levels or the presence of a disinfectant, a dormant cyst with a wavy outer wall and a rounded, stellate, polygonal, or oval inner wall is formed.[7] Mattar and Byers clearly described the morphological changes that occur during excystment.[8] Cyst walls contain cellulose while the trophozoite does not.[9] This difference can be useful in differentiating between the two. Cyst formation seems to provide protection against colonization by potentially endosymbiotic bacteria.[10]

Occurrence in the environment

Acanthamoebae have been found in soil, fresh water, public water supplies, swimming pools, bottled water, mineral water, brackish water, sea water, ventilation ducts, air-conditioning units, industrial cooling water, compost, sediments, vegetables, mushrooms, fish, reptiles, birds, mammals, atmosphere, medical instrumentation, and sewage.[11, 12] Swimming pool water can contain upwards of 100,000 amoebae per liter, with *Acanthamoebae* being the most prevalent species, and a relatively high percentage of the amoebae in swimming pool water is pathogenic when compared to the population in other waters. *Acanthamoebae* are resistant to relatively high temperatures, high osmolarity, and a large pH range.[9] In water treatment, sublethal doses of disinfectant have been shown to induce encystment.[13, 14] The cysts are resistant to

dehydration, starvation, disinfection, and extreme variations in temperature. *Acanthamoebae* cysts over 100 years old have been shown to recover when placed in an appropriate medium or bacterial food source. They can also survive transient temperatures as low as 0°C up to 80°C.[15, 16] Freon, methylene oxide and autoclaving have been found to effectively destroy cysts.[17]

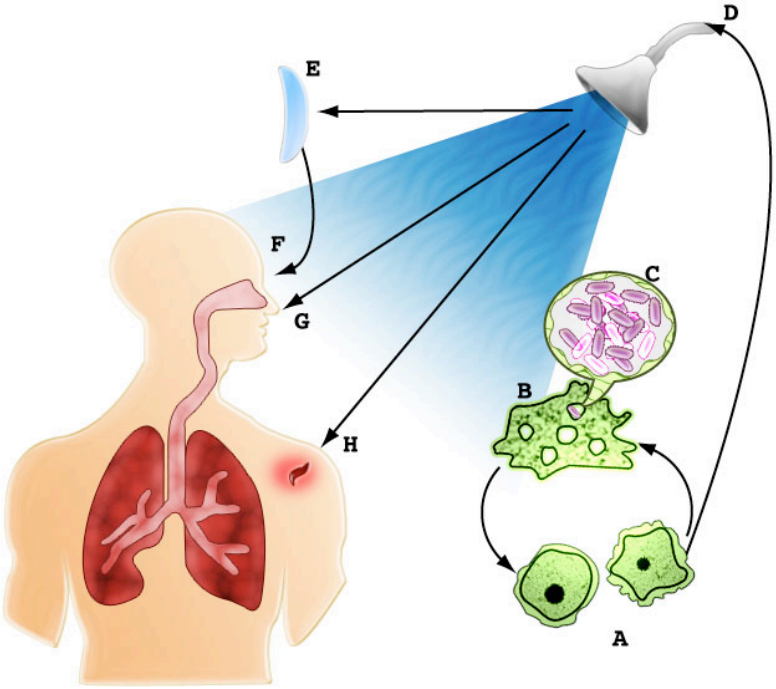

Figure 1-1. Exposure diagram for Acanthamoeba spp.

A. Cysts of *Acanthamoebae*. B. Trophozoite of *Acanthamoebae*. Trophozoites can encyst any time conditions become unfavorable for growth. Vesicles within trophozoites transport wastes but can also harbor bacteria. C. A single vesicle may contain 20-1500 bacteria. D. The heat and moisture of shower plumbing is conducive to *Acanthamoebae* growth and is a common exposure route. E-F. Contact lens wearers are particularly vulnerable to keratitis infection, and *Acanthamoebae* can adher to contacts any time they are exposed to water or air containing cysts or trophozoites. G. Inhaling mists containing *Acanthamoebae* with internalized pathogens, like *L. pneumophila*, can lead to infections of the lungs. H. For those with a compromised immune system, exposure to *Acanthamoebae* through skin lesions can allow access to the blood stream and result in a granular amoebic encephalitis (GAE) infection.

Pathogenicity

In humans *Acanthamoebae* have been isolated in throats, nasal cavities, and intestines, as well as from infected brain and lung tissue, skin wounds, and corneas.[18] Species within the *Acanthamoebae* genus were first suspected of causing human disease in 1958 during safety trials for a polio vaccine.[16] We now know that *Acanthamoebae* can cause infections of human skin, lungs, nose, eye and brain. However, not all *Acanthamoeba* species are pathogenic, as will be discussed later.

In addition to osmotolerance and thermotolerance, the presence of serine and cysteine proteases is an indicator of pathogenicity; these proteases cause the degradation of host tissues and proteins and allow infections to occur.[16, 19] In water artificially heated by industrial cooling applications, *Acanthamoebae* are significantly more likely to be pathogenic.[20] While thermotolerance is widely accepted to be a good indicator of pathogenicity, it is not necessarily a valid technique for all species of *Acanthamoebae*.[21] The temperature of the cornea is much lower than that of the rest of the body, allowing amoeba that would not survive a thermotolerance test to be the causative agent of a keratitis infection and may be the reason that keratitis infections are more prevalent than granular amoebic encephalitis (GAE).[18]

Classification

There are approximately 20 species of *Acanthamoeba,* which have previously been broken into three classification categories based primarily on their cyst morphology (Group I, II, and III). Large cysts with rounded outer walls that are clearly distinct from the inner walls characterize group 1. Group II cysts have a variable shape and are smaller; group III cysts are smaller than group II cysts and have poorly separated walls.[22] Though this classification system is sometimes problematic due to morphology changes based on water conditions it is still commonly used (Table 1-1).

Recently, several of the species identified by morphology as unique have been challenged. For instance, Kong and Chung used riboprinting to identify clinical isolates and found that strains of *A. mauritaniensis, A. divionensis,* and *A. paradivionensis* have identical riboprints to that of *A. rhysodes*.[23] Classification based on rRNA gene

sequences provides a simple and reliable identification method. This method allows the genus *Acanthamoebae* to be divided into 16 separate genotypes (T1-T15, with T2a and T2b), and the classification of species of *Acanthamoeba* via this method continues.[24-28] Within this classification, the T4 genotype is most often associated with human illness.[9]

Table 1-1. *Acanthamoeba spp.* Classification [9, 16, 22, 24, 26, 29]

Species	Group Number	Sequence Type	Associated Diseases, if Applicable
A. astronyxis	I	T7	GAE
A. castellanii	II	T4	GAE, Keratitis
A. commandoni	I	T9	
A. culbertsoni	III	T10	GAE, Keratitis
A. divionensis	II	--	GAE
A. echinulata	I	--	
A. griffini	II	T3	Keratitis
A. hatchetti	II	T11	
A. healyi	III	T12	GAE
A. jacobsi	III	T15	
A. lenticulata	III	T5	
A. lugdunensis	II	T4	
A. mauritaniensis	II	T4	
A. palestinensis	III	T2	GAE
A. paradivionensis	II	--	
A. pearcei	I	T3	
A. polyphaga	II	T4	Keratitis
A. pustulosa	III	T2	
A. quina	II	--	
A. rhysodes	II	T4	GAE, Keratitis
A. royreba	III	T4	
A. stevensoni	II	T11	
A. triangularis	II	T4	
A. tubiashi	I	T8	

B. Acanthamoeba Keratitis

Acanthamoebae are causative agents of Acanthamoeba keratitis, an eye infection that can potentially infect an otherwise healthy individual and cause blindness. It is especially prevalent in contact lens wearers (CLWs). Acanthamoeba keratitis is not a reportable disease and can be difficult to distinguish from other microbial keratitis cases.

Therefore, estimates of the incidence of this disease vary widely. Incidence estimates among CLWs in the US range from 1.36 to 13.6 cases per million people while incidence in the UK is 17.53 to 21.14 per million. Incidence of keratitis in Hong Kong and continental Europe is similar to that of the UK.[30, 31] The slightly higher incidence rate in the UK is attributed in part to the customary use of water storage tanks on rooftops; these storage tanks provide an ideal environment for the proliferation of microorganisms.[31]

CLWs are particularly vulnerable to infection because contact lens wear often results in corneal abrasions that allow amoebae entry into the eye. The amoebae can also become trapped between the eye and the lens and can adhere to contact lenses, allowing for repeated exposure to the eye. CLWs can be especially vulnerable to infection if they fail to follow proper lens care instructions. These include wearing the lenses for a period longer than that for which they are designed, showering or swimming while wearing contact lenses, cleaning lenses with a homemade solution, allowing lenses or lens cases to come in contact with tap water, using only a one-step hydrogen peroxide cleaning agent, or failing to replace contact lens cases every 3-6 months.[32, 33] While CLWs make up a significant percentage (85% according to most sources) of infected persons, any corneal trauma presents an opportunity for infection.

Humans are exposed to pathogenic amoebae through both residential and recreational waters and air. Amoebae can be transported in water vapor or on dust particles. Common residential water exposure pathways include tap water, showers, hot tubs, bottled water, and non-sterile waters used to store contact lens. Showers are particularly good conduits for amoebae infection because, in addition to direct exposure from amoebae in the water, amoebae cysts may also be small enough to travel via aerosols (tiny droplets of water). Kilvington et al. used a combination of DNA analyses to definitively link cases of keratitis to amoebae strains in hot and cold taps in households of infected persons. [31] The most prevalent route of exposure for keratitis is through contact lens use; for secondary infections from *Acanthamoebae* endosymbionts, the most likely route of exposure is via aerosols in steam or mists of contaminated water.

Symptoms of Acanthamoeba keratitis include eye pain, a typical corneal ring, photophobia, and blurred vision.[29] The prognosis is usually good if properly identified early on. Acanthamoeba keratitis can be diagnosed by plating corneal samples on media specific to the amoebae. This method is time consuming and problematic because fast diagnosis is paramount in successful treatment of the disease. Many ophthalmologists are now using scanning confocal microscopy to visualize high-contrast images of corneal sections in order to diagnose patients with acanthamoeba keratitis. In addition, researchers have used PCR assays of tears to identify *Acanthamoebae* infections and to track the success of treatment.[16] Treatment can be very difficult and can take a long time, however, due to the possibility for cyst formation. The cysts are resistant to antibiotic disinfection, and every last one must be removed in order to prevent a recurrence. The average treatment takes five months and surgical intervention is required in 50% of cases.[31] Acanthamoeba keratitis infections that are not caught early or that are unsuccessfully treated may result in blindness. Keratitis infections have rarely resulted in death of the patient.

C. Granular Amoebic Encephalitis

Acanthamoeba spp. are also named as a causative agent of granular amoebic encephalitis (GAE), an opportunistic, chronic infection of the nervous system. The first cases of GAE were diagnosed in 1972 by Jager and Stamm.[7] GAE only affects those with depressed immune systems or otherwise debilitating, chronic health problems such as lupus erythematosus, HIV, and tuberculosis, and it is almost always fatal. Smoking, dialysis, chemotherapy, and treatment with steroids can also leave one vulnerable to infection. Amoebae causing GAE may enter the body through mucus membranes or breaks in the skin and reach the nervous system via transport through blood vessels. The exact incubation period is unknown, though it is thought to be weeks to months. Symptoms of GAE include personality changes, confusion, seizures, nausea, headache, and dizziness.[29] There have been no known cases of exposure to laboratory cultures that led to GAE infection.

D. Endosymbionts

In addition to being pathogenic in their own right, *Acanthamoeba sp.* can also act as hosts to pathogenic bacteria. These bacteria inside of amoeba are often called endosymbionts because of their symbiotic relationship with the amoeba. *Acanthamoebae* were first found to harbor bacteria in 1954, and the first endosymbiotic bacterial relationships were noted in 1975 with pathogenic endosymbionts first noted in 1978.[9] Winiecka-Krusnell and Linder estimate that 25% of *Acanthamoebae* in the environment and in humans carry endosymbionts.[13] Table 1-2 contains a list of bacteria and one yeast known to be either obligatively or facultatively endosymbiotic with *Acanthamoebae*. Enteroviruses are also suspected to be hosted by amoebae, though this has not been proven. They have been found, however, on amoeba surfaces, which suggests that they may be key to the survival and transport of enteroviruses in the environment.[10] Bacteria endosymbiotic with *Acanthamoebae* tend to be from one of the following three families: *Alphaproteobacteria, Bacteroidetes,* and *Chlamydiae*.[34]

Table 1-2 A List of *Acanthamoeba* Endosymbionts, * indicates pathogenicity. [10, 13-15, 29, 35-43]

*Acanthamoeba polyphaga mimivirus**	*Caedibacter acanthamoebae*	*Escherichia coli O157**
*Achromobacter xylosoxidans**	*Campylobacter jejuni**	*Flavobacterium spp.*
Actinobacter sp.	*Caulabacteriaceae*	*Francisella tularensis**
*Aeromonas sp.**	*Chlamydia pneumoniae**	*Hafnia alvei*
*Afipia broomae**	*Chromobacterium haemolyticum*	*Helicobacter pylori**
*Afipia felis**	*Chryseobacterium spp*	*Klebsiella spp*
Alcaligenes spp	*Citrobacter sp.*	*Kluyvera cryocrescens*
Amoebophilus asiaticus	*Clostridium frigidicarnis*	*Legionella (L.) pneumophila**
Ancylobacter sp.	*Comamonas acidovorans**	*L. anisa **
Bacillus sp.	*Coxiella burnetii**	*L. bozemanii*
*Bosea sp.**	*Cryptococcus neoformans (yeast)*	*L. drancourtii**
*Bradyrhizobium japonicum**	*Cytophaga spp*	*L. drozanskii**
B. liaoningense	*Delftia acidovorans*	*L. dumoffii*
Brevundimonas spp	*D. tsuruhatensis*	*L. fallonii**
*Burkholderia cepacia**	*Dysgonomonas spp*	*L. feelei*
Burkholderia picketti	*Enterobacter sp.**	*L. gormanii*
*Burkholderia pseudomallei**		*L. hackeliae*
		*L. lytica**
		L. micdadei
		L. oakridgensis
		*L. rowbothamii**

*Listeria monocytogenes**	*M. mucogenicum*	*Providentia alcalifasciens*
*L. seeligeri**	*M. neoaurum*	*P. rettgeri*
Methylophilaceae	*M. peregrinum*	*Pseudomonas aeruginosa**
Mezorhizobium amorphae	*M. phlei**	*P. otitidis**
Microbacterium spp	*M. porcinum*	*P. fluorescens**
*Mobiluncus curtisii**	*M. septicum*	*P. putida*
Morganella morganii	*M. simiae**	*P. mosselii*
*Mycobacterium (M.) avium**	*M. smegmatis**	*Ralstonia sp.*
M. habana	*M. szulgai*	Rasbo bacterium*
M. abscessus	*M. terrae*	*Rhanella aquatilis**
M. aurum	*M. tusciae*	*Rhizobiaceae*
M. bovis	*M. ulcerans**	*Rhodococcus sp.*
M. bovis BCG	*M. xenopi*	*Rhodospirillaceae*
*M. chelonae**	*Obesumbacterium proteus*	*Rickettsiales*
*M. fortuitum**	*Ochrobactrum pseudintermedium*	*Salmonella enterica**
M. fuerthensis	*Odyssella thelassonicensis*	*S. typhimurium**
M. gastri	*Oligotropha carboxidovorans*	*Serratia ficaria*
M. goodi	*Pandoraea pnomenusa*	*S. fonticola**
M. gordonae	*Pantoea agglomerans*	*S. marcescens**
M. immunogenum	*P. ananatis**	*S. proteamaculans*
M. intracellulare	*Paracaedibacter symbiosis*	*Shigella sonnei**
M. kansasii	*P. Acanthamoebae*	*Shigella boydii**
M. lentiflavum	Parachlamydiacea	*Simkania negevensis**
*M. leprae**	*Parachlamydia acanthamoebae**	*Sphingomonadaceae*
M. mageritense	*Procabacter Acanthamoeba*	*Stentotrophomonas sp.**
M. malmoense		*MRSA**
*M. marinum**		*Vibrio cholerae**
*M. massiliense**		*Xanthomonaceae*
M. monacense		*Yersinia enterocolitica**

Acanthamoebae engulf these bacteria during phagocytosis, but the bacteria have developed an evolutionary response that allows them to live within the amoebae rather than being consumed. Bozue and Johnson describe the use of coiling phagocytosis, in addition to the use of conventional phagocytosis, as a way to engulf both virulent and avirulent strains of bacteria.[44] Cirillo et al. determined that the entry of bacteria into an amoeba in this way made them more likely to be ingested by human macrophages through coiling phagocytosis.[4] Winieka-Krusnell and Linder reported that

Acanthamoebae secrete material that promotes faster replication of endosymbionts, particularly *Legionella spp*.[13]

The fate of bacteria inside *Acanthamoebae* varies. Some bacteria remain in the amoebae without reproducing. Other bacteria are able to consume resources inside the amoebae and replicate. Some of these replicating bacteria are able to leave the amoebae in vesicles without destroying the amoebae, while still others replicate until they run out of nutrients and then lyse, or rupture, the cell membrane and spill out.[15] Bacteria that are able to become endosymbionts may be able to do so because they prevent lysosomal fusion with the amoebae.[45] *Legionellae* use a pore-forming technique to lyse the cell wall of the *Acanthamoebae* trophozoite. The gene allowing this pore formation, icmT, has been identified. If it is removed the bacteria remain trapped inside the amoeba cell following replication.[10, 46]

Amoeba commonly produce vesicles, small sacs surrounded by a lipid bilayer, to store and transport cellular wastes. They originate inside the amoeba and are excreted to eliminate waste. In 24 hours, *Acanthamoeba polyphaga* can release up to 25 vesicles which contain 20-200 pathogenic bacteria according to an estimate by Berk et al. (See Figure 1-1).[47] However, Rowbotham estimated that 365-1,483 bacteria may be housed in each vesicle. More than 90% of vesicles containing pathogenic bacteria are small enough to be inhaled (less than 5 microns in diameter), and these vesicles can travel for miles on the wind from a source like a cooling tower.[47, 48]

Endosymbionts can be passed to daughter amoebae during division and can remain viable following encyst- and excystation.[14] Heinz et al. first reported the existence of multiple endosymbionts within one host amoeba. Two species of bacteria, *Procabacter* sp. and *Parachlamydia* sp., were found within a single *Acanthamoeba* sp. isolated from an Austrian lake. Lateral gene transfer is a well-documented phenomenon among Chlamydiae bacteria, and the authors of that study suggest that the existence of multiple bacteria within a single host could create a “hot spot” for this transfer.[49] Even anaerobic bacteria like *Molibuncus curtisii* have been known to proliferate with *Acanthamoebae* spp, allowing pathogenic and nonpathogenic bacteria to thrive in oxygenated water systems in which they would not normally survive.[13]

Pathogenic bacteria that have developed the ability to live symbiotically with *Acanthamoebae* are typically able to survive within cysts, making them effectively immune to disinfection, nutrient limitation, antibiotics, and extreme temperatures.[10, 36] In fact, *Legionella pneumophila* has been found to survive disinfection with up to 50 mg/L free chlorine within cysts of *A. polyphaga.*[50] Winieka-Krusnell and Linder reported that antibiotic resistance in amoebae can be passed on to hosted bacteria like *L. pneumophila* and *M. avium.*[13]

Unfortunately, the biological changes that allow the pathogenic bacteria to live within the *Acanthamoebae* also make them more virulent. Goy et al. found that strains of *Mycobacteria kansasii* that were known to be pathogenic grew more successfully within *Acanthamoebae* than did nonpathogenic strains.[51] In addition, a single pass through an amoeba selects for clone bacteria that are better equipped to infect other amoebae, allowing for faster proliferation of the bacteria in a water system.[52] Cirillo et al. reported that *M. avium* grown in *Acanthamoeba castellanii* was eight to ten times better able to invade new amoebae. In addition, the bacteria was significantly more able to infect the intestine, liver, and spleen of beige mice, an indication that they would be able to infect humans.[45]

The processes used by bacteria to inhabit amoebae are the same as those used to enter human macrophages.[38, 53, 54] *L. pneumophila* grown within *Acanthamoebae* were 1000 times better able to invade human carcinoma cells than those grown on agar.[4] *M. avium* and *L. pneumophila* grown in *Acanthamoebae* are also able to enter epithelial cells more easily.[4, 45]

Temperature may be a particularly important determining factor in whether or not an endosymbiotic relationship is formed. At temperatures of around 20°C, *Acanthamoebae* consume *Legionellae*. At temperatures of 37°C or above, *Legionellae* are protected from phagocytosis and survive within the host.[16] Ohno et al. found that, at 15°C, *A. castellani* expel all their vesicles containing *L. pneumophila* and encyst to protect themselves from infection. Additionally, Ohno et al. reiterate that below 20°C *A. castellani* is able to digest *L. pneumophila* and state that *L. pneumophila* infects and replicates freely within *Acanthamoebae* at temperatures above 25°C.[55] With *O.*

thessalonicensis, a stable relationship is formed at 20°C, whereas amoeba lysis occurs at 30 and 37°C.[10] Temperature is also important for the fate of *Parachlamydia acanthamoeba* within *Acanthamoebae*. At temperatures of 25-30°C, *P. acanthamoeba* resides within *Acanthamoebae* without causing lysis of the amoebae. However, at temperatures of 32-37°C, *P. acanthamoeba* is lytic towards its host--even though the growth rate of *P. acanthamoeba* is not increased by the higher temperatures.[56] This is very important for human health because *P. acanthamoeba* is a pathogen that acts as a causative agent of pneumonia, and the lysis of the amoeba cell results in the simultaneous release of a large number of bacteria.

Growing viable but noncultivable (VBNC) pathogenic bacteria within *Acanthamoebae* may allow sufficient replication to make these bacteria cultivable.[57] In addition, living within an amoeba may cause physiological changes that will allow a previously noncultivable bacteria to be cultured.[14, 36] On a positive note, this may allow for the identification of new species. For example, Wang found that cocultivation of mycobacteria with *A. culbertsoni* in an environmental sample led to the cultivation of previously unknown, uncultivable mycobacteria.[58] Unfortunately, cocultivation of pathogenic species like *L. pneumophila* that are VBNC also results in resuscitation of the bacteria, increasing cultivability and regenerating pathogenic potential.[57] Even after *L. pneumophila* cells were inactivated by treatment with up to 1 mg/L monochloramine, they could be resuscitated by *A. castellanii*.[59]

Conversely, large amounts of bacteria in a sample can out-compete *Acanthamoebae* for nutrients. Wang and Ahearn found that a ratio of 100 bacteria or more to one amoeba over 48 hours was enough to suppress the growth of *Acanthamoeba castellanii*. Even gram-negative bacteria that serve as good food sources for *Acanthamoebae,* like *E. coli*, can inhibit amoebic growth. The authors speculate that, once the amoebae engulf as many bacteria as they can, excess bacteria adhere to the surface of the amoebae, dropping the pH at the surface significantly. Additionally, production of a phospholipase by the bacteria may result in the inhibition or death of *Acanthamoebae*.[60]

Pseudamonas aeruginosa was particularly effective at inhibiting growth; Wang and Ahearn quote another study which found that 92.5% of amoebae were killed in coculture with *P. aeruginosa* after only 48 hours.[60] Singh used a chloroform extraction technique to obtain a crude extract of pyocyanin from a culture of *P. aeruginosa*. He found that the crude extract was more effective at killing amoebae at lower concentrations than both chemically pure pyocyanin and autoclaved crude extract of pyocyanin.[61] This suggests that an additional component in the crude extract may enhance the virulence of *P. aeruginosa* towards amoebae. Qureshi et al. found that *P. aeruginosa* adhered to the surface of *Acanthamoebae* almost immediately and was not internalized. In addition to cultures containing amoeba and live *P. aeruginosa* exhibiting inhibition, cultures of *Acanthamoeba* exposed to filtered broth only containing *P. aeruginosa* exoenzymes and no bacteria cells also showed inhibition. There are several candidate exoenzymes that may be responsible for this behavior. Phospholipase C, various proteases such as elastase, and pyocyanin are all secreted by *P. aeruginosa* and may cause membrane lesions in *Acanthamoebae*.[62] These studies were conducted over a relatively short period of time (most only 48 hours), and none looked at the effects of suspended bacteria versus *P. aeruginosa* in a biofilm. Both extended study time and biofilm presence may reveal significantly different behavior of *Acanthamoebae* and *P. aeruginosa* by-products.

1.2 Incidence of Acanthamoebae in the Environment

This section describes the occurrence of *Acanthamoebae* in the environment. While *Acanthamoebae* can be found in soil and air, this section will focus largely on water pathways for human exposure.

A. Sources of *Acanthamoebae* in the Environment

Acanthamoebae are found in all phases of the natural environment. They live in soils and water and have been cultured from air samples. Their ability to form cysts allows them to be resistant to freezing and desiccation.

B. Occurrence in the United States

Acanthamoebae were found in seven percent of amoebae isolates from the James River in Virginia, primarily in the spring and early summer. They were most commonly found at the soil-water interface in an area of the river downstream from a power plant where the water was warmer by an average of 3°C. [63] A study of tap water in South Florida found amoebae in 19.4% of samples, and *Acanthamoebae* in 2.8%. The authors suggest that such a high concentration of cultivable amoebae is evidence that they are actively growing in the system rather than just surviving the treatment process.[64]

A study by Joslin et al. in the Chicago area has found a marked increase in Acanthamoeba keratitis cases in recent years. The study paired increased risk with specific geological locations. The authors suggest that the increased risk correlates with increased distance from the treatment plant, since distance from the treatment plant was more than 35 miles for some patients. They also suggest that the risk may be rising recently due to changes in disinfectant in order to comply with increasingly demanding disinfection by-product standards.[30] However, data obtained from the City of Chicago showed no change in disinfectant chemicals or concentrations used from February 2000 to December 2006. The City of Chicago uses chlorine gas as the primary and secondary disinfectant with a residual target of 1 mg/L as the water exits the plant. In addition, fluoride tracer studies of the distribution system conducted by the water authority show a 48-hour residence time.[65]

C. Worldwide Occurrence

Table 1-3. Studies of *Acanthamoebae* occurrence worldwide.

Study	Conditions	Results
Barbeau and Buhler[66]	Surveyed dental units for Acanthamoebae presence	Concentration of amoebae was 300 times greater than that in incoming water
Hoffmann and Michel[67]	Tested for Acanthamoebae in 6 German water treatment plants	Acanthamoebae found year round, reservoirs contained 34 strains, 14 of which were thermophilic, those isolated from later steps of water treatment facilities were more likely to be thermophilic
Thomas et al.[37]	Tracked Acanthamoebae	Acanthamoeba spp. were

	populations through a Paris water treatment plant	found in river influent, in the biofilm in the sand filter and at distant points in the distribution system
Tsvetkova et al.[68]	Tested clays and soils, salt and fresh water (artificial and natural) sources for free living amoebae in Bulgaria	Found Acanthamoebae in all waters tested, though in low numbers in the black sea samples, and in 93% of wastewater treatment plant samples
Leiva et al.[69]	Tested for Acanthamoebae presence in recreation and tap water in Nicaragua	29% of water tank samples, 19% of tap water samples, 21% of wells, 13% of streams contained Acanthamoeba, several living at $\geq$ 40°C
Seal et al.[33]	Tested cold water storage tanks and main lines for Acanthamoebae in the UK	Faucets fed from cold water storage were much more likely to contain amoeba then those fed by main lines, presence of limescale may increase amoeba concentrations
Kilvington et al.[31]	Tested taps fed by cisterns and taps fed by main lines in keratitis patient homes in the UK	Larger numbers of amoebae were cultured from the taps fed by cisterns
Boost et al.[70]	Tested homes for presence of Acanthamoebae in Hong Kong	10% of homes tested positive, homes without storage tanks, even homes older than 20 years, did not test positive
Lorenzo-Morales et al.[19]	Used osmotolerance, thermotolerance and PCR tests on Acanthamoebae isolated from sea and fresh water to test for pathogenicity in Spain	Identified Acanthamoebae in 88 of 148 tap waters with 61 of these demonstrating pathogen potential and 27 being both pathogenic and virulent. Also found Acanthamoebae in 24 of 60 sea waters, all were pathogenic
Lorenzo-Morales et al.[71]	Tested river, sea, and tap water in Jamaica for Acanthamoebae	36.1% of tap water, 26.4% of river water, and 49.6% of seawater samples were positive, approx. 60% of the

		fresh water samples were pathogenic
Lorenzo-Morales et al.[72]	Tested freshwater sources associated with human activity in areas of Egypt for Acanthamoebae	43.24% of samples contained Acanthamoebae, 69% of positive samples were pathogenic
Rivera et al.[73]	Tested air samples in Mexico City, Mexico for protozoan species	11 species of Acanthamoebae were identified
Rodriguez-Zaragoza and Magana-Becerra[74]	Collected air samples in San Luis Potosi, Mexico to test for Acanthamoebae	23 Acanthamoeba spp were found, 31% had invasive capacity toward human cells

1.3 Environmental Factors Affecting Acanthamoebae Growth

This section describes the effects of environmental factors such as nutrients and chemical and physical water characteristics on *Acanthamoebae* growth.

A. Chemical and Physical Water Characteristics

Acanthamoebae can survive in water, air, and soil by consuming natural carbon sources and bacteria, cyanobacteria, fungi, and other protozoa in the environment.[75] *Acanthamoebae* can also survive on easily assimilable organic carbon. They grow rapidly in the 14 g/L total organic carbon available in a culture medium (PYG) whose composition is discussed in further detail in the Laboratory Methods section below. While this amount of carbon is significantly higher than that normally found in treated water, this suggests that some concentration of organic carbon available in potable water may be sufficient to sustain an amoebae population, even in the absence of a bacterial food source.

Acanthamoebae concentrations are higher when the conductivity of the water is greater than 2000 $\mu S/cm^2$.[76] In a study of nine strains of *Acanthamoebae* spp., all grew well within the pH range of 3-9.5, with the best growth between 7 and 8.5. In addition all tested strains grew well at temperatures ranging from 10-37°C, with the pathogenic varieties surviving at higher temperatures ($\geq$37°C).[21] However, *Acanthamoebae* cysts have been observed to survive temperatures from 0-80°C.[18, 23, 74] Kyle and Noblet

reported that high levels of amoebae in environmental samples often correlated with high attenuance, large amounts of bacteria, and large populations of blue-green algae. The oxygen produced by the blue-green algae during photosynthesis attracted the amoebae to layers lower than they would ordinarily prefer. Large numbers of *Acanthamoebae* were also found near the surface of open reservoirs following heavy rains; the amoebae were attached to the clay particles in the runoff. Dramatic seasonal differences in amoebae distribution were also noted due to chemical changes in the water.[77] *Acanthamoebae* can also withstand high amounts of pressure. *A. polyphaga* has been found in ocean sediments at a depth of 2500 meters.[78]

Tsvetkova et al. suggested that the amount of oxygen in the water source is less important to *Acanthamoeba* growth than is the presence of an abundance of bacteria and that the low concentration of amoeba in the Black sea samples suggests the salt concentration reduces organisms ability to reproduce, though all samples of amoebae found in the Black Sea were able to survive temperatures greater than 37°C—a possible marker of pathogenicity.[68]

Acanthamoebae was the most prevalent of all genera isolated from an air sample in Mexico City, Mexico. This level of diversity in air samples proves their ability to resist a variety of atmospheric conditions and pollution.[73] While the authors hypothesized that more amoebae would be found in the rural areas, they found that the suburban and urban areas had higher concentrations of *Acanthamoebae* due to poor sanitary conditions like waste disposed on the streets and standing rain water.[74]

B. Water Treatment and Premise Plumbing

In a study of a drinking water treatment system, *Acanthamoebae* were found to have colonized the sand filter of a water treatment plant, allowing for the occasional release of significant numbers of amoebae continuing through the plant. Indeed, on two occasions the authors saw no decrease in amoeba concentration following sand filtration even though the turbidity was reduced below 0.2 NTU.[37] This demonstrates the importance of monitoring biofilm formation in a treatment plant and is a potential problem for treatment plants using biofilm to remove organic carbon. *Acanthamoebae* isolated from steps further in a water treatment system were very likely

to be thermophilic (6 of 8 strains).[67] This is an important distinction to make since the thermophilic strains are more likely to be pathogenic.

Recently, a lawsuit was brought against a dentist for a keratitis infection acquired during treatment after a dental assistant sprayed the patient in the eye with water. The lawsuit was decided in favor of the dentist, but the case underlines the importance of removing *Acanthamoebae* from the water system.[79] The ability of researchers to match DNA of an infectious strain to the *Acanthamoebae* in possible source locations will likely make future lawsuits more successful.[33] The threats posed by *Acanthamoebae* in premise plumbing are significant and should be carefully studied to protect human health.

Other than the copper toxicity discussed below, no research has been done on the effect of plumbing materials on the growth of *Acanthamoebae* or the effect of *Acanthamoebae* on these materials. The use of cold-water storage tanks in homes is common in several countries outside of the United States. Three studies found that bathroom taps fed from tanks or cisterns were more likely to contain *Acanthamoeba* spp. than taps fed from main lines.[31, 33, 70] In addition, pipes having significant lime-scaling were more likely to test positive for *Acanthamoebae* by direct culture. The authors suggest that limescale may increase amoebae concentrations by trapping the cysts as the scale is formed. This would allow the cysts to become viable at a later time when bacteria are present. The limescale may also function to inactivate disinfectants.[33] A similar mechanism in the sediment layer of hot water tanks may allow for high concentrations of amoebae.

1.4 Disinfection Studies Involving Acanthamoeba spp.

A. Chlorine Disinfection Studies

Free chlorine has been the disinfectant of choice for many years and continues to be used widely for drinking water treatment. The free chlorine residual in a typical distribution system is approximately 0.5 mg/L, though this residual can disappear at the far reaches of the system or within buildings. Table 1-4 below is a summary of experimental parameters of studies using free chlorine to treat *Acanthamoebae*.

Table 1-4. Summary of chlorine disinfection studies.

Concentrations of chlorine are reported as free chlorine unless otherwise indicated.

Organism(s)	Concentration(s) (mg/L)	Contact time	Media or Water	pH	Temp.	Author, Year
A. castellanii, suspended culture	Residuals maintained at .5, 1, 2, 4, 10, 20	60, 120, 180 min and 24hr	chlorine demand free buffer	7	25°C	King, 1988 [80]
A. culbertsoni cysts, suspended culture	2.25, 4, 8, 16 &, 40- residuals measured at 1, 1.75, 5, 9, and 40 respectively	time until negative presence test	chlorine solution in DI water	7.3-7.4	25°C	DeJonckheere, 1976 [81]
A. polyphaga cysts, suspended culture	2, 4, 8 & 16- residuals measured at 1, 3.5, 6, and 16 respectively	time until negative presence test	chlorine solution in DI water	7.3-7.4	25°C	DeJonckheere, 1976 [81]
Acanthamoeba sp. isolated from river water, established biofilm in a pipe system	2.5, continuous application	106 days	tap water, flushed daily	7.6	not stated	Thomas, 2004 [82]
Two thermotolerant Acanthamoeba sp., suspended culture	0, 2, 5, 10, 20, 50, 100- residuals remained within 15% of initial conc	0, 10, 30, 60 min	tap water	not stated	37°C	Storey, 2004 [83]
A. polyphaga, monolayers	0-1024 as NaOC, residual conc not noted	0, 14, 22, 46 hours	PY medium	7	37°C	Garcia, 2007 [52]
Acanthamoeba cysts, suspended culture	8, 15, 25, 50 and 100, residuals measured at sample time to determine Ct	5, 15, 30, 60, & 120 minutes	dechlorinated drinking water	7.5	20°C	Loret, 2008 [84]
A. castellanii & A. culbertsoni, suspended culture	0.80-1.25, residual of all approx. 0.25	30 minutes	Sterile phosphate buffer	7.0	25°C	Cursons, 1980 [85]
A. polyphaga trophozoites and cysts	1-100, residual not indicated	2, 8, & 18 hours	Page's amoeba solution	6.9 & 8.0	30°C	Kilvington, 1990 [86]

Acanthamoeba castellanii survived at residual chlorine levels as high as 10 mg/L for 24 hours. Coliform bacteria cocultured with *Acanthamoeba* survived concentrations of 2-4 mg/L chlorine, while *K. pneumoniae* cocultured with amoeba was only inactivated after 120 minutes in 10 mg/L free chlorine. All of these bacteria were inactivated in under one minute without the presence of *Acanthamoeba*. King et al. also suggested that exposure to chlorine disrupts amoebic digestion of bacteria that would otherwise be

digestible, allowing for a residual population in water.[80] These results demonstrate the profound chlorine resistance lent to bacteria by coculture with amoeba.

In one study concentrations of NaOCl less than or equal to 128 mg/L over 46 hours had no effect on *A. polyphaga* infected with *Legionella pneumophila*. This supports the conclusion that infection of amoebae by endosymbiotic bacteria actually increases amoebic resistance to disinfection. In addition, amoebae that were uninfected were more likely to encyst following exposure to NaOCl than infected amoebae.[52]

DeJonckheere and van de Voorde suggest that the chlorine residual concentration is less important in determining the amount of time it takes to destroy amoebae than the strength of the initial chlorine exposure. However, data that supports this statement is not presented, so the meaningfulness of this statement is unclear. None of the concentrations of free chlorine that they tested were able to inactivate the cysts of pathogenic *A. culbertsoni* in less than 24 hours, while 16 mg/L inactivated the avirulent *A. polyphaga* strain in one hour. The pathogenic strain was still cultivable following 3 hours of exposure to 40mg/L free chlorine.[81] The fact that pathogenic strains of *Acanthamoebae* are often more resistant to chlorine than avirulent strains has important implications for drinking water treatment.

Storey et al. reported that exposure to 10 mg/L free chlorine for 30 minutes resulted in a 2-3 log reduction in cysts.[83] Cursons et al. reported that 1.25 mg/L of free chlorine was sufficient to remove all *Acanthamoeba* spp.[85] This discrepancy from other works may be due to the use of a phosphate buffer solution rather than an environmental sample or the use of a plaque assay to enumerate the amoebae remaining in the sample. The plague assay has been shown to underestimate the presence of viable cysts.[87]

Thomas et al. found that 2.5 mg/L chlorine was sufficient to significantly reduce planktonic amoebae in a pipe system, but the amoebae population was able to rebound to initial levels following cessation of chlorination, suggesting protection from the remaining biofilm.[82]

Kilvington et al. reported that more than 50 mg/L of free chlorine was necessary to inactivate *A. polyphaga* cysts.[86] Confirming this result, Loret et al. found culturable

Acanthamoebae cysts even after exposure to 100 mg/L for two hours. A concentration of at least 25 mg/L was necessary to see any reduction in cyst viability.[84] Hyperchlorination is not successful as a long-term amoebae control because amoebae continue to enter the system in the treated water and reemerge. In addition to being costly, there are many drawbacks for hyperchlorinating distribution systems including pipe corrosion, formation of carcinogenic disinfection by-products, and the difficulty involved in maintaining a consistent, high chlorine concentration.[52]

B. Monochloramine Disinfection Studies

Only two studies have examined disinfection using monochloramine (Table 1-5).

Table 1-5. Summary of monochloramine disinfection studies.

Organism(s)	Concentration(s) (mg/L)	Contact time	Media or Water	Cl:NH_4 ratio	Temp.	Author, Year
Acanthamoeba sp. isolated from river water, pipe system flushed every day, established biofilm	0.5 mg/L total Cl, continuous application	35 days	tap water	2:1	--	Thomas, 2004 [82]
2 thermotolerant Acanthamoeba, suspended culture	0, 2, 5, 10, 20, 50, 100 mg/L, residual maintained within 15% of original conc	0, 10, 30, 60 min	tap water	not given	37°C	Storey, 2004 [83]

Thomas et al. found that 0.5 mg/L monochloramine was not able to reduce the amoebae population in a laboratory scale pipe system.[82] Storey et al. found that *Acanthamoebae* cysts survived treatment with 100 mg/L of combined chlorine following an exposure time of ten minutes.[83] Chlorine and monochloramine seem equally ineffective at reducing *Acanthamoebae* at levels currently used in drinking water treatment.

C. Temperature Disinfection Studies

Table 1-6. Summary of temperature disinfection studies.

Organism(s)	Degrees C	Contact time	Media or water	Author and Year
Two thermotolerant Acanthamoeba sp.	40, 50, 60, 70, 80	0, 10, 30, 60 min	tap water	Storey, 2004 [83]

Storey et al. found that cocultivation with *Acanthamoebae* increased the thermotolerance of *L. pneumophilia* by 10 to 100 times when compared to the thermotolerance of *L. pneumophila* alone. *Acanthamoebae* cysts remained viable even up to a temperature of 80°C.[83]

D. Other Chemical and Physical Disinfection Studies

Table 1-7. Summary of disinfection studies involving other chemical disinfection methods.

Disinfecting Agent	Organism(s)	Concentration(s) (mg/L)	Contact time	Media or water	Temp.	Author and Year
electrochlorination	Acanthamoeba sp. isolated from river water, pipe system flushed every day, established biofilm	2.5 as free chlorine, continuous application	106 days	tap water	not stated	Thomas, 2004 [82]
chlorine dioxide	Acanthamoeba sp. isolated from river water, pipe system flushed every day, established biofilm	0.5, continuous application	106 days	tap water	not stated	Thomas, 2004 [82]
chlorine dioxide	Acanthamoeba cysts	0.5, 2, 5 and 7	5, 15, 30, 60, & 120 minutes	Dechlor. drinking water	20°C	Loret, 2008 [84]
chlorine dioxide	A. castellanii & A. culbertsoni, suspended culture	2.5-3.4, residual of 0.6-0.75	30 minutes	Phosphate buffer solution	25°C	Cursons, 1980 [85]
ozone	Acanthamoeba sp. isolated from river water, pipe system flushed every day, established biofilm	0.5 at contact inlet, continuous application	106 days	tap water	not stated	Thomas, 2004 [82]
ozone	Acanthamoeba cysts	0.5, 0.8, 0.9, 1.2, & 1.7	5, 15, 30, 60, & 120 minutes	Dechlor. drinking water	20°C	Loret, 2008 [84]

ozone	A. culbertsoni & A. castellanii, axenic cultures	Initial concentration of 6.75, final of 0.08	30 minutes	Phosphate buffer solution	25°C	Cursons, 1980 [85]
UV	Acanthamoeba cysts	26, 36, 213, 618 & 1212 mJ/cm^2	5, 15, 30, 60, & 120 minutes	Dechlor. drinking water	20°C	Loret, 2008 [84]
UV	A. castellanii cysts	10, 20, 40, & 60 mW-s/cm^2	Not explicitly stated	Cysts plated on Fulton agar	25°C	Chang, 1985 [88]
UV	Acanthamoeba sp. isolated from wastewater and an A. culbertsoni reference sample from ATCC	Approx. 0, 5, 15, 20, 30, 40, 60, 90, 115, 145, 175 mW-s/cm^2	Not explicitly stated	Sterilized secondary effluent from a wastewater plant containing amoebae	30°C	Maya, 2003 [89]
copper/silver ionization	Acanthamoeba sp. isolated from river water, pipe system flushed every day, established biofilm	.8/.02	106 days	Tap water	not stated	Thomas, 2004 [82]
copper/silver ionization	A. polyphaga	.1mg Ag/L and 1.0mgCu/L separately and combined	0, 14, 30, 190 days	Synthetic water	25°C	Hwang, 2006 [90]
bromine	A. culbertsoni	.4-1.0	24 hours	Synthetic pool water	--	DeJonckheere, 1976 [81]

iodine	A. culbertsoni	2-5	24 hours	Synthetic pool water	--	DeJonckheere, 1976 [81]
iodophore	A. culbertsoni	2-5	24 hours	Synthetic pool water	--	DeJonckheere, 1976 [81]
polyhexamethylene biguanide, benzisothiazolone, & 5-chloro-N-methylisothiazolone	A. polyphaga	2,8, 16 respectively	5 days	ABCD broth	35°C	Barker, 1992 [91]
Biocides MBC 350, MBC 120, MBC 215, & MBC 115	A. hatchetti ATCC culture & an environmental sample	Multiple concentrations tested	24 hours	Tris-buffered saline solution	25°C	Srikanth, 1993 [92]
Phagozyme, Rivascop, Endosporine, and Anioxyde	A. polyphaga	Undiluted biocide and 10% biocide in PAS	30 min, 3 and 24 hours	Undiluted biocide and 10% biocide in PAS	28°C	Greub, 2003 [93]
caspofungin	A. polyphaga, A. castellanii, A. culbertsoni	16-500 mg/L	1, 4, 12, 24, and 48 hours	PYG diluted with saline	30°C	Bouyer, 2007 [94]

Thomas et al. found that electro-chlorination and chlorine dioxide, at the concentrations listed above, in a pipe system with an established biofilm were able to reduce the planktonic amoeba population, though not below the detection limit, and the amoeba rebounded quickly once treatment ended.[82]

Unfortunately, the concentration of chlorine dioxide typically applied for drinking water applications had no effect.[84] A concentration of 2.9 mg/L was unable to completely sterilize samples of *A. castellanii* and *A. culbertsoni*.[85]

Ozone at a concentration of 0.5 mg/L also reduced planktonic amoebae in a pipe system with an established biofilm, but it was unable to eradicate the organisms from the system and allowed them to rebound quickly once treatment was stopped.[82] In a separate study ozone concentrations up to 1.7mg/L reduced the amoebae population by 1 to 2 logs. [84] Ozone at an initial concentration of 6.75 mg/L has been shown to be effective at reducing but not completely removing *Acanthamoebae* trophozoites.[85]

The UV treatment used by Loret et al. was effective to 3 to 4 log reductions but did not remove the amoebae completely.[84] UV irradiation at typically used levels for water treatment were ineffective against cysts of *A. castellanii*; in fact, the cysts were 15 times more resistance to UV disinfection than bacteria.[88] Maya et al. report that *Acanthamoebae* are among the most resistant microorganisms to disinfection by UV light. An offset UV fluence of at least 30 mJ/cm^2 was required to affect the virulence of the amoebae, deviating from the usual first-order kinetics assumption. A 20 minute exposure time and doses higher than 173 mW-s/cm^2 was necessary for total inactivation.[89] For 1 log of removal using monochromatic UV radiation, 40 mJ/cm^2 were necessary; for 2 logs, 71 mJ/cm^2 were required; for 3 logs, 119 mJ/cm^2 were necessary, and for 4 logs, 167 mJ/cm^2 were needed.[95] In addition to the high doses and exposure times required for UV irradiation to completely eradicate *Acanthamoebae* from water supplies, studies have indicated that some microorganims have developed the ability to repair DNA damage caused by UV light in response to exposure to sunlight.[96] This may be particularly important for water treatment facilities processing

water from rivers and other open reservoirs because prolonged exposure to sunlight increases the possibility of photo-reactivation.[95]

Silver is an effective biocide due to its ability to interfere with potassium release, breaking hydrogen bonds, reducing an organism's ability to absorb nutrients, inhibiting cell division, and disrupting DNA transfer. Copper has also been used as a biocide. Hwang et al. found that exposure to 1.0mg/L copper in a synthetic drinking water (Table 1-8) did not have much effect on *A. polyphaga*. Silver was much more effective alone in reducing both planktonic and endosymbiotic bacteria than copper alone. However, when copper and silver were used together, Hwang could not cultivate any of the cocultivated endosymbiotic bacteria following treatment, suggesting that the amoeba were inactivated as well.[90]

Table 1-8. Water chemistry for the Hwang et al. disinfection study.[90]

Component	Amount
Glucose	1.0 mg TOC/mL
KNO_3	995.07 μg/L
KH_2PO_4	129.92 μg/L
Na_2SO_4	44.29 μg/L
$CaCl_2 \bullet 2H_2O$	18.34 μg/L
$MgCl_2 \bullet 6H_2O$	41.81 μg/L
$FeCl_3 \bullet 6H_2O$	9.68 μg/L
KCl	19.07 μg/L
$CoCl_2 \bullet 6H_2O$	0.40 μg/L
$CuCl_2 \bullet 2H_2O$	0.54 μg/L
$MnSO_4 \bullet 5H_2O$	21.94 μg/L
$ZnCl_2$	0.21 μg/L
$(NH_4)_6Mo_7O_{24} \bullet 4H_2O$	0.13 μg/L
pH	7.0

The copper/silver ionization treatment in treated river water (Table 1-9) utilized by Thomas et al. was not successful in reducing the amoeba population.[82]

Table 1-9. Water chemistry for the Thomas et al. disinfection studies.[82]

Component	Amount
pH	7.6
Turbidity	0.08 NTU
TOC	0.7-0.8 mg/L

Dissolved oxygen	8.6%
Oxygen saturation	95%
Conductivity	598 μS/cm
Hardness	21 French Grade
Ca^{2+}	109.8 mg/L
Cl^-	32 mg/L
SO_4^{2-}	69 mg/L
Fe total	<20 μg/L
Cu	<2 μg/L
HCO_3^-	256 mg/L

DeJonckheere and Van de Voorde found that *A culbertsoni* cysts were not inactivated by any of the tested bromine, iodine and iodophore concentrations after 24 hours of exposure.[81]

Barker et al. exposed *A. polyphaga* to three commercial biocides. They reported that almost all of the amoeba cells survived treatment with 8 mg/L benzisothiazolone (BIT), while approximately 30% of cells survived exposure to 2 mg/L polyhexamethylene biguanide (PHMB). A concentration of 16 mg/L 5-chloro-N-methylisothiazolone (CMIT) was most effective; very few amoeba cells recovered following this treatment. These concentrations were chosen because they were sufficient to inhibit cell growth. Unfortunately, these concentrations do not correspond to what would be used in commercial applications. While CMIT was most effective in inactivating cells, the concentration used here was 6 mg/L higher than that typically used. PHMB is usually used at a concentration of 10 mg/L, and BIT is typically applied at 150 mg/L. While use of CMIT and BIT could be a cause for concern due to their toxic qualities, PHMB is not considered a health hazard, though use as a potable water disinfectant would require further study as to the effects of chronic exposure. PHMB is currently used in swimming pool disinfection and as a preservative for contact lenses, and it provided effective treatment against amoebae in this study.[91]

Srikanth and Berk also tested the effectiveness of four biocides commonly used to disinfect cooling towers. The first was a thiocarbamate compound, MBC 350 (50% potassium dimethyldithiocarbamate); the second was 5% tributyltin neodecanoate mixed with quaternary ammonium compounds labeled MBC 120. The third is MBC 215, an

isothiazolin compound (5-chloro-2-methyl-4-isothiazolin-3-one). Finally, they tested MBC 115, a quaternary ammonium compound alone (poly[oxy-ethylene(dimethyliminio)ethylene (dimethylimino)ethylene dichloride]). Srikanth and Berk tested these biocides on an *A. hatchetti* isolated from a cooling tower and an isolate purchased from ATCC. They found the strain from the cooling tower was significantly more resistant to disinfection by all of the tested biocides except MBC 120. Importantly, they found that concentrations of the biocides lower than that recommended by the manufacture were actually capable of stimulating the growth and reproduction of the amoebae. The isothiazolin compound was found to be the least toxic towards *A. hatchetti*, while MBC 120 was the most effective. Srikanth and Berk found that it was possible for the amoebae to survive and reproduce at disinfectant levels within those recommended by the manufacturers. The authors also pointed out that it is important to know where the isolates from ATCC came from because the environment they evolved in could be very important to how they react to disinfectants.[92] The fact that biocides in low concentrations could actually increase numbers of pathogenic amoebae in a cooling tower system should have a large impact on how carefully they are dosed. If compounds like these are used in a water treatment scheme, this knowledge will also be important.

Greub and Raoult investigated several biocides used to disinfect bronchoscopes: Phagozyme whose active compounds are quaternary ammonium and encapsulated enzymes, Rivascop whose active compounds are quaternary ammonium and biguanides, Endosporine containing 2% glutaraldehyde, and Anioxyde which contains peracetic acid. All of the agents were effective after 24 hours against trophozoites. After 30 minutes of exposure, Phagozyme was the only biocide capable of killing all of the trophozoites at a 10% dilution; undiluted Anioxyde also killed all of the trophozoites at 30 minutes of exposure time. Phagozyme was also the most effective against cysts. Undiluted Rivascop and Endosporine left viable trophozoites even after three hours of exposure.[93]

Bouyer et al. found that caspofungin, an echinocandin fungicide, was somewhat effective against the trophozoite forms of *A. castellanii, A. polyphaga,* and *A. culbertsoni* at a concentration of 16 mg/L after 24 hours. The cysts of *A. castellanii* and *A. polyphaga* were 100% eradicated at a concentration of 500 mg/L. A small percentage of

treated cysts of *A. culbertsoni* survived this treatment and rebounded when the amount of caspofungin was decreased. Topical treatment of keratitis in a rabbit model showed that even concentrations as high as 5 mg/mL were not toxic to the patient.[94]

Kilvington found that less than 5 mg/L of the following compounds were sufficient to inactivate *Acanthamoebae* after eight hours of contact: chlorinated phenolic thioether, isothiazolones, and methylenebis thiocyanate at a pH of 6.9 or 8. Polyhexamethylene biguanide and peracetic acid were effective at levels less than 15 mg/L. Concentrations of 200 mg/L or higher of bromo nitropropane diol for eight hours were necessary to remove *Acanthamoebae*; 25 mg/L were necessary with an 18 hour exposure time. Bromo nitropropane diol was particularly ineffective against cysts of *Acanthamoebae*, requiring more than 10,000 mg/L for inactivation. Chlorinated phenolic thioether was effective against cysts at the lowest concentration, only 30 mg/L.[86]

Loret et al. also looked at the removal efficiencies of physical water treatment steps for the removal of *Acanthamoebae* under typical operational conditions. Conventional clarification and filtration achieve a 2-log reduction of *Acanthamoebae*, and ultrafiltration is capable of removing greater than 4 logs.[84]

In addition to chemical methods of disinfection, Vernhes et al. have shown that pulsed electric fields are capable of inactivating a species of *Naegleria* by physically damaging the cells, resulting in permeabilization of the cell membrane. A field intensity of 0.25 kV/cm or greater was necessary to achieve permeabilization. It was effective against both trophozoites and cysts, though to a greater extent for the trophozoites. The method was most effective when the electric field was applied parallel to the water flow.[97] While this method has not yet been tested on *Acanthamoeba* spp. specifically, it may prove a very effective water treatment option for removal of amoebae. Pulsed electric fields could be used in conjunction with traditional disinfection chemistry without additional harmful by-products and may be a cost effective solution.

Polat et al. recently completed a study on the cytotoxic effects of garlic extract on both trophozoites and cysts of *Acanthamoebae*. They were looking for a new treatment for keratitis and found that concentrations of garlic extract as low as 0.78 mg/mL completely destroyed *Acanthamoebae* trophozoites in a sample over 72 hours. A dose of

3.90 mg/mL was necessary to eradicate all of the *Acanthamoebae* cysts in a 72 hour period. Higher concentrations (≥7.81 mg/mL) were tested and worked even more quickly, but they were found to have cytotoxic effects on the rabbit corneal epithelial cells tested and were, therefore, undesirable for treatment.[98] While garlic flavored water would likely be undesirable for water utility consumers, further investigation into the actual compounds responsible for this activity against *Acanthamoebae* could result in an effective, organic treatment of *Acanthamoebae* in water distribution systems.

E. *Acanthamoebae* Disinfection in Human Tissue

In keratitis patients, hexamidine, chlorhexidine and propamidine in combination, and chlordexidine have been demonstrated to be successful in destroying *Acanthamoebae*. The addition of phenylmethylsulfonylflouride to amoeba-containing media has been found to severely decrease the cytolytic activity of the amoebae, and mannose has been shown to inhibit the trophozoite's ability to attach to cells and to consume red blood cells and yeast.[16] Though these compounds have not been studied in regard to water treatment, they may provide useful insight into new methods. Cycloheximide has also been demonstrated to prevent cysts from emerging into trophozoites by inhibiting the incorporation of proline into proteins.[8]

1.5 Laboratory Methods for Detection of Acanthamoebae

A. Environmental Sampling Methods

Acanthamoeba spp. are routinely found in every environmental medium: soil, water, and air.

Soil can be tested for *Acanthamoebae* by suspending it in Page's amoebic solution, then plating small dots or lines of the suspension on agar coated with a layer of gram-negative bacteria.[99] This method is not specific for *Acanthamoebae*.

Water samples may be tested directly or may be obtained through filtration or centrifugation of large volumes of water in order to concentrate samples into large enough numbers of amoebae for quantification. Filtration results in the loss of fewer viable trophozoites when compared with centrifugation. The National Standard Method

published by the United Kingdom's Health Protection Agency details the method for each of these.[99] This is often necessary because amoebae often naturally occur in water at a rate of only one amoeba per liter of water. Shoff et al. found that the most reliable place to sample for *Acanthamoebae* in homes is to test the water storage tank of toilets because it is constantly supplied by cold water from the municipal source and is largely undisturbed and not cleaned, whereas faucets are fed with a mix of hot and cold water.[64]

Several air sampling methods have been developed to measure microorganisms. The Anderson sampler is used to deposit airborne amoebae onto a plate of non-nutritive agar smeared with *E. coli*. The Greenburg-Smith and Litton-type air samplers trap airborne microorganisms in water reservoirs, which can later be analyzed using lab culture or molecular identification techniques.[100] Air samples collected by Ramirez et al. using a modified impinger were bubbled into Bold's basal medium and were then plated on *E. coli*-layered NNA.[73]

B. Morphological Identification, Enumeration, and Lab Culture Methods

Acanthamoebae can be enumerated using a hemacytometer, standard plaque assay, quantitative microtiter method, and the counting of track-forming units growing on nonnutritive agar with an *E. coli* top layer.[101]

Acanthamoeba spp. from environmental sources can be cultured on a non-nutritive media spread with a top layer of gram-negative bacteria, typically *E. coli* or *Enterobacter aerogenes* (formerly *Klebsiella aerogenes*). The presence of amoebae on a plate is indicated by a clear hole or trail in the opaque layer of bacteria where the amoebae have eaten away the bacteria. Khan gives a very detailed description of this process.[9] These plates can be used to quantify the *Acanthamoebae* in a sample by utilizing serial dilution and a Most Probable Number (MPN) table.

Beattie et al. found the method described by Perrine et al.[102] to be reproducible in studies of the efficacy of multipurpose contact solution.[101] Unfortunately, the MPN method may miss cysts, as well as trophozoites damaged during sample collection and can only give a probable estimation of cell numbers.[87] Isolated strains of *Acanthamoebae* sp. can be enriched in a Peptone, Yeast Extract, and Glucose (PYG) agar

or can be maintained on the following mammalian cells: African green monkey kidney, human embryonic lung, human embryonic kidney, B103 rat neuroblastoma, and L929 fibroblasts.[16] If an axenic, or uncontaminated, culture is desired, bacterial contamination can be removed by the addition of penicillin-streptomycin or gentamicin, which are not harmful to *Acanthamoebae*, even in higher doses. Amphotericin B can be used to remove fungal contamination, though it is toxic to *Acanthamoebae* in high doses.[103]

To determine whether or not a strain is pathogenic, *Acanthamoebae* can be incubated for 12-24 hours on plates containing a monolayer of rabbit corneal epithelial cells. Staining with eosin following incubation allows researchers to assess the cytopathic effect of the amoebae. In addition, non-nutritive agar containing 0.5 to 1.0 M mannitol can be used to differentiate between pathogenic and nonpathogenic *Acanthamoebae*. The mannitol causes the plates to have a high osmolarity, inhibiting the growth of nonpathogenic amoebae.[21] Osmotolerance seems to be a better indicator of pathogenicity than thermotolerance, because several species of *Acanthamoebae* that are capable of causing keratitis are killed at temperatures of 37°C or higher, as discussed in the Pathogenicity section above.

In order to create cysts from healthy trophozoites, Neff's solution is most often used. Briefly, cells in stationary phase growth are immersed in a buffered salt solution at a pH of 7.0 during induction and then raised to pH 8.9 during wall synthesis at a temperature of 29-30°C. Oxygen saturation is also imperative.[104]

For short-term storage, *Acanthamoebae* grown on agar plates can be placed in 4°C storage as long as they are kept under moist conditions. For long term storage, 3-4 x 10^6 cells per milliliter can be placed in a DMSO freezing medium and stored at -70°C.[9]

C. Molecular Methods of Identification and Enumeration

While the cyst walls of most protozoa are made of chitin, the cyst wall of *Acanthamoeba* sp. contains cellulose, which may provide an additional physical barrier to disinfection, extremes of pH, temperature, dessication, and anoxia.[15] Linder et al. suggest that this quality can be used to differentiate between *Acanthamoebae* and other pathogenic amoebae in an environmental sample. Since it is impossible to tell the

difference between cell walls containing chitin and those containing cellulose using microscopy alone, Linder used an anti-cellulose binding domain and flourescein-labeled antibodies in conjunction with *Trichoderma reesei* cellulose binding domains to effectively stain *Acanthamoebae*. They could then use immunofluorescence to successfully distinguish between *Acanthamoebae* and other protozoa in an environmental sample (*Naegleriae, Hartmanellae, G. intestinalis,* and *Entamoeba dispar*). Unfortunately the marker does not react with intact cysts; the cellulose is contained in the inner side of the cyst wall, so the inner cell wall must be exposed. Other problems with the marker include making these markers available to researchers in the field and the necessity of removing nonspecific background fluorescence.[105]

Several researchers have described primers and probes for the identification and quantification of *Acanthamoebae* using polymerase chain reaction (PCR) and qPCR. PCR has been used to identify as low as one to five cells in a sample. The ACARNA primers (forward primer 5'-TCC CCT AGC AGC TTG TG-3' and reverse primer 5'-GTT AAG GTC TCG TTC GTT A-3') have been used successfully by multiple researchers to identify *Acanthamoebae* at a genus level with a resulting PCR byproduct of approximately 272 bp.[21, 70] qPCR is used to quantify amoebae and has the advantage of giving an absolute number rather than an estimate. Riviere et al. successfully used the TaqAcF1 (CGACCAGCGATTAGGAGACG), TaqAcR1 (CCGACGCCAAGGACGAC), and the probe TaqAcP1 (TGAATACAAAACACCACCATCGGCGC) with T_m of 59, 60, and 70°C to quantify *Acanthamoebae* in environmental samples.[87]

Importantly, the DNA extraction technique chosen for *Acanthamoebae* analysis prior to PCR analysis is very important in reducing the possibility of false negative tests. DNA extraction of *Acanthamoebae* can be difficult due to the resistance of *Acanthamoebae* cysts and nuclear proteins to lysis and to the difficulty of opening cysts without breaking up DNA strands. Goldschimdt et al. found that a treatment with Proteinase K for ten minutes prior to using a QIAmp® or MagNA Pure® kit markedly improved the detection limit for *Acanthamoebae*.[106] While PCR and qPCR are more selective and objective measures of cells in an environmental sample, a major

disadvantage of the technique is that it cannot be used to distinguish between nucleic acids from live, dead, or VBNC cells. Riviere et al. found that the DNA yield from cysts is three times lower that of trophozoites; therefore, a sample with a large amount of cysts may be underestimated by qPCR by a factor of at least three.[87] It is often possible to use these techniques in combination with traditional solid culture techniques to get a good idea of what fraction falls into each category.[59] Though PCR is a very useful technique for species identification, it may be time consuming, and it can be difficult for 18S rRNA to discriminate between closely related organisms.

Kong and Chung used PCR-RFLP of a riboprint of *Acanthamoebae* genes and morphological grouping to more quickly identify unknown samples of *Acanthamoebae*. This technique multiplies a small section of a gene rather than requiring a researcher to sequence the entire gene and is much faster than Mt DNA RFLP and alloenzyme analysis. Riboprinting is not as accurate when intron is present in the DNA.[23]

Kohsler et al. attempted to use internal transcribed spacers (ITS1) to classify *Acanthamoebae* more quickly and with more precision when working with closely related strains but found that the ITS1 technique did not result in a correlation to species as identified by morphology.[107]

In addition to identification of *Acanthamoeba* sp., PCR primers can be used to determine pathogenic capacity by identifying those amoebae containing serine or cysteine proteases--indicators of pathogenic potential.[19] Unfortunately, these primers only work with axenic cultures of the amoeba, and they can take several weeks to prepare.[21]

Due to the subjective nature of morphological and solid culture analysis, inexperienced researchers attempting the identification of *Acanthamoebae* are more likely to achieve accurate results using molecular methods. A study by Behets et al. found a high level of correlation between data collected on free-living amoebae counts using morphological, immunological, and biochemical molecular methods.[76]

DNA analysis has also been used to identify the source of keratitis infections. Cox-PCR sequence comparison and HindIII mtDNA RFLPs were used by Kilvington et al. to match clinical isolates to isolates from tap water sources. Unfortunately, these analyses also require an axenic culture, which can be difficult or impossible to aquire for

some environmental isolates.[31] These analyses may prove useful in negligence cases in the future, where a strain of *Acanthamoebae* causing a keratitis infection could be linked to a service industry location.

While isoenzyme patterns have been shown to have limited effectiveness, amplified ribosomal DNA restriction analyses (ARDRA) is very sensitive, cost effective and can identify a single pathogenic cell without prior cultivation.[108]

1.6 REGULATION

A. EPA Contaminant Candidate List (CCL)

The United States Environmental Protection Agency (USEPA) is required by the Safe Drinking Water Act (SDWA) to establish a list of contaminants to assist in setting priorities for its drinking water program. The first list, called the Candidate Contaminant List (CCL), was published in 1998 and included *Acanthamoebae* as a microbial contaminant.[109] The EPA decided against forming regulatory standards for *Acanthamoebae* and removed it from the list. The authors cited a lack of evidence of the role of tap water in causing incidences of keratitis, as well as a lack of studies on host relationships with pathogenic organisms. In addition, the incidence rate of keratitis is less than the 1:10,000 risk of infection per year that has been set as the EPA goal for water supplies.[29]

1.7 CONCLUSIONS

The risks associated with the presence of *Acanthamoebae* in drinking water treatment plants, distribution systems, and premise plumbing are significant and should be of continued concern to researchers and regulators. While the diseases associated with *Acanthamoebae* are serious, the potential of dangerous, pathogenic endosymbionts to survive in the drinking water system because of the presence of the amoebae presents a significant potential danger.

Acanthamoebae readily survive in the conditions provided by drinking water systems. They can adapt to an extreme range of temperatures and pH and can survive indefinite exposure to doses of disinfectant currently used in water treatment via cyst

formation. Use of chlorine based disinfectants, ozone, and UV has proven ineffective at long-term control of *Acanthamoebae*.

The use of PCR for identification of *Acanthamoebae* and of qPCR for quantification of amoebae is the most reliable and objective method available today. The relative ease of the analysis makes it ideal for less experienced researchers and is recommended for use in future experiments.

In order to eliminate the threat posed by *Acanthamoebae* in drinking water systems, further research into novel disinfection techniques is required. Additional work with chemical disinfectants not currently used in the water treatment process is also warranted, though cost is often prohibitive in the widespread use of these chemicals.

Another area recommended for future research is field studies of *Acanthamoebae*. It would be very useful to collect samples in different areas of a distribution system and under different conditions to identify the prevalence of *Acanthamoebae* in drinking water in the United States and also to identify any endosymbiotic bacteria that the amoebae are harboring. It will be necessary to obtain this data before further recommendations regarding regulations can be made.

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Chapter 2: Preliminary Investigation into the Role of Organic Carbon and Disinfectants in *Acanthamoeba polyphaga* Amplification

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2.0 INTRODUCTION

Acanthamoeba polyphaga is a known causative agent of Acanthamoeba keratitis--an infection of the eye that can lead to blindness if not treated quickly. It can infect otherwise healthy individuals. It can survive in air, soil, and water. *Acanthamoeba polyphaga* can encyst during times of unfavorable environmental conditions, making it resistant to changes in temperature, pressure, pH, dessication, and disinfection. When conditions are favorable for survival, the amoebae excyst and once again become pathogenic.

Several species of *Acanthamoebae* are known to host bacteria, called endosymbionts, that are commonly found in drinking water. The list of possible endosymbionts includes pathogens like *Legionella pneumophila*, *Pseudomonas aeruginosa*, and MRSA. These bacteria are able to survive and even thrive within the host organism in environments where they would normally perish. Even in potable water conditions that they would not normally survive, the bacteria flourish within host *Acanthamoebae*.

This work is a preliminary investigation of *Acanthamoeba polyphaga* persistence in simulated potable water tanks. The effects of natural organic matter and assimilable organic carbon availability and the effect of disinfectants commonly used in drinking water were evaluated.

2.1 MATERIALS AND METHODS

A. Culture of Acanthamoeba

A culture of *Acanthamoeba polyphaga* (ATCC 30871) was obtained from the lab of Dr. Joseph O. Falkinham, III. The culture was maintained on PYG media (per liter: 20 g peptone, 1 g yeast extract, 1 g sodium citrate, 10 mL- 0.4 M $MgSO_4$, 8 mL- 0.05 M $CaCl_2$, 10 mL- 0.005 M ferric ammonium sulfate, 10 mL- 0.25 M Na_2HPO_4, 10 mL- 0.25 M KH_2PO_4, 50 mL of 2 M glucose) in tissue culture flasks (25 cm^2, BD Falcon).[1]

B. Enumeration

Microscopy

Enumeration was attempted using a light microscope and a Petroff counter. This method had a minimum detection limit of approximately 50,000 cfu/mL, which is higher than the population we were attempting to observe. We also used an inverted light microscope to count fields of amoebae in situ. The inverted light microscope allowed us to see the *A. polyphaga* in the tissue culture flasks without disturbing them, but the method is inherently subjective and time consuming.

Plating techniques

An R2A agar (Difco) was used to perform heterotrophic plate counts (HPC) to monitor the amount of heterotrophic bacteria present in each sample. Plates were incubated at room temperature for one week before colonies were counted.[2]

When the microscopy method of enumerating *A. polyphaga* was deemed too subjective, a Most Probable Number (MPN) method was utilized. Several research groups report that they have used variations of this method to successfully quantify *Acanthamoebae*.[3-7] To accomplish this, plates with a bottom layer of R2A and a top layer of nonnutrient agar containing live *E. coli* were made. Following a serial dilution in Page's Amoebic Saline, 0.01mL aliquots of sample containing *Acanthamoebae* were plated on each quarter of the MPN plates.[8] The plates were incubated for ten days in the dark at room temperature and were checked every two days. Plates were considered positive when cleared zones were visible in the *E. coli* lawn. Unfortunately, data obtained using this determination method was subjective, and the method was labor intensive.

The MPN method relies heavily on the researcher's ability to correctly identify a sample as positive or negative. To determine if a plate is positive or negative for amoebae, the researcher must determine if a cleared zone exists. Occasionally, cleared zones can be created by the force of the sample being pipeted onto the *E. coli* lawn or by the tip of a pipet scratching the surface of the lawn. While the difference is recognizable to an experienced user, data collected by new users may not be accurate. In addition to

these problems, cleared zones are not specific to *Acanthamoeba polyphaga,* so any contaminating amoebae would be counted.

The MPN method uses the combination of a dilution series and a presence test to determine the probable concentration in a sample. Each dilution is plated five times to improve the statistical probability that samples containing amoebae will be identified as such. The data is reported based on how many plates tested positive at each dilution, so if at 1 mL 4 of 5 plates tested positive, at 0.1 mL 3 of 5 plates tested positve, and at 0.01 mL 1 of 5 plates tested positive, the data would be recorded as 4-3-1. This combination is assigned a specific MPN index number allowing quick calculation of the probable amoebae concentration in the sample.

One false determination can make a large difference in the reported amoebae concentration. For instance, incorrectly reading a combination of positives as 4-3-1 instead of 5-3-1 means recording the data as 110 amoebae per 100 mL of sample instead of 33 amoebae per 100 mL of sample. These numbers are not significantly different from each other, but a difference of this magnitude could change whether or not a sample compared to this one is reported as statistically different or not. In addition, the method is not *Acanthamoebae* specific so any contaminating amoebae would be counted as *Acanthamoebae*.

Polymerase Chain Reaction

A polymerase chain reaction (PCR) method has been demonstrated to provide a relatively easy, objective method of identifying *Acanthamoebae* at the genus level. The method described by Boost et al. has been used successfully by several researchers and by our lab to identify *Acanthamoeba* spp.[9] Quantitative Polymerase Chain Reaction (qPCR) is an objective and effective way to enumerate *Acanthamoebae*. The method described by Riviere et al. has been used to successfully count amoebae in samples.[10]

C. Flask Preparation for Experiments

Tissue culture flasks (225 cm^2, Corning) were inoculated with water from a residential water heater in order to establish a representative biofilm. Water changes were accomplished using a dump-and-fill method three times a week during inoculation, which lasted two weeks. Successful biofilm inoculation was confirmed visually and by HPC of

water samples on R2A agar. The base water used is described in Table 2-1 below. The pH of the bulk water was adjusted to 7.2 prior to use.

Table 2-1. Components of *Acanthamoebae* experiment base water.

Concentration	Added as
2 ppm as N	9.44 mg/L $(NH_4)_2SO_4$
1 ppm as Mg	4.96 mg/L $MgSO_4$
4.9 ppm as Ca	18.12 mg/L $CaCl_2$
10 ppm as K	19.06 mg/L KCl
1.03 ppm as P	4.68 mg/L Na_2HPO_4
122 ppm as HCO_3^-	167.96 mg/L $NaHCO_3$
20 ppm as SO_4^{2-}	0.19 mL/L H_2SO_4
5 ppb as Cu	0.99 μg/L $CuCl_2$
0.1 ppb as Co	0.22 μg/L $CoCl_2 \bullet 6H_2O$
5.6 ppb as Mn	15.4 μg/L $MnSO_4 \bullet H_2O$
1.7 ppb as Mo	3.62 μg/L $Na_2MoO_4 \bullet 2H_2O$
2.6 ppb as Zn	6.41 μg/L $ZnSO_4 \bullet 7H_2O$
0.1 ppm as Fe	0.273 mg/L $FeSO_4 \bullet 7H_2O$

To grow enough amoebae for inoculation, *A. polyphaga* was transferred into a 225 cm^2 flask containing 400 mL of PYG five days prior to use. In order to lessen the shock of transfer from a rich medium into a simulated drinking water, 25% of the media was replaced with base water after two days of growth; one day prior to use, 50% of the solution was replaced with base water. On the fifth day, the flask wall was gently scraped with a plastic cell scraper, and 10 mL aliquots of media containing *A. polyphaga* were put in test tubes. These tubes were centrifuged, and the supernatant was decanted to remove the extra nutrients. The cells were then suspended in sterile distilled water. Flasks containing 400 mL of base water were inoculated with 10 x 10^6 cells of *A. polyphaga*, enumerated using a Petroff counter.

D. Carbon nutrient study

This study used a defined stock of simple assimilable organic carbon (AOC) as well as ozonated natural organic matter (NOM) to simulate the organic carbon available in a drinking water system. The defined stock AOC solution was composed of equal parts (as carbon) of sodium acetate, glucose, and oxalyic acid. The NOM was obtained

from a concentrated stock solution. Before use, it was ozonated to break up long carbon chains and filtered with a 0.45 μm pore size cellulose acetate membrane (Micro Filtration Systems). The filtration step was necessary to make it easier to count *Acanthamoebae* under a microscope, since larger particles remaining after ozonation interfered with counting. The carbon concentration of the ozonated and filtered NOM was measured using a portable total organic carbon analyzer (Sievers Model 800).

Each condition was tested in triplicate, and composite samples were collected for analysis. The base water was prepared in bulk and adjusted to a pH of 7.2, and the appropriate amount of carbon for each condition was added to base water so that all of the flasks in each set received the same amount of carbon. The water was changed using a dump-and-fill method. The carbon concentrations used are detailed in Table 2-2 below. In addition to these conditions, three more tissue culture flasks were inoculated with *A. polyphaga* in which no biofilm had been established and to which no organic carbon was added. The expectation was that *A. polyphaga* would disappear rapidly from this control, labeled COO (Table 2-2). For the first three months of experimental conditions, distilled water was used. Following the observation that this water contained approximately 1mg/L of total organic carbon, water filtered through a Barnstead NANOpure™ Analytical Deionization System was used.

Table 2-2 Carbon concentrations dosed. The NOM carbon concentration was measured following ozonation and filtration and dosed accordingly.

Condition	Condition Label	AOC (ppb as C)	NOM (ppb as C)
1	C0, C00	0 ppb	0 ppb
2	C5	5 ppb	5 ppb
3	C50	50 ppb	50 ppb
4	C300	300 ppb	300 ppb
5	C1K	1000 ppb	1000 ppb
6	C3K	3000 ppb	3000 ppb
7	C10K	10000 ppb	10000 ppb
8	C30K	30000 ppb	30000 ppb
9	AOC	600 ppb	0 ppb
10	NOM	0 ppb	600 ppb

Each week, HPC and MPN counts of *A. polyphaga* were performed.

E. Disinfectant study

Tissue culture flasks of amoebae were prepared and inoculated as described above. The water used in the disinfection study was the base water described in Table 2-1 at a pH of 7.2 with 300 ppb each of AOC and NOM. The only exception was the condition containing chlorine, which contained the base water described in Table 2-1 without the ammonia. Seven conditions were tested in triplicate flasks using composite sampling (Table 2-3). Chlorine dosing was ramped up on a preset schedule (Table 2-4).

Table 2-3. Experimental matrix for disinfectant study.

	Condition
1	Control, no disinfectant, base water & carbon
2	Control water plus 1.3 mg/L Cu^{2+}
3	Control water plus 1 ppm NH_4
4	Control water plus 3 ppm NH_4
5	Control water plus chlorine
6	Control water plus 1 ppm NH_4 plus chlorine to form chloramines
7	Control water plus 3 ppm NH_4 plus chlorine to form chloramines

Table 2-4. Experimental matrix for chlorine addition.

Week	1	2	3	4	5	6	7	8	9	10	11	12
Cl^- dosed (mg/L)	0	0.1	0.3	1	1	3	3	10	10	25	60	90

Prior to use, the chlorine and ammonia concentrations in each experimental water were tested using the HACH spectrophotometer. The water was changed once a week using a dump-and-fill method to provide fresh nutrients to the amoebae. Composite samples for each condition were collected. HPC and MPN plate counts were used to enumerate bacteria and amoebae in each sample.

2.2 RESULTS

A. Carbon nutrient study

Contrary to expectations, five months of study indicated that the varied carbon concentrations did not decisively control the *A. polyphaga* concentrations in the biofilm or in the suspended water. There were no temporal trends. For example, throughout the study the sample labeled C30K had higher counts than the sample labeled COO, but only 9 of 17 (53%) of the samples collected each week were statistically different at a 95%

confidence level. The C10K sample had equal or greater amounts of amoebae than the COO sample according to the MPN levels in 16 of 17 weeks, but only two of these samples were statistically different than the COO sample. In Table 2-5 below, a typical data set for this study is detailed with the lower and upper 95% confidence levels. This data set was collected three months after the experiment began. It was also deemed highly unreasonable for the control sample without carbon to have high counts. Moreover, while population dynamics are likely complex, it also seemed unreasonable that there was no clear simple trends between organic carbon dosing and MPN over the carbon range from 0 to 30,000 μg/L.

Table 2-5. Typical data set during the carbon nutrient study from week 12.

		95% Confidence Limits	
	MPN/100 mL	Lower	Upper
C00	350,000	160,000	820,000
C0	350,000	160,000	820,000
C5	340,000	160,000	800,000
C50	280,000	120,000	690,000
C300	1,600,000	600,000	5,300,000
C1K	900,000	300,000	2,900,000
C3K	220,000	100,000	580,000
C10K	350,000	160,000	820,000
C30K	9,000,000	3,000,000	29,000,000
AOC600	280,000	120,000	690,000
NOM600	1,600,000	600,000	5,300,000

In this data set, only the C30K sample is statistically higher than the COO sample at a confidence level of 95%.

However, in another representative data set without any change in the water conditions (Table 2-6), the C30K sample was not statistically different than the sample without any organic carbon and pre-existing biofilm (COO).

Table 2-6. A second representative data set where the C30K sample amoebae concentration is not significantly different than the C00 sample (Week 17).

		95% Confidence Limits	
	MPN/100 mL	Lower	Upper
C00	80,000	30,000	250,000
C0	140,000	60,000	350,000
C5	170,000	70,000	480,000
C50	34,000	16,000	80,000
C300	280,000	120,000	690,000
C1K	350,000	160,000	820,000
C3K	900,000	300,000	2,900,000
C10K	900,000	300,000	2,900,000
C30K	280,000	120,000	690,000
AOC600	27,000	12,000	67,000
NOM600	220,000	100,000	580,000

There are two possible explanations for these results. The first explanation is that despite the statistical rigor of the MPN method, the error and confidence levels are higher than expected (Tables 2-5 and 2-6). Alternatively, it is possible that organic carbon plays no role in the concentration of *Acanthamoeba* that were growing in the reactor. In addition, the inherent variability of the MPN method makes repeated testing with replication necessary to draw definitive conclusions about the role of carbon in *Acanthamoebae* growth.

While HPC numbers are variable because the number determined via plating depends on how much of the biofilm detaches during the water change, they can provide insight into the health of the system and the effect of changes in the system. One month following inoculation of the carbon study with *A. polyphaga*, a representative HPC data set shows no trend in HPC growth as the carbon concentration was varied (Table 2-7).

Table 2-7. Representative HPC data set. Samples collected one month following inoculation with *A. polyphaga.*

Condition	HPC (cfu/mL)
C00	288,000
C0	181,000
C5	106,000
C50	213,000
C300	177,000

C1K	178,000
C3K	202,000
C10K	218,000
C30K	262,000
AOC600	580,000
NOM600	330,000

After three months of experimental conditions, the water source was switched from distilled water to low TOC water. This produced an immediate drop in HPC (Table 2-8).

Table 2-8. Heterotrophic plate counts in the carbon study flask effluent the week before and three weeks after the change to low TOC water.

	HPC before change (cfu/mL)	HPC after change (cfu/mL)
COO	246,000	56,000
CO	212,000	75,000
C5	202,000	72,000
C50	230,000	93,000
C300	670,000	156,000
C1K	510,000	420,000
C3K	650,000	550,000
C10K	700,000	1,260,000
C30K	4,000,000	830,000
AOC600	680,000	420,000
NOM600	520,000	185,000

The change of water source for the flasks resulted in a drop in HPC on the order of 10X for the lower levels of carbon, suggesting that the carbon in the distilled water had been artificially inflating the amount of bioavailable carbon in the system and masking the effect of the imposed carbon limitation.

Two months following this change, the large plastic carboy holding the bulk water for up to two weeks was identified as imparting large amounts of carbon into the water (up to 500 ppb TOC). Unfortunately, no data was recorded to measure the drop in HPC resulting from changing this plastic storage to a glass container, but the assumption is that the concentration would have dropped again by some amount.

B. Disinfectant study

The number of *A. polyphaga* present in the sample receiving copper disinfectant is significantly lower than the control receiving no disinfectant on weeks 3-9 and week 11 at a 95% confidence level, suggesting that 1.3 mg/L of copper ion may be an effective disinfectant of *A. polyphaga* (Figure 2-1).

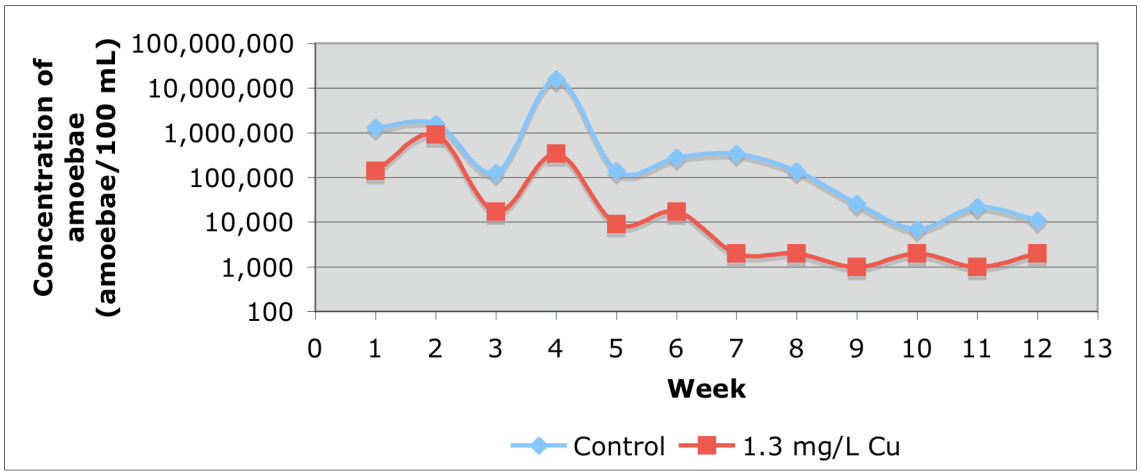

Figure 2-1. Graph of copper disinfectant.

Data presented in logarithmic scale. Data points below the detection limit for the method (2000 amoebae/ 100 mL) were graphed as 1000 amoebae/ 100 mL.

At a 95% confidence level, data suggests that free chlorine may be an effective disinfectant against *A. polyphaga* at a dose of 10 mg/L (weeks 8 and 9) and at 50 mg/L on week 11. The concentrations of amoebae in the samples dosed with chloramines were significantly lower than the control concentration at a 95% confidence level only on week 11 at a dose of 50 mg/L. However, because of the inherent variability and general lack of trustworthiness of the MPN method used to collect this data, it would be necessary to repeat the testing with replication to draw definitive conclusions. The concentration of the amoebae in the control flasks was low on weeks 10 and 12, so even though the concentration of the amoebae in the flasks receiving disinfectant was lower than the control concentration, it was not statistically significant (Figure 2-2).

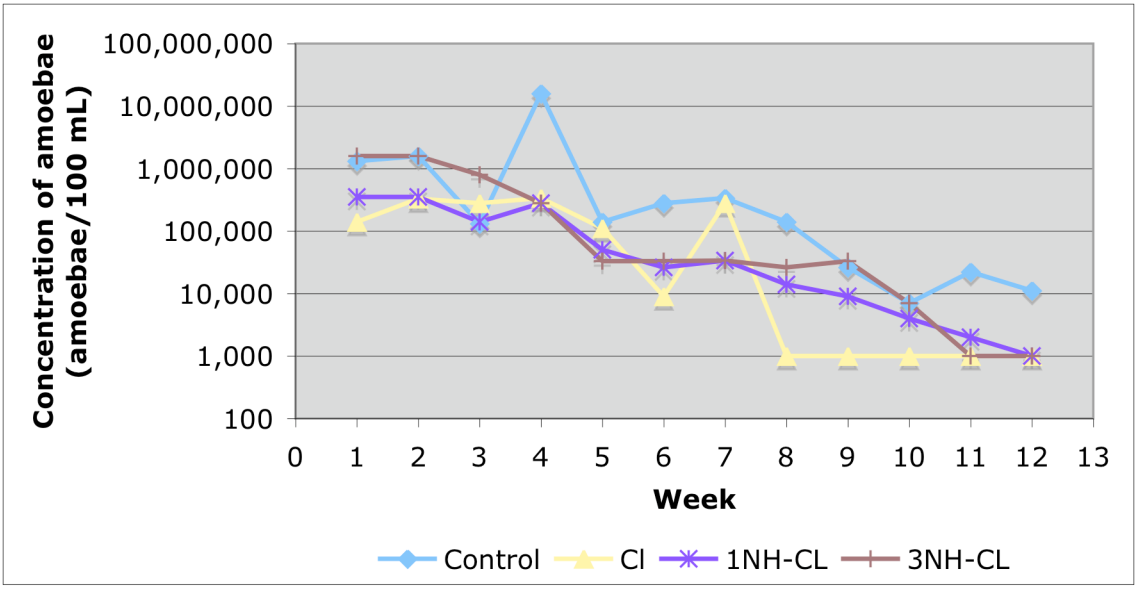

Figure 2-2. Graph of chlorinated disinfectants.

This graph is presented in logarithmic scale to make the difference in magnitude more visible. Data points below the detection limit for the method (2000 amoebae/100 mL) were graphed as 1000 amoebae/ 100 mL.

However, these results were determined using the MPN enumeration method and may not be significant because of the aforementioned issues regarding variability of the MPN method and the contamination issues discussed below. In addition, the MPN method is thought to miss a majority of *Acanthamoebae* cysts in a sample, which are still viable but not easily cultivable.[10] This is a major limitation for any study of *Acanthamoebae* in potable water using the method because failing to remove a single cyst will allow recolonization of the system.

A disinfectant residual study found that 1 mg/L of free chlorine decayed completely in 24 hours. Chloramines were 90% decayed in 24 hours and were completely gone in 48 hours.

2.3 Carbon Study Problems

Several potential problems have been identified that may have affected the accuracy of the carbon nutrient experiment. The current research began by using plastic containers to hold the base water for the experiments prior to use. Subsequent tests revealed high concentrations of carbon being added to the water unintentionally due to

contact with plastic. As much as 500 ppb of TOC was added to bulk water left standing in a plastic container for approximately ten days. A new set of experiments was initiated with glass containers but new results from these reactors were not obtained during this work.

In addition, the distilled water used in the first three months of the experiment had a significantly higher carbon concentration than the low TOC water used for the remainder of the experiment. This allowed a potentially large amount of carbon unintentionally added to the system. This may have inflated the number of amoebae in the system, masking potential responses to the imposed carbon limitation. Table 2-8 above shows that changing the water source resulted in a drop in the HPC concentration of an order of magnitude.

Further research into work done with *A. polyphaga* indicates that the carbon added to the system as AOC and NOM may be sufficient to sustain *A. polyphaga* without previously establishing a biofilm. While the presence of a well-developed biofilm provides a more realistic environment to compare with that in premise plumbing, it is not necessary for the carbon nutrient study. In this study, water collected from a residential water heater (assumed to contain an established biofilm) was collected weekly and mixed with the base water and 600 ppb of carbon. The water in the flasks was changed three times a week, receiving seed water from the water heater each time, for a month. A heterotrophic plate count performed on two randomly selected flasks prior to inoculating the flasks with *A. polyphaga* showed a bacteria concentration of approximately 15,000 cfu/mL. This biofilm, established on the wall of the flasks, was most likely a large reservoir of organic carbon that overwhelmed the effect of the carbon concentrations that were dosed. If all of the carbon dosed into the flasks was retained and the HPC is used to estimate the amount of carbon available in the biofilm, hundreds of grams of carbon could have been continually recirculated in the system. The new bacteria and amoebae in the system could have fed on the waste products from this biofilm, obscuring the effect of the carbon limitations imposed on the system.

2.4 LESSONS LEARNED

During the course of this experiment several observations were made that will be useful to future researchers. First, unexpectedly, the distilled water used in the lab tested positive for amoebae using the MPN method. The distilled water amoebae levels measured as high as 13,000 amoebae per 100 mL in one test but was typically 2,000 per 100 mL. Since 2000 amoebae per 100 mL is the detection limit of our MPN method, the presence of a smaller number of amoebae would go unnoticed but would still affect the accuracy of our research.

This potential interference is particularly important to note in the disinfectant study, because the number of amoebae measured by the MPN technique was often less than 20,000 amoebae per 100 mL. The presence of amoebae in distilled water invalidates any research done using the MPN method because care was not taken to determine which plates turned positive because of *A. polyphaga* and which plates were contaminated with unknown amoebae. Future researchers should autoclave the distilled water prior to use in the experiments. While this approach was not tried in this study, other groups have reported autoclaving as a successful way to remove amoebae.[11]

Due to the possible interferences outlined above, an effective quality control regimen is of utmost importance when using the MPN method. Each time samples are plated several controls should also be plated in order to identify problems early on. These controls should include an autoclave-sterilized deionized water sample and a sample of the water going in to the flask before and after the chemicals are added. This will help pinpoint potential interferences before they occur.

The carbon limitation study of *Acanthamoebae* would also have benefited from a more rigourous QA/QC process. Consistent monitoring of the amount of carbon in the synthetic water both prior to and following the addition of chemicals and, in instances where the water was stored for a length of time, the amount of carbon in the water prior to use in the amoebae flasks would have provided for a much earlier determination of the carbon interferences in the study.

The water samples containing the highest concentrations of disinfectant used were not neutralized because it was assumed that the residual would be zero. However, the remaining disinfectant was strong enough to destroy the *E. coli* field at the highest

concentration of the serial dilutions. It would be wise to neutralize any disinfectant using a small amount of sodium thiosulfate to remove this potential problem.

Finally, those working with *Acanthamoebae* in the future should make an effort to use PCR and qPCR to identify and enumerate amoebae because the MPN method is unreliably subjective, particularly for inexperienced researchers, and it allows contaminating amoebae to be counted as *Acanthamoebae*. In addition, the method currently used in our lab has a significantly higher detection limit than that commonly reported for PCR.

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CHAPTER 3: INVESTIGATION OF CIRCUMSTANCES REQUIRED FOR THE GROWTH OF SULFATE REDUCING BACTERIA IN PREMISE PLUMBING

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3.0 INTRODUCTION

Pinhole leaks in copper pipe cost home and business owners an estimated $1 billion annually in the United States alone.[1] Expenses include costs of replumbing entire buildings, water damages, and mold growth. One factor that is strongly believed to be involved in initiation and propagation of leaks is the growth of sulfate reducing bacteria (SRB).

SRB are anaerobic bacteria that reduce sulfates to sulfides. Previous work demonstrated that the presence of sulfides in water increases copper corrosion. Copper pipes in water containing sulfides leached five to fifty times as many corrosion by-products as copper pipes in water without sulfides. After placing copper coupons in water containing sulfides with a pH of 9.2, Jacobs et al. found that the coupons experienced a 5% weight loss, which correlates to a significant decrease in the pipes expected lifespan. Copper coupons in solutions containing sulfides formed a copper sulfide scale. Even after these coupons were placed in sulfide free water, they exhibited ten times the corrosion rates of coupons that were never exposed to sulfides. Chlorine exacerbated the corrosion of the pipes in water dosed with sulfides. Case studies quoted in this paper note that a majority of homes experiencing copper tubing failure were unoccupied for at least a month at a time.[2] Long term exposure ($\geq$ 90 days) to low sulfide concentrations, even as small as 0.007 mg/L, are capable of inducing localized attacks to copper alloys.[3] Sulfides are produced by SRB in potable water systems in amounts of this magnitude (Equation 1).

Equation 1:

$$\text{Carbon Source (i.e. } CH_3CH_2COOH) + SO_4^{-2} \xrightarrow{SRB} S^{-2} + CO_2 + H_2O + HCO_3^- + H^+$$

A number of independent methods can be used to identify sulfide and SRB induced corrosion occurring within a real plumbing system. Forensic evaluation using surface spectroscopy methods can identify the presence of elemental sulfur either in

corrosion scale or down within an active pit. Since spectroscopy techniques can not differentiate the state of sulfur, an inorganic spot test can confirm sulfides.[4] When sulfide bearing compounds are submerged in a sodium azide-iodine solution, nitrogen gas bubbles immediately form which are readily visible in this solution. Sulfate reducing bacteria can be identified and quantified with biological activity reaction tests (BART) spiked with representative water, preferentially that which had been stagnant for at least 8 hours. When available, fresh tubercles can be extracted from pipe samples and introduced into the BART tests in order to improve the likelihood of capturing live bacteria in these culture tests.

Four areas of concern were identified for this work. It was important to understand how SRB reacted when they were inoculated into pipes, what conditions they needed and what evidence could be measured proving their growth, their ability to grow in copper pipe, and their propagation strategies. The combination of these factors that allow SRB to grow on copper premise plumbing pipes are not well defined and require elucidation before mechanistic studies of pitting by SRB can be undertaken. This work is aimed at systematically evaluating effects of nutrients, dissolved oxygen, and stagnation time on colonization of copper pipes by SRB bacteria.

3.1 MATERIALS AND METHODS

A. Copper pipe nutrient study

Ten foot lengths of type M, ½" diameter hard copper pipe were cut into 2' segments. These segments were deburred and then polished using sand paper. Each end of each pipe was fitted with a size 0, inert silicon stopper (Labpure). The pipes were weighed prior to use in order to allow later determinations of weight loss and were then divided into groups of three for triplicate testing of each condition. Pipes were inoculated for three weeks with water known to contain sulfate-reducing bacteria until a positive SRB-BART test (Drycon Bioconcepts, Inc.) indicated that the pipes were colonized. The water chemistry and nutrient levels to each pipe were then changed to the target conditions (Table 3-1), and the water was changed weekly using a dump-and-fill protocol to simulate infrequent water use in the premise plumbing.

Table 3-1. Water chemistry for sulfate reducing bacteria experiments.

All experiments received the base water with the trace elements. Only elements of the control recipe were varied.

	Ingredient	
Base Water		
	0.041 g/L $CaCl_2$	
	8.33 mg/L $NaHCO_3$	
Trace elements & vitamins (added to recipe after seven months)		
	0.325 μL HCl	
	75 μg/L $FeCl_2 \bullet 4H_2O$	
	3 μg/L H_3BO_3	
	5 μg/L $MnCl_2 \bullet 4H_2O$	
	6 μg/L $CoCl_2 \bullet 6H_2O$	
	3.5 μg/L $ZnCl_2$	
	1.25 μg/L $NiCl_2 \bullet 6H_2O$	
	0.75 μg/L $CuCl_2 \bullet 2H_2O$	
	1.25 μg/L $Na_2MoO_4 \bullet 2H_2O$	
	0.5 μg/L Biotin	
	2.5 μg/L *p*-aminobenzoic acid	
	2.5 μg/L Vitamine B_{12}	
	5 μg/L Thiamine	
Control Recipe	Target concentration	Dosed as
	900 ppb C	7.37 mg/L $Ca(C_3H_6O_3)_2$ (calcium lactate)
	1 ppm NH_3^- as N	0.114 mg/L NH_4OH
	1 ppm NO_3^- as N	7.26 mg/L KNO_3
	30 ppm SO_4^{2-}	44.63 mg/L Na_2SO_4
	15 ppb PO_4^{3-}	0.0687 mg/L Na_2HPO_4
	20 ppm Si	177.94 mg/L Na_2SiO_3

The pH of the solutions was adjusted to 9.2±0.5. The trace elements and vitamins are a $1/20^{th}$ dilution of those used in the Postgate Medium G.[1] A total of 19 conditions were tested in triplicate, except for the control condition which began with seven pipes, for a total of 61 pipes. For each condition the concentration of a single ingredient of the control water was varied with one exception. The control condition contained both ammonia and nitrate, but for the conditions varying ammonia or nitrate, the other

ingredient was not added. For instance, the condition with 1 ppm ammonia had 0 ppm nitrate, and the condition with 1 ppm nitrate contained 0 ppm ammonia. The concentrations tested are listed below (Table 3-2).

Table 3-2. Concentrations of ingredients used to create experimental conditions.

Component				
Lactate	0ppb	100ppb	300ppb	2700ppb
Sulfate	0ppm	3ppm	10ppm	100ppm
Phosphate	0ppb	5ppb	45ppb	1000ppb
Ammonia	0ppm	1ppm		
Nitrate	0ppm	1ppm	3ppm	
Silica	0ppm			

The pipes were stored horizontally in the same radial orientation each time and at room temperature. Samples were collected at each water change to measure the amount of copper leaching from the system using inductively coupled plasma mass spectroscopy (ICP-MS). The pH of the effluent was also measured and recorded. Occasionally, samples were retained for testing sulfate consumption on the ion chromatograph (IC), for testing sulfide production using a HACH protocol, for counting the heterotrophic bacteria population, and for SRB-BART tests to test for SRB presence. All samples were tested as composite samples of the triplicate pipes.

B. Sequencing and Progression of Experiments to Stimulate SRB Growth

As the experiment progressed over a period of 16 months, several changes and additions were made in the experimental conditions in an attempt to stimulate the colonization of the pipes by the SRB.

Table 3-3. Progression of experiments.

Changes to the copper nutrient study pipes are in chronological order (1-10).

Parameter Varied	Reason it was of interest
1) Increased nutrients	Higher levels of lactate may encourage biofilm development, resulting in rapidly consumed dissolved oxygen and stimulating SRB growth.

2) Frequency of water changes	Various lengths of time between water changes model premise plumbing conditions and may optimize conditions for SRB growth
3) Australian Inoculum	Water from Australian pipes producing blue water may do so because of the presence of a particular strain of SRB
4) Sand Inclusion	High levels of silica in water may promote SRB growth
5) Valve Pipes	Eliminating the dump-and-fill method may reduce oxygen exposure to the biofilm
6) Chlorinated Pipe	Low levels of chlorine may encourage pit formation
7) Copper Pipe with Stagnant Zone	A volume of water continuously without dissolved oxygen may be required for SRB growth
8) Ultra High Nutrients	Nutrients specifically intended to produce large numbers of SRB may result in aggressive corrosion
9) SRB-BART Inclusion	Nutrients in an SRB-BART may encourage SRB growth in the small section of pipe and serve as an indicator of growth
10) Imposed Galvanic Current Experiment	Forcing one section of the pipe to become anodic may remove oxygen and increase nutrient concentrations to stimulate SRB growth
11) Sulfide Decay Study	Sulfide production may not be noticeable in copper due to sulfide decay but may be noticeable in CPVC
12) CPVC Pipes	Sulfide production by SRB as an indicator of growth may be traceable in CPVC
13) Pit Wire Experiment	Localized colonization of SRB in a small area may cause corrosion of connected pipes

Frequency of Water Changes

One factor examined was the frequency of the water changes. At one extreme, if the water is never changed, the bacteria will be limited by a lack of necessary components and growth will slow or stop. At another, if water changes are too frequent,

the dissolved oxygen concentration would not drop to 0 mg/L as is necessary for SRB to grow. In an attempt to find an optimal frequency, water change intervals of three per week, two per week, and one per week were tested.

Copper pipe with Stagnant Zone

After 10 months, 3" sections were cut from the bottom of each pipe and were reconnected according to the example pictured below in order to form a stagnant zone (Figure 3-1). The general idea was to create a small volume in the pipe in which oxygen was persistently absent where SRB could flourish. A small silicon stopper with a hole 1-2 mm in diameter was placed in between the two pipe sections, allow for some transport of nutrients and ions into the small volume of stagnant water. The sections of the pipe were then electrically connected to allow for flow of natural corrosion currents between the two sections of copper tube. The current was monitored after water changes. In this case the pipe was stored vertically to prevent mixing that might result from salt buildup in the lower pipe section.

Figure 3-1. Photo of a pipe with the stagnant zone connection at the bottom.

Ultra High Nutrients

For experiment, one copper pipe and one CPVC pipe previously receiving control water were filled with Postgate Medium G at each water change.[5] This medium is specifically designed to encourage rapid SRB growth. The nutrient concentrations are significantly higher in this medium than those seen in potable water conditions and those in this experiment (Table 3-4).

Table 3-4. Nutrient concentrations in Postgate Medium G used in this experiment. The pH of the solution was adjusted to 9.2±0.5.

Base Medium	
	1 L distilled water 3.0 g $NaSO_4$ 1.2 g NaCl 0.3 g KCl 0.3 g NH_4Cl 0.4 g $MgCl_2 \bullet 6H_2O$ 0.2 g KH_2PO_4 0.15 g $CaCl_2 \bullet 2H_2O$ 0.3125 g calcium lactate
Trace elements (Added 1mL of this solution to 1L base medium)	
	993 mL distilled water 6.5 mL HCl 1.5 g $FeCl_2 \bullet 4H_2O$ 0.06 g H_3BO_3 0.1 g $MnCl_2 \bullet 4H_2O$ 0.12 g $CoCl_2 \bullet 6H_2O$ 0.07 g $ZnCl_2$ 0.025 g $Na_2MoO_4 \bullet 2H_2O$ 0.015 g $CuCl_2 \bullet 2H_2O$ 0.025 g $NiCl_2 \bullet 6H_2O$
Vitamin solution (Added 1mL of this solution to 1L base medium)	
	1000 mL of distilled water 0.01 g Biotin 0.05 g *p*-Aminobenzoic acid 0.05 g Vitamin B_{12} 0.1 g Thiamine

Imposed Galvanic Current Experiment

A voltage of 0.5 V was applied overnight to three pipes with the stagnant zone connection so that the anode was the smallest section of pipe and the cathode was the larger segment. The dissolved oxygen, pH, and lactate concentrations were later measured on both ends of the pipe.

Additional nutrients and conditions tested

Increased Nutrients. After two months under experimental conditions, the lactate concentration in all of the pipes receiving lactate was increased by a factor of 10 to encourage faster biofilm growth. Pipes were also re-inoculated with SRB after five months of exposure to experimental conditions.

Australian Inoculum and Under-deposit Corrosion. Three pipes were inoculated with the effluent from three, ¼" diameter, one-foot sections of pipe from Australia, which released blue water in the field and had previously tested positive for SRB. This effluent water was supplemented with control water in order to completely fill the pipes.

Sand Inclusion. Approximately two milliliters of sand was added to the bottom of two of the original control pipes and one of the Australian water inoculated pipes. This setup with sand was intended to model conditions of relatively high silica levels that are often found in pipes delivering blue water.

Valve Pipes. Two additional pipes from the same batch were fitted with CPVC valves and filled with control water at each water change (Figure 3-2). During a water change, water was replaced as it was removed in an attempt to minimize the contact between air, and therefore oxygen, and the biofilm on the pipe walls.

Figure 3-2. Valve pipe setup.

Chlorinated Pipe. Another control pipe was dosed at each water change with 0.15 mg/L free chlorine in an effort to encourage pit formation.

Ultra High Nutrients. Eleven months after the experiment began, one pipe that was previously filled with control water was filled with Postgate medium G at each water change.[5] Postgate Medium G contains nutrients specifically designed to promote sulfate-reducing bacteria growth.

SRB BART inclusion. Four new, unused SRB-BARTs were filled with distilled water and agitated until the media on the bottom of the BART was in solution. Four individual pipes were selected from the copper pipe nutrient study to receive this solution (one from each of the following: No Si, 2700 La, 100 SO_4, and 1000 PO_4). The water in

the small section of the pipe was removed, and one of the SRB BART solutions was poured into each one. Then the pipe was refilled with the water that was removed from the small section.

CPVC pipe nutrient study

A second set of three control pipes was created in CPVC pipes in two foot lengths with a diameter of ½". One of these pipes was switched to Postgate Medium G following 5.5 months as a control pipe. In addition to these control pipes, a separate group of CPVC pipes were set up with the same experimental matrix as in the copper pipe nutrient study outlined above. The pipes were inoculated for three weeks with water known to contain a high concentration of SRB. These pipes were started with experimental conditions eight months after the copper pipe nutrient study began. CPVC pipes with a diameter of ½" and a length of one foot, cut from 10' lengths, were utilized for this experiment. The initial mass for each pipe was recorded. Each condition was tested in triplicate and the water was changed at the same time and using the same water that the copper pipes received.

Sulfide decay study

In order to compare the amount of sulfide decay in CPVC, soft and hard copper, lengths of each pipe were obtained from a local hardware store. Due to the different diameters of the pipes, calculations were performed so that each pipe had the same surface area. Each pipe was filled with control water, spiked with a known concentration of Na_2S and stopped at both ends with silicon stoppers. The pipes stood vertically, and measurements were taken from the top of the pipes. As soon as samples were pipeted from the pipes, the excess length was cut off to minimize the interaction of the sulfides with air. The sulfide concentration in each sample was measured on a HACH spectrophotometer. Measurements were taken at intervals to create a sulfide decay curve.

Pit wire experiment

Four 6" copper wires with a known diameter were cut from a spool of copper wire and enclosed in an inert plastic sleeve and glued so that water could not contact any part of the wire other than the tip, which was exposed. The tip was sanded to remove any glue and to ensure that there was a flat surface. These wires were inoculated in a beaker

containing water from positive SRB BARTs for a period of five weeks. Following their incubation two of the wires were equipped with a small sleeve extending approximately 3mm past the exposed tip to limit the amount of oxygen exposure to the tip during water changes. These wires were then inserted into silicon stoppers with the tip extending between 1"-1.5" from the end of the stopper and glued to stop water from leaking out. The stoppers were then inserted into the bottoms of a separate set of four 2' pipes from the same batch as those described above. The pipes were filled with control water with the exception of having 2700ppb of lactate instead of 900ppb with a pH of 9.2. A ring stand was used to store the pipes vertically. Each pipe and the exposed end of its copper wire were connected electrically. It was expected that a corrosion current would be induced.

3.2 RESULTS

Copper leaching in copper pipe studies

Copper leaching was monitored for all of the copper pipes used in these studies. The copper data is labeled with the component concentration that varied from the control water. In the copper pipe nutrient study, the 0 NH3 and 0 NO3 pipes were replicates. The large difference between the copper concentrations of 0 NH3 and 0 NO3 demonstrates the amount of variability present, even when using composite sampling (Figure 3-3).

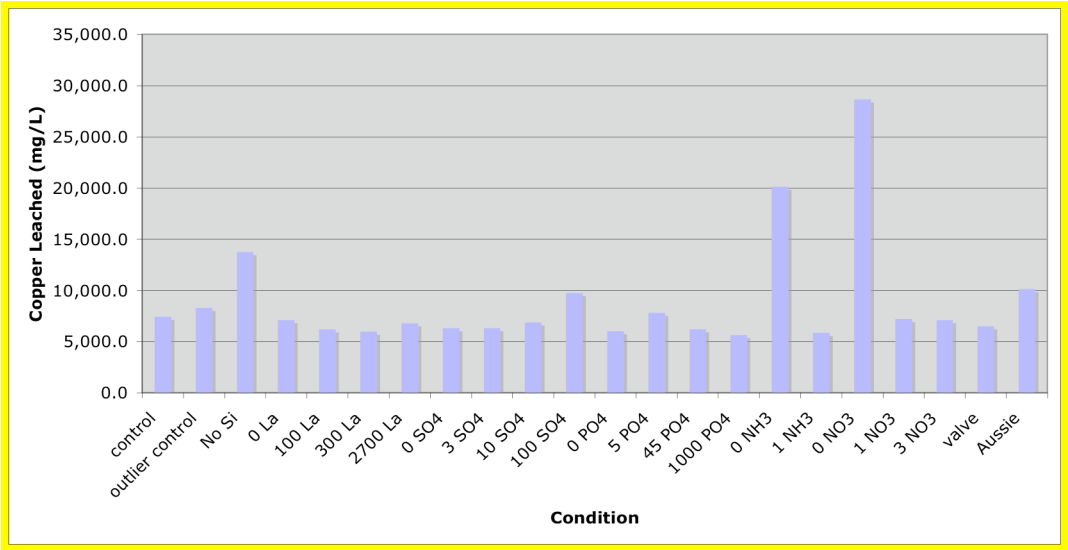

Figure 3-3. Total concentration of copper leached following the creation of the stagnant zone.

The magnitude of the 0 NH3 and 0 NO3 in comparison with the other conditions, especially given that they are replicates, indicates that nitrogen may have an inhibitory effect on SRB corrosion (Table 3-5).

Table 3-5. Copper leached matrix.

The copper concentrations of the conditions that do not contain nitrogen are significantly higher than the conditions containing nitrogen.

	0 ppm Nitrate	1 ppm Nitrate
0 ppm Ammonia	20,100 mg/L 28,600 mg/L	7,200 mg/L
1 ppm Ammonia	5,860 mg/L	7,440 mg/L

Increased nutrients

SRB-BART tests taken two months in to the copper nutrient study were negative for the presence of SRB. This led to the decision to increase the lactate concentration with the hypothesis that a higher amount of available carbon would increase the biofilm in the pipes and would, in turn, drive the oxygen concentration down more quickly, creating a more favorable environment for the SRB. Heterotrophic plate counts (HPCs) taken three months after this change showed high concentrations of heterotrophic bacteria for all conditions, typically on the order of 10^5 cfu/mL.

Frequency of water changes

After one week of stagnation, the dissolved oxygen in the pipes approached 0 mg/L.

Australian Inoculation

In the pipes from Australia, the production of blue water did not continue using this experimental water, nor was the blue water phenomenon induced in the pipes receiving the water coming out of the Australian pipes. The heterotrophic bacteria count for the composite sample of these pipes was fairly high, 2,260,000 cfu/mL, but this was not unusual when compared to the HPCs of the other conditions. An SRB-BART taken for the pipes receiving the Australian water indicated an SRB concentration of approximately 31 cfu/mL after eight months of experimental conditions.

Sand inclusion

The inclusion of a high amount of silica in the form of sand did not result in additional corrosion or SRB growth.

SRB presence test and enumeration

SRB-BART samples were taken for each condition after eight months of experimental conditions and monitored for a positive result. The resulting SRB concentrations are summarized in Table 3-6 below. SRB-BARTs consistently turned black along the ball at the top of the tube for each condition, indicating that the SRB in the pipes were facultative anaerobes rather than obligate anaerobes.

Table 3-6. SRB concentration determined using SRB-BARTs.

Condition	SRB (cfu)	Condition	SRB (cfu)
Control	18,000	0 PO_4	79,000
Sand	18,000	5 PO_4	200
No Si	5,000	45 PO_4	1,200
0 La	5,000	1000 PO_4	18,000
100 La	18,000	0 NH_3	18,000
300 La	550	1 NH_3	1,200
2700 La	18,000	0 NO_3	5,000
0 SO_4	18,000	1 NO_3	18,000
3 SO_4	18,000	3 NO_3	68
10 SO_4	6	AI	550
100 SO_4	550		

These results answer the first and second of the four questions posed in this research. It demonstrates the fact that, once SRB are inoculated into pipes, they can survive and that they survive as facultative anaerobes, allowing them to survive in an environment that is routinely exposed to oxygen.

Chlorinated pipe

No visual or olfactory evidence of SRB growth was recorded for the chlorinated pipe, and it only leached an average amount of copper. The hypothesis that the presence of a small concentration of chlorine would encourage tubercle formation was not correct.

Valve pipes

An SRB-BART test collected for the composite effluent of these pipes turned positive after sitting for several months. While this result does not have any statistically significant meaning, it may indicate a very small SRB presence.

Ultra High Nutrients

The Ultra High Nutrients test using Postgate Medium G greatly increased the amount of copper leaching from the pipe. The concentration of copper leached from the Postgate pipe is almost an order of magnitude higher than the pipes without nitrogen and almost two orders of magnitude higher than the concentration of copper leached from the control condition.

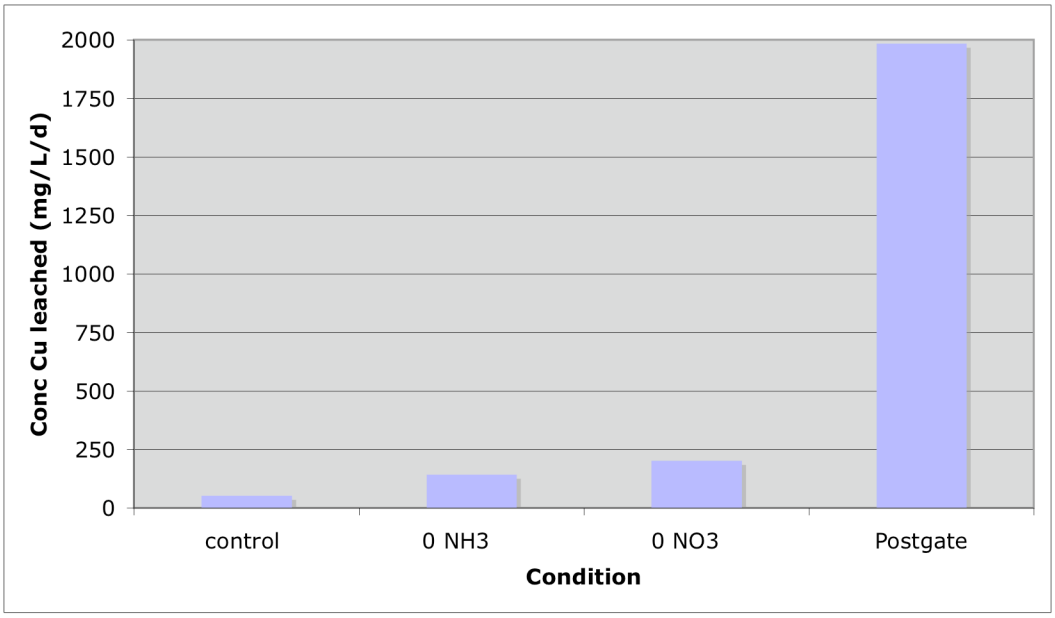

Figure 3-4. Concentration of copper leached (mg/L/d) following the creation of the stagnant zone normalized per day for select conditions.

These results were normalized per day in order to allow direct comparison of the pipe containing Postgate Medium G, which was started 14 days later.

In addition to the high levels of copper leaching, the ultra high nutrient pipes also produced measurable amounts of sulfides. After 14 days of stagnation the copper pipe effluent contained 12 ppb of S^{2-}. The CPVC pipe receiving ultra high nutrients produced 12 ppb after 10 days of stagnation and 240 ppb of sulfides after 14 days. The samples used to obtain these results were not filtered but did use the sample prior to the addition of the color indicator as the blank, rather than DI water. While the cloudiness of the Postgate Medium solution may have interfered with the numbers here, comparative tests with a later sample have shown that using the sample as the blank prior to adding the color indicator provides results very near those of samples that were filtered and used DI

water blanks. In addition to the HACH test providing evidence of sulfides, the CPVC pipe after 14 days had a distinct sulfide smell. The production of sulfides serves as an indication that SRB are growing successfully under these high nutrient conditions.

The concentration of sulfides produced by the copper pipe was most likely higher than could be measured using the HACH method (see the sulfide decay test results below). According to Gudas and Hack, this concentration of sulfides is sufficient to induce localized corrosion on copper.[3]

SRB-BART inclusion

Due to a death in the family, the pipes that received the SRB-BART inclusion were not checked for two weeks. However, when checked, the solution was dark black in all of the pipes tested, indicating significant SRB growth. This test proved that SRB resist the biocidal properties of copper and that they can grow in copper pipe systems.

Imposed galvanic current experiment

The data collected from the galvanic nutrient transportation study did not support our hypothesis that the dissolved oxygen and pH would drop in the anode with respect to the cathode, while the sulfate concentration would rise. However, the lactate concentration did rise in the anode with respect to the cathode as was expected (Table 3-7). Concentration of nutrients necessary for SRB growth at an anodic area by an imposed galvanic current provide a rationale for why researchers in the field looking at SRB-induced corrosion see results similar to those in the ultra high nutrient study in this work.

Table 3-7. Data for the imposed galvanic current experiment.

	Initial water	Anode following current application	Cathode following current application
pH	9.2	9.19	8.84
Sulfate (mg/L)	3.17	*sample lost in IC malfunction*	3.18
TOC (mg/L)	3.19	7.89	1.48
DO (mg/L)	8.5	4.5	4.4

The sulfate and lactate ions were expected to travel to the anode because of their negative charge, providing increased amounts of food to promote bacteria growth. Our hypothesis was based on basic electrochemistry. When a current is applied to a solution, anions in the solution travel to the anode, which accepts electrons from the negative ions.

Cations in the solution travel to the cathode, which donates electrons. The voltage in this study was kept low with the intention of preventing hydrolysis. Oxygen should be removed at the cathode and also at the anode as a reaction secondary to the creation of copper ion.

Sulfide decay study

Figures 3-5, 3-6, and 3-7 show the results of the sulfide decay study for each pipe material tested.

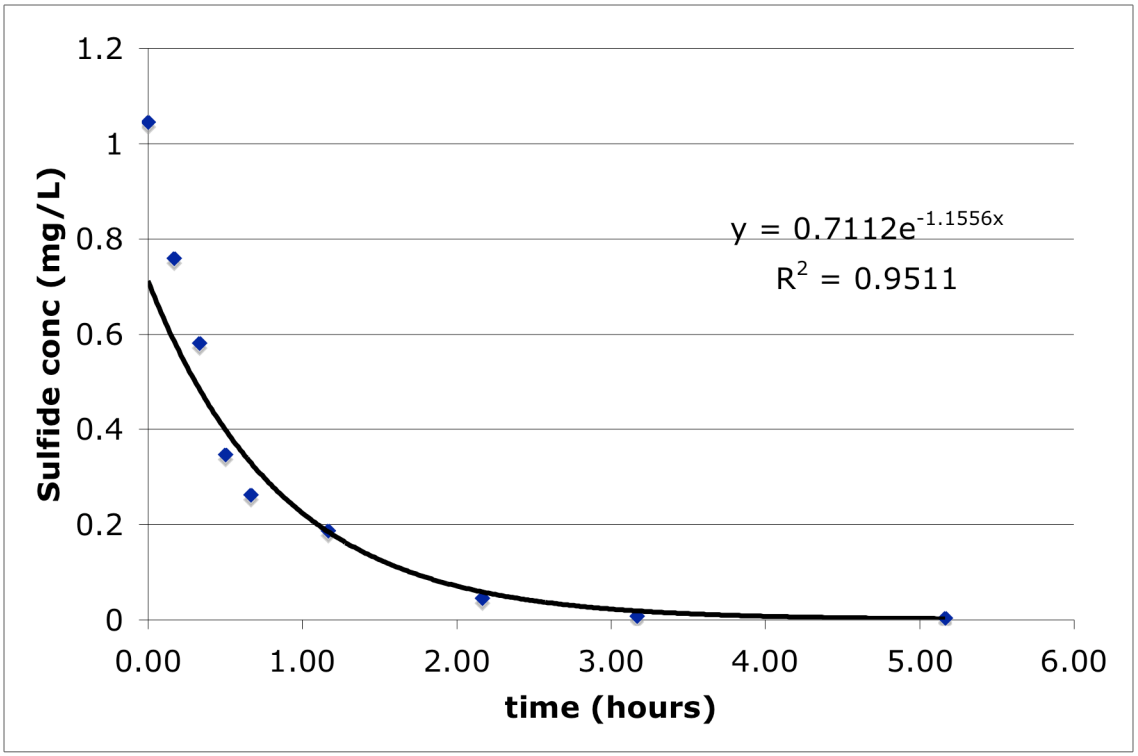

Figure 3-5. Sulfide decay curve for the soft copper pipe, normalized by surface area to volume ratio.

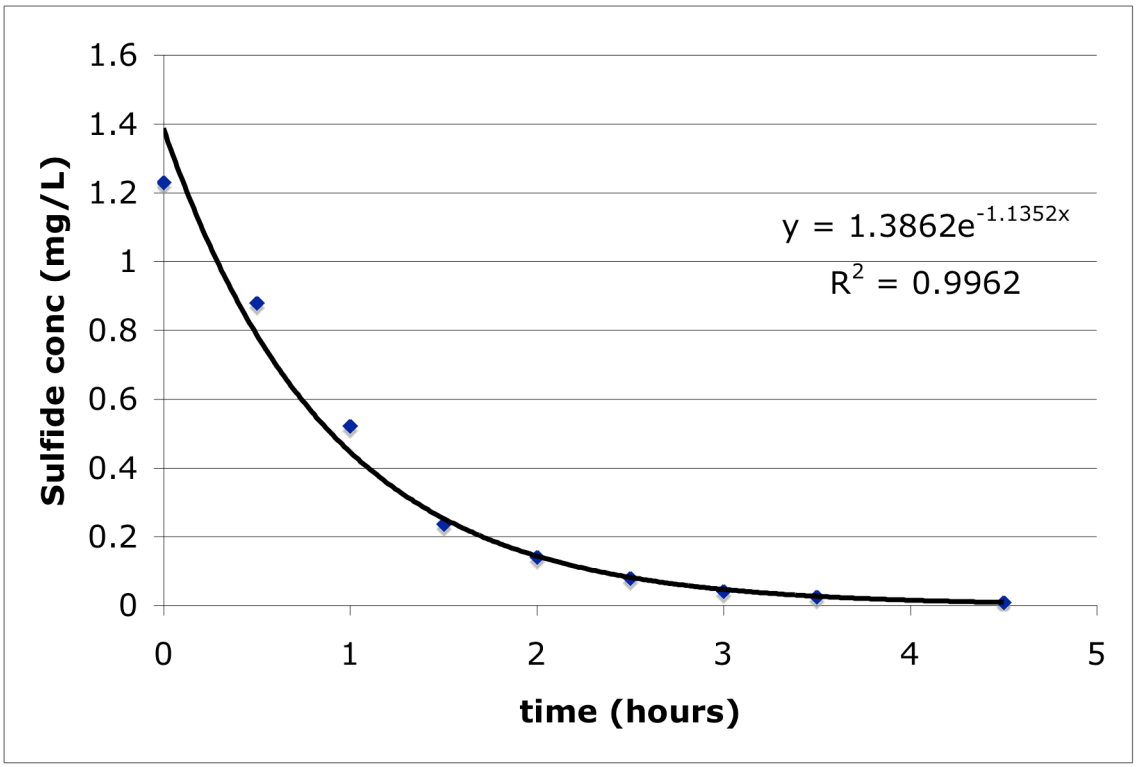

Figure 3-6. Sulfide decay curve for the hard copper pipe, normalized by surface area to volume ratio.

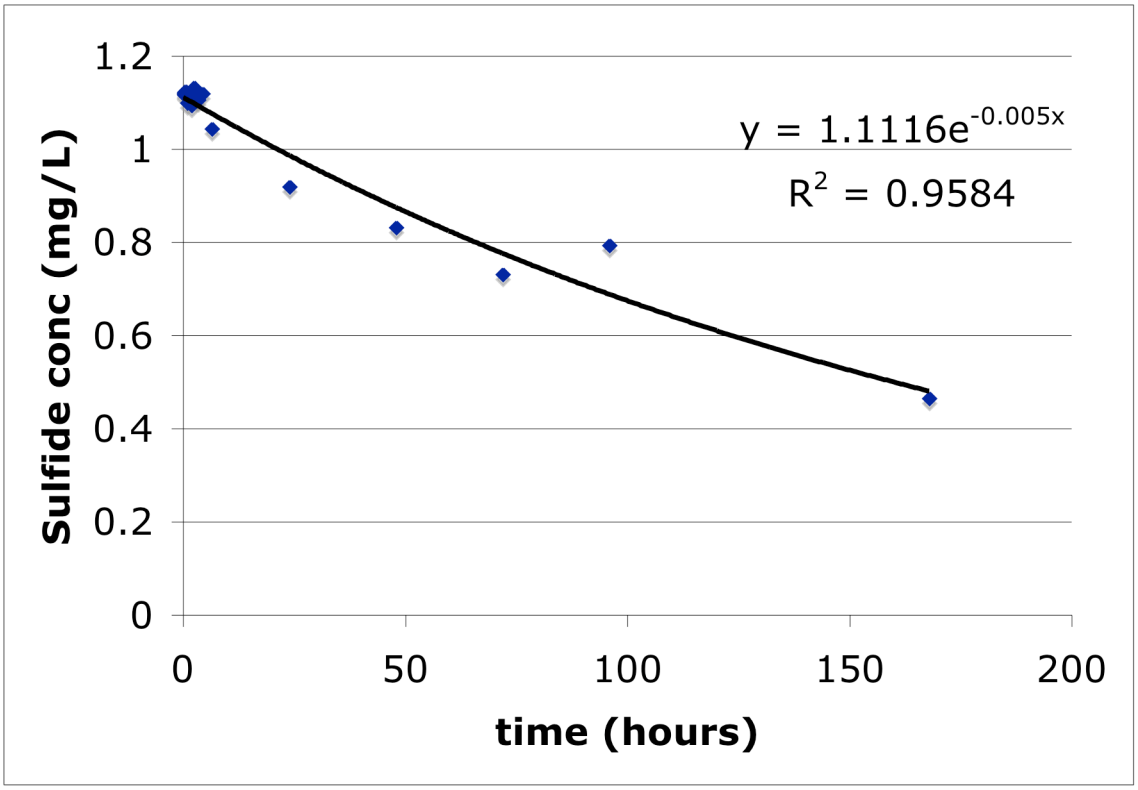

Figure 3-7. Sulfide decay curve for CPVC pipe, normalized by surface area to volume ratio.

Figures 3-5 and 3-6, above, clearly demonstrate the speed at which even high concentrations of sulfides decay within copper pipes. The CPVC pipe retained a high concentration of sulfides for an extended period of time. This explains why it is so difficult to detect SRB in copper pipes using sulfide measurements or smell.

CPVC pipes

An SRB-BART test on a composite sample of the pipe receiving control water turned positive 28 days after it was taken. While this result is not statistically significant, it does suggest a very small SRB presence. Following two months of experimental conditions, an SRB-BART taken for the pipes receiving 45 ppb of phosphate ($45PO_4$) and 27 mg/L of lactate (2700La) indicated an SRB population of approximately 31 cfu/mL.

Pit wire experiment

The pit wire experiment ran for eight months. No significant corrosion currents and no weight loss were measured during the course of this study. High concentrations of heterotrophic bacteria (approx. 10^5 cfu/mL) were measured five months and eight

months into the experiment, following the increase in lactate concentration by plating dilutions on R2A. SRB-BART tests were performed on all four of the pipes. Only one tested positive with a concentration of 5,200 cfu/mL.

3.3 CONCLUSIONS

SRB pose a serious but poorly understood threat to premise plumbing. While the nutrient studies attempted in this research have not yet yielded clear indications of the circumstances required for SRB growth in pipes, a great deal of insight has been gained. The four areas of concern that were identified above were explored. This work found that SRB, when inoculated into copper pipes, survived in the system and resisted the biocidal potential of copper. Sulfides were produced by the ultra high nutrient pipes, and the SRB-BARTs showed that the bacteria growing in these pipes were facultative anaerobes. This work also provides a possible explanation for the similarity between the behavior of SRB in the field and the behavior in the ultra high nutrient study in the form of the galvanic current experiment. The formation of an anode following a constriction or bend in a premise plumbing system may encourage SRB growth. These findings are a large step forward in advancing our understanding of how these bacteria survive in potable water plumbing. However, questions remain. The fact that tubercles were not formed in these experiments shows that there is a crucial piece of the puzzle yet missing. The mechanism that causes these tubercles to form and pits to be initiated will be an important advance for the next researcher.

The ultra high nutrients present in Postgate Medium G resulted in significantly increased copper corrosion. While this condition would never be present in a real water system, future research should explore why this medium resulted in such high copper leaching. The high nutrient levels in Postgate Medium G may have resulted in a large number of SRB that caused significant copper corrosion. If this was the case, exploring the same ratio of nutrients at lower concentrations may be useful. However, the high concentration of salts in this medium may also have had a great deal of influence on the amount of copper leaching (Table 3-4). Sulfate and nitrate salts, in particular, have been shown to cause pitting of copper materials when in high concentrations.[6]

In addition, the fact that nitrogen seems to be inhibitory to SRB growth should be explored. The increased corrosion in the 0 NH3 and 0 NO3 pipes was more noticeable

following the creation of the stagnant zone. Perhaps there is a bacterial competition in the small section of pipe that is not favorable for SRB.

It may be beneficial for future researchers to limit the number of conditions tested at one time and to focus on producing more replicates that would allow for greater statistical power. Finally, while a longer length of time between water changes is less representative of daily water usage in buildings, there are occasions when water remains in pipes for a long period of time without being flushed (vacations, vacancy). These long stretches may allow a protective biofilm to form, shielding the SRB from disinfection and shearing. By extending the amount of time between water changes, researchers would mimic this condition and would also allow the oxygen level in the pipes to remain low longer and may encourage SRB growth.

3.4 REFERENCES

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