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Silver disinfection studies

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Abstract

In recent years, the antimicrobial properties of silver have gained recognition and use in a wide array of applications. A review of the current drivers for new disinfection technologies and of the past uses of silver as an antimicrobial agent is provided. General metal-microbe interactions are discussed including metal mechanisms in biological systems and the factors that affect metal toxicity. Our understanding of metal toxicity is limited and conflicting reports regarding toxicity can be found in the literature. A review of several previously conducted silver toxicity tests is provided to highlight some of these discrepancies. Finally, laboratory testing was conducted to identify silver's efficacy for inactivation of microbial contaminants of concern in drinking water. Specifically, testing was conducted to confirm reported bactericidal properties of silver on *E. coli* and to investigate the ability of silver to reduce the infectivity of *Cryptosporidium*, a chlorine-resistant pathogen.

Silver-loaded ion-exchange fibers (24, 40, 20 mg at 122mg Ag/g fiber) were shown to produce 100% disinfection of *E. coli* within 5 minutes. The fibers were also shown to release silver into solution and all amounts tested resulted in the finished product water containing aqueous silver concentrations above the current drinking water limit of 0.1mg/L.

The toxicity of the silver ion (Ag^+) species to *E. coli* and *Cryptosporidium* was tested over a range of concentrations (0.5M, 0.05M, 0.005M, 0.93μM, and 9.3μM) in laboratory grade water. For all concentrations tested 100% disinfection of *E. coli* was observed

within 15 minutes. Viability and infectivity assays were performed on *Cryptosporidium* for all concentrations. It is hypothesized that at high concentrations (0.5M and 0.005M) silver ions are having an effect on the cell well based on the observation that treated oocysts have a diminished ability to react with a *Cryptosporidium*-specific surface stain. Conflicting data was obtained for both the viability and infectivity assays at lower concentrations, and thus definitive conclusions cannot be drawn at this time. While additional testing needs to be completed, the results of this testing show that silver ions represent a potential disinfectant for microbial contaminants of concern in drinking water.

Introduction

It is well known that human health and water quality are intrinsically linked. For developed countries such as in the U.S., disinfection of drinking water is one of the major advances in public health in the 20th century. In the U.S., the Safe Drinking Water Act (SDWA) was established in 1974 with the primary purpose of maintaining safe drinking water quality to protect public health. Chlorine is the principal drinking water disinfectant due to its low cost, efficacy of bacterial disinfection and simple application. Since its introduction as a water disinfectant in 1908, there have been countless lives spared from waterborne diseases including cholera and typhoid. It has been reported that the use of chlorine in drinking water has saved more lives than penicillin and antibiotics combined (Shuval et al 1995). In addition to its bactericidal properties, cchlorination offers the principal advantage over other forms of disinfection through the ability to incorporate a chlorine residual in the water that can continue to provide disinfection capabilities after the water leaves the treatment plant and while in the distribution lines. However, since the discovery of ddisinfection by-products (DBPs) in 1970s, concerns regarding the toxicity and health effects of treating water by chlorination have increased (Rook 1974).

DBPs are formed when chlorine reacts with naturally-occurring organic matter in the water to form unintended byproducts. DBPs include several compounds categorized as trihalomethanes and classified possible human carcinogens (Christman 1998). In November 1998, the U.S. Environmental Protection Agency (USEPA) issued the final Stage 1 Disinfectants and Disinfection Byproducts Rule that reduced the maximum

contaminant level (MCL) for total trihalomethanes from 100 to 80μg/L for public drinking water systems (USEPA 2006a).

Another disadvantage to chlorination is that it is not effective against certain protozoan cysts, most notably those of *Giardia lamblia* and *Cryptosporidium.* The past decade has ushered in a heightened awareness of the potential public health risks posed by these protozoan parasites that are resistant to chlorination. Once thought to be an opportunistic pathogen of immuno-compromised individuals, *Cryptosporidium* has proven to be one of the most commonly identified intestinal pathogens throughout the world (WHO 2002). The Center for Disease Control (CDC) reports that during the past two decades, *Cryptosporidium* has become recognized as one of the most common causes of waterborne disease, from recreational water and drinking water, in the humans in the U.S. The parasite is found in every region of the United States and throughout the world (CDC 2007).

Cryptosporidium is a protozoan pathogen that exists in the environment as an oocyst. Figure 1 depicts the various life-stages of *Cryptosporidium.* An oocyst is a thick-shelled spore phase that protects the infectious sporozoites from potentially detrimental environmental conditions. Infection causes the disease Cryptosporidiosis, which is a diarrheal disease for which there is no medical cure. While there have been recent developments for healthy individuals, there is currently no alleviation for the highest risk population, specifically immuno-compromised individuals. In addition to being resistant to chlorine, the oocysts small size, 2-4μm in diameter, allows it to pass through most

drinking water filtration systems. Therefore, the majority of drinking water treatment plants are ineffective at removal.

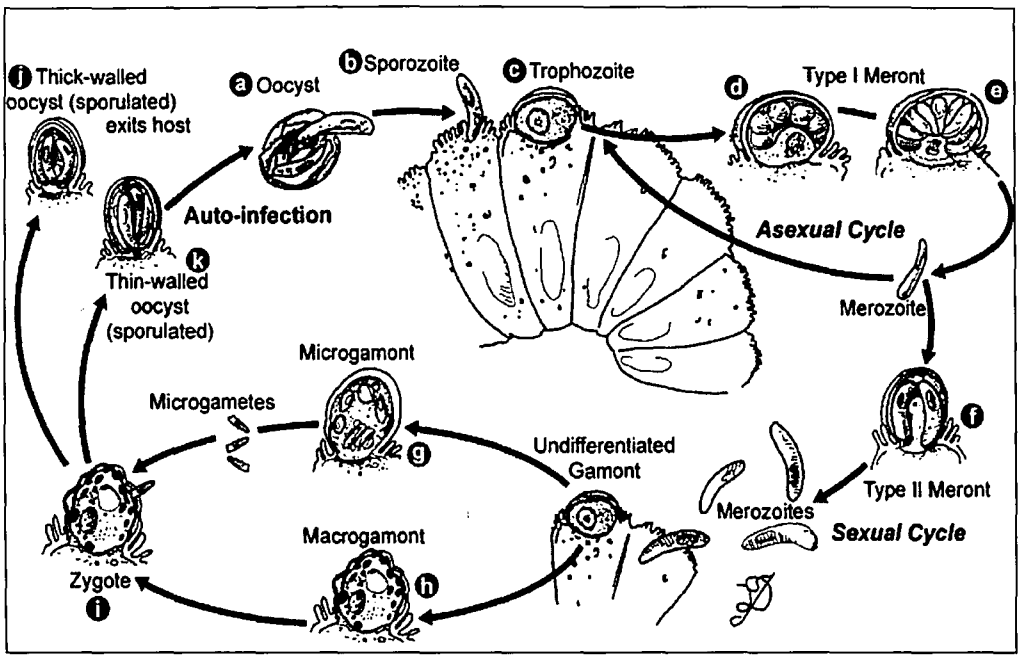


Figure 1. Life cycle of *Cryptosporidium parvum* and *C. hominis*. (Strickland 2000)

In response to the rising risk of exposure to this pathogen, the USEPA passed two additional rules in January 2006: the Long Term 2 Enhanced Surface Water Treatment Rule (LT2ESWTR) and the Stage 2 Disinfection Byproduct Rule. The rules strengthen protection against microbial contaminants, especially *Cryptosporidium*, and at the same time, reduce potential health risks of DBPs (EPA 2006a, EPA2006b). Implementation of the LT2ESWTR was initiated in October 2006 requiring all public drinking water systems to monitor current *Cryptosporidium* levels. The rule required all public drinking water systems to monitor baseline levels of *Cryptosporidium* in their influent water. After a baseline is established, all systems will have three years to comply with any additional treatment requirements. Finally, a second round of monitoring is required in six years to determine if source water conditions have changed significantly. The USEPA estimates that implementation and compliance with the LT2ESWTR will range

from $92 to $133 million with the majority of the cost (99%) falling on public water systems (USEPA 2006b). The high costs are associated with implementation of either ozone or UV-radiation systems into existing systems, which are the two most commercially-viable options for treating *Cryptosporidium* in large systems.

For developing countries, the challenge of providing clean, safe water is even more daunting. The World Health Organization reports that over 1.1 billion people do not have access to drinking water from improved sources (WHO 2006) and the vast majority of these people live in Africa and Asia. With the number likely to climb dramatically in the near future, there is an urgent need for appropriate water treatment technologies for developing countries (UNICEF 2004). The Millennium Development Goals (MDGs), adopted in 2000 by 189 countries, are eight goals aimed at reducing poverty and have been designed with measurable targets within 15 years (target date of 2015). One of the MDGs is to halve, by 2015, the proportion of people without sustainable access to safe drinking-water and sanitation (WHO 2005).

Implementation of advanced technology in developing nations has been criticized as ineffective and wasteful (Schumacher 1975). It has been found that when certain water disinfectant technologies are implanted in developing countries, they tend to sit idle due to insufficient resources, lack of operating expertise, and/or prohibitive maintenance costs. Alternately, indigenous technologies are often too rudimentary and rarely achieve satisfactory disinfection; rather it is recommended that intermediate technologies that are more advanced than indigenous methods but more practical than sophisticated alternatives should be investigated and implemented (Schumacher 1975). In fact, the

United Nations Environment Program has acknowledged the need for the development of intermediate technologies and has encouraged decision makers in local and national governments and other organizations to implement environmentally sound intermediate technologies (UNEP 2004).

Successful achievement of meeting the goals and targets for both developed and developing nations will depend on multidisciplinary approaches that leverage the knowledge and experience from the past while devising new methods for the future. New innovative water purification and disinfection methods are necessary. If silver proves successful for disinfecting bacteria and chlorine-resistant microorganisms from drinking water supplies, the technology could prove useful for drinking water disinfection at the personal, household, and municipal levels.

Background Information

Silver's antibacterial properties have been known for centuries and has been used in a multitude of applications. The first recorded description of water disinfection by silver was by Raulin in 1869, observing that *Aspergillus niger* could not grow in silver vessels (Laubusch 1971). In 1884, the German obstetrician C.S.F. Crede observed that there was a 79% relationship between blind children and maternal venereal disease. He subsequently introduced a prophylactic 1% silver nitrate eye solution for newborns for the prevention of ophthalmia neonatorum. Following its introduction, the incidence of eye disease in newborns dropped to about 0.2%. His treatment was a milestone in clinical

prophylaxis and became a government regulation throughout most of the world (Rentz 2007). By 1897, silver nitrate began to be used in the U.S. to prevent blindness in newborns and is still used today.

One of the earliest uses of silver for water purification was the development of Katadyn silver, a porous metallic, spongy mesh that attempts to maximize surface area, in 1928. The silver meshwork also contained a small quantity of gold or palladium. Katadyn silver has been used inside flasks and storage containers and with water filters (Katadyn 2007).

In the 1970s based on proven success of 0.5% silver nitrate solutions for reducing burn infections, Charles L. Fox developed silver sulfadiazine, a water insoluble salt which dissolves only slowly in biological fluids. The intention was that low concentrations of silver ions (Ag^+) would be attained, chloride would not be readily precipitated and the antibacterial activity would be maintained (Fox 1974). Controlled trials confirmed its usefulness and non-toxic effects to skin cells; it became the primary treatment for burns and is still used to the present day.

With the exception of its use to prevent infections in burn patients and to prevent blindness in newborns, the development of antibiotics and the widespread implementation of chlorination for water disinfection overshadowed silver's antimicrobial properties and it fell out of mainstream use as a disinfectant. It was until the 1960s, when NASA used an electrolysis system to introduce silver ions into the water supply onboard the Apollo shuttles that industry's interest in silver and its antimicrobial

properties for new and emerging water purification technologies reemerged. NASA still leverages silver for the CO_2 chemsorption process on board the Challenger space shuttles and the international space station (NASA 2004).

Today silver's antibacterial properties are exploited in a wide range of applications. It is used in pools to reduce to the amount of chlorine needed. Samsung has recently released a line of washing machines, air conditioners, air purifiers and refrigerators that use the *Silver Nano Health System*TM technology (Samsung 2004). The Samsung's *Silver Nano Health System*TM technology uses a silver anode assembly to release silver ions into a bypass stream that is blended into the water supply to achieve better bacterial control than conventional systems. Silver, often in conjunction with copper, ions are introduced in warm water systems of hospitals and nursing homes to prevent the growth of *Legionella* bacteria and to prevent fouling in water lines for boilers in many industrial applications.

There are several manufacturers (e.g., Pur, Brita, and Katadyn) of small, or point-of-use, commercially-available water purification units that incorporate some form of silver into their products. In addition, pottery-based filtering systems designed for use in rural, developing countries also employ the use silver (Lantange 2001a). While silver is a component in the aforementioned products, the manufacturers cannot claim that the silver is actually disinfecting the water to a safe a level for drinking. Since the silver is almost always applied to the filter media, it is reported that the silver prevents bacterial growth on the filter media thereby improving performance and prolonging the useful life.

Project Objective

The objective of the proposed work was to test the efficacy of silver to disinfect water contaminated with *E. coli* and *Cryptosporidium* to identify whether silver could be a potential alternative to chlorination. Initial testing used silver-loaded ion-exchange fibers to introduce silver into the system and resulting effects to *E. coli* were measured. The second phase of testing was designed to assess the effects of different silver speciations and concentrations on *E. coli* and *Cryptosporidium* in order to develop a better understanding of the mechanism of disinfection. The *E. coli* testing was performed to confirm and reproduce reported toxic concentrations and contact times. The effects of silver on *Cryptosporidium* oocysts, protozoan pathogens which are resistant to conventional chlorine disinfection, have not been reported, and testing was performed to investigate resulting impacts on viability and infectivity.

Identifying the species of silver that are important for disinfection will help elucidate the mechanism of silver disinfection (i.e., microbial surface reaction vs. microbial uptake) and contribute to the development of an effective treatment alternative for water disinfection. Results from this project will attempt to answer the following questions for the various species of silver: (1) Which silver speciation is effective for disinfection of *E.coli* and/or *Cryptosporidium*? (2) What is the optimum silver concentration for disinfection? (3) Is the contact time a factor in disinfection efficacy? (4) How does water quality (e.g., pH, turbidity, temperature, quantity/quality of microbial contamination, etc.) impact the effectiveness of disinfection? (5) Do microorganisms become resistant to the silver over time? (6) Do any of the silver treatments offer a

residual effect for disinfection? Answers to these questions are necessary to assess the potential success of silver as an alternative water disinfection treatment option.

The information and potential disinfection alternative which could result from this work would impact water utilities and water consumers, at the municipal, household, and personal water treatment levels and in both developed and developing countries. For developed regions, the need to minimize chlorine disinfection by-products in drinking water supplies, coupled with the increasing threat of chlorine-resistant waterborne pathogens, has led to a renewed interest in alternative water disinfection technologies. For rural, developing areas, sustainable drinking water disinfection which (i) leverages the antimicrobial properties of silver, (ii) requires no additional power source, (iii) results in minimal leaching of silver to the effluent water, and (iv) is affordable would be a powerful tool for providing safe drinking water to populations in need. Thus, the development of a new, alternative disinfectant that is effective against chlorine-resistant microbes like *Cryptosporidium* would be a contribution to the protection of public health across the globe.

Metal-Microbe Interactions

Life depends upon multiple metals. It is estimated that approximately one-third of all enzymes require a metal for maintaining structure and/or initiating functionality (Madigan 2006). Yet only ten metals are known to be essential nutrients and most, except for iron, are only required in very small quantities and thus are classified as

micronutrients (Sigel 1986). Other non-essential metals have the ability to take the place of essential metals or to react with other parts of biological cells often resulting in a toxic effect to the organism. This is the double role that metal ions play in the physiology of organisms; some are indispensable for normal life, while the majority of them are toxic at high enough concentrations.

General Mechanisms of Metal Toxicity

The mechanisms by which metal ions become toxic are difficult to definitively identify for any single metal ion. Because such a large number of proteins require a metal ion for stabilization and/or activation, it is not known which exact proteins will cause the most consequential damage to the organism. In addition, many metal ions can exert toxicity on biological systems by multiple biochemical pathways simultaneously, which further complicates the ability to identify the mode of action. Even with these complexities, five mechanistic categories of metal toxicity have been suggested.

The first mechanism is substitutive metal-ligand binding, in which one metal ion replaces another at the binding site of a specific biomolecule, thereby altering or destroying the biological function of the target molecule. An example is the replacement of Ni for Mg in some redox-active metalloproteins or in DNA, which destroys their function and/or may lead to DNA damage, respectively (Nieboer et al. 1996).

Secondly, metal species can participate in an array of reactions with thiols and disulphides, so destroying the biological function of proteins that contain sensitive sulfur groups (Stohs et al. 1995, Zannonii et al. 2007). These reactions frequently require and

produce reactive oxygen species (ROS), which are by-products of normal metabolism (Stohs et al. 1995). Thiol groups (R-SH) are often involved in the binding of substrates to specific carriers and this transport mechanism can be impaired by toxic metal species. The destruction of sensitive thiol groups on proteins by metal species may also impair protein folding or the binding of other cofactors, thereby destroying the normal activity of the protein (Nieboer et al. 1996).

The third mechanism is the participation of certain transition metals in catalytic reactions, known as Fenton-type reactions, which also produce ROS (Stohs et al. 1995). In general, ROS are transient and highly reactive compounds that can damage all biological macromolecules (Pomposiello et al. 2002). Collectively, the reactions from the second and third mechanisms can place the cell in a state of oxidative stress, and the increased levels of ROS can damage DNA, lipids and proteins (Geslin et al. 2001).

The fourth mechanism entails interference with membrane transport processes. Metal ions must gain entry into cells through transporters or by binding to lipophilic carriers, as cell membranes are not permeable charged species (Foulkes 1998). Toxic metal ions can competitively inhibit a specific membrane transporter by occupying binding sites or by using and/or interfering with the membrane transport process that is normally reserved for an essential substrate (Foulkes 1998).

Recent evidence suggests that some metal species are reduced by oxidoreductase DsbB, which draws electrons from the bacterial transport chain through the quinone pool (Borsetti et al. 2007). Thus the reduction of the metal species indirectly siphons electrons

from the respiratory chain, thereby destroying the proton motive force of the cell membrane (Lohmeier-Vogel et al. 2004).

Silver Mechanisms

For silver, the exact antimicrobial mode of action is not known, and even the chemistry of Ag+ in biological systems is often contradiction between reports (see Past Silver Toxicity Studies section) however, the following possible mechanisms of silver toxicity have been suggested:

- Alters enzyme conformations via silver complexes with electron donors containing sulfur, oxygen or nitrogen (thiols, carboxylates, phosphates, hydroxyl, amines, imidazoles, indoles) (McDonnell 1999);
- Inhibition of the oxidation of glucose, glycerol, fumarate, and succinate;
- Complexation with purine and pyrimidine nucleotides (nitrogen-containing bases) (Ahearn 1995) and prevents DNA replication (McDonnell 1999);
- Inhibition of DNA-ligase and/or –polymerase (Ahearn 1995); and
- Interference with the proton motive force (Dibrov et al., 2002).

Factors Affecting Toxicity

Many factors can influence the reactions of organisms toward metals; consequently a concentration which in one situation is benign may become toxic as the conditions change. This has invariably led to conflicting reports of toxic metal concentrations since the ranges of innocuous and toxic effects may easily overlap.

One of the most important factors that determine the biological availability of a metal is its physiochemical state, or species. Metals exist as a variety of chemical species in natural waters. Metal species dissolved in water may occur as free ions, or aquo-ions, or as complexes. Free metal cations are generally surrounded by coordinating water molecules and so have been termed "aquocations," although by convention the water molecules are ignored when writing chemical reactions involving metal cations. There are a variety of chemical reactions that metals may undergo upon entering aquatic systems. These include acid-base reactions, as well as complex formation with inorganic ligands [such as carbonate (CO_3^{-2}), hydroxide(OH^-), chloride (Cl^-), and sulfide(S^{-2})], organic matter such as humic substances, or biological ligands (Sigel 1986), see Figure 2. In addition metals can undergo oxidation-reduction, as well as chemical precipitation reactions. Metals are also involved in surface reactions with colloids, particulates, and suspended and bedded sediments. The preferential bonds these metals may form are possible explanations for their toxicity. Their toxicity ranges from very acutely toxic (at concentrations on the order of parts per billion or parts per trillion) for some forms of copper (Cu^+), Silver (Ag^+), or chromium (Cr^{+6}), to relatively non-toxic metals such as tin (Sn). It is important to remember that all of these metals form a number of compounds that have widely varying toxicity, so a detailed knowledge of the speciation of any metal is necessary to reliably predict ecotoxicological effects.

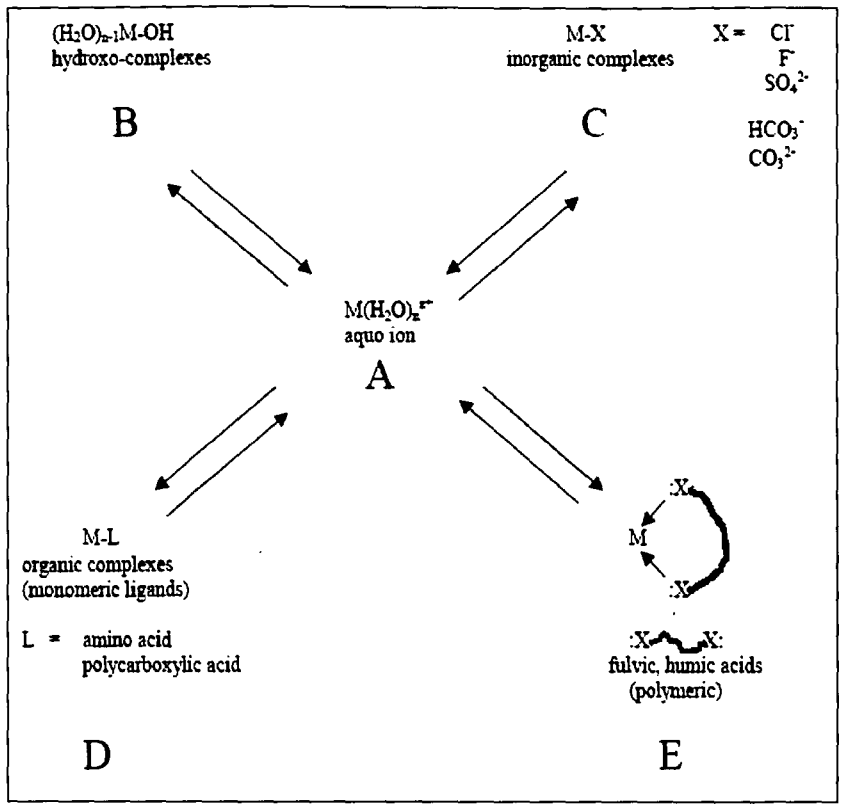


Figure 2. General scheme showing metal speciation in solution. (CNTC 2000)

The stability of silver (Ag(I)) complexes is dependent on the ligand donor atom N<<P; O<<S; F<Cl<Br<I and Ag(I) is categorized as a class b or "soft" lewis acid. Soft lewis acids are characterized by low charge density and low electronegativity that tend to form strong complexes that are stabilized by covalent interactions. In general, soft metal cations are more toxic than borderline metal cations, which are more toxic than hard metal cations. Since covalent bonding is the hallmark of the soft lewis acid, it is reported that metal toxicity is enhanced by the possibility of forming strong complexes with biomolecules (Sigel 1986).

Concentration is also an important factor in toxicity; even essential trace metals, such as Cu or Zn, can be prove to be detrimental or ultimately lethal if the concentration is either to low or two high. Thus it follows that non-essential metals (such as Cd and Ag) present

in low concentrations may be tolerated but with increasing levels may eventually prove lethal. Because the form in which the metal exists often is difficult, most studies have measured the total concentration of the metal, which does not correlate well with toxicity (CNTC 2000). This has invariably lead to conflicting reports of toxic metal concentrations, and may explain why two studies examining the same total concentration of the metal may report quite different results for the same test organism (see Past Toxicity Studies section).

Factors which may individually or collectively impact toxicity of a particular metal are temperature, pH, dissolved oxygen, light, redox potential and salinity of the ambient environment (Peterson et al. 1984). For example at low pH, metals generally exist as free cations, but at an alkaline pH, like that of seawater, they tend to precipitate as insoluble hydroxides, oxides, carbonates, or phosphates. Individually or collectively, these variables may either increase or decrease the toxicity of a specific metal. This may be achieved by changing the physiochemical nature of the metal in a solid or liquid medium, and thus changing its availability and consequently toxicity. The interactions of salinity and temperature with toxicity are not always clear. An increase in temperature has resulted in an increase in toxicity in some cases, but a reduction in other instances. Increased toxicity at higher temperature may be explained by increases in the energy demand, which would result in enhanced respiration of the organism, but decreased toxicity at high temperature has not been satisfactorily explained (Sigel 1986).

In addition to influencing the form of the metal, these factors may also contribute to impacts on the physiology of the organism. The condition of organism and its

environment may greatly influence or facilitate the toxic impact of a metal on the particular organism. Influential physiological variable of an organism impacted on by toxic metals are its developmental stage, age, size, nutritional state, physical and metabolic activity, and behavioral responses to contaminants (Campbell 1995). Toxicity may also be enhanced by facilitating a change in the uptake rate of the metal by an organism leading to deviations from the generally optimal condition.

In addition, the composition of the environment often increases the complexity of toxicity. High concentrations of certain nutrients, such as phosphorus, may reduce toxicity because of the formation of insoluble phosphates. Extracellular products have been reported to reduce metal toxicity in laboratory cultures of phytoplankton when the culture density is high (Kurek et al. 1991). However, the importance of extracellular products in the natural environment is not clear, because dilution effects are considerable.

Ultimately, all these factors will affect the metal's speciation and subsequently its bioavailability (Campbell 1995). It follows that metal speciation must be considered in the design and interpretation of experiments intended to evaluate metal bioavailability and toxicity.

Past Silver Toxicity Studies

A review of past silver toxicity studies has highlighted a number of inconsistencies regarding effective concentration and speciation; localization of silver in the cells; and,

even the resulting chemistry of the Ag+ in the test environment. While not intended to be a comprehensive summary, those issues most relevant to the current work are discussed.

Silver Chloro-Complexes

One previously mentioned factor that can affect the metal speciation and toxicity to organisms is the salinity of the environment, but the impact of salinity on toxicity is not always clear. This is especially true when dealing with microorganisms in cultured environments. While silver accumulation in aquatic organisms has been primarily attributed to the bioavailability of the free silver ion (Ag^{+}), there conflicting reports of both increased and decreased toxicity due to the presence of chloride ions (Cl^{-}).

In 1999, Reinfelder published results from a study using phytoplankton and waters with varying chloride concentrations concluded that the neutral chloride complex, silver chloride ($AgCl^{0}$), is the principal bioavailable species (Reinfelder 1999). In 2004 another report was published citing $AgCl^{0}$ as the principal bioavailable species for gulf toadfish (Wood, 2004).

For bacteria, specifically *E. coli*, an increase in the bactericidal action of 100mM silver nitrate was observed when combined with 100mM of sodium chloride obtaining 100% kill in 30 minutes (Matsumura 2003). Some have suggested that the observed synergistic bactericidal effect between the silver ion and highly concentrated chloride ions is due to the formation of soluble silver chloro-complexes, $AgCl^{2-}$ and $AgCl^{3-}$ (Gupta et al.). Other

studies that have performed similar testing have reported that no synergistic action of chloride with silver was observed (Matsumura 2003).

Solid Silver Species Toxicity

Other studies have attempted to disprove the idea that silver even has to present in solution at all to induce a toxic effect by testing the ability of inert silver surfaces to induce a bactericidal effect. In 1993, Heinig performed studies on silver deposited on inert surfaces and showed that bacteria and viruses were killed on contact without the need for the release of metals into the water (Heinig 1993). Heining suggested that a silver-alumina surface could promote a catalytic interaction with oxygen, which resulted in bactericidal activity.

Others have also observed antimicrobial activities at metal surfaces without apparent release of toxic metal ions. Ahearn tested the adherence of bacteria to silver-coated surfaces and found that silver surfaces that released silver ions were both biocidal and inhibited bacterial adherence to the surfaces. In contrast, silver-coated surfaces that could not be shown to release silver ions permitted initial bacterial adherence followed by killing and detachment of the bacterial cells (Ahearn 1995). Thus, the mechanism of silver inactivation remains unclear since silver has been shown to be an effective disinfectant when in solution as well as when fixed to solid surfaces.

Localization of Metal and Mechanism

Another area of debate with regard to silver toxicity is the localization of silver accumulation within cells. Often times, researchers use the point of accumulation within the cell of a metal species to infer about the mechanism of toxicity that is taking place. Early experiments found that found that most silver was associated with the cell primarily as discrete particles at or near the cell walls of the bacteria (Mullen 1989, Clement 1994). Based on these results, it was hypothesized that the silver ion primarily affects the function of membrane-bound enzymes, such as those in the respiratory chain, through binding to thiol groups (Yamanaka 2005). For a long time it was believed that silver only interacts with the cell wall and does not actually enter the cell.

In experiments with *Ps. Aeruginosa*, it was reportedly found that less than 0.5% of the silver was actually in the lipid fraction, up to 3% in the RNA fraction, up to 12% in the DNA fraction and the remainder was bound to the cell "residue" (proteins and polysaccharides). The distribution of silver in *Citrobacter intermedius* was 5% in the cytoplasm, about 27% in the cell membrane and about 68% in the cell wall fraction; electron micrographs showed large electron dense granules associated with the cell envelope. (Clement et al 1994) Based on these and other reports that the majority of the metal accumulation was being observed at the surface of the cell, it became generally accepted that damage to membrane function was the primary mode of action for silver.

In 1998, Efrima demonstrated the silver accumulation could take place both inside and on the surface bacterial cells after exposure to silver nitrate solution (Efrima 1998). The study successfully showed that silver can distribute uniformly within, or, alternatively, form a rough coating over cells. It was reported that the localization of silver accumulation was dependent upon exposure to a reducing agent. When *E. coli* cells were exposed to a reducing agent (sodium borohydride) first and then exposed to silver ions accumulation occurred on the surface. In contrast, when the bacteria were exposed to the silver first followed by the introduction of a reducing agent to the system then the majority of the silver was taken up into the cells.

Later observations were made that indicated that the bactericidal actions of the silver ion are caused primarily by its interaction with the cytoplasm in the interior of the cell. In studying the effects on *E. coli*, it was reported that the silver ion appeared to penetrate through membrane transport channels without causing damage to the cell membranes. It was hypothesized that once in the cytoplasm the silver ion denatures the ribosome and suppresses the expression of enzymes and proteins essential to ATP production. And that it was this inability to continue to produce ATP that rendered the cell unable to sustain the membrane structures and result in the cell lyses (Yamanaka 2005). These results contrasted the early hypothesis that silver ions primarily affect the function of membrane-bound enzymes, such as those used in the electron transport chain during respiration.

Understanding that there are a multitude of factors that can affect metal speciation and the resulting toxicity to microorganisms, still an attempt was made to summarize the reported toxic concentrations of silver to *E.coli* in order to develop a range of testing

concentrations for the current work. Reported effective toxic silver concentrations ranged from 20mM to nM levels for laboratory grade water (Butkus et al. 2005, Kim et. al 2002, Estevao 2003, Reinfelder 1999, Ahearn 1995, Matsumura 2003) and from 0.05M to 0.005M for water with turbidities ranging from 30 to 100 NTU (Efrima 1998).

> "In conclusion, a great deal remains to be learned about the mode of action of antiseptics and disinfectants. Although significant progress has been made with bacterial investigations, a greater understanding of these mechanisms is clearly lacking for other infectious agents. Studies of the mechanisms of action of and microbial resistance to antiseptics and disinfectants are thus not merely of academic significance. They are associated with the more efficient use of these agents clinically and with the potential design of newer, more effective compounds and products." (McDonnell 1999)

Description of Project

The overall goal of the project is to determine the efficacy of silver as a potential alternative for water disinfection. Two main testing strategies were incorporated. First, ion exchange fibers were used to introduce silver into a batch test system. Following challenges with controlling the amount of silver being released into the system, the testing system was reevaluated and redesigned in order to evaluate the effect of different silver speciation and concentrations on the test organisms. The microorganisms tested included *E. coli* (bacteria) for the ion exchange fiber tests and both *E. coli* and *Cryptosporidium* (protozoa) for the silver speciation testing.

Testing of Ion Exchange Fibers

Ion exchange fibers were initially loaded with the monovalent charged silver ion (Ag+) to introduce silver into the system. Bacterial counts on agar plates were used to test reported bactericidal effects of silver. Testing was executed in a batch setup leveraging various amounts of fibers in order to compare the amount of fibers and contact time required in order to achieve 100% disinfection of ~10^6 CFU/mL bacterial loading. *E. coli* was selected as the test organism since it is the most commonly used indicator for the presence of fecal material for water treatment systems.

Materials and Methods

Bacteria preparation. Individual *E. coli* colonies were isolated via the streak plate method on LB agar plates. LB agar broth was inoculated with a single colony and incubated at 37°C for 24 hours. The *E. coli* concentration of the broth was estimated using a spectrophotomer. Based on the target starting concentration for each experiment, the required broth volume was placed in a 1.5mL microcentrifuge tube and centrifuged at 13,000 rotations per minute (13K rpm) for 4 minutes. The supernatant was aspirated and discarded; the resulting pellet was resuspended in water purified in a highgrade Milli-Q Plus De-ionizer system (Millipore).

Fiber Preparation. The Fiban K-1 fibers (UP Unitechprom BSU, Belarus) were used for all tests. The fibers are composed of a polymetric matrix consisting of a polypropylene fiber with graft copolymer of styrene and divinilbenzene and have sulfonic ($SO_3^-H^+$) functional groups, see Figure 3. The fibers have a diameter of ~40μm and have a loading capacity of 3meq/g (dry weight). Prior to laboratory use, fibers were pretreated with 1 N HCl for 1 h, thoroughly rinsed with distilled water, and then air-dried.

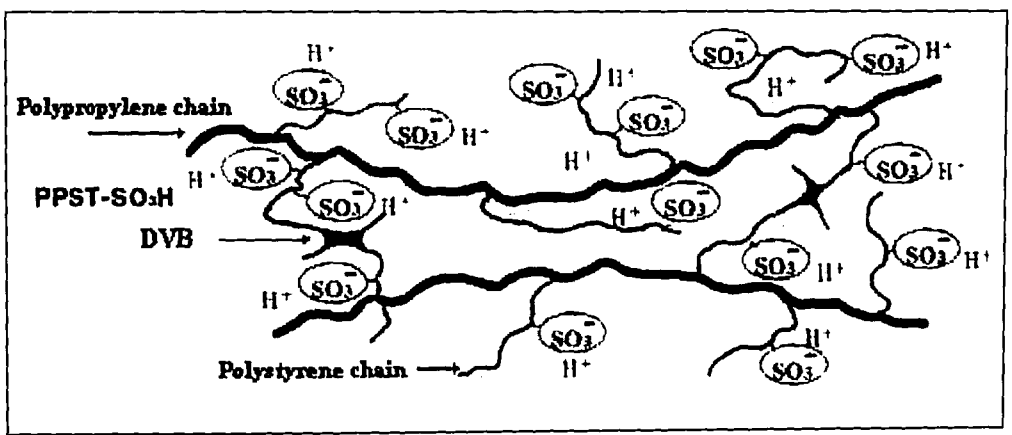


Figure 3. Schematic presentation of the structure of sulfonic cation exchanger, Fiban® K-1 fibers.

To load silver onto the fibers silver nitrate was used because it readily dissociates, as depicted in the following equation

$$(AgNO_3 \leftrightarrow Ag^+ + NO_3^-) \quad (1)$$

The fibers used were cation ion exchange fibers and thus the fixed sulfonic group readily reacted with the free silver ion in solutions, as follows:

$$F\text{–}SO_3H^+(s) + Ag^+(aq) \Rightarrow F\text{–}SO_3^-Ag^+(s) + H^+(aq) \quad (2)$$

F denotes the fiber matrix.

First, 368mg of fibers were added to 492μL 1N $AgNO_3$ (53mg of Ag) in 1L of Millipore water. The fibers were continuously mixed in the solution for 24 hours to maximize the silver loading to the fibers. Next the fibers were placed in 80mg/L solution of sodium borohydride ($NaBH_4$) to reduce the silver from Ag^+ to Ag^0. The final step of the fiber preparation consisted of a rinse in Millipore water. After each step (loading, reduction, and rinse) the solution containing fibers was filtered through an 8μm filter to separate out the fibers and the filtrate was analyzed for silver using atomic adsorption spectroscopy (AA). Based on the resulting AA measurements of each filtrate from each step, the total amount of silver loaded to the fibers was ~45mg, which equates to ~122mg Ag/g fiber.

Antimicrobial activity was assessed using traditional colony counts for *E. coli* on LB agar plates. The procedure was carried out as follows: starting with a concentration of ~10^6 colony forming units per mL (CFU/mL) of *E. coli* in 500mL of Millipore water, varying amounts of fibers were added (240, 40, and 20 mg). Figure 4 is a schematic of the batch test setup utilized for testing the silver-loaded fibers in *E. coli* contaminated water. For each run, 10^6 CFU/ml of *E. coli* in 500mL of Millipore water was tested as a control. Flasks were continuously stirred throughout the experiment using magnetic stir bars and stir plates. 10mL samples were taken at each sampling time after addition of the fibers, including a time zero (prior to addition of the fibers for quantification of initial bacteria concentration).

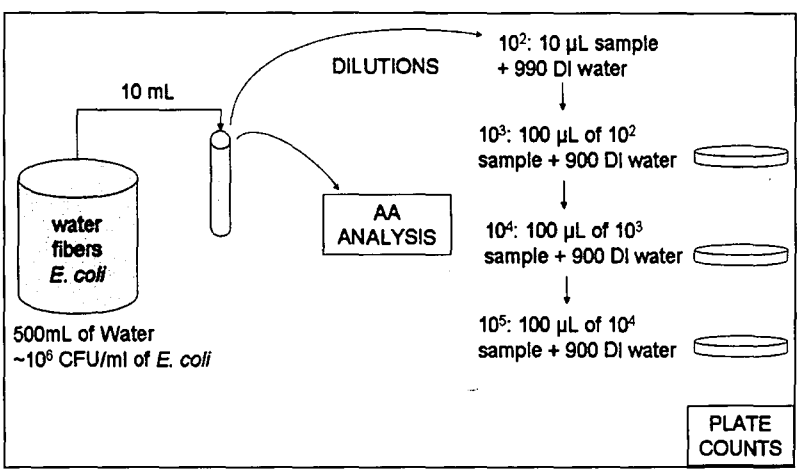


Figure 4. Batch Test Setup for Silver-loaded Fibers and *E. coli*

A minimum of 1mL was used to make dilutions for the plates counts and a minimum of 3mL was analyzed via atomic adsorption (AA) to obtain the silver concentration (mg/L) of the sample. Samples were filtered through an 8µm filter prior to the AA analysis to ensure complete removal of fibers. Serial dilutions of 1:10^2, 1:10^3, and 1:10^4 were made. 50µL of each dilution was spread over LB agar plates. Plates were incubated at 37°C for

24 hours and the resulting CFUs were counted. Only plate counts that were statistically significant, those with 30-300 CFUs per plate, were used in the calculations of *E. coli* concentrations.

Results

The initial tests performed with the ion-exchange fibers tested the effects of 240, 40, and 20 mg of fibers for runs 1, 2, and 3, respectively. The same batch of fibers were used for all three runs and had a silver loading of 122mg Ag^{+}/g fiber. The relative *E. coli* concentrations at the various sampling times are shown in Figure 5. 100% disinfection was achieved within 5 minutes after the addition of the fibers.

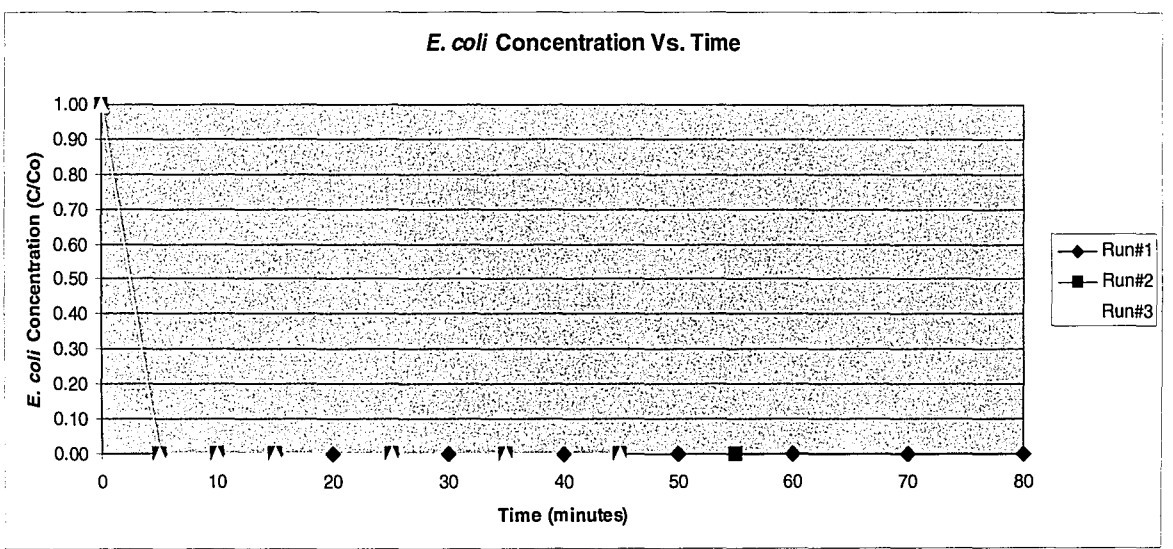


Figure 5. *E. coli* Concentrations for Silver-loaded Fiber Testing. Amount of fibers added for each: 240mg for run 1, 40mg for run 2, and 20mg for run 3.

The results of the AA analysis are shown in Figure 6. There was a substantial increase in the amount of silver in solution, or that had desorbed from the fibers, for run 1 compared to runs 2 and 3. Run 1 had the largest amount of fibers added, 240mg, and at the 60 minute sampling time the silver concentration was 10.086 mg/L, which corresponds to a

14% loss of silver from the fibers. Whereas, runs 2 and 3, with 40 and 20mg of fibers respectively, only had 1.8% and 1.4% of silver loss from the fibers.

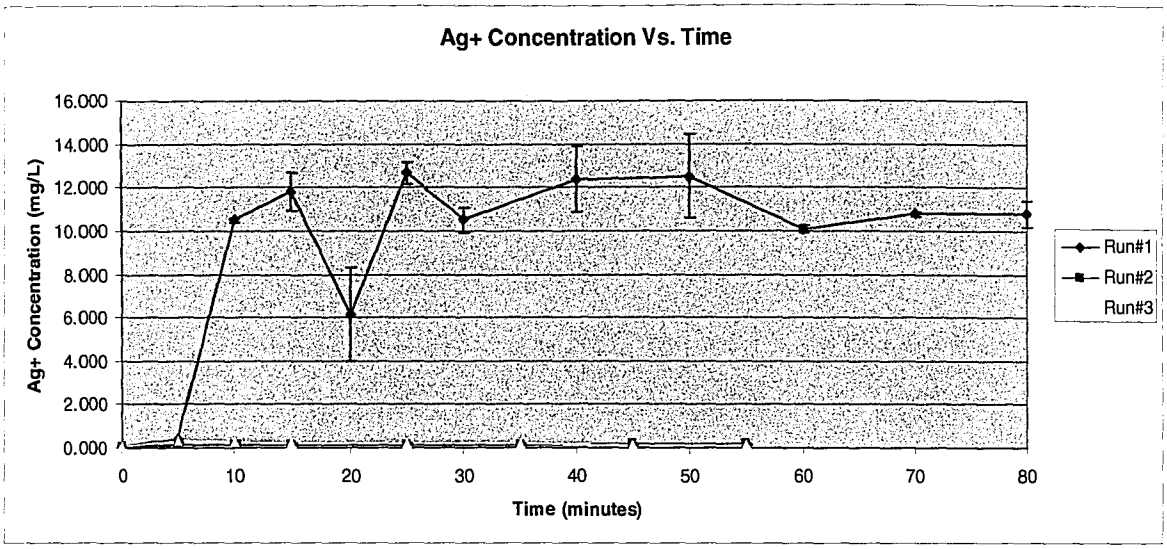


Figure 6. Silver Concentration in Solution from Silver-Loaded Fiber Testing for 240mg (run #), 40mg (run#2), and 20mg (run#3) of fibers.

Figure 7 is a plot of only the silver concentration for runs 2 and 3 (run 1 removed) so that the resulting trends can be analyzed. The results show that the silver in solution increases substantially within the first 5 minutes of contact. In fact, the silver concentration of the water for both runs was above the USEPA drinking water limit of 0.1mg/L (Ag+) at this first sampling time. While the silver concentration for run 3 with 20mg of fibers appears to be stabilizing around 0.17mg/l, stabilization does not occur until >35minutes.

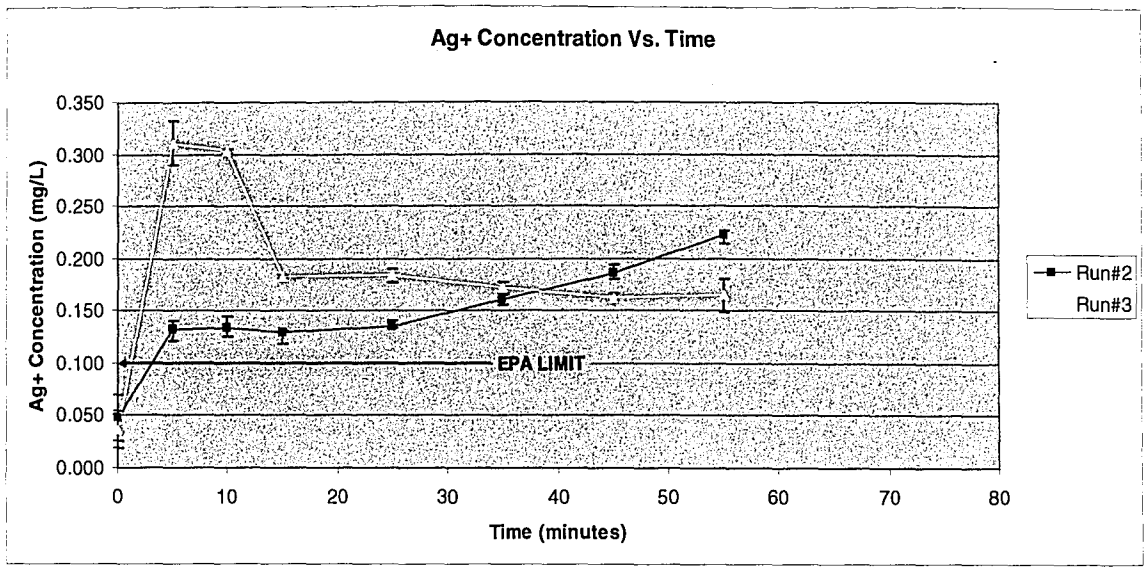


Figure 7. Silver Concentration in Solution from Silver-Loaded Fiber Testing for 40mg (run2) and 20mg (run 3) fibers.

Another set of tests were performed in which the sampling times were reduced (<5 minutes) in an attempt to identify when disinfection was occurring. Both runs used 40mg of fibers. The same batch of fibers were used for both runs and had a silver loading of 122 mg Ag^{+}/g fiber (same batch of fibers used in the aforementioned runs as well). Figure 8 and 9 show the resulting *E. coli* and silver concentrations, respectively.

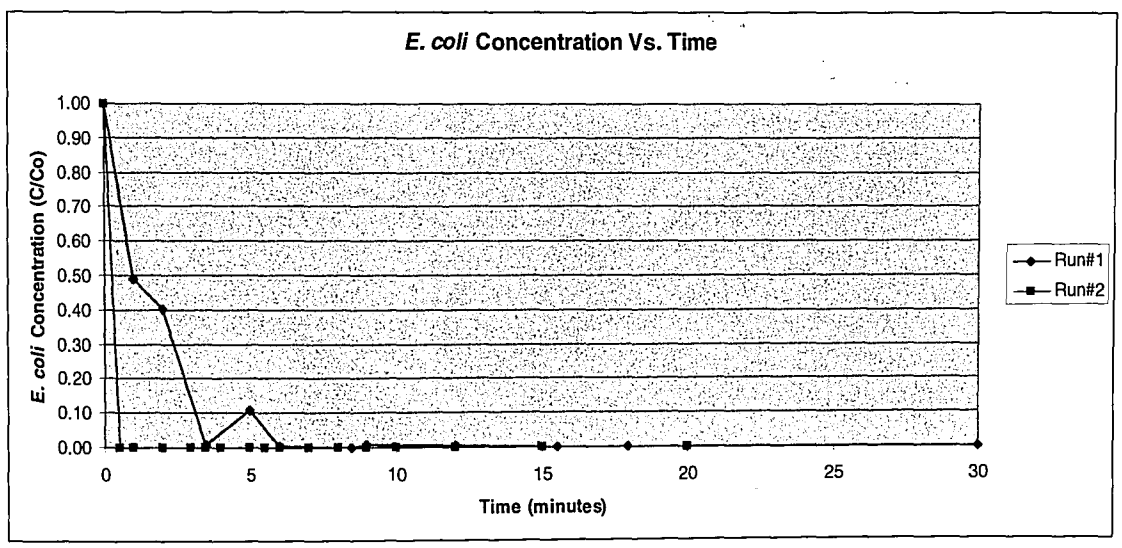


Figure 8. *E. coli* Concentrations for Silver-loaded Fiber Testing for 40mg of Fibers

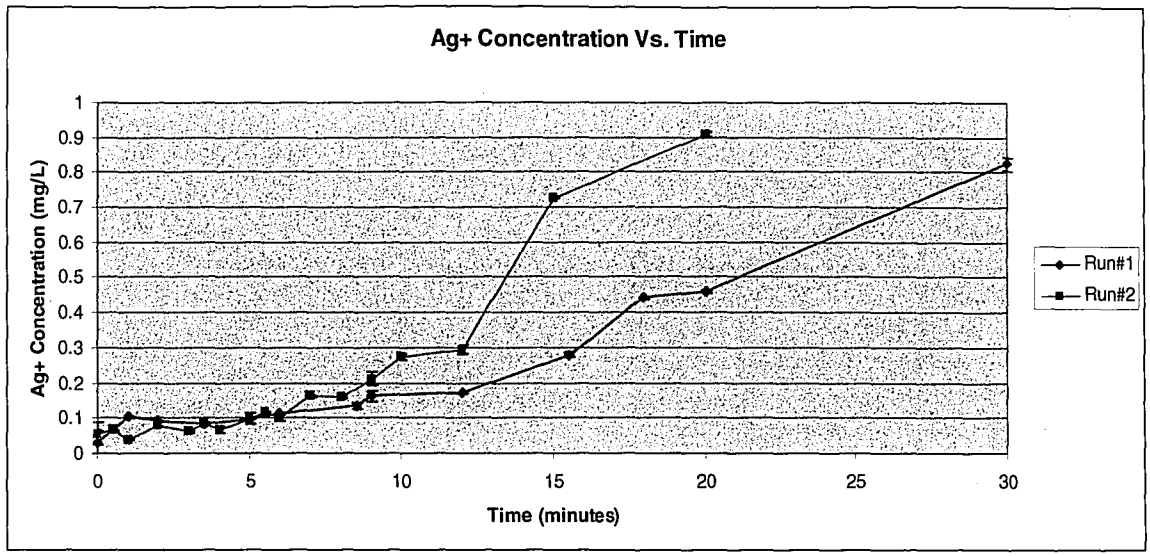


Figure 9. Silver Concentration in Solution from Silver-Loaded Fiber Testing for 40mg

Again these results confirm that 100% disinfection is obtainable within a 5 minute contact time with the fibers. However, the silver concentration is still right around the drinking water limit.

Conclusions

Testing results confirmed that silver is an effective disinfectant against *E. coli*. Ion-exchange fibers (24, 40, 20 mg at 122mg Ag/g fiber) were successful at inducing 0% survival within 5 minutes. However, an unexpected result was that the aqueous silver concentration was increasing to levels above the USEPA drinking water limit. The elevated silver concentrations also result in an estimated ingestion rate 0.16mg/d above the WHO guideline of a maximum of 0.08mg/d over a 70yr lifetime (WHO 2002).

A method that introduces a disinfecting agent that is then required to be removed after disinfection is not a feasible alternative to chlorination. Based on these results, the method for introducing the silver into the system and the entire test design was

reevaluated. It was decided to focus on gathering data in order to identify whether silver exerts any toxic effects on *Cryptosporidium* before trying to design a new method of application. Experiments were redesigned to focus on attempting to identify which species of silver is the most toxic and potentially identify the mode of action.

Silver Speciation Testing

The scientific literature contains a wide range of concentrations and often conflicting conclusions regarding silver speciation and resulting toxicity (see Past Silver Toxicity Studies section). And even though for over 15 years it has been readily accepted that the total aqueous concentration of a metal is not a good predictor of its bioavailability (Campbell 1995), it is still used by most regulatory agencies for metal discharge limits and/or guidance values. As previously discussed the speciation of a metal can greatly influence its bioavailability and subsequently its toxicity. It follows that metal speciation must be considered in the design and interpretation of experiments intended to evaluate metal bioavailability and toxicity (CNTC 2000).

The silver speciation testing was designed to evaluate the disinfection capabilities of various speciations of silver, namely the free ion, Ag^{+}, the uncharged neutral chloride complex, $AgCl^{0}(aq)$, and the higher order chloro-complexes of silver, $AgCl^{2-}$, $AgCl_3^{2-}$, and $AgCl_4^{3-}$. These speciations were chosen based on previous work that demonstrated that the uncharged chloride complex, $AgCl^{0}(aq)$, is the principal bioavailable species of inorganic silver in phytoplankton and that the speciation of inorganic silver in natural

waters is primarily a function of chloride concentration and is not significantly affected by changes in pH (Reinfelder 1999).

Manipulation of the chloride concentration can provide a means of controlling the silver speciation in the laboratory. Thus, at low chloride concentrations (<1mM), the dominant silver species is the free ion, Ag^+. As the chloride concentration increases, in the range of 1 to 16mM (typical of natural brackish waters), silver speciation is dominated by the uncharged neutral chloride complex, AgCl(aq). At even higher chloride concentrations (representative of natural seawater), the higher order chloro-complexes of silver become dominant.

The effects of the free silver ion (Ag^+) speciation were tested in a series of batch experiments using Millipore water spiked with *Cryptosporidium* and *E. coli* (10^3 oocysts/ml and 10^6 cfu/ml, respectively). Silver speciation will be controlled by the chloride concentration of the water. For the free ion (Ag^+) tests, the microorganisms will be exposed to varying concentrations of $AgNO_3$ (0.5M, 0.05M, 0.005M, 0.93μM, and 9.3μM) in Millipore water.

A wide range of silver concentrations have been reported as being effective for inducing toxic effects on *E. coli*, as high as 0.05 and 0.005M for waters with high turbidity and as low as 20mM to nM levels for laboratory grade water. The concentrations selected, specifically 0.05M, 0.005M, 0.93μM, and 9.3μM, represent a range of values for which would readily expect to see bactericidal effects. Since the effects on *Cryptosporidium*

have not been reported, the 0.5M concentration was added to the test matrix as the upper bound for this round of toxicity testing.

Water samples were analyzed at various times after introduction of the silver for the percent inactivation of *E. coli* and *Cryptosporidium.* For the *E. coli* analysis, plate counts were used to identify the concentration of live *E. coli* cells in the water at each time point. For the *Cryptosporidium* analysis, two viability assays and one infectivity assay were performed at each time point. *Cryptosporidium* viability was estimated using excystation and vital dye staining assays, and infectivity was determined using an *in vitro* cell culture assay. Each assay was performed in triplicate.

Materials and Methods

Experimental Design. Starting from a 1N solution of $AgNO_3$, dilutions (0.5M, 0.05M, 0.005M, 0.93μM, and 9.3μM) in Millipore water were made to avoid silver salt precipitation. The capacity of $AgNO_3$ preparations to inhibit bacterial growth was studied at all concentrations prepared. Control samples consisted of appropriate concentrations of the test organism in Millipore water with no $AgNO_3$ addition.

Antimicrobial Activity

Bactericidal Effects

Antimicrobial activity was assessed using traditional colony counts for *E. coli* on LB agar plates and infectivity and viability assays for *Cryptosporidium.* For *E. coli* testing, the procedure was carried out as follows: starting with a concentration of ~10^6 CFU/ml of *E. coli* in 100mL of Millipore water, appropriate amounts of 1N $AgNO_3$ solution was added

to obtain the desired silver concentrations (0.5M, 0.05M, 0.005M, 0.93μM, and 9.3μM). For each run, 10^6 CFU/ml of *E. coli* in 100mL of Millipore water was tested as a control. Flasks were continuously stirred throughout the experiment using magnetic stir bars and stir plates. 1mL samples were taken after addition of the $AgNO_3$ at times of 15, 30, and 60 minutes. In an attempt to remove additional aqueous silver from exposure to the bacteria, the samples were immediately centrifuged at 13K rpm for 5 minutes; the supernatant was removed and the bacteria were resuspended in Millipore water. Serial dilutions of $1:10^2$, $1:10^3$, and $1:10^4$ were made. 50μL of each dilution was spread over LB agar plates. Plates were incubated overnight at 37°C and resulting CFUs were counted.

Pathogen Inactivation

Cryptosporidium Parvum oocysts were obtained from Waterborne, Inc. (Iowa isolate, mouse shed) and consisted of oocysts in 1X phosphate buffered saline (PBS). Each run included selected silver nitrate treatments, a control and a positive stock sample. The starting concentration for all treatments and the control was ~10^5 oocysts/mL. The silver concentrations were obtained by diluting appropriate amounts of 1N $AgNO_3$ solution with Millipore water. The control was oocysts in Millipore water on the bench top for duration of the test and a positive stock sample was prepared at the time of sample processing. The stock solution was kept at 4°C until sample processing. All assays were preformed in triplicate and the resulting average of the stock solution sample was used as the baseline infectivity and viability. 50mL samples were taken after the desired contact times. In an attempt to remove additional aqueous silver from exposure to the bacteria,

the samples were washed twice with Millipore water before processing for the infectivity and viability assays. A wash cycle consisted of centrifugation at 13K rpm for 5 minutes and removal of the supernatant. The final oocyst pellet was left remaining in ~10-20μL of water and was then processed by one of the three protocols outlined below: Tri-Stain, Excystation, or Infectivity.

Viability Assays

Tri-Stain Method

The Tri-Stain method estimates infectivity by determining the number of potentially infectious oocysts based on a measure of the permeability of the oocyst cell wall. Permeability is measured by the inclusion or exclusion of two fluorogenic vital (nucleic) dyes, specifically 4',6-diamidino-2-phenylindole (DAPI) and propidium iodide (PI). DAPI is a smaller molecule relative to PI and intact oocyst cell walls are permeable to DAPI; therefore, staining of the sporozoite nuclei is expected. In contrast, PI is a larger molecule and can only permeate the oocyst and stain the sporozoite nuclei if cell wall integrity has been compromised. The third stain, MeriFluor (Meridian BioScience, Cincinnati, OH) detection reagent contains a mixture of fluorescein isothiocyanate (FITC) labeled monoclonal antibodies that attach to the cell wall antigens of *Cryptosporidium* oocysts.

Oocysts are viewed by direct microscopic visualization of treated samples and their comparison to the initial untreated population. Oocysts are identified by MeriFluor staining yielding an apple green fluoresce when viewed under a FITC filter block (490-

500-nm excitation, 510-530-nm emission); DAPI staining of sporozoite nuclei gives a characteristic sky blue fluorescence under a UV filter block (350-nm excitation, 450-nm emission); and PI stained sporozoite nuclei fluoresce bright red under a green filter block (500-nm excitation, 630-nm emission), see Figure 10. Table 1. outlines the categories for classifying the results of the tri-stain viability assay. Oocysts are positively identified by viewing the sample under a FITC filter block; they fluoresce green. Once an oocyst has been located, it is viewed under both the UV and green filter blocks. Oocysts are considered DAPI positive if they fluoresce blue under the UV filter block and are considered PI positive (PI+) if they fluoresce bright red under the green filter block. Confirmed oocysts, those that fluoresced apple green under the FITC filter block, but that did not fluoresce under the UV nor the green filter block, DAPI negative PI negative (DAPI- PI-) were classified as "empty," or dead oocysts.

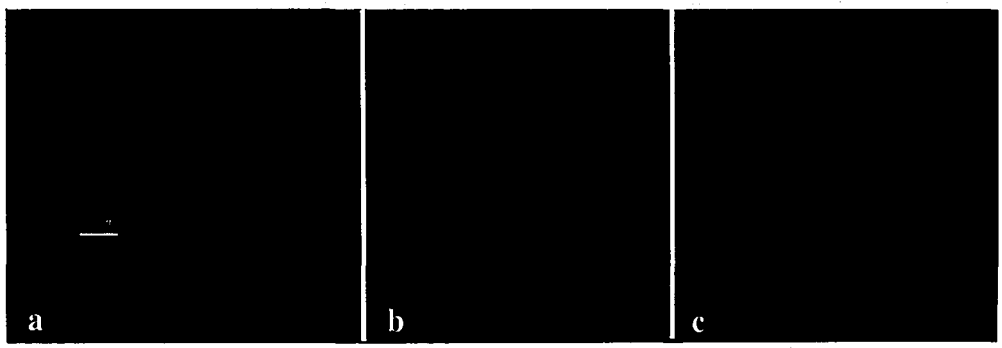


Figure 10. Fluorescence Micrograph of oocysts stained with (a) Merifluor (b) DAPI and (c) PI.
(Photos (a) and (b) USEPA 2008, Photo (c) by Author)

Table 1. Classification Scheme for the Tri-Stain Viability Assay

Viable	Non-Viable	Empty	Total Count
DAPI + PI-	DAPI+ PI-	DAPI- PI-	100

500-nm excitation, 510-530-nm emission); DAPI staining of sporozoite nuclei gives a characteristic sky blue fluorescence under a UV filter block (350-nm excitation, 450-nm emission); and PI stained sporozoite nuclei fluoresce bright red under a green filter block (500-nm excitation, 630-nm emission), see Figure 10. Table 1. outlines the categories for classifying the results of the tri-stain viability assay. Oocysts are positively identified by viewing the sample under a FITC filter block; they fluoresce green. Once an oocyst has been located, it is viewed under both the UV and green filter blocks. Oocysts are considered DAPI positive if they fluoresce blue under the UV filter block and are considered PI positive (PI+) if they fluoresce bright red under the green filter block. Confirmed oocysts, those that fluoresced apple green under the FITC filter block, but that did not fluoresce under the UV nor the green filter block, DAPI negative PI negative (DAPI- PI-) were classified as "empty," or dead oocysts.

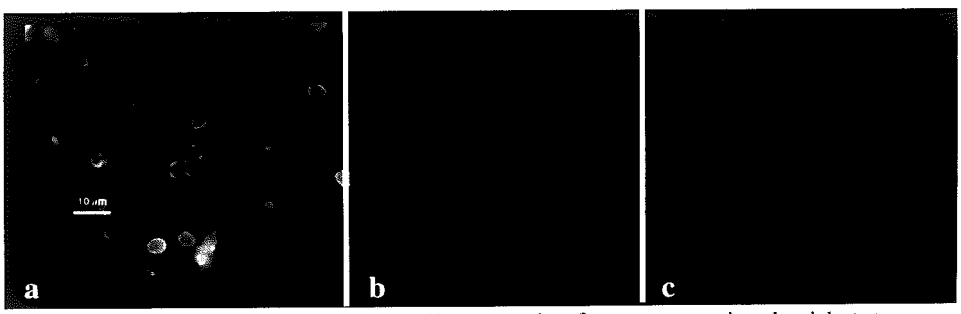


Figure 10. Fluorescence Micrograph of oocysts stained with (a) Merifluor (b) DAPI and (c) PI.
(Photos (a) and (b) USEPA 2008, Photo (c) by Author)

Table 1. Classification Scheme for the Tri-Stain Viability Assay

Viable	Non-Viable	Empty	Total Count
DAPI + PI-	DAPI+ PI-	DAPI- PI-	100

The protocol described is based on previously described assays (Campbell et al. 1992, Jenkins et al. 1997). Solutions of DAPI (2mg/ml in absolute methanol) and PI (1mg/ml in 0.1 M PBS, pH 7.2) were prepared and stored at 4°C in the dark. 10µL DAPI solution and 10µL PI solution was added to 20µL of the oocyst suspension in a 1.5mL microcentrifuge tube. The tube was mixed via a vortex mixer and incubated in the dark at 4°C for 30 minutes. Following incubation, the suspension was centrifuged at 13K rpm for 4 minutes and the supernatant was aspirated and discarded. A rinse of 200µL of Millipore water was added and the solution was again vortexed, centrifuged and the resulting supernatant removed. The final pellet was resuspended in a total volume of 10µL of Millipore water.

The entire specimen (10µL oocyst suspention) was placed on a MeriFluor-treated slide well, spread evenly with a sterile inoculating loop, and left to dry completely at room temperature. The oocysts were then fixed to the slide by adding ~15µL of 100% methanol per well and again allowed to dry completely at room temperature. One drop of MeriFluor detection reagent to each well and was spread gently over entire well with sterile inoculating loop, as necessary. The slide was then incubated in a dark humid chamber at room temperature for 30 minutes. Following the incubation, the slide was rinsed with 1X MeriFluor wash buffer. One drop of MeriFluor mounting media was added to each well and a cover slip was applied. Slides were stored in the dark at 4°C for up to 12 hours before examination by fluorescence microscopy.

in vitro Excystation (IVE) Method

The *in vitro* excystation (IVE) method estimates infectivity by determining the number of potentially infectious oocysts based on simulating the conditions of the mammalian gastrointestinal tract. Oocysts exposed to an acid pretreatment at elevated temperatures will excyst and release their infectious sporozoites, whereas metabolically inactive (nonviable) oocysts will fail to excyst under such conditions. Excystation is tracked by direct microscopic visualization of treated samples and their comparison to the initial untreated population (stock).

The protocol described is based on a previously described assay (Robertson et al. 1993). A 10% bleach solution (50µL sodium hypochlorite in 450µL Millipore water) was added to the oocyst suspension in a 1.5mL microcentrifuge tube. The tube was vortexed and incubated on ice for 1 minute. Following incubation, the suspension was centrifuged at 13K rpm for 4 minutes and the supernatant was aspirated off and discarded. A rinse of 50µL of Millipore water was added and the solution was again vortexed, centrifuged and the resulting supernatant removed. Millipore water was added to obtain a total volume of 50µL to which 50µL of 1.5% taurocholic acid was then added. The solution was vortexed and incubated at 37°C for 1 hour. Following incubation, the suspension was centrifuged at 13K rpm for 4 minutes and the supernatant was aspirated and discarded. A final rinse of 500µL of Millipore water was conducted, along with the subsequent centrifugation and supernatant aspiration. The final pellet was resuspended in a total volume of 10µL of Millipore water.

The entire specimen (10μL oocyst suspension) was placed on a MeriFluor-treated slide well, spread evenly with a sterile inoculating loop, and left to dry completely at room temperature. The oocysts were then fixed to the slide by adding ~15μL of 100% methanol per well and again allowed to dry completely at room temperature. One drop of MeriFluor detection reagent to each well and was spread gently over entire well with a sterile inoculating loop, as necessary. The slide was then incubated in a dark humid chamber at room temperature for 30 minutes. Following the incubation, the slide was rinsed with 1X MeriFluor wash buffer. One drop of MeriFluor mounting media was added to each well and a cover slip was applied. Slides were stored in the dark at 4°C for up to 12 hours before examination by fluorescence microscopy.

Infectivity Assay

Cell culture infectivity offers the ability to estimate the infectivity of oocysts without the use of live animal hosts. For this assay, human intestinal cells are grown on chamber slides and a known number of oocysts are added to each well. After an incubation period, a stain with a fluorescence label designed to react with all the life stages of *Cryptosporidium* is applied to the slides. Slides are viewed by fluorescence microscopy and the number of infections is recorded. The number of infections from the treated samples is then compared to the initial untreated population (stock).

The protocols for *in vitro* cell culture infectivity described below are based on previously described assays (Slifko 1997, Rochelle et al. 2002).

Cell Maintenance and Growth

Human illeocecal adenocarcinoma cells (HCT-8 cells) were maintained in RPMI 1640 supplemented with 4 mM L-glutamine, 30 mM HEPES (pH 7.3), 100μg/mL of penicillin, 100μg/mL of streptomycin, 100μg/mL of kanamycin, and 0.25μg/mL of amphotericin B (Rochelle et al. 2002). Cell culture growth medium was supplemented with 10% fetal bovine serum (FBS) for maintenance of uninfected cells (maintenance media) and with 2% FBS for maintenance of cells (growth media) following inoculation with oocysts. The HCT-8 cells were maintained in 25-cm^2 cell culture flasks in a 5% CO2 atmosphere at 37°C and 100% humidity. The maintenance media was changed every 2 or 3 days and cells were grown to at least 80% confluence. The cells were removed from the inside surface of the flask by incubating in a 1x Trypsin solution (diluted in PBS) for 10 minutes at 37°C in the CO_2 atmosphere. The cell suspension was pipetted vigorously through a 1000-μl pipette tip repetitively until the cells were separated (about 10 times). The cells were centrifuged at 13K rpm for 4 minutes, and the pellet was resuspended in maintenance media. The cell suspension was then either added to new cell culture flasks for additional cell growth or added to 8-well chamber slides to be infected with treated oocysts. Typically 1 flask containing a ~80% monolayer of cells was used for up to a maximum of 5 chamber slides.

Oocyst Pretreatment (prior to infecting cells on chamber slides)

A 10% bleach solution (100μL sodium hypochlorite in 900μL Millipore water) was added to the oocyst suspension in a 1.5mL microcentrifuge tube. The tube was mixed via a vortex mixer and incubated at room temperature for 8 minutes. Following incubation,

the suspension was centrifuged at 12K rpm for 4 minutes and the supernatant was aspirated and discarded. A rinse of 1mL of Millipore water was added and the solution was again vortexed, centrifuged and the resulting supernatant removed. 80-100μL of growth media (RPMI maintenance media as described above with 2% FBS) was added to the final pellet and vortexed to disperse the pellet. Oocyst concentration was calculated using triplicate hemacytometer counts. The resulting concentration was used to calculate the volume to obtain 100 oocysts, the infection volume for each well. Each sample was plated in three wells, leaving a control well per sample to monitor cell health, resulting in the ability to plate 2 samples per slide. After the appropriate infection volume was added, the slides were incubated for 48 hours in a 5% CO_2 atmosphere at 37°C prior to staining and enumeration.

Staining & Enumeration of Infections

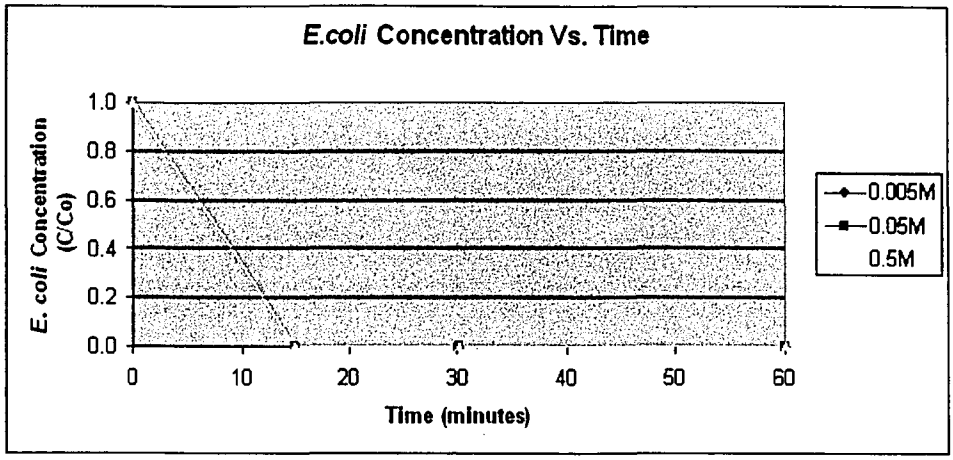
After the 48-hour incubation period, the overlying media was aspirated and discarded. The infected monolayers were fixed with 100% methanol and incubated at room temperature for 10 minutes. 40μL of a 1:20 dilution of Sporo-Glo (Waterborne, Inc., New Orleans, LA) in dilution buffer was added to each well and incubated in a humid chamber for 45 minutes. Sporo-Glo is a fluorescence label that reacts with sporozoites, merozoites, and all other intracellular reproductive stages of *Cryptosporidium.* Following the incubation period, the chamber wells were removed from the slides and rinsed in 1X PBS. To reduce non-specific background fluorescence, counterstain (C101) (Waterborne, Inc., New Orleans, LA) was added and allowed to react for 1 minute. Slides were again rinsed in 1X PBS and mounting media was added to the wells. The

cells were examined by fluorescence microscopy. The various life-cycle stages of *Cryptosporidium* fluoresce an apple-green color against a relatively dark background of uninfected cells when viewed under a FITC filter block. The number of infections per well (per 100 oocysts) was recorded.

Results

Bactericidal Effects

The effects of various $AgNO_3$ concentrations on *E.coli* were tested and the results are presented in Figure 11. For all concentrations tested (0.5M, 0.05M, 0.005M, 0.93μM, and 9.3μM), 100% disinfection was observed within 15 minutes. Tests exposing the bacteria to silver concentrations of 0.005M, 0.05M, and 0.5M were performed in triplicate, while silver concentrations representing the EPA drinking water limit and 10 times the limit (0.93μM and 9.3μM, respectively) were performed twice. These results were anticipated based on the reported range of toxic silver concentrations for *E. coli*. The importance of these tests was to verify the bactericidal properties of silver over a wide range of concentrations for relatively short contact times.



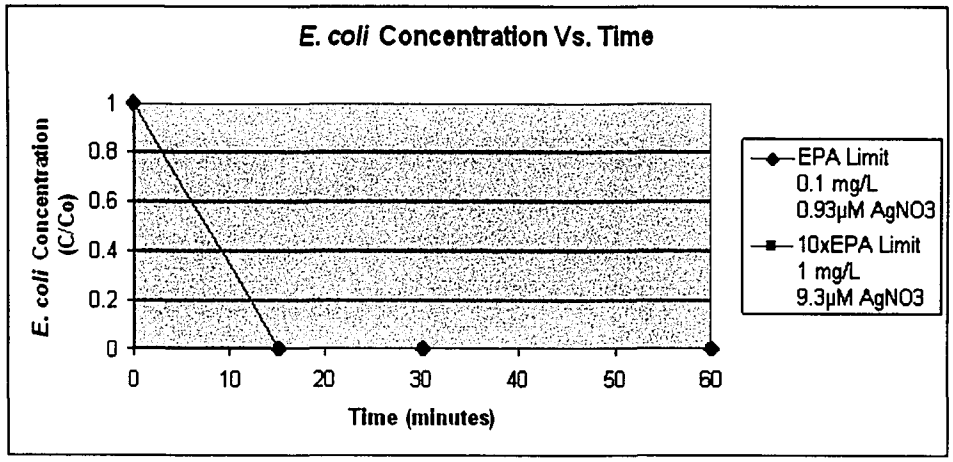


Figure 11. Results of Silver Nitrate Effects on *E.Coli*

Pathogen Inactivation

A summary of the usable data obtained from the viability and infectivity assays performed are presented in Figures 12 and 13. Assay/concentration combinations with gray shading indicate that the tests were not performed for that run. Of the assay/concentration combinations that were performed, an "X" indicates that reliable data were obtained.

			Ag+ Concentration		
Run	Time	Assay	0.005M	0.05M	0.5M
1	30min & 60min				
		Excystation	X	X	X
		Infectivity			
2	30min & 60min				
		Excystation			
		Infectivity	X		
3	2 hours				
		Excystation		X	
		Infectivity			
4	2 hours				
		Excystation			
		Infectivity	X	X	X
5	2 hours				
		Excystation			
		Infectivity		X	X
6	2 hours				
		Excystation	X	X	X
		Infectivity			
	Total				
		Excystation	2	3	2
		Infectivity	1	2	2

Figure 12. Usable Data from Testing Performed on *Cryptosporidium* for Silver Concentrations of 0.005M, 0.005M, and 0.5M

			Ag+ Concentration	
Run	Time	Assay	EPA Limit 0.1 mg/L 0.93µM AgNO3	10xEPA Limit 1 mg/L 9.3µM AgNO3
7	1 hour			
		Excystation		
		Infectivity	X	X

Figure 13. Usable Data from Testing Performed on *Cryptosporidium* for Silver Concentrations within the EPA Drinking Water Limit Range

All assays (tri-stain viability, excystation viability, and infectivity) were performed a minimum of three times for the higher silver concentrations (0.005M, 0.005M, and 0.5M). For the lower silver concentrations (0.93µM and 9.3µM), the infectivity assay was performed once.

Due to the lack of reported effects of silver on *Cryptosporidium*, the higher silver concentrations were tested to identify whether silver exposure would cause a noticeable effect at all. The inability to obtain data from some of the testing runs is the result of

			Ag+ Concentration		
Run	Time	Assay	0.005M	0.05M	0.5M
1	30min & 60min	Tri-Stain			
		Excystation	X	X	X
		Infectivity			
2	30min & 60min	Tri-Stain			
		Excystation			
		Infectivity	X		
3	2 hours	Tri-Stain	X	X	
		Excystation		X	
		Infectivity			
4	2 hours	Tri-Stain			
		Excystation			
		Infectivity	X	X	X
5	2 hours	Tri-Stain			
		Excystation			
		Infectivity		X	X
6	2 hours	Tri-Stain	X	X	
		Excystation	X	X	X
		Infectivity			
	Total	Tri-Stain	2	2	0
		Excystation	2	3	2
		Infectivity	1	2	2

Figure 12. Usable Data from Testing Performed on *Cryptosporidium* for Silver Concentrations of 0.005M, 0.005M, and 0.5M

			Ag+ Concentration	
Run	Time	Assay	EPA Limit 0.1 mg L 0.93µM AgNO3	10xEPA Limit 1 mg L 9.3µM AgNO3
7	1 hour	Tri-Stain		
		Excystation		
		Infectivity	X	X

Figure 13. Usable Data from Testing Performed on *Cryptosporidium* for Silver Concentrations within the EPA Drinking Water Limit Range

All assays (tri-stain viability, excystation viability, and infectivity) were performed a minimum of three times for the higher silver concentrations (0.005M, 0.005M, and 0.5M). For the lower silver concentrations (0.93µM and 9.3µM), the infectivity assay was performed once.

Due to the lack of reported effects of silver on *Cryptosporidium*, the higher silver concentrations were tested to identify whether silver exposure would cause a noticeable effect at all. The inability to obtain data from some of the testing runs is the result of

several issues regarding the protocols. These issues are presented and discussed below in context with the results from each assay. One issue that is not assay dependent and that is important to consider when evaluating the results is that each run was performed on different batches of oocysts. Since *Cryptosporidium* is not able to be grown in culture, oocysts are obtained from infected animal specimens (mice) from a commericial source (Waterborne, Inc., New Orleans). Past experience in the laboratory has shown that viability and infectivity are reduced as the time from shedding from the animal host is increased. Thus, acquisition of oocysts is dependent upon the schedule of the outside vendor and tests were performed within two weeks of receipt of a lot of oocysts.

Tri-Stain Viability

Usable results were obtained from two separate runs (run #3 and #6); they are shown in Figures 14 and 15. For each run the oocysts were exposed to 0.005M and 0.05M silver concentrations for a contact time of 2 hours.

For some of the early runs where the tri-stain assay was performed, including run#3 shown in Figure 14, two potential sources of error/loss were identified. First, a large number of oocysts in the stock and the control samples were being observed as containing neither DAPI nor PI (DAPI- PI-), effectively categorizing them as empty, or dead, oocysts. The initial protocol had prescribed that the oocyst solution be dried at 37°C prior to staining and it was hypothesized that this elevated temperature was inducing excystation of oocysts and thus effectively increasing the numbers of empty oocysts in the final counts. In addition, the low recoveries were potentially attributed to

loss of oocysts during staining and the subsequent rinse cycle of the DAPI and PI to the oocysts after they had dried on the MeriFluor slide. The protocol was revised to introduce the stains in solution (see Material and Methods section for Silver Speciation Testing).

In addition, there are no results reported for either of these runs for the 0.5M treatment due to the fact that no oocysts were found upon microscopic examination of the slide. It is hypothesized that this is due to large interactions of the silver with the oocyst wall that interfere with the ability of the MeriFluor to stain the oocyst and possibly even physically damage to the oocyst wall. See the Excystation Results section below for more detail.

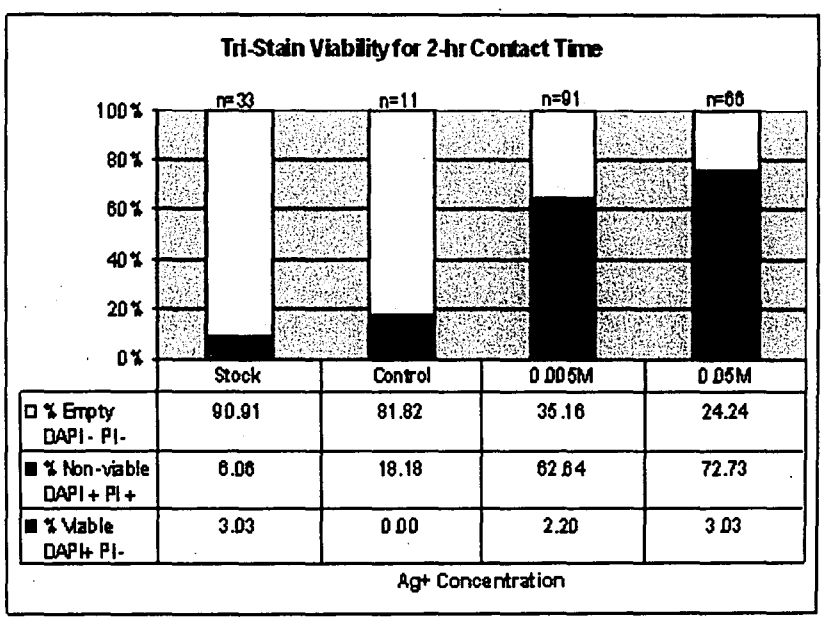


Figure 14. Tri-Stain Results for 2-hour Contact Time with 0.005M and 0.05M Silver Concentrations (Run#3).

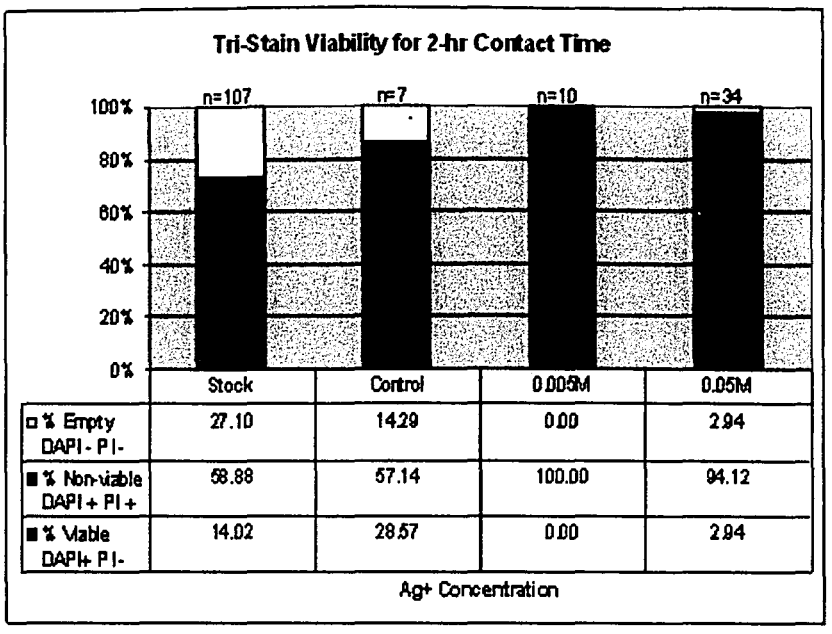


Figure 15. Tri-Stain Results for 2-hour Contact Time with 0.005M and 0.05M Silver Concentrations (Run#6).

Review of the data shown in Figure 15, for Run#6, shows that there is the potential for the silver to be having an effect on the oocysts. If the empty oocysts are not included, then the percent viability for the stock, control, and 0.05M treatment are 43%, 24%, and 3%, respectively.

Excystation Viability

For the excystation viability testing, two separate runs produced usable data; run#1 at contact times of 30 minutes and 1 hour, and run#6 at contact time of 2 hours. These results from run#1 and run#6 are shown in Figures 17 and 18, respectively. Initial review of the results obtained from a contact time of 30 minutes and 1 hour (Figure 17) suggest that the ability of oocysts to excyst is decreasing as the silver concentration is increased. However due to the low recoveries (n) for each sampling time/concentration, and the fact that multiple runs were not completed so error bars for the counts could not

be included, it is difficult to conclude whether the data show a true trend in the reduction of the oocysts' ability to excyst.

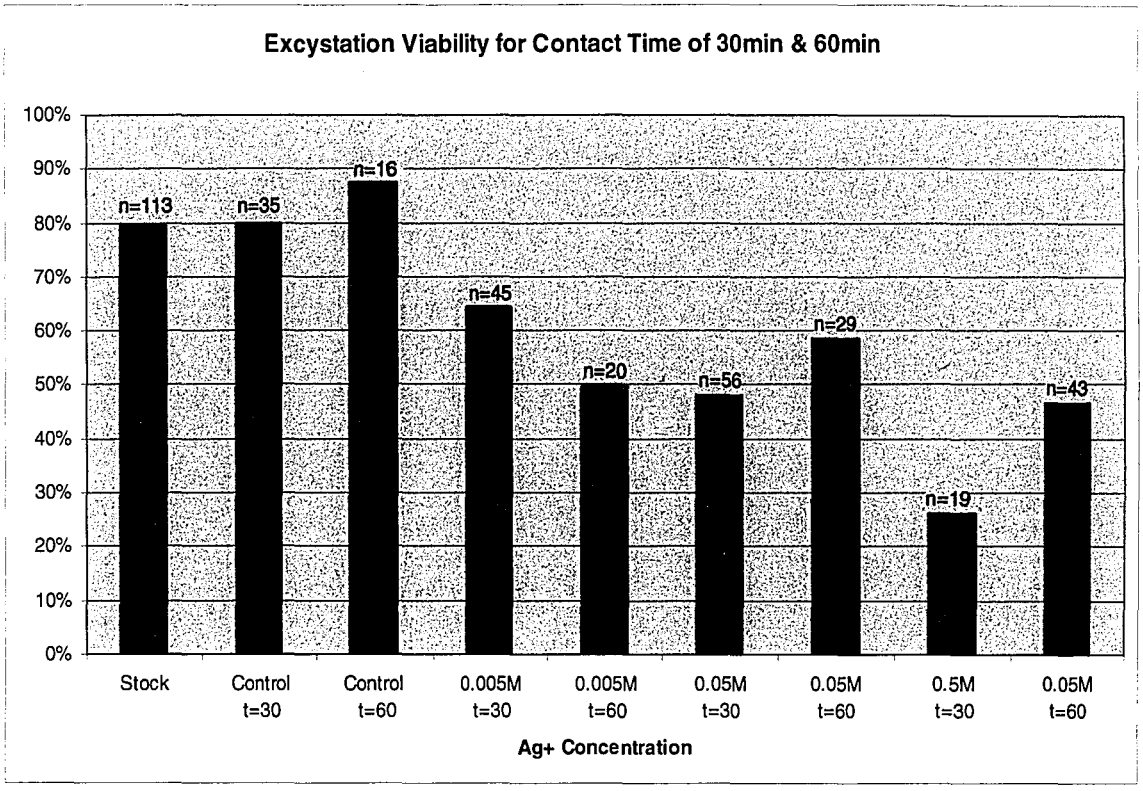


Figure 17. Excystation Results for Silver Concentrations of 0.005M, 0.05M, and 0.5M at Contact Times of 30 minutes an 1 hour (run#1)

Increasing the contact time to 2 hours produced less of an effect on the excystation results as is shown in Figure 18, a potential reason for this is the very low oocyst recoveries (<10 oocysts) achieved for the 0.005M and 0.05M tests. If these samples are discounted, then it appears that there may still be a reduction in excystation, as the stock and controls exhibited approximately 80% excystation and the 0.5M treatment only showed 47% excystation, equating to an approximate 41% reduction in excystation capability.

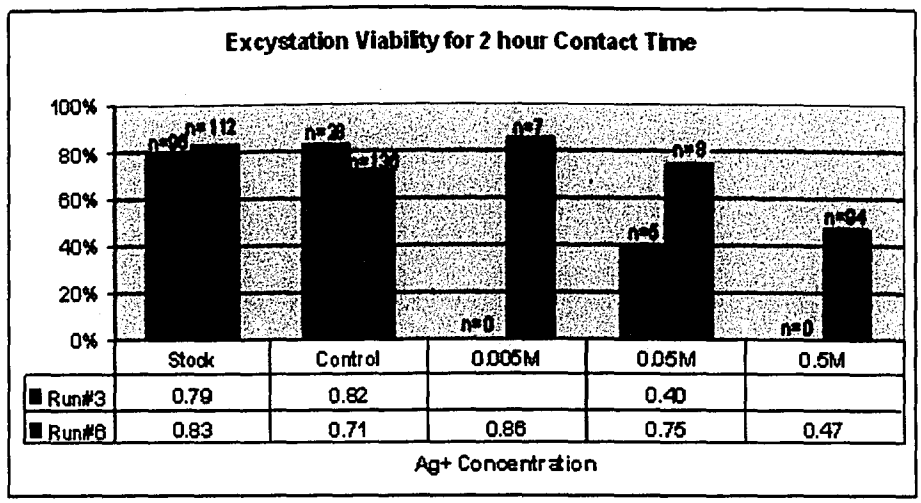


Figure 18. Excystation Results for Silver Concentrations of 0.005M, 0.05M, and 0.5M at Contact Time of 2 hours (run#6)

In addition, during visual microscopic examination, many of the oocysts in the 0.5M and 0.05M treatments were observed with only partial MeriFluor staining (Figure 19). It is hypothesized that this is due to interactions of the silver with the oocyst wall, interfering with the ability of the MeriFluor to stain the oocyst and possibly even causing physical damage to the oocyst wall. The low recoveries (n) exhibited in the results could be due to the fact that the oocysts were there but were not stained and therefore unidentifiable.

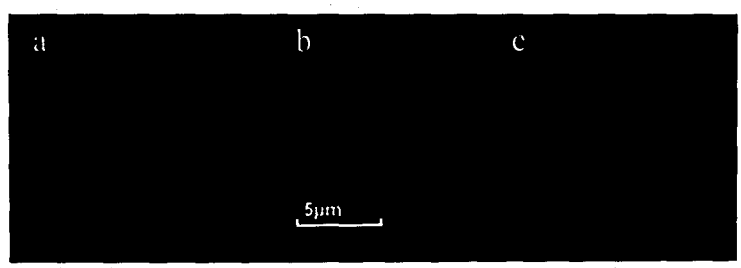


Figure 19. MeriFluor stained oocysts (a) intact with complete staining, (b) excysted with complete staining, and (c) excysted with incomplete staining.
(Photo (b) USEPA 2008, Photos (a) and (c) by author)

Due to the inability of the oocysts to stain completely, the applicability of this assay at high silver concentrations is questionable. In fact, both the tri-stain and excystation,

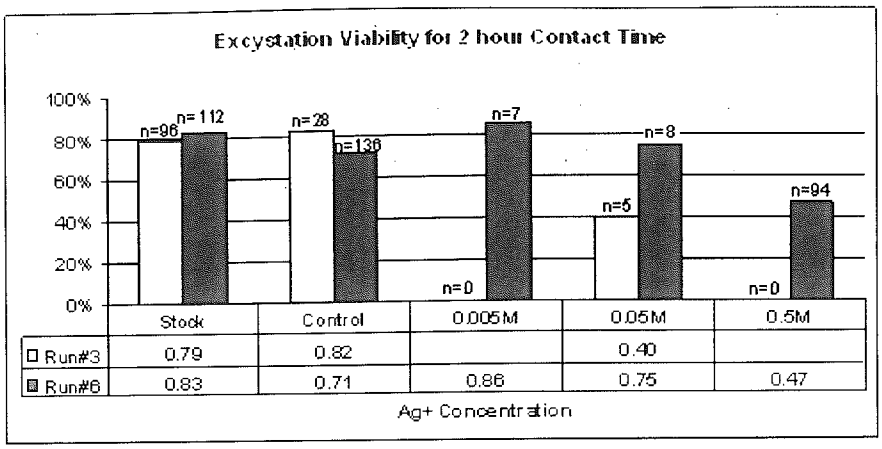


Figure 18. Excystation Results for Silver Concentrations of 0.005M, 0.05M, and 0.5M at Contact Time of 2 hours (run#6)

In addition, during visual microscopic examination, many of the oocysts in the 0.5M and 0.05M treatments were observed with only partial MeriFluor staining (Figure 19). It is hypothesized that this is due to interactions of the silver with the oocyst wall, interfering with the ability of the MeriFluor to stain the oocyst and possibly even causing physical damage to the oocyst wall. The low recoveries (n) exhibited in the results could be due to the fact that the oocysts were there but were not stained and therefore unidentifiable.

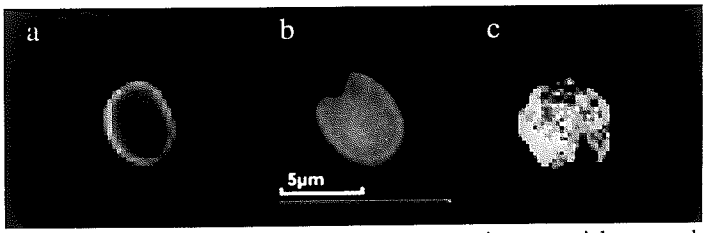


Figure 19. MeriFluor stained oocysts (a) intact with complete staining, (b) excysted with complete staining, and (c) excysted with incomplete staining.
(Photo (b) USEPA 2008, Photos (a) and (c) by author)

Due to the inability of the oocysts to stain completely, the applicability of this assay at high silver concentrations is questionable. In fact, both the tri-stain and excystation,

assays, utilize MeriFluor staining for identification of oocysts, and the results could have been skewed due to potential inability of treated oocysts to stain properly. However, it is clear from these results that silver is having an impact on the oocyst wall even if the exact mechanism cannot be identified at this point in time.

An attempt was made to quantify the numbers of completely stained (intact) oocysts and those oocysts that exhibited an inability to stain completely (damaged) in order to obtain an idea of the potential impact this damage could be having on the results. Figure 20 shows the results of the counting and supports the hypothesis that the applicability of this assay at high silver concentrations (>0.005M) may not be valid.

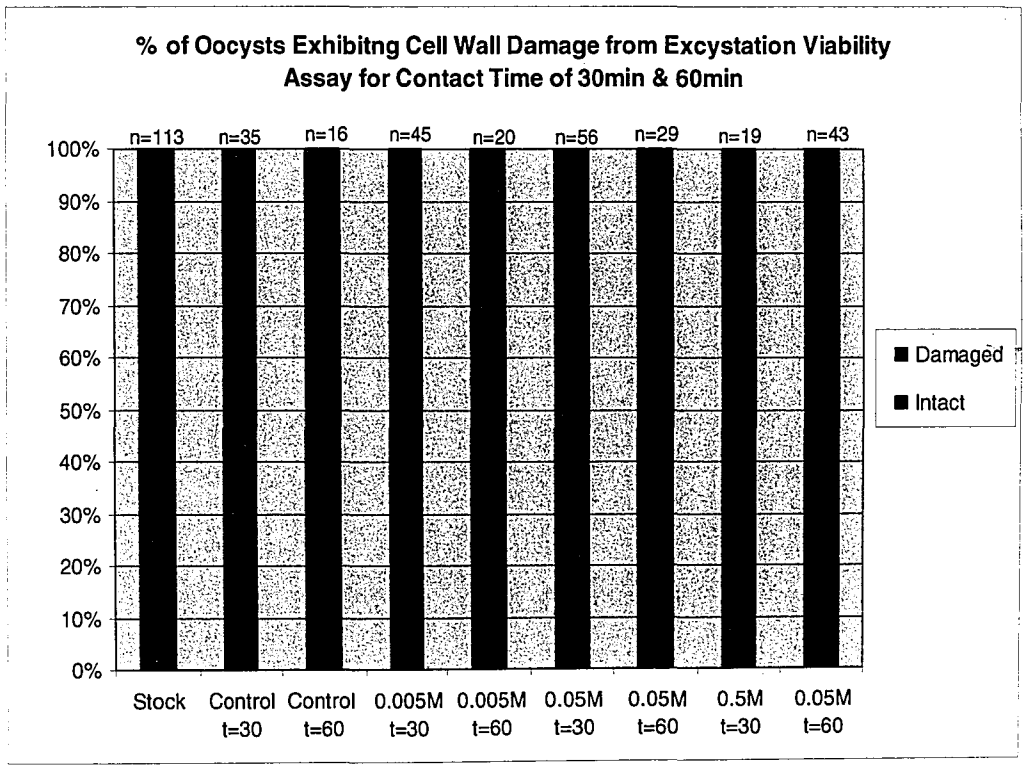


Figure 20. Percent of Sample Exhibiting Cell Wall Damage

Infectivity

For the infectivity testing, three separate runs produced usable data run#2 at contact times of 30 minutes and 1 hour and runs#4 and #5 at contact time of 2 hours (results shown in Figures 21 and 22, respectively). For run#2, no results were obtained for the two highest silver concentrations (0.5M and 0.05M) because there were no cells on slides. In fact the cells were observed to be "washing off" of the slides during processing. It was hypothesized that the oocyst solutions from these treatments contained enough silver that they were exerting a toxic effect on the cells. An additional rinse cycle was added to the protocol and cell health on the subsequent runs (run#6) was normal.

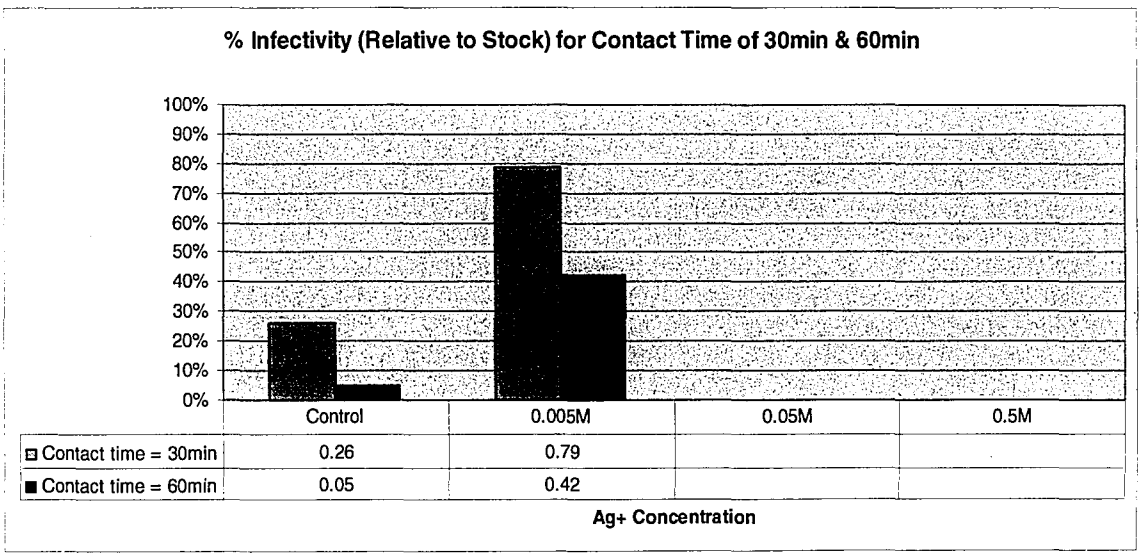


Figure 21. Infectivity Results for Silver Concentrations of 0.005M, 0.05M, and 0.5M for Contact Times of 30 minutes and 1 hour

Review of the data obtained from run#2 (Figure 21) suggests that infectivity may not be affected by the 0.005M treatment. However, the infectivity of the stock for this run (6.3%) was much lower than is normally expected. In addition, the control infectivity for this run was substantially lower than that of the 0.005M treatment.

Run#4 (Figure 22) had a stock infectivity of 19.3%, and the results support the hypothesis that it is possible that the 0.005M treatment is not having a large effect on infectivity. Because run#5 only had a stock infectivity of 5.2%, the results of this run are suspect (Figure 22). However, preliminary results from run#2 coupled with run#4 suggest that 0.005M $AgNO_3$ may not be affecting the infectivity of the oocysts.

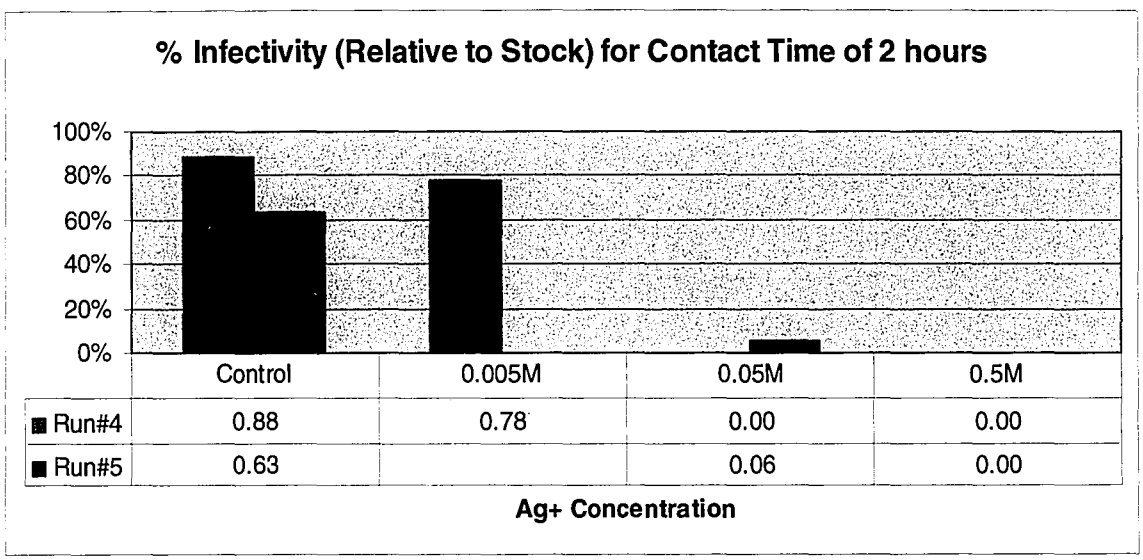


Figure 22. Infectivity Results of 0.005M, 0.05M, and 0.5M for Contact Time of 2 hours

The final data set obtained for infectivity was for a 1 hour contact time with treatments of silver concentrations corresponding to 0.1mg/L, the USEPA drinking water limit, and 1mg/L, ten times the EPA limit. The results of this run are shown in Figure 23. It appears that these much lower silver concentrations are still able to impart a negative effect on the infectivity of the oocysts. However, when viewed in comparison with the previous infectivity data, the 0.1 and 1mg/L treatments appear to be having a larger impact on infectivity than the higher (>+5000 times) silver concentrations.

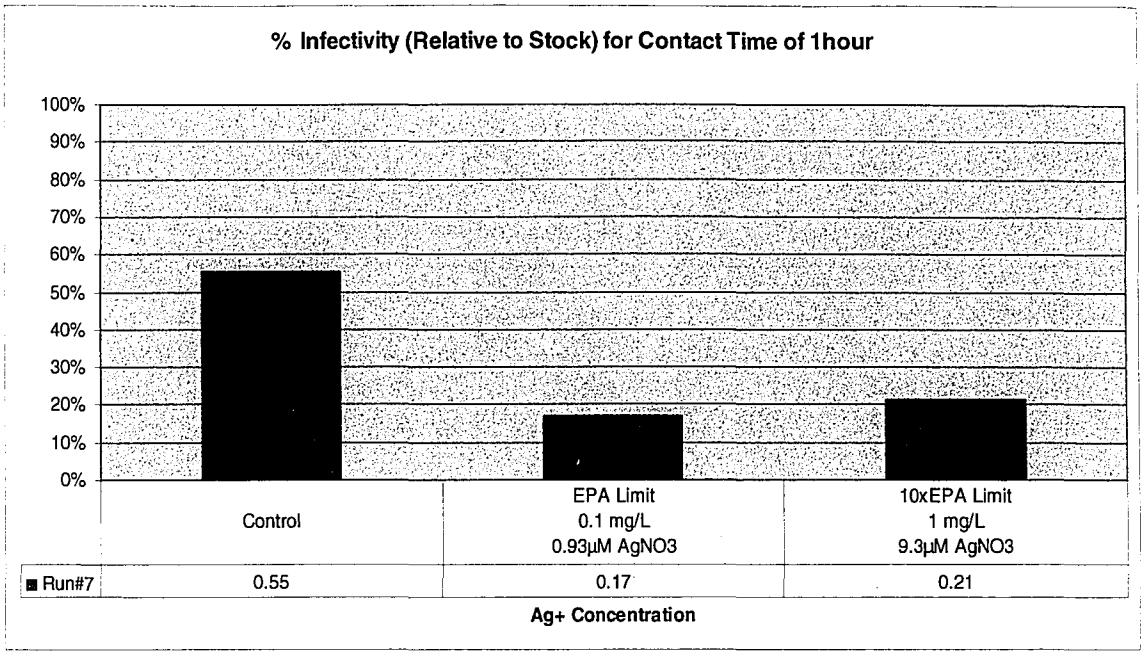


Figure 23. Infectivity Results of 0.1 and 1mg/L for Contact Time of 1 hour

Conclusions

The testing was successful in obtaining repeatable bactericidal effects of all the silver concentrations on *E. coli*, however, the effects of silver on *Cryptosporidium* remain unclear. This work has effectively identified that silver, at a high enough concentration, will impart some effect on the oocyst wall such that the successful application of surface stain is diminished. At lower concentrations, the effects of silver are not clear. Due to the conflicting infectivity data, additional runs need to be completed in order to draw a definitive conclusion regarding silver's impact on infectivity.

Future Work

Silver Speciation Testing

In order to draw a sound conclusion regarding silver toxicity and *Cryptosporidium* additional runs need to be completed. In particular, the entire range of silver

concentrations, from 0.5M to the EPA limit, need to be test in one run so that same lot of oocysts is used. Executing a run in this fashion will effectively reduce the number of variables, such as stock infectivity, that have been shown to be a problem. In addition, the test will need to be performed a minimum of three times in order to obtain an estimate of error. Once statistically valid and repeatable results are obtained for the silver ion effects on *Cryptosporidium*, testing will continue to look at other potentially toxic species.

Testing of the various silver-chloro complexes will be executed by varying the NaCl concentrations of the test water to mimic the levels of chloride in natural waters. For freshwater simulation, the chloride concentration will be 0.2mM; for brackish waters, chloride concentrations of 1, 7.5, and 15mM will be tested; and for seawater, a 550mM chloride concentration will be used.

The anticipated benefit from the results of this testing will be to identify whether any of the silver-chloro complexes are more bioavailable and thus more toxic to *E. coli* and/or *Cryptosporidium*. The same assays, as described previously, will be performed on the test microorganisms; and, all assays will be performed in triplicate (as a minimum) in an attempt to obtain repeatable, statistically valid results. Silver speciation calculations will be performed using the chemical speciation model MINEQL.

It is also anticipated that the silver speciation testing will continue to include disinfection efficacy under more complex environmental conditions. In particular, additional experiments will be performed using Lehigh River water (collected throughout the year

during baseflow and after storm events) in place of Millipore water and measuring coliform bacteria concentrations after disinfection. Some Lehigh River samples will also be spiked with *Cryptosporidium* oocysts, which are unlikely to be present in large quantity during base flow conditions, to determine the efficacy of oocyst disinfection by the different silver species under these more complex environmental conditions. Temperature, pH, specific conductivity, turbidity, and coliform density of the Lehigh River water will be measured before each test to assess the impact of these water quality parameters on disinfection efficacy. In addition, Millipore water will be adjusted to different combinations of pH, temperature, and turbidity and used in controlled experiments to further elucidate the impact of water quality on disinfection performance.

Silver Application Testing

Additional future work includes testing to evaluate the disinfection efficacy of solid phase silver. Several water purification systems in current use in developing countries use colloidal silver (CS) solutions on ceramic based filters. Testing will focus on the Potters For Peace (PFP) ceramic filter that uses a collodial silver solution applied to the inside of the filter. Testing to date has shown that there is no measurable loss of silver from the system into the purified water (Lantagne, 2001a). Previous work, however, has identified that the collodial silver reduces the pore size and structure of the filter medium, which reduces flowrate and increases the incidence of clogging (Fahlin, 2003). The intended outcome from optimizing the colloidal silver application is to extend the life of the filter by reducing clogging, maintain a high quality effluent, and keep costs at or below current levels.

The purpose of this work will be to test various combinations of filter materials and methods of silver application to fully utilize the entire path of water flow through the filter, minimize loss of silver into the effluent water, and maximize disinfection rates. While the PFP filters are solely manufactured with colloidal silver, other ceramic purifiers, such as grogs and candles, are also in use and leverage other application methods, specifically silver nitrate and silver chloride (Harvey 2007). In addition, research has shown that potential recontamination in the filter spigot and/or clean water receptacle can reduce a system's disinfection efficacy (Lantagne, 2001b). Thus, in addition to testing various silver application methods on a range of ceramics used in the manufacture of filter media, this study will also attempt to apply silver to the surfaces of concrete and plastic, materials routinely used for the clean water receptacle, to identify potential improvements in system disinfection.

A series of batch experiments will be performed using various types of container materials and three silver application techniques. For the container materials, two general types will be tested: filter materials and clean water receptacles. To test the disinfection efficacy of silver applied to the ceramics used in filters, grogs, and candles, pots made from terra cotta, white kaolin and black clay will be used. To test the disinfection efficacy of silver applied to the clean water receptacle, plastic and cement containers will be used. Currently the various silver solutions, collodial silver (CS), silver nitrate ($AgNO_3$) and silver chloride (AgCl), are applied to the filter materials either by immersion in the solution or by paint brush (Harvey 2007, Campbell 2005). To evaluate

the impact of application method, varying concentrations of CS, $AgNO_3$ and AgCl solutions will be applied via immersion and painting to the various containers. In order to evaluate the leaching properties of the containers, Millipore water will be placed in each silver-treated container, and samples will be taken at various times and analyzed for aqueous silver concentration.

To test silver disinfection efficacy of the various container/application combinations, Millipore water spiked with *Cryptosporidium* and *E. coli* (10^3oocysts/ml and 10^6cfu/ml, respectively,) will be placed into the containers. Water samples will be analyzed at various times after placement in the silver treated container for the percent inactivation of *E. coli* and *Cryptosporidium*. For the *E. coli* analysis, plate counts will be used to identify the concentration of live *E. coli* cells in the water at each time point. For the *Cryptosporidium* analysis, two viability assays and one infectivity assay will be performed at each time point. *Cryptosporidium* viability will be estimated using excystation and vital dye staining assays, and infectivity will be determined using an in vitro cell culture assay. Each assay will be performed in triplicate. Atomic absorption spectroscopy will be used to verify the aqueous silver concentration at each sampling time.

Conclusion

Silver is a promising disinfectant for use in a wide-range of water treatment applications, from small, point-of-use to large community-based distribution systems. The testing conducted to-date has confirmed the bactericidal properties of silver on *E. coli*; however the question remains as to whether silver has the ability to provide disinfection of *Cryptosporidium*.

Silver-loaded ion-exchange fibers (24, 40, 20 mg at 122mg Ag/g fiber) were shown to produce 100% disinfection of *E. coli* within 5 minutes. The fibers were also shown to release silver into solution and all amounts tested resulted in the finished product water containing aqueous silver concentrations above the current drinking water limit of 0.1mg/L.

The toxicity of the silver ion (Ag^+) species to *E. coli* and *Cryptosporidium* was tested over a range of concentrations (0.5M, 0.05M, 0.005M, 0.93μM, and 9.3μM) in laboratory grade water. For all concentrations tested 100% disinfection of *E. coli* was observed within 15 minutes. Viability and infectivity assays were performed on *Cryptosporidium* for all concentrations. It is hypothesized that at high concentrations (0.5M and 0.005M) silver ions are having an effect on the cell well based on the observation that treated oocysts have a diminished ability to react with a *Cryptosporidium*-specific surface stain. Conflicting data was obtained for both the viability and infectivity assays at lower concentrations, and thus definitive conclusions cannot be drawn at this time. While

additional testing needs to be completed, the results of this testing show that silver ions represent a potential alternative disinfectant for microbial contaminants of concern in drinking water.

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