ABSTRACT

Lindsey Dawn Witmer: Efficacy of BacSan as a Point-of-Supply Drinking Water Disinfectant
(Under the Direction of Mark D. Sobsey, Ph.D.)

Point-of-supply disinfection is one promising means of providing safe drinking water to the nearly one billion people lacking access around the world. BacSan is an aqueous solution of natural ionic minerals that may serve as an effective option for point-of-supply disinfection; charged ions in the solution may inactivate microbes by binding DNA and/or disrupting cells walls and other structures. This project evaluated BacSan disinfection by varying concentration and time to meet the performance criteria set by the US EPA and WHO for the reduction of *Escherichia coli*, *Klebsiella terrigena*, male-specific coliphage MS-2, and Poliovirus type 1. Appreciable reductions of microbial indicators were observed. For *E. coli* B, a reduction of ≥6.8 $\log_{10}$ (≥99.9999%) was achieved (24 hours, 13.3 µl BacSan/L), for *K. terrigena*, a reduction of 8.5 $\log_{10}$ (99.9999997%) was achieved (24 hours, 20 µl BacSan/L), for MS-2, a reduction of >5.5 $\log_{10}$ (>99.9997%) was achieved (24 hours, 20 µl BacSan/L), and for Poliovirus-1, a reduction of 1.321 $\log_{10}$ (92.89%) was achieved (24 hours, 28.6 µl BacSan/L). This study also evaluated the potential health risks posed to users of BacSan. It was found that BacSan contributed concentrations of nitrates and copper above those considered safe for human health, according to the US EPA and WHO. BacSan also contributed concentrations of aluminum and zinc at levels that may cause negative aesthetic effects in treated water. We conclude that BacSan significantly reduces concentrations of some representative indicator microorganisms in raw water, though it may cause health risks in some populations.

For Eve

And for every child around the world who is killed prematurely because humankind's learning curve with regard to love and kindness is so tragically inadequate.

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1. Introduction

More than one billion people, or close to one in every five persons on Earth, lack access to an improved water source (WHO 2004). An improved water source can include a well, borehole or collected rainwater, but such improved sources do not necessarily deliver microbiologically or chemically safe water. This significant lack of access to clean water has meant that every year 1.6 million people die from diarrheal disease related to unsafe water, sanitation, and hygiene (USAID 2007). The vast majority of these deaths occur in children under 5 years of age; these problems represent the third highest cause of death in children worldwide (WHO 2008).

The economic effects of unclean water are also felt deeply. According to the World Health Organization (WHO), the health burden resulting from lack of access to clean water translates into the loss of an estimated 5.5 billion productive days per year and costs of over US$11 billion in medical treatment costs (UNICEF 2006). Further, children in the developing world are, in many places, chronically infected with parasitic protozoa and worms as a result of drinking unclean water. Such infections significantly interfere with a child's nutrient absorption in its most formative years, leading to malnutrition and failure to thrive. The consequences of these very prevalent infections are dire, as the child's development both intellectually and physically is often irreversibly and substantially thwarted (Sobsey 2008). The deleterious health effects of consuming unsafe drinking water thus perpetuate the cycle of poverty in which many of world's citizens are currently entrenched.

Increasing access to clean water forwards five of the eight Millennium Development Goals, including goals 1, 4, 5, 6 and 7. These goals are, respectively, to

eradicate extreme poverty and hunger; reduce child mortality; improve maternal health; combat HIV/AIDS, malaria and other diseases; and halve the number of people without access to safe drinking water by 2015.

The ideal long-term solution to providing clean water is to create private, piped connections to households from a municipal water source. A centrally treated and regulated water source can best guarantee that water entering households meets minimal quality standards. Unfortunately, this is not a feasible short-term goal due to the lack of infrastructure and the political and economic instability of those nations whose citizens are most in need of access to clean water.

The most feasible short-term alternatives for providing clean water lay in household water treatment and safe storage (HWTS) interventions. Such interventions can lead to dramatic improvements in drinking water quality, making an immediate difference to the lives of those who rely on water from polluted lakes, streams and, in some cases, unsafe "improved" sources such as wells or rainwater catchment systems. Disinfectants, physical and/or chemical treatment options, and various water treatment technologies, can provide effective household water treatment. When these are combined with safe storage, good hygiene practices and wide implementation, they can significantly increase the amount of individuals with access to safe water.

This research project evaluated the effectiveness of BacSan, an aqueous solution of natural ionic minerals, including silver, gold, aluminum and copper that may serve as an effective option for point-of-supply drinking water disinfection in developing countries. BacSan is a highly concentrated disinfectant meant for the treatment of large volumes of water. It has the potential to be a viable alternative to chlorine; a product that

is highly effective against target microbes but is often rejected by users due its effect on the taste of water and its formation of toxic by-products.

This project evaluated various concentration and times to determine the conditions required for BacSan to meet the performance criteria set by the US EPA and WHO for the reduction of five target microbes, including two bacteria and three viruses. In order to determine this, four one-liter volumes of raw water containing 10 µL (1:35,000), 13.3 µL (1:50,000), 20 µL (1:75,000), or 28.6 µL (1:100,000) of BacSan were measured for concentrations of added *Escherichia coli*, *Klebsiella terrigena*, male-specific coliphage MS-2, and Poliovirus type1 initially and after 10, 30, 90, 240 and 1440 minutes.

This project also evaluated the possibility of deleterious taste and health effects resulting from the use of BacSan. To determine these possibilities, US EPA and WHO guidelines were utilized.

This report presents and interprets the findings from these studies.

2. Objectives

2.1. Overall Research Aims

- To evaluate the effectiveness of BacSan as a point-of-supply water disinfectant based on its ability to reduce concentrations of two indicator bacteria (*E. coli* and *K. terrigena*), a fecal indicator virus (male-specific coliphage MS-2) and two pathogenic viruses (Poliovirus-1 and Rotavirus SA-11) in water
- To determine what volume and contact time of BacSan is required to achieve the 6-log10 performance criterion for bacteria as set by the US EPA, WHO, and NSF-International

- To determine what volume and contact time of BacSan is required to achieve the 4-log10 performance criterion for viruses as set by the US EPA, WHO, and NSF-International
- To determine if BacSan contributes chemical constituents to treated water above safe levels of exposure as determined by the US EPA and WHO
- To determine if BacSan contributes chemical constituents to treated water above levels of exposure that would result in deleterious health and/or aesthetic effects as determined by the US EPA and WHO

2.2. Overall Approach

- BacSan was added to raw surface water at concentrations of 1:100,000; 1:75,000; 1:50,000; and 1:35,000 in water containing high concentrations of target microorganisms
- Concentrations of target microbes were determined initially and after 10, 30, 90, 240, and 1440 minutes
    - Concentrations of bacteria were determined using membrane filtration assays (US EPA method 1603)
    - Concentrations of MS-2 were determined using single agar layer infectivity assays (US EPA method 1602)
    - Poliovirus and Rotavirus SA-11 concentrations were determined using most probable number (MPN) mammalian cell culture infectivity assays

- A negative control, consisting of raw surface water containing target microbes but without BacSan, was assayed simultaneously to determine the extent of microbial reductions that were due to BacSan as opposed to natural die-off
- Existing chemical data was used to determine the safety and aesthetic effects of drinking water treated with BacSan

3. Background

3.1. Waterborne Disease in Developing Countries

3.1.1. Definitions

3.1.1.1. Waterborne Disease

Waterborne disease is caused by exposure to pathogenic microorganisms through the ingestion of contaminated water; this can occur through drinking, physical contact, or ingestion of food containing contaminated water. Exposure typically occurs through the fecal-oral route of transmission, though in some cases waterborne diseases can result from organisms that proliferate in water but are not fecally associated, such as *Legionella* bacteria. Waterborne infectious organisms typically cause enteric diseases classically manifest as diarrhea. Cases of diarrhea are 500% more prevalent in the developing world than in the developed world (Sobsey 2008).

Most waterborne disease is caused by microbes, as opposed to chemicals. The four classes of microbes are bacteria, viruses, protozoan, and helminthes. Bacteria are cellular prokaryotes that range from 0.5-2.0 μm in diameter. Viruses are composed of nucleic acid surrounded by a protein coat; they are the smallest and structurally simplest of the classes of microbes, ranging from 0.02-0.3 μm in diameter. Protozoan are

eukaryotes that range from 2 µm-2 mm in diameter. Helminthes, or worms, are muticellular parasites transmitted through their eggs; these eggs range from 25-150 µm.

Waterborne diseases compose one classification of disease transmission routes related to water. Other routes include water-washed, water-based, and water-related diseases. Water-washed diseases are caused by a lack of access to sufficient volumes of water for use in personal hygiene. Water-washed diseases include insect infestations such as from lice, ticks, or mites. Other diseases of the skin and eyes can also be water-washed infections, as they can be a result of direct contact with water contaminated to a high concentration due to an overall lack of water. Water-based diseases are caused by parasites found in organisms living in water. Helminthes generally cause diseases classified as water-based. Water-related diseases are caused by insect vectors that breed in water; malaria and Yellow Fever are examples of water-related diseases.

3.1.1.2. Developing Countries

Developing countries include those nations that earn a moderate or low score on the Human Development Index (HDI), a measure of human development created by the United Nations. The HDI is a compound indicator of statistical indexes including but not limited to per capita income, gross domestic product, life expectancy, and literacy rate. Developing countries exhibit a low standard of living as a consummation of factors such as low income, high population growth, and limited industrialization. The term does not infer that the same standard of living or specific or even similar challenges are faced by all nations that fall within this category.

The term "developing countries" as used in this report may also be referred to as less developed countries, least economically developed countries (LEDCs),

underdeveloped nations, undeveloped nations, or third world nations. The term LEDC is often utilized to moderate the euphemistic nature of alternative terms or to refer to those nations that cannot be considered developing. The term "third world," as opposed to the term "first world" in relation to the richest nations, is no longer in common usage because such terms are considered ethnocentric with a bias favoring Western culture. Critics counter that the term "developing" in reference to the poorest nations is a misnomer; they hold that "re-developing" is often a more accurate term, as many of the nations referred to as "developing" once had highly-developed economies and societies prior to colonialism.

The term "developing country" is clearly imperfect, as well as imprecise, as is its opposite term, "developed country," which will be used in reference to highly industrialized nations with high scores on the HDI (e.g. The United States). However, in recognition of these weaknesses, such terms will be used in this report for their simplicity and accessibility.

According to the International Monetary Fund (IMF), there are a total of 128 developing nations in the world. Of these, there are 45 developing nations in Africa, 21 in North and Central America, 12 in South America, 30 in Asia, and 20 in Oceania (IMF 2008). As of 1998, of the 6 billion people on Earth, 4.8 billion, or 80%, lived in the developing world (US AID, 1999).

3.1.2. Transmission of Infectious Agents and Outcomes of Infection

Pathogens can enter the body through the respiratory tract, enteric (gastrointestinal) tract, genitourinary tract, skin, or eyes. Such entry can result from direct person-to-person or person-to-animal (in the case of zooinoses) contact, or indirect contact through fomites, droplets, or airborne particles. Entry can also occur through ingestion of contaminated water or food, or through contact with disease vectors. Other potential sources of pathogens include transmission through intrauterine or transplacental routes, organ transplants, or exposure to blood and/or blood products. Figure 1 diagrams the most common routes of transmission of infectious agents.

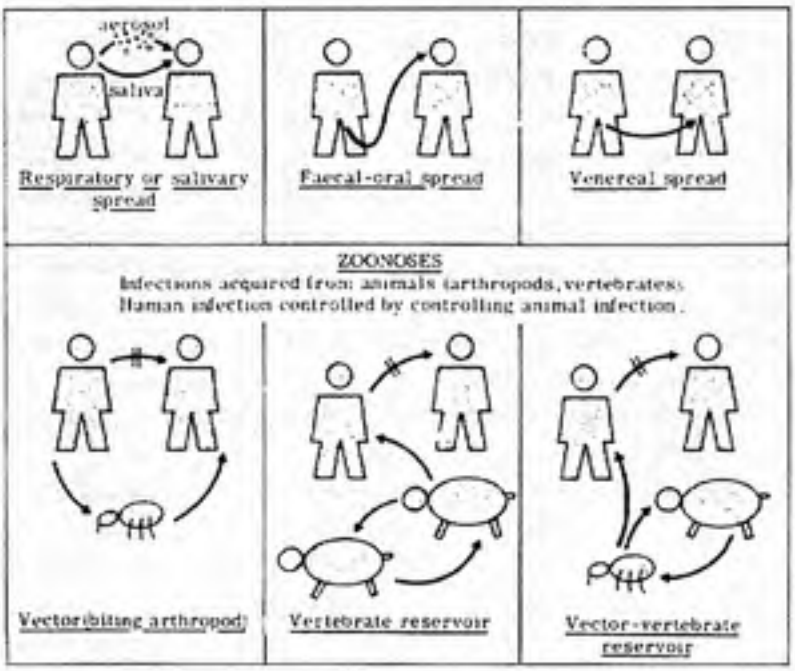


Figure 1. Transmission routes of infectious agents (Sobsey 2008)

The transmission of infectious agents is influenced by agent (microbe) factors, environmental factors, and host factors.

Agent factors include the existence of sufficient resources and reservoirs for microbe breeding, as well as the microbe's ability to persist and to be transported in the

environment. Another factor is the agent's ability to enter a portal in potential hosts and to reach and proliferate at target sites within the host. The success of the microbe is further dependent on its ability to be excreted from the host in order to infect others; this in part explains why the classical manifestation of waterborne infections is diarrheal disease. The virulence and infectious dose of the agent also affects its survival.

There are many environmental factors that influence the transmission of infectious agents. The existence of appropriate reservoirs, vectors, and vehicles (e.g. fomites or bodily fluids) for agents is necessary. Suitable physical, chemical, and biological conditions for the survival of a given agent must also be present. Temperature, relative humidity, and sunlight are examples of physical factors. Chemical factors such as the existence of nutrients or antimicrobial chemicals are also influential, as are biological factors like local parasitism or vector activity. Many environmental factors that are deleterious to the survival of infectious agents in the developed world are rarely present in much of the developing world. For example, household climate control, antimicrobial cleaners, and dwelling and working spaces sealed from the environment are often very rare in the developing world, especially in rural settings. As such, agents are free to proliferate in the environment and in close quarters with human activity, increasing the odds of exposure, infection and disease.

Host factors that affect disease transmission include the host's age, nutritional status, and overall immunocompetence and health. The genetic makeup of the host can also impact the virulence of infectious agents, as can the behavior and habits of the host (e.g. hand washing or water filter use). Because most individuals in the developing world often are of poor nutritional and health status, they are more likely to be infected, and it is

more likely that this infection will result in serious illness. The disease burden in the developing world is also much higher than in the developed world; this causes a cycle of poor health status as individuals with pre-existing diseases are more likely to be immunodepressed or immunoincompetent, resulting in increased susceptibility to infectious agents, and thus further increased disease burden and mortality. Further, lack of sanitation and sources of clean water in the developing world, as well as lack of education about the importance of each, results in a situation in which chronic exposure to infectious agents is highly likely.

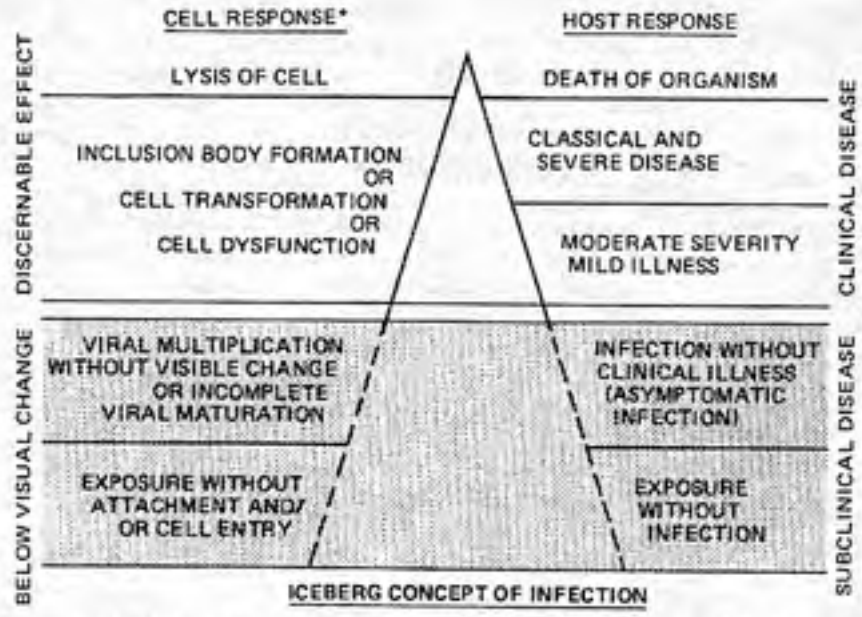


Figure 2. Iceberg concept of infection for viruses (Sobsey 2008)

Exposure to an infectious agent does not always result in illness. A host can be exposed to an agent, but the agent may not successfully infect the host. An agent may successfully infect the host, but not have enough of a deleterious impact on the host to result in disease. Otherwise, an agent can cause illness, and this may end in the host eliminating the agent or the agent causing the death of the host. The "Iceberg concept of

infection" in Figure 2 illustrates this concept for viruses. For the reasons outlined above, the disease outcomes at the tip of the iceberg are much more likely in the developing world than in the developed world.

3.1.3. Prevalent Waterborne Pathogens

The most common waterborne pathogens cause enteric (gastrointestinal) infection and diarrhea. Important enteric viruses include enteroviruses, such as polios, echos, and coxsackie viruses, as well as Hepatitis A and E viruses, rotavirus, adenovirus, reovirus, and norovirus. Prevalent enteric bacteria include *Salmonella typhi*, *Campylobacter spp.*, *Vibrio cholerae*, *Shigella* spp., and *Escherichia coli*. Important protozoan parasites include *Cryptosporidium parvum*, *Cyclospora cayetanensis*, and *Giardia lamblia*. Prevalent pathogenic helminthes include Nematodes (roundworms), hookworms, and Cestodes (tapeworms).

Table 1 outlines the most significant waterborne pathogens, their persistence, infectivity, and other traits relevant to their public health significance. The long persistence and high infectivity of protozoan parasites and viruses, such as the rotavirus and poliovirus (an enterovirus) examined in this study, is particularly noteworthy. The ability of some bacteria, such as *Legionella*, to proliferate, rather than simply persist in water supplies, presents a unique public health challenge as well.

Pathogen	Health significance	Persistence in water supplies[a]	Resistance to chlorine[b]	Relative infectivity[c]	Important animal source
Bacteria					
Burkholderia pseudomallei	Low	May multiply	Low	Low	No
Campylobacter jejuni, C. coli	High	Moderate	Low	Moderate	Yes
Escherichia coli – Pathogenic[d]	High	Moderate	Low	Low	Yes
E. coli – Enterohaemorrhagic	High	Moderate	Low	High	Yes
Legionella spp.	High	Multiply	Low	Moderate	No
Non-tuberculous mycobacteria	Low	Multiply	High	Low	No
Pseudomonas aeruginosa[e]	Moderate	May multiply	Moderate	Low	No
Salmonella typhi	High	Moderate	Low	Low	No
Other salmonellae	High	May multiply	Low	Low	Yes
Shigella spp.	High	Short	Low	Moderate	No
Vibrio cholerae	High	Short	Low	Low	No
Yersinia enterocolitica	High	Long	Low	Low	Yes
Viruses					
Adenoviruses	High	Long	Moderate	High	No
Enteroviruses	High	Long	Moderate	High	No
Hepatitis A virus	High	Long	Moderate	High	No
Hepatitis E virus	High	Long	Moderate	High	Potentially
Noroviruses and sapoviruses	High	Long	Moderate	High	Potentially
Rotaviruses	High	Long	Moderate	High	No
Protozoa					No
Acanthamoeba spp.	High	Long	High	High	No
Cryptosporidium parvum	High	Long	High	High	Yes
Cyclospora cayetanensis	High	Long	High	High	No
Entamoeba histolytica	High	Moderate	High	High	No
Giardia intestinalis	High	Moderate	High	High	Yes
Naegleria fowleri	High	May multiply[f]	High	High	No
Toxoplasma gondii	High	Long	High	High	Yes
Helminths					
Dracunculus medinensis	High	Moderate	Moderate	High	No
Schistosoma spp.	High	Short	Moderate	High	Yes

Table 1. Waterborne pathogens and their significance in water supplies (WHO 2008)

3.1.4. Indicator Microorganisms

Rather than directly measuring pathogenic microbial concentration in water, indicator microbes are often used. The presence and amount of microbial contamination in water is estimated with use of indicators because these can be safer to work with and easier to detect and quantify than pathogens.

An ideal indicator is present in higher concentrations and is more persistent than its pathogenic counterpart, though its presence is correlated with the presence of the

pathogen. Ideal indicators are present in all types of water, are easily detected and are harmless to humans and animals. Indicators should also have constant characteristics and not exhibit independent growth in the environment.

The most common bacterial indicator is *Escherichia coli*, and the most common viral indicator is the male-specific coliphage, MS-2. There are currently no highly acceptable indicators identified for protozoan parasites. In this study, viral reductions by BacSan will be measured through its reduction of the indicator virus, MS-2, and bacterial reductions by BacSan will be measured through its reduction of the indicator bacteria, *E. coli* and *K.terrigena.* BacSan's reduction of two prevalent pathogenic viruses, poliovirus, an enterovirus, and rotavirus, will also be measured.

3.1.5. Diarrheal Disease

There are four billion acute cases of diarrheal disease and 1.6 million deaths caused by the disease annually, 90% of which occur in children. Diarrheal disease is a leading cause of illness and death among the world's children, second only to respiratory infection (Institute for OneWorld Health 2008). Of these cases of diarrheal disease, 88% are attributed to unsafe water supply and inadequate sanitation and hygiene (WHO 2004). Besides high levels of morbidity and mortality, the burden of diarrheal disease caused by inadequate water and sanitation results in the loss of 644 million workdays per year (UNESCO and WHO 2006). The economic ramifications of diarrheal disease likely extend far beyond this statistic, however. Cases of diarrhea within the first two years of life can have irreparable long-term deleterious effects on the physical and intellectual development of children due to reduction in appetite and decreased nutrient absorption. A 2007 study found that diarrhea and associated weight loss in infancy causes persisting IQ

deficits at eight years (Emond 2007). This loss of potential human capital goes uncalculated. Further, diarrheal disease is also often a comorbidity factor in deaths due to diseases such as HIV/AIDS and malaria.

Death from diarrheal disease is usually a result of dehydration. Symptoms of diarrheal disease include thirst, restlessness, sunken eyes, and decreased skin turgor and can progress to diminished consciousness, rapid and feeble pulse and low or undetectable blood pressure (Institute for OneWorld Health 2008).

Though it is a global problem, diarrheal disease is especially prevalent and deadly in the developing world.

3.1.6. Drinking Water Standards

International drinking water guidelines are set by the World Health Organization (WHO) as the basis for regulation and standard-setting on the national level. These settings address microbial, chemical, radiological and acceptability aspects of water safety. Such standards depend on risk assessments and associated calculations used to estimate tolerable risk levels or concentrations of contaminants. In setting tolerable risk levels for microbial contaminants the WHO includes estimates of the likelihood of infection per organism, per year, and per day, based on the consumption of a liter of water per day at various microbial contamination levels (WHO 2006). The tolerable risk level is based on the amount of contamination that would cause the maximum acceptable level of Disability Adjusted Life Years (DALYs). DALYs are a unit of health care status based on the loss of health and years of life due to disability from disease (NICHSR, 2007). The WHO holds that an acceptable level of contamination is that which results in a maximum of 10^{-6} DALYs per person per year.

Standards that meet or exceed those set by the World Health Organization are enforced throughout most of the developed world. In the United States, drinking water standards are set by the US Environmental Protection Agency (US EPA) and the National Sanitation Foundation (NSF) International. Due to the lack of infrastructure and the relative political and economic instability of developing nations, drinking water standards are rarely, if ever, enforced.

Relevant to the assessment of drinking water treatment products such as BacSan are standards set by the WHO, EPA, and NSF-International on the minimum percent reductions of various contaminants that must be met in order for such a product to be considered effective. WHO standards hold that protozoan parasites should be reduced by 99.9% (3-log $_{10}$), bacteria by 99.9999% (6-$\log_{10}$), and viruses by 99.99% (4-$\log_{10}$) (WHO 2006). These standards are also based on the maximum acceptable level of 10^{-6} DALYs per person per year. Such performance targets are typically higher for the developing than the developed world because the disease burden is higher in the developing world due to increased susceptibility caused by poor health status and higher proportions of children under the age of five.

In additional to microbial guidelines, the US EPA maintains guidelines on the maximum contaminant levels for organic and inorganic chemicals, disinfectants, and disinfectant biproducts. These guidelines take the form of National Primary Drinking Water Regulations (NPDWR) and National Secondary Drinking Water Regulations (NSDWR). The NPDWR sets legal limits for the amounts of certain contaminants allowed in drinking water, based on the adverse health effects of consuming greater than this amount of the contaminant. The NSDWR sets guidelines that are not legally binding

for contaminants that will not cause negative health effects but may cause negative cosmetic effects to the consumer (e.g. skin or tooth discoloration) or negative aesthetic effects (e.g. strong taste or odor) to the water. These regulations are particularly pertinent when evaluating a disinfectant for its potential to cause adverse health effects due to its chemical make-up.

3.1.7. Household Water Treatment Options

The burden of disease caused by lack of clean water in the developing world, coupled with the recent international spotlight shone on this disease burden through the Millennium Development Goals and the Water for Life Decade of 2005-2015, has motivated an upsurge in research on and development of point-of-use (POU) and point-of-supply (POS) household water treatment options. Recent systematic reviews established that such household-scale water quality interventions can be effective in reducing the burden of diarrheal disease, with mean reductions of 39% - 44% in users versus non-users (Clasen et al 2006; Fewtrell et al 2005). Point-of-use interventions, however, have been found to be more effective than point-of-supply interventions in reducing disease incidence. According to a recent meta-analysis on water treatment interventions, the relative risk, or risk of disease in exposed populations versus unexposed populations, for POS interventions is 0.89 (95% CI: 0.42-1.90), while the relative risk for POU interventions is 0.61 (95% CI: 0.46-0.81) (ibid.). This implies that users of POS interventions are 11% less likely than non-users to become ill, while users of POU interventions are 39% less likely than non-users to become ill. The potential for recontamination of water between its source and its consumption likely underlies this

discrepancy. Table 2, below, outlines the microbial effectiveness of the leading household water treatment (HWT) technologies.

HWT Technology	Virus	Bacteria	Protozoa	Residual
Chlorination	Medium	High	Low	Yes
Solar Disinfection	High	High	High	Safe Storage
BioSand Filter	Unknown	Med-High	High	No
Ceramic Filter	Unknown	Med-High	High	No
Filtration & Chlorination	Medium	High	Unknown	Chlorine
Flocculation & Chlorination	High	High	High	Chlorine

Table 2. Microbial effectiveness of household water treatment (HHWT) technologies (Lantagne, Quick et al 2007)

3.1.8. Safe Storage, Sanitation and Hygiene

Household treatment of water must be coupled with safe storage, sanitation and hygiene in order to be optimally effective in reducing the transmission of waterborne disease, though it has been proven effective without other measures.

Safe storage implies that water is stored between treatment and consumption such that it is protected from recontamination. Safe storage also refers to the way that water is withdrawn from storage for use. For example, a spigot attached to a jug of treated water is likely to prevent recontamination more than if water was withdrawn by dipping a cup into the treated water, where microbes on the hands of the user or on the cup could cause recontamination of the entire supply.

Adequate sanitation is a necessary preventative step for water contamination. It is estimated that while 1.1 billion people lack access to an improved water source, 2.4 billion do not have access to improved sanitation (WHO 2004). As a consequence of inadequate or nonexistent sanitation facilities, such as toilets or wastewater treatment

systems, there is little to no containment of fecal matter or other pathogenic vehicles, thus increasing the contamination of local water sources.

Another significant means of preventing waterborne disease transmission is through hygienic practices. For example, a large-scale school hand washing program in China was found to significantly reduce absenteeism. Children in control groups experienced a median of 2.0 episodes of diarrhea and 2.6 days of absence per 100 student-weeks, while children in the hand washing program experienced a median 1.2 episodes and 1.2 days of absence per 100 student-weeks (Bowen et al 2007). This is only one of many studies that show that hygienic habits are a highly effective way to reduce contact with pathogens and thus reduce disease prevalence.

3.2. Microorganisms Studied

This report examines the effectiveness of BacSan, a point-of-supply disinfectant intended for use in the developing world, in reducing the microbial load of drinking water. This report sought to examine BacSan's effectiveness against the indicator bacteria *E. coli* and *K. terrigena*, as well as MS-2, a male-specific coliphage and common viral indicator, and Poliovirus-1 and Rotavirus SA-11, two common pathogenic viruses.

3.2.1. *Escherichia coli*

3.2.1.1. Historical and Public Health Significance

Coliform bacteria are the most common indicators of fecal contamination, and *E. coli* is the most feces-specific of these bacteria. Most strains of *E. coli* do not readily replicate in the natural environment, adding to their reliability as indicators of fecal contamination (Sobsey 2008). The concentration of *E. coli* in water is considered to be a

reliable indicator of fecal contamination and the extent of that contamination. Though there are some strains of *E. coli* that are pathogenic, nonpathogenic strains are most often used for laboratory assays. *E. coli* is easy to work with because it grows quickly and has simple nutritional requirements. Further, each strain of *E. coli* has been thoroughly characterized genetically and otherwise and can thus be distinguished by various methods and easily cultured and enumerated, as well.

3.2.1.2. Physical Characteristics and Taxonomy

E. coli is a rod-shaped, facultative anaerobic bacterium. There are six strains of *E. coli* that cause enteric infections, including enteroadherent *E. coli* (EAEC), enteroaggregative *E. coli* (EAggEC), enterohaemorrhagic *E. coli* (EHEC), enteroinvasive *E.coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enterotoxigenic *E. coli* (ETEC). There are two strains that cause extraintestinal infections, including uropathogenic *E. coli* (UPEC), and neonatal menigitis *E. coli* (NMEC). *E. coli* belongs to the proteobacteria phylum, the class of gamma proteobacteria, order of enterobacteriales and family enterobacteriaceae.

3.2.2. *Klebsiella terrigena*

3.2.2.1. Historical and Public Health Significance

Klebsiella is a coliform bacterium and a common indicator of fecal contamination due to its prevalence in the human enteric tract. *Klebsiella* may also colonize the skin or pharynx in humans. *K. terrigena* expresses similar virulence factors as the pathogenic and most medically significant species of *Klebsiella*, *K. pneumonia*; it is considered a non-pathogenic indicator of this and other pathogenic species of *Klebsiella* as well as other

fecally-derived contaminants. It is a well-characterized bacterium with simple nutritional requirements that make it easy and quick to culture in a laboratory setting.

3.2.2.2. Physical Characteristics and Taxonomy

Klebsiella terrigena is one of 7 bacterial species of the *Klebsiella* genus. Like *E. coli*, it belongs to the proteobacteria phylum, the class of gamma proteobacteria, order of enterobacteriales and family enterobacteriaceae. It is a nonmotile, rod-shaped, gram-negative bacterium with a polysaccharide capsule.

3.3.3. MS-2

3.2.3.1. Historical and Public Health Significance

MS-2 is significant not for its infectivity or direct impact on human health, but more for its compatibility as a surrogate for pathogenic viruses in public health studies. The size, shape, and nucleic acid composition of MS-2 is similar to hepatitis A and poliovirus, two significant public health threats. MS-2, however, is not pathogenic and is thus safe for use in laboratory studies. It easily and rapidly propagates in the presence of *E. coli*, which makes it easy to enumerate, particularly through the Single Agar Layer (SAL) method. Further, MS-2 is one of the smallest viruses and this makes it particularly useful in the testing of water filtration systems.

3.2.3.2. Physical Characteristics and Taxonomy

The MS-2 virus is a non-pathogenic ribonucleic acid (RNA) coliphage commonly used as a viral indicator. MS-2 is negatively charged, 0.025 μm in size and icosahedral in shape. It contains a single strand of RNA with 3,569 nucleotides. MS-2 belongs to the genus *Levivirus* and family *Leviviridae*.

3.2.4. Poliovirus

3.2.4.1. Historical and Public Health Significance

The disease caused by poliovirus, poliomyelitis, or sudden paralysis, was first described in writing by Michael Underwood in 1789. However, some, notably John Paul (1971), point to an Egyptian stone carving dating between 1580 and 1350 B.C. that appears to depict a young man afflicted with the disease. Thus, the history of poliovirus and its effects on mankind may pre-date written history.

In the past two centuries, poliovirus has been the subject of a great deal of research, as outbreaks of the disease dating to the early 1800s in Europe have presented a particularly salient public health issue. Between 1840 and the 1950s, the world experienced a global epidemic of poliovirus infection. However, the sub-microscopic nature of poliomyelitis' causative organism resulted in doctors speculating as late as the second half of the 19th century that the disease was caused by things such as teething, upset stomach and trauma (ibid.). Finally, in 1908, two Austrian physicians, Karl Landsteiner and E. Popper identified the polio virus. Jonas Sulk created the first effective polio vaccine in 1952; the currently used oral polio vaccine, created by Albert Sabin, replaced this vaccine in 1962. The use of polio vaccines, combined with surveillance efforts and effective sanitation and hygiene measures, have been highly effective in eradicating the disease in the developed world. However, outbreaks of poliomyelitis occurred as recent as 2005 in Sudan and Nigeria, indicating that the virus remains a threat in much of the developing world.

3.2.4.2. Physical Characteristics and Taxonomy

Poliovirus is considered the simplest of those viruses with public health significance, consisting of only a single-stranded RNA genome and a protein coat, or capsid. It is icosahedral in shape and its genome is about 7,500 nucleotides long. Poliovirus is in the *Enterovirus* genus and the *Picornaviridae* family. Three serotypes of poliovirus exist, PV1, PV2, and PV3. Each serotype has a slightly different capsid protein, though all three forms are extremely infectious. PV1, or poliovirus-1, is the most common form of poliovirus; a non-pathogenic strain of this serotype was used in this research.

3.2.4.3. Disease Transmission and Symptoms

Poliovirus is a waterborne disease transmitted primarily through the fecal-oral route in humans. In 95% of cases of poliovirus infection, no symptoms are experienced. However, even without signs or symptoms, infected individuals may shed the virus intermittently in their stool for several weeks and in some cases, for months. In areas with poor sanitation, a single person with poliovirus can potentially infect hundreds of others before the first symptomatic case of polio appears. Polio symptoms can range from mild, flu-like symptoms such as sore throat, nausea, fever, vomiting, and constipation or diarrhea, to its classical manifestation, severe and sudden paralysis of the limbs (Mayo Clinic, 2008). Polio can also cause aseptic meningitis.

3.2.5. Rotavirus SA-11

3.2.5.1. Historical and Public Health Significance

Currently, almost every child in the world is infected with rotavirus at least once before the age of five (Cincinnati Children's Hospital, 2008). In the United States, approximately three million cases of gastroenteritis a year are caused by rotavirus, resulting in an estimated 500,000 visits to the doctor and 50,000 hospitalizations a year. In a developed nation such as the US, 20 to 40 deaths occur each year from rotavirus infection, while in developing nations, an estimated 500,000 children die of rotavirus infection annually (ibid.). This elevated morbidity is likely the result of higher prevalence of the disease due to inadequate sanitation, lack of clean water, lack of medical treatment, and more children under the age of five, combined with a variety of host factors conducive to severe infections. Richard Ward, PhD, and David Bernstein, MD, developed a rotavirus vaccine that was approved by Mexico in 2004, though it is not yet commercially available around the world.

3.2.5.2. Physical Characteristics and Taxonomy

Rotavirus refers to a genus of icosahedral, double-stranded RNA viruses in the family *Reoviridae*. There are seven species of rotaviruses, referred to as species A, B, C, D, E, F and G. Rotavirus A is endemic worldwide and the most common rotavirus; it is responsible for 90% of human infections. A non-pathogenic strain of this serotype was utilized in this research.

3.2.5.3. Disease Transmission and Symptoms

Rotavirus is a highly contagious and primarily waterborne disease transmitted through the fecal-oral route in humans and animals. While only 10-100 infectious particles are required for transmission of the disease, the feces of an infected person can contain more than 10 trillion infectious particles per gram (Cliver 1997).

Symptoms of the disease range from mild to severe and can include vomiting, fever, and watery diarrhea. Dehydration is the most common cause of death from diarrhea, and severe cases of this symptom are more common in rotavirus infections than in those caused by bacterial pathogens.

An individual infected with rotavirus is asymptomatic for an incubation period of about two days before symptoms appear. Rotavirus infection is also frequently asymptomatic in individuals who have been previously infected. Thus, there exists a high frequency of individuals carrying the virus unknowingly. This fact, coupled with the ease of infection transmission, is responsible for the immense disease burden caused by rotavirus, particularly in areas without adequate sanitation.

3.3. BacSan

BacSan is a highly concentrated point-of-supply disinfectant produced by the Water Development Program (WDP), a for-profit company that operates out of the United Kingdom. It is meant for the treatment of large volumes of water, specifically for "use in polluted reservoirs, rivers, and other water sources" (WDP 2006). The manufacturer holds that BacSan is "all natural and chemical free," and decreases turbidity and disinfects water while maintaining a residual to prevent recontamination (ibid.). This report aims to examine these claims, particularly evaluating BacSan's ability to reduce

concentrations of bacteria and viruses in treated water, maintain a residual, and not cause deleterious health or aesthetic effects.

BacSan has the potential to be an alternative to chlorine, as this highly effective disinfectant has fallen out of favor recently due to the discovery that chlorine creates toxic disinfection byproducts, trihalomethanes (THMs). Individuals also prefer not to use chlorine because of the characteristic odor and taste it produces in water. If BacSan is found neither to be toxic to human health nor to cause negative aesthetic effects while producing adequate microbial reductions, then it could be a very promising chlorine alternative.

3.3.1. History and Target Users

BacSan was introduced by WDP along with its Point-of-Use counterpart, OneDrop, in 2006. Both products are intended primarily for use in the developing world, though the manufacturer acknowledges other uses, as well. According to WDP, these products can be used to remove the biofilm in pipes, canals and water systems without causing corrosion. They can also be used as an algacide. This report principally evaluates BacSan's utility as a disinfectant for use in the developing world.

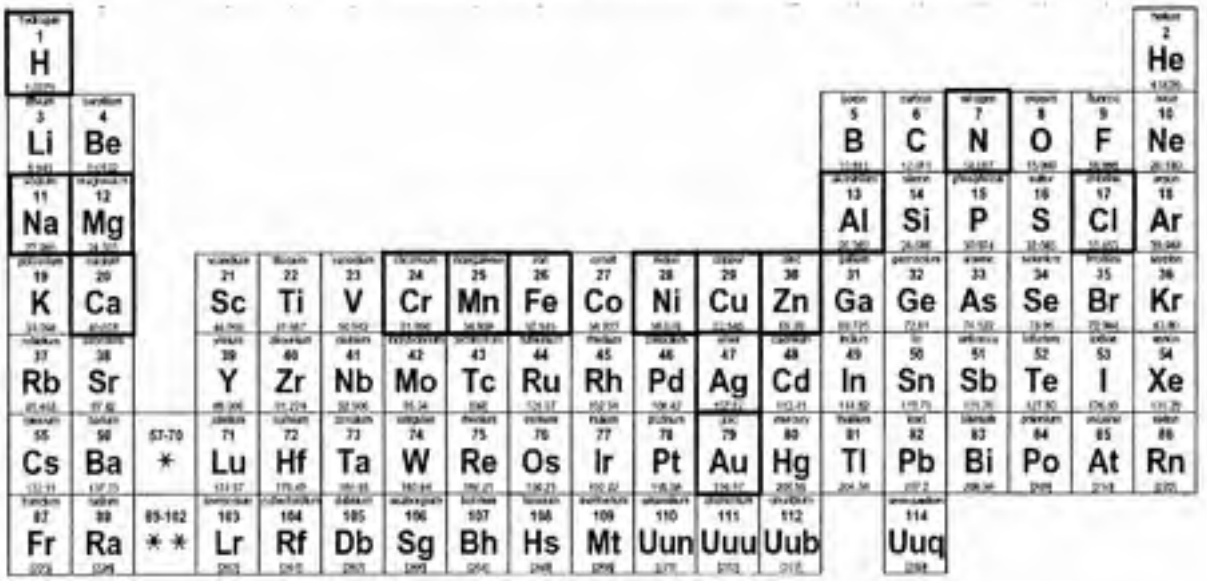
3.3.2. Composition

BacSan is composed of a mixture of chemical elements in various volumes as shown in Table 3, below. It is unknown how many of these elements are present in their ionic forms or other forms, or to what extent. The smallest tested volume of BacSan was 10 µL and the largest was 28.6 µL per liter of raw water.

Table 3. Ingredients in BacSan

Element	Molecular Weight	Ionic Charge	mg/L BacSan	mg/10µL BacSan	mg/28.6µL BacSan
Silver	107.87	1+	5	5E-06	1.4E-05
Copper	63.546	1+	95000	0.095	0.2717
Gold	196.97	1+	0.02	2E-08	5.72E-08
Aluminum	26.982	1+	6300	0.0063	0.01802
Calcium	40.074	2+	23	2.3E-05	6.6E-05
Chromium	51.996	1+	38	3.8E-05	0.00011
Iron	55.845	2+	170	0.00017	0.00049
Magnesium	24.305	2+	16	1.6E-05	4.6E-05
Manganese	54.938	2+	10	0.00001	2.9E-05
Sodium	22.99	1+	7.6	7.6E-06	2.17E-05
Nickel	58.693	2+	29	2.9E-05	8.3E-05
Zinc	65.39	2+	39,000	0.039	0.11154
Nitrates	varies	varies	280,000	0.28	0.8008
Chloride	35.453	1-	61	6.1E-05	0.00017

Figure 3. Position of BacSan ingredients on the Periodic Table of the Elements



3.3.2.1. Silver

In the late 1800s, Carl von Nageli observed that algae disappeared when exposed to small amounts of copper or silver. Since this observation, and some will hold that even

thousands of years prior to this event, silver has been known and successfully used as an antimicrobial agent; the antimicrobial properties of silver may underlie the fact that eating utensils are composed of this element in much of the western world. Some bacteria have developed resistance to silver in areas where they are regularly exposed to large amounts of the chemical, such as in areas surrounding silver mines or in hospital burn wards, though this is not a widespread phenomena (Rodriguez et al 2007). The mechanism(s) by which silver inactivates microbes is unknown, though it is speculated that silver in its ionic form, Ag^+, may bind DNA or inhibit the function and/or form of the cell membrane (ibid.). However, despite our incomplete understanding of how silver works, the element has been used widely and successfully in disinfectants for over a century and more recently as an additive to household water filters, such as Brita and Pur.

3.3.2.2. Copper

Copper has a history similar to silver's in its use as a disinfectant. It has been shown that storing contaminated water in copper containers can significantly decreases microbial load (Mehta et al 2008). Copper, like silver, exists naturally as a cation, Cu^+, and though its mechanism as a disinfectant is also not completely understood, it is thought to work similarly to silver in disrupting cell membranes and nucleic acid. As disinfectants, silver and copper are often used in concert because they operate synergistically; their effects together are greater than each of their individual effects.

3.3.2.3. Gold

Gold, or Ag^+ in its ionic form, though in the same group on the periodic table as both copper and silver, does not share a storied history for its use as a disinfectant, but

rather as a status symbol. Gold has been historically used as the primary construction material of objects and containers used by high status individuals, and it is possible that gold's antimicrobial properties could in part underlie this fact. However, though gold has not been traditionally used or studied as a disinfectant, the synergistic effects between cations such as gold (i.e. that between copper and silver) have been well documented (Rodriguez et al 2007), and it is thus logical that gold could work in concert with copper, silver and other ions as a successful disinfectant.

3.3.2.4. Aluminum

Some sources claim that aluminum was used as an astringent for dressing wounds by the ancient Greeks and Romans, though in the modern world, aluminum was not isolated until 1827 by Friedrich Wohler. Since this time, aluminum has been used for purposes from casting statues to wrapping food. Aluminum is currently included in many disinfectants; one of the earliest patents for the use of aluminum in a compound for the disinfection of water was issued in 1950 to Morris Carrell (FPO 2007).

3.3.2.5. Calcium

Calcium in the form of calcium hypochlorite, or $Ca(ClO)_2$, a type of chlorine, has long been used as a disinfectant, though it was not until the early 1990s that it became a commercial success as a water disinfectant, namely in the form of a product known as "Pool Shock". It is unknown what form calcium takes in BacSan, however.

3.3.2.6. Iron

The role of iron as a disinfectant has been recently explored. It is unknown in what form iron takes in BacSan, but iron (VI) and iron (V), are strong oxidants that can

react with pollutants and in the process reduce to iron (III), a non-toxic byproduct. These reactions are relatively fast though they increase in speed at lower pH. Low doses of Fe (VI) can inactivate a wide variety of microorganism and it also possesses coagulation properties (Sharma 2004). Fe (V) is often used to inactivate microorganisms with Fe (VI) resistance; Fe (V) reacts with microbes three to five times faster than Fe (VI) (ibid).

3.3.2.7. Magnesium, Nickel, Zinc, Chloride and Chromium

Magnesium in the form of magnesium borate, magnesium salicylate and magnesium sulfate are used as antiseptics in the medical field. Nickel salts are typically used as antiseptics, and are known to arrest fermentation and plant growth. Zinc sulphate and zinc chloride have been utilized as bactericides, and most notably as late in antimicrobial, alcohol-based handwashing products. Chloride is a well-known and oft-used disinfectant for use on surfaces, in antiseptic wipes, and other disinfection products. Chromium has not been widely utilized as a disinfectant, though it is a well-known pollutant. The US EPA (2008) holds that the maximum allowable level of chromium in drinking water is 0.1 mg/L, and the WHO holds that it is 0.05 mg/L.

3.3.2.8. Sodium

Sodium is used as a disinfectant in the form of iodbenzoic, iodosobenzoic, and iodoxybenzoic acids, sodium chloride, and sodium bicarbonate. Sodium bicarbonate was found to be a highly effective antiseptic for medical use during World War II, particularly in Poland during the insurrection in Warsaw. At this time, doctors had no access to their usual antiseptics and instead used 5% sodium bicarbonate solution, the only potential

antiseptic they could find, and found it to be highly effective. Sodium chloride is commonly used in mouthwash and as a general cleaner.

3.3.2.9. Nitrates

The nitrate ion has the molecular formula NO_3-, though the term nitrate can refer to any organic compound with a nitrate substituent, e.g. $RONO_2$, where R is any hydrocarbon. Silver nitrate is used as a disinfectant in podiatry and is also placed in the eyes of infants at birth to prevent blindness. Nitrates are a byproduct of the production of BacSan and for this reason remain in the final product at high concentrations.

3.3.3. Potential Mechanisms of Microbial Inactivation by BacSan

BacSan may inactivate microbes via the combined synergistic effects of each of the elements in solution, as well as the compounds they may form together. Silver is likely a main active ingredient, as its effectiveness as a disinfectant in small volumes has been well-researched and confirmed, as has its synergy with other ions such as those present in BacSan, particularly copper (Rodriguez et al 2007). There are five generally accepted mechanisms by which silver is believed to work (ibid.); these mechanisms may be generalizable to the other elements of BacSan.

1. Silver may electrostatically bind to negatively charged peptidoglycans on bacteria cell walls (Thurman and Gerba 1989). Among other disruptions, this would interfere with proton transport and thus energy production.
2. Cells may uptake silver due to its resemblance to essential metals of similar ionic size or charge (Slawson et al 1992; Solioz and Odermatt 1995).

3. Silver may bind essential enzymes and other cellular proteins, causing a decrease or loss of function (Slawson et al 1992).
4. Silver may replace the hydrogen bonds between the two strands of DNA, stabilizing the helix and preventing DNA replication and cell division (Modak and Fox 1973; Richards 1981; Thurman and Gerba 1989).
5. Silver may bind what would otherwise be electron-donating groups, undermining cell metabolism and energy production (Grier 1983; Modak and Fox 1973).

Compounding these mechanisms are the documented antimicrobial synergies among many of the elements present in BacSan. It is believed that oxidizers, such as chromium compounds and iron, may disrupt the cell wall and facilitate the flow of metals into the cell and thus the subsequent deactivation of DNA (Armon et al 2000; Straube et al 1995; Yahya et al 1992). Further, this role of oxidizers may reverse silver resistance in bacteria, as it has been shown that silver resistance works by blocking silver from entry through the cell wall via binding silver on the cell wall (Deshpande and Copade 1994; Shakibaie et al 1999). It has also been shown that the effects of silver and copper together are greater in the presence of small amounts of free chlorine (chloride) and that these three elements together achieve greater effects than would be predicted by any of them alone (Landeen et al 1989; Yahya et al 1990), and even greater than would be achieved by high levels of chlorine (Beer et al 1999). By capitalizing on such findings, BacSan may achieve the effectiveness of chlorine without the negative side effects that result from using large volumes of the latter disinfectant. Besides copper and chlorine, silver has also been shown to have synergistic effects with zinc. Lin et al (1998) demonstrated

that both silver and zinc alone were effective against *L. pneumophila*, but that the two elements together produced greater results than either of them alone.

3.4. Water Sources Used in Study

Raw water from the Orange Water and Sewage Authority (OWASA) Water Treatment Plant in Carrboro, NC, was collected. Water that originated from University Lake was collected May 21, 2007; July 16, 2007; and March 17, 2008. Water that originated from Cane Creek was collected on August 23, 2007. Water collected in 2007 was utilized in *E. coli, K. terrigena*, and MS-2 assays. Water collected in 2008 was utilized in poliovirus-1 and rotavirus assays. In all cases, water was stored in a 4°C refrigerator in a cubitainer until it was used.

Table 4. Source water parameters[1]

Parameter	UL 5/21/07	UL 7/16/07	CC 8/23/07	UL 3/17/08
Color (CU)	34	17	17	33
Ortho-P (mg/L)	<0.10	<0.10	<0.10	<0.10
Iron (mg/L)	0.25	0.13	0.33	0.48
Fluoride (mg/L)	<0.10	<0.10	<0.10	<0.10
Total Coliform (Colonies/100mL)	2203	534	762	3671
E coli	Absent	Present	Absent	Absent
Heterotrophic Plate Count (AVG CFU/mL)	215	740	325	>5700
Turbidity (NTU)	7.37	5.19	1.54	20.91
pH	6.82	7.01	6.57	6.65
Alkalinity (mg/L)	28.00	34	26	16
Hardness (mg/L)	28.00	32	23	27

[1]data from Orange Water and Sewage Authority
[2]Average of 12 trials over 12 hours
[3]Average of 2 trials over 12 hours
UL: University Lake
CC: Cane Creek
CFU: colony-forming unit
NTU: Nephelometric turbidity unit

4. Methods

4.1. Microbial Stock Preparation

4.1.1. *Escherichia coli* B

An overnight culture of *E. coli* B was grown by combining tryptic soy broth (TSB) with streptomycin/ampicillin (0.015% w/v each) and a small volume of a known *E. coli* B stock culture (obtained from the Mark D. Sobsey laboratory collection) and incubating with shaking at 37 °C overnight. The enrichment culture was then centrifuged (Sorvall Instruments RC-3B refrigerated centrifuge, DuPont Company, Medical Instruments, Wilmington, DE) at 2500 x g (3000 rpm) for 10 minutes at 4 °C to pellet the cells. The supernatant was removed and TSB containing 20% glycerol (v/v) was added. The sample was vortexed, aliquoted into desired volumes, and frozen at -80 °C. Prior to each experiment, an individual stock was thawed and centrifuged (Sorvall Instruments RC-3B refrigerated centrifuge, DuPont Company, Medical Instruments, Wilmington, DE) at 2500 x g (3000 rpm) for 10 minutes at 4 °C. The cell pellet was then resuspended in a 10 ml volume of the raw surface water to be used in the experiment (see 4.2. below) by vortexing for two minutes.

4.1.2. *Klebsiella terrigena*

The same procedure described in 4.1.1. was followed to grow up stock cultures of *K. terrigena* (obtained from the Mark D. Sobsey laboratory collection), however centrifugation (Sorvall Instruments RC-3B refrigerated centrifuge, DuPont Company, Medical Instruments, Wilmington, DE) was performed at 8000 x g (5000 rpm) to successfully pellet cells.

4.1.3. MS-2 Coliphage

4.1.3.1. Host Preparation

An overnight culture of *E. coli* Famp was grown by combining TSB containing streptomycin/ampicillin (0.015% w/v each) and a small volume of a known *E. coli* Famp stock culture (obtained from the Mark D. Sobsey laboratory collection) and incubating with shaking at 37 °C overnight. A small volume of this overnight culture was then inoculated into fresh TSB containing streptomycin/ampicillin (0.015% w/v each).

4.1.3.2. MS-2 Propagation and Purification

A 100 µL aliquot of a known MS-2 coliphage stock (obtained from the Mark D. Sobsey laboratory collection) was added to the log phase *E. coli* Famp culture. This mixture was incubated with shaking for approximately three hours, or until the OD_{520} was between 0.1 and 0.5. The enrichment was then divided into two equal volumes and chloroform was added to equal half the enrichment volume. The tubes were shaken vigorously for five minutes and were then centrifuged (Sorvall Instruments RC-3B refrigerated centrifuge, DuPont Company, Medical Instruments, Wilmington, DE) at 2500 x g (3000 rpm) for 20 minutes at 4 °C. The supernatant containing virus stock was removed and transferred to a large Petri dish within a biological hood to allow evaporation of remaining chloroform. After 30 minutes, aliquots of desired volumes of stock were frozen at -80 °C. Prior to each experiment, an individual stock was thawed.

4.1.4. Poliovirus type 1, strain LSc (Poliovirus-1)

A T-25 flask containing a monolayer of BGMK cells at 90% confluence was selected by visual inspection under a dissecting microscope. Maintenance media was

removed. A 100 microliter volume of Poliovirus type 1 stock culture (ATCC #VR-59; obtained from the Mark D. Sobsey laboratory collection) was diluted 1:1000 in 4900 µL maintenance media and added to the flask. The maintenance media referenced here and throughout this report was composed of a solution of 7.5% bicarbonate, hepes, L-glutamine, non-essential amino acids, sodium pyruvate, 4M $MgCl_2$, fetal calf serum, genatamycin, kanamycin, and nystatin. The flask was incubated at 37 °C for one hour and gently rocked by hand every 15 minutes to keep the inoculum distributed evenly over the monolayer. Fifty milliliters of maintenance media was then added and the flask was returned to the incubator until the cell layer exhibited cytopathogenic effects (>90% destruction upon visual inspection). The flask was next frozen at -20 °C for one week and then thawed at room temperature with agitation. The thawed liquid was transferred into a 15-mL conical tube to which two milliliters of PBS was added. Any remaining cells were scraped from the flask and added to the centrifuge tube. The liquid was centrifuged (Sorvall Instruments RC-3B refrigerated centrifuge, DuPont Company, Medical Instruments, Wilmington, DE) at 6000 x g (4500 rpm) for 30 minutes at 4 °C. The supernatant of virus was purified via chloroform extraction as was done for MS-2 and outlined in section 4.1.3.2. The virus stock was stored at -20 °C.

4.1.5. Rotavirus SA-11

A T-25 flask containing a monolayer of MA-104 cells at 90% confluence was selected by visual inspection under a dissecting microscope and maintenance media was removed. A 100 µL volume of Rotavirus SA-11 stock culture (ATCC #VR-899; obtained from the Mark D. Sobsey laboratory collection) was diluted 1:1000 in 4900 µL maintenance media and added to the flask. The maintenance media was comprised of a

solution of 7.5% bicarbonate, hepes, L-glutamine, non-essential amino acids, sodium pyruvate, 4M $MgCl_2$, fetal calf serum, genatamycin, kanamycin, and nystatin. The flask was incubated at 37 ˚C for one hour and gently rocked by hand every 15 minutes to keep the inoculum distributed evenly over the monolayer. Fifty milliliters of maintenance media was then added. The flask was returned to the incubator until the cell layer exhibited cytopathogenic effects (>90% destruction upon visual inspection). The flask was next frozen at -20 ˚C for one week and then thawed at room temperature with agitation. The thawed liquid was transferred into a 15-mL conical tube to which two milliliters of PBS was added. Any remaining cells were scraped from the flask and added to the centrifuge tube. The liquid was centrifuged (Sorvall Instruments RC-3B refrigerated centrifuge, DuPont Company, Medical Instruments, Wilmington, DE) at 6000 x g (4500 rpm) for 30 minutes at 4 ˚C. The supernatant of virus was purified via chloroform extraction as was done for MS-2 and outlined in section 4.1.3.2. The virus stock was stored at -20 ˚C.

4.2. Experimental Design

Five liters of raw surface water (Orange Water and Sewer Authority (OWASA), Carrboro, NC; see characteristics in Table 4) was brought to room temperature and turbidity was measured (Hach model 2100N turbidimeter). An appropriate volume of previously prepared microbial stock (*E. coli* B, *K. terrigena*, MS-2 coliphage, poliovirus-1, or rotavirus; see above) was added to the five liters and stirred via a magnetic stir bar for 30 minutes. The water was then dispensed into five one-liter volumes. An aliquot of seeded water was removed and the initial concentration of target microbe was determined (see 4.2.1 – 4.2.3 below). Different volumes of BacSan (as determined by the

manufacturer) were then added to each of four one-liter volumes: 10 µl (1:100,000), 13.3 µl (1:75,000), 20 µl (1:50,000), and 28.6 µl (1:35,000). The fifth one-liter volume did not receive BacSan treatment and served as a negative control to determine the natural microbial die-off over time. Each of the five volumes were then stirred with a sterile pipet for 30 seconds. Following stirring, water volumes were left undisturbed to allow flocculant formation. At 10, 30, 90, 240 and 1440 minutes (24 hours), an aliquot of water was removed from half-way down the water column of each of the five volumes of water and assayed to enumerate the target microbe (see 4.2.1 – 4.2.3 below).

4.2.1. *Escherichia coli* B

To determine the initial *E. coli* B concentration and *E. coli* B concentrations at each of the time points (10, 30, 90, 240, and 1440 minutes), a ten-fold dilution series of sample was prepared in phosphate buffered saline (PBS) and nine milliliters of appropriate dilutions were membrane filtered (45 µm, 47 mm diameter) (Millipore HAWG 047 S6 filters, 47 mm diameter, 0.45 µm pore size) in duplicate. PBS alone was membrane filtered and served as a negative control. Filters were placed on MacConkey agar plates and incubated at 37 ˚C for 18-24 hours (Eaton 2005).

Following incubation, pink colonies were enumerated and the concentration of *E. coli* B in the undiluted sampled was calculated (colony forming units (CFU)/ml). Log_{10} reductions were determined by subtracting the log_{10} concentration at each time point from the log_{10} concentration of the initial concentration. When no CFU were detected in the total volume assayed, the lower detection limit of the assay was reached. In this case, a log_{10} reduction value was calculated using 1 CFU in the total sample volume assayed.

This is indicated by the use of a less-than symbol (<) for the $\log_{10}$ concentration and a greater-than symbol (>) for the $\log_{10}$ reduction.

4.2.2. *Klebsiella terrigena*

The same method and maintenance media described for *E. coli* B (4.2.1) was followed for analysis of *K. terrigena*.

4.2.3. MS-2 Coliphage

A log phase culture of *E. coli* Famp was prepared by combining TSB, streptomycin/ampicillin (0.015% w/v each), and a small volume of an overnight *E. coli* Famp culture (preparation described in 4.1.3.) and incubating with shaking at 37 °C for approximately three hours, or until the OD_{520} was between 0.1 and 0.5. To determine the initial MS-2 concentration and MS-2 concentrations at each of the time points (10, 30, 90, 240, and 1440 minutes), a ten-fold dilution series of sample was prepared in TSB and appropriate dilutions were assayed in duplicate using the Single Agar Layer Method (US EPA Method 1602, modified). Quickly, 18 milliliters of appropriate dilutions were mixed with two milliliters of log phase host *E. coli* Famp and approximately 18 milliliters of molten double-strength tryptic soy agar (TSA) containing streptomycin/ampicillin (0.015 % w/v each) and 4M $MgCl_2$ (0.0038 % w/v each). The solution was mixed gently, poured evenly into two empty Petri dishes, and incubated inverted at 37 °C for 18 – 24 hours. Unseeded raw surface water diluted in TSB was assayed and served as a negative control.

Following incubation, plaques, or clear lysis zones in the agar, were enumerated and concentrations of MS-2 in the undiluted samples were calculated (plaque forming

units, (PFU)/ml). Log_{10} reductions were determined by subtracting the log_{10} concentration at each time point from the log_{10} concentration of the initial concentration. When no PFU were detected in the total volume assayed, the lower detection limit of the assay was reached. In this case, a log_{10} reduction value was calculated using 1 PFU in the total sample volume assayed. This is indicated by the use of a less-than symbol (<) for the log_{10} concentration and a greater-than symbol (>) for the log_{10} reduction.

4.2.4. Poliovirus type 1, strain LSc (Poliovirus-1)

Buffalo green monkey kidney (BGMK) cells (obtained from the Mark D. Sobsey laboratory collection) were prepared in 24-well plates. Cell monolayers were checked for 90% confluence via visual inspection under a dissecting microscope and maintenance media was removed. To determine the initial poliovirus concentration and poliovirus concentrations at each of the time points (10, 30, 90, 240, and 1440 minutes), a ten-fold dilution series of sample was prepared in maintenance media. One hundred fifty microliters of appropriate dilutions were transferred into each of five wells. One hundred fifty microliters of maintenancemedia alone was added to a sixth well to serve as a negative control. The BGMK cell culture plates were incubated in a CO_2 incubator at 37 °C for one hour to allow for viral adsorption to the cells. One milliliter of maintenancemedia was then added to each well and plates were incubated in a CO_2 incubator at 37 °C for 48 hours.

Following incubation, wells were examined under a dissecting microscope for cytopathogenic effects (CPE), characterized by cells clumping into spheres and separating from the monolayer. Greater than or equal to 90% CPE in a well was considered a positive well, while less than 90% CPE was considered a negative well

(Estes et al. 1979). The ratio of negative and positive wells per dilution was used to calculate the most probable number (MPN) of poliovirus in the undiluted sample (Blodgett 2006). When no CPE was detected in the total volume assayed, the lower detection limit of the assay was reached. In this case, a $\log_{10}$ reduction value was calculated using 1 CPE in the total sample volume assayed. This is indicated by the use of a less-than symbol (<) for the $\log_{10}$ concentration and a greater-than symbol (>) for the $\log_{10}$ reduction.

4.2.5. Co-spiking Experiments with Poliovirus type 1, strain LSc (Poliovirus-1) and Rotavirus SA-11

Initially, co-spiking experiments with poliovirus and rotavirus were conducted. To determine the initial concentrations and concentrations at each of the time points (10, 30, 90, 240, and 1440 minutes) for each virus, two separate samples were taken. The method described in 4.2.4 was followed for enumeration of poliovirus; rotavirus enumeration is described in 4.2.5.1.

4.2.5.1. Rotavirus SA-11

MA-104 cells were prepared in 24-well plates. Cell monolayers were checked for 90% confluence via visual inspection under a dissecting microscope. Maintenance media was removed. To wash serum from the cell monolayer, 150 microliters of rotavirus diluent was added to each well and well trays were rocked gently to ensure complete coverage of the monolayer with the diluent. The rotavirus diluent was composed of 91 mL of phosphate-buffered saline, 2 mL of a 100X solution of gentamicin and kanamycin, and 7 mL of a 30% solution of beef extract. After 30 seconds, the diluent was aspirated

from the wells. Trypsin working stock, a solution of trypsin powder (Type IX-S from porcine pancreas, Sigma-Aldrich catalogue #T-0303) and sterile 0.9% NaCl (0.0005% w/v), was added to the sample to result in a final trypsin concentration of 0.4 µg/mL. The sample was incubated at room temperature for one hour to allow the trypsin to fully digest the rotavirus coat. Poliovirus antisera (American Type Culture Collection, #VR-1001 SA/HO) was then added 1:100, based on the initial concentration of virus, and the sample was incubated at 37 ˚C for 30 minutes to allow for complete deactivation of poliovirus in the sample (Almeida et al. 1978).

To determine the initial rotavirus concentration and rotavirus concentrations at each of the time points (10, 30, 90, 240, and 1440 minutes), a ten-fold dilution series of sample was made in rotavirus diluent. One hundred fifty microliters of each appropriate dilution was transferred into each of five wells and 150 microliters of rotavirus maintenance media alone was added to a sixth well to serve as a negative control. The MA-104 cell culture plates were incubated at 37 ˚C for one hour to allow for viral adsorption to the cells. One milliliter of rotavirus maintenance media was then added to each well and plates were incubated at 37 ˚C for four days.

Following incubation, wells were examined under a dissecting microscope for cytopathogenic effects (CPE), characterized by cells clumping into spheres and separating from the monolayer. Greater than or equal to 90% CPE on a well was considered a positive well while less than 90% CPE was considered a negative well (Estes et al. 1979). The ratio of negative and positive wells per dilution was used to calculate the most probable number (MPN) of rotavirus in the undiluted sample (Blodgett 2006). When no CPE was detected in the total volume assayed, the lower detection limit

of the assay was reached. In this case, a $\log_{10}$ reduction value was calculated using 1 CPE in the total sample volume assayed. This is indicated by the use of a less-than symbol (<) for the $\log_{10}$ concentration and a greater-than symbol (>) for the $\log_{10}$ reduction.

4.2.6. Neutralization Control

For each co-spiking experiment, a neutralization control was included to ensure that complete inactivation of poliovirus by antisera was achieved. Maintenance media was removed from BGMK cells (90% confluence) and 150 microliters of sample treated with poliovirus antisera (1:100) was added to one well. The BGMK cell culture plate was incubated at 37 °C in a CO_2 incubator for one hour to allow for viral adsorption to the cells. One milliliter of maintenancemedia was then added to the well and the plate was incubated at 37 °C for 48 hours.

Following incubation, the well was examined under a dissecting microscope for cytopathogenic effects (CPE), characterized by cells clumping into spheres and separating from the monolayer. Any CPE observed on the BGMK cells would indicate incomplete deactivation of poliovirus, as rotaviruses are unable to infect the BGMK cell line. CPE would also indicate the presence of potentially false-positive rotavirus results, as poliovirus will infect MA-104 cells more rapidly than rotavirus.

4.3 Virus Optimization Assays

Prior to virus studies, a cytotoxicity and an interference assay were conducted to determine if BacSan affected the susceptibility of host cells to infection and/or if BacSan affected the infectivity of the viruses, respectively.

4.3.1. Cytotoxicity Assay

Buffalo green monkey kidney (BGMK) cell monolayers in six-well cell culture plates were checked for 90% confluence, as determined by visual inspection under a dissecting microscope, and maintenance media was aspirated. A 1:35,000 dilution of BacSan (the highest dilution of BacSan used in these laboratory studies) was prepared in a one-liter volume of room temperature raw surface water (OWASA, Carrboro, NC) and was stirred for 30 seconds. After 10 minutes, a ten-fold dilution series of raw surface water containing BacSan was prepared in PBS. To determine cytotoxicity due to raw surface water alone, a ten-fold dilution series of raw surface water without BacSan was also prepared in PBS. A 200 microliter sample of each dilution of raw surface water containing BacSan was added to each of five BGMK cell wells, while 200 microliters of the corresponding dilution of raw surface water without BacSan was added to a sixth well. BGMK cell culture plates were carefully swirled to ensure that the added samples covered the cell monolayer. Plates were incubated at 37 °C in the presence of CO_2 for one hour. Five milliliters of maintenancemedia were then added to each well and plates were incubated in a CO_2 incubator at 37 °C for 48 hours.

Following incubation, wells were examined under a dissecting microscope for damage to the cell monolayer. The wells on which the raw surface water containing BacSan was plated were compared to the wells on which the raw surface water without BacSan was plated. Damage to the cell layer from water containing BacSan would indicate if BacSan alone is toxic to the BGMK cell monolayer and at what concentration damage occurs. Toxicity of BacSan to BGMK cells in the absence of polioviruses would

suggest that BacSan may possibly lead to false-positive results when viral assays are conducted.

4.3.2. Interference Assay

Buffalo green monkey kidney (BGMK) cell monolayers in 24-well cell culture plates were checked for 90% confluence, as determined by visual inspection under a dissecting microscope, and maintenance media was aspirated. A 1:35,000 concentration of BacSan (the highest concentration of BacSan used in these laboratory studies) was prepared in a 100 milliliter volume of room temperature raw surface water (OWASA, Carrboro, NC) and was stirred for 30 seconds. A ten-fold dilution of the raw surface water containing BacSan was prepared in maintenance media. To determine interference due to raw surface water alone, a ten-fold dilution series of raw surface water without BacSan was also prepared in maintenance media. In duplicate, one hundred fifty microliters of each appropriate dilution of raw surface water containing BacSan was transferred into each of six wells. One hundred fifty microliters of maintenance media alone was added to each of six wells to serve as a negative control. Similarly and in duplicate, 150 microliters of each appropriate dilution of raw surface water not containing BacSan was transferred into each of six wells. Again, 150 microliters of maintenancemedia alone was added to each of six wells to serve as a negative control. The BGMK cell culture plates were incubated in a CO_2 incubator at 37 °C for one hour.

A serial ten-fold dilution series of poliovirus-1 was prepared in maintenance media /raw surface water, raw surface water only, and maintenance media only solutions were aspirated from each well. One hundred fifty microliters of each appropriate dilution of poliovirus-1 stock were transferred into each of the pre-treated well types. One

hundred fifty microliters of maintenance media alone was added to a separate well to serve as a negative control. The BGMK cell culture plates were incubated in a CO_2 incubator at 37 °C for one hour to allow for viral adsorption to the cells. One milliliter of maintenancemedia was then added to each of the pre-treated well types and plates were incubated in a CO_2 incubator at 37 °C for 48 hours.

At 24 and 48 hours, wells were examined under a dissecting microscope for cytopathogenic effects (CPE), characterized by cells clumping into spheres and separating from the monolayer. Greater than or equal to 90% CPE in a well was considered a positive well, whileless than 90% CPE was considered a negative well (Estes et al. 1979). The ratio of negative and positive wells per dilution was used to calculate the most probable number (MPN) of poliovirus in the undiluted sample (Blodgett 2006). When no CPE was detected in the total volume assayed, the lower detection limit of the assay was reached. In this case, a $\log_{10}$ reduction value was calculated using 1 CPE in the total sample volume assayed. This is indicated by the use of a less-than symbol (<) for the $\log_{10}$ concentration and a greater-than symbol (>) for the $\log_{10}$ reduction.

The MPNs calculated for each of the pre-treated well types (raw surface water with BacSan, raw surface water only, and maintenance media only) were compared to determine any discrepancies between the infectivity of Poliovirus-1 on BGMK cells exposed to BacSan and/or raw surface water relative to cells unexposed to either. If a higher or lower MPN was calculated when cells were exposed to raw surface water and BacSan compared to those exposed to maintenance media only, this would indicate that exposure to this solution, as occurs in experimental protocol, increases or decreases,

respectively, the susceptibility of cells to virus infectivity. If a higher or lower MPN was calculated when cells were exposed to raw surface water only compared to those exposed to maintenance media only, this would indicate that exposure to raw surface water increases or decreases, respectively, the susceptibility of cells to virus infectivity. If the BacSan and raw water solution led to different MPN results than the raw water only, this would indicate whether BacSan or raw water, or both, are responsible for affecting the susceptibility of cells to virus infectivity.

4.4. Risk Considerations Associated with BacSan

The amounts of chemical constituents BacSan contributes to water at concentrations of 1:100,000, 1:75,000, 1:50,000, and 1:35,000 were compared to the US EPA Regulations and WHO Guidelines for Drinking Water Quality.

4.4.1. Measurement of BacSan Residual Over Time

To confirm the concentration of copper in BacSan, undiluted BacSan was serially ten-fold diluted in deionized water. Select dilutions were analyzed for copper concentration (mg/L or parts per million (ppm) using a calibrated HACH Copper Pocket Colorimeter II Test Kit, as described by the manufacturer.

To determine the residual of BacSan over time, four one-liter volumes of room temperature raw surface water were dosed with 1:100,000, 1:75,000, 1:50,000, and 1:35,000 dilutions of BacSan and were stirred for 30 seconds. Immediately following stirring (time 0), samples from each beaker were removed, diluted if necessary, and subjected to copper analysis using a calibrated HACH Copper Pocket Colorimeter II Test Kit, as described by the manufacturer. This procedure was repeated at 10, 30, 90, 240,

and 1440 minutes. The beakers were not moved or stirred during the testing period so as to not disturb flocculant. Concentrations at each time point were compared to the initial (time 0) concentration.

4.3. Data Analysis

K. terrigena, E. coli concentrations were calculated as described in US EPA Method 1603. MS-2 concentrations were calculated as described in US EPA Method 1601. Log_{10} reduction values at each timepoint were calculated by subtracting the log_{10} of the concentrations at each timepoint from the log_{10} of the initial concentrations. When no colony forming units (CFU) or plaque forming units (PFU) were detected in the total volume assayed, the lower detection limit of the assay was reached. In this case, a log_{10} reduction value was calculated using 1 CFU or 1 PFU in the total sample volume assayed. This is indicated by the use of a less-than symbol for the log_{10} concentration and a greater-than-or-equal-to symbol ($\geq$) for the log_{10} reduction. Overall log_{10} reduction values were calculated using total number of CFU or PFU for each concentration of BacSan at each time point in all experiments and total volumes assayed.

Poliovirus-1 and Rotavirus SA-11 concentrations were calculated by enumerating wells positive for cell lysis and utilizing the MPN calculation described in the *Bacteriological Analytical Manual* (BAM) that employs Thomas' Rule and Haldane's method (Blodgett 2006). From the MPN was calculated the Log_{10} concentration of virus. Log_{10} reduction values at each timepoint were calculated by subtracting the log_{10} of the concentrations at each timepoint from the log_{10} of the initial concentrations.

5. Results

5.1. *Escherichia coli*

Six trials were conducted to determine the $\log_{10}$ reductions in *E. coli* reached by various concentrations of BacSan over time, up to 1440 minutes (24 hours). The curve of natural bacterial die-off served as a standard for comparison against those beakers with added BacSan. Table 5 presents the natural $\log_{10}$ reductions in *E. coli* concentration over time. Overall, the $\log_{10}$ reduction of *E. coli* over 10, 30, 90 and 240 minutes remained within 0.33-$\log_{10}$ of zero. After 1440 minutes, a 2.302-$\log_{10}$ reduction was reached, on average.

Table 5. Log_{10} *E. coli* reduction over time with 0 uL BacSan

0 µL BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	-0.121	ND	0.146	0.318	ND
2	-0.032	-0.003	ND	-0.05	0.778
3	-0.081	0.105	-1	0.602	ND
4	0.038	0.01	0.299	0.64	3.825
5	-0.71	-0.99	-0.95	-0.433	ND
6	0.361	-0.372	-0.142	0.04	ND
Mean	-0.091	-0.250	-0.329	0.233	2.302
SD	0.349	0.452	0.611	0.414	2.155

ND: no data
SD: standard deviation

Tables 6, 7, 8 and 9 present $\log_{10}$ reductions in *E. coli* concentration over time for those beakers containing 10 µL (1:100,000), 13.3 µL (1:75,000), 20 µL (1:50,000), and 28.6 µL (1:35,000) of BacSan, respectively. The values that meet or exceed the 6-$\log_{10}$ performance criteria are in bold. *E. coli* reductions reached the 6-$\log_{10}$ performance criterion for bacteria on average after 1440 minutes (24 hours) at each concentration. *E. coli* reductions reached the 6-$\log_{10}$ performance criterion for bacteria within 95%

confidence intervals after a minimum contact time of 240 minutes (4 hours) at concentrations of 1:50,000 and 1:35,000.

Table 6. Log_{10} *E. coli* reduction over time at a 1:100,000 concentration of BacSan

10 µL (1:100,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	ND	ND	2.713	5.679	ND
2	0.591	2.723	4.196	ND	**6.301**
3	-0.050	0.209	1.581	4.068	ND
4	0.000	0.576	2.132	4.757	**>6.712**
5	-0.822	-0.326	1.903	3.978	ND
6	0.718	1.758	3.734	4.495	ND
Mean	0.087	0.988	2.710	4.595	**>6.507**
SD	0.613	1.236	1.050	0.684	0.291

ND: no data
SD: standard deviation

Table 7. Log_{10} *E. coli* reduction over time at a 1:75,000 concentration of BacSan

13.3 µL (1:75,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	2.278	ND	3.561	5.658	ND
2	ND	ND	3.95	ND	**>6.823**
3	ND	1.112	2.985	5.798	ND
4	-0.022	1.244	3.622	5.297	**>6.712**
5	0.03	2.14	4.103	4.258	ND
6	0.413	2.131	3.501	4.005	ND
Mean	0.675	1.657	3.620	5.003	**>6.768**
SD	1.086	0.555	0.391	0.821	0.078

ND: no data
SD: standard deviation

Table 8. Log_{10} *E. coli* reduction over time at a 1:50,000 concentration of BacSan

20 µL (1:50,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	ND	ND	ND	ND	ND
2	ND	3.267	4.467	ND	**6.778**
3	0.916	2.115	4.219	**6.602**	ND
4	0.694	2.952	ND	5.757	**>6.712**
5	1.839	3.839	3.951	4.295	ND
6	2.168	3.633	3.721	ND	ND
Mean	1.404	3.161	4.090	5.551	**>6.745**
SD	0.711	0.677	0.324	1.167	0.047

ND: no data
SD: standard deviation

Table 9. Log_{10} *E. coli* reduction over time at a 1:35,000 concentration of BacSan

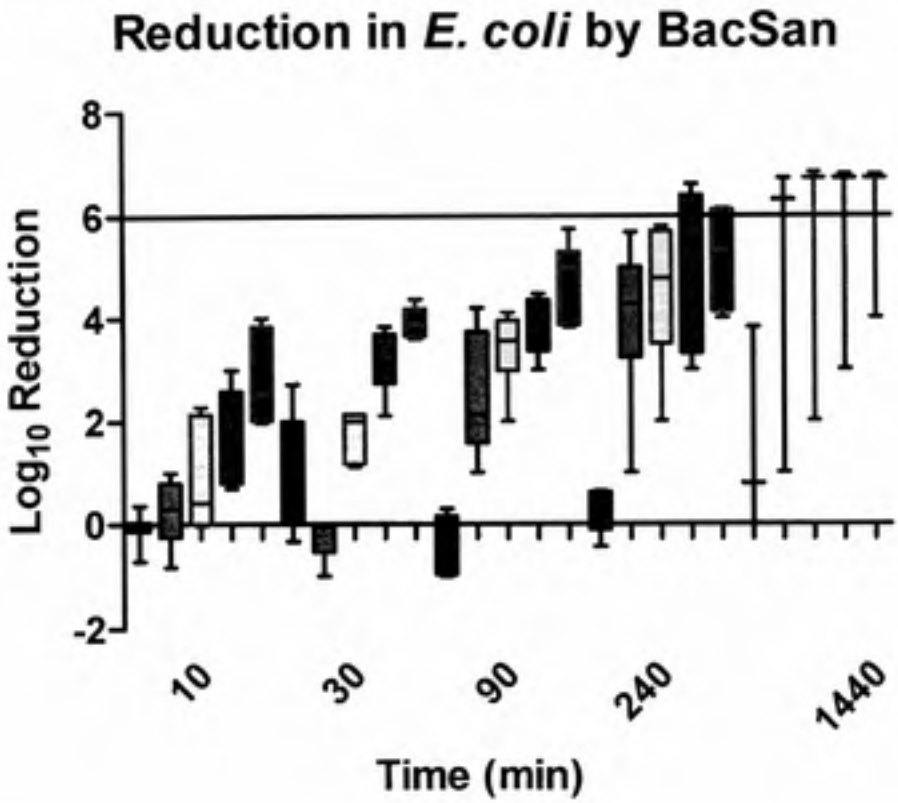
28.6 µL (1:35,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	ND	ND	5.749	**6.135**	ND
2	ND	3.709	5.013	ND	**6.778**
3	2.087	ND	4.994	5.824	ND
4	1.971	3.867	5.297	**6.110**	**>6.712**
5	2.538	4.362	3.871	4.839	ND
6	3.671	3.600	3.831	4.168	ND
Mean	2.567	3.885	4.793	5.415	**>6.745**
SD	0.776	0.337	0.779	0.874	0.047

ND: no data
SD: standard deviation

Figure 4 summarizes the log_{10} reductions of *E. coli* reached by each concentration of BacSan over time. The bars with bright blue outline represent log_{10} reductions in *E. coli* in the beaker with 0 µL BacSan, the orange bars with red outline represent 10 µL (1:100,000), yellow with green outline 13.3 µL (1:75,000), green with purple outline 20 µL (1:50,000) and bright blue with light blue outline 28.6 µL (1:35,000). See Appendix A for individual graphs of the efficacy of each concentration of BacSan against *E. coli*. A

horizontal line at a $\log_{10}$ reduction of 6 signifies the US EPA, WHO, and NSF-International performance criterion for bacteria.

Figure 4. $\log_{10}$ *E. coli* reductions over time with 0 uL, 10 uL, 13.3 uL, 20 uL and 28.6 uL BacSan



5.2. *Klebsiella terrigena*

Four trials were conducted to determine the $\log_{10}$ reductions in *K. terrigena* reached by various concentrations of BacSan over time, up to 1440 minutes (24 hours). The curve of natural bacterial die-off served as a standard for comparison against those beakers with added BacSan. Table 10 presents the natural $\log_{10}$ reductions in *K. terrigena* concentration over time. Overall, the natural $\log_{10}$ reduction of *K. terrigena* over 10, 30, 90 and 240 minutes remained within 0.360 $\log_{10}$ of zero. After 1440 minutes, a 1.307-$\log_{10}$ reduction was reached, on average.

Table 10. Log_{10} *K. terrigena* reduction over time with 0 uL BacSan

0 µL BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	-0.942	ND	-1.205	-1.138	0.462
2	0.246	0.089	0.047	0.857	ND
3	0.061	0.025	-0.095	-0.038	2.310
4	-0.081	-0.105	-0.188	0.088	ND
Mean	-0.179	0.003	-0.360	-0.058	1.386
SD	0.526	0.099	0.571	0.822	1.307

ND: no data
SD: standard deviation

Tables 11, 12, 13 and 14 present-log_{10} reductions in *K. terrigena* concentration over time for those beakers containing 10 µL (1:100,000), 13.3 µL (1:75,000), 20 µL (1:50,000), and 28.6 µL (1:35,000) of BacSan, respectively. The bold values are those that meet or exceed the 6-log_{10} performance criteria. On average, over the four trials, *K. terrigena* reductions came within 0.032-log_{10} of reaching a 6-log_{10} reduction at 240 minutes at a concentration of 1:50,000. At a concentration of 1:35,000, a 6-log_{10} reduction was exceeded at 240 minutes on average.

Table 11. Log_{10} *K. terrigena* reduction over time with 1:100,000 concentration of BacSan

10 µL (1:100,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	-0.559	-0.719	0.134	-0.214	-0.340
2	ND	ND	0.114	2.677	1.396
3	4.143	ND	1.905	4.185	3.411
4	4.910	4.213	5.571	5.857	5.138
Mean	2.831	1.747	1.931	3.126	2.401
SD	2.961	3.487	2.568	2.578	2.383

ND: no data
SD: standard deviation

Table 12. Log_{10} *K. terrigena* reduction over time with 1:75,000 concentration of BacSan

13.3 µL (1:75,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	-1.075	ND	3.894	4.658	3.831
2	0.201	ND	ND	3.554	ND
3	0.078	0.146	3.502	5.217	ND
4	-0.238	1.436	3.845	**6.936**	ND
Mean	-0.259	0.791	3.747	5.091	3.831
SD	0.575	0.912	0.214	1.411	NA

ND: no data
SD: standard deviation

Table 13. Log_{10} *K. terrigena* reduction over time with 1:50,000 concentration BacSan

20 µL (1:50,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	1.204	ND	4.394	5.380	ND
2	-0.535	ND	ND	4.660	ND
3	0.146	0.877	4.322	7.217	**8.498**
4	1.118	3.760	4.820	**6.656**	ND
Mean	0.483	2.319	4.512	5.978	**8.498**
SD	0.831	2.039	0.269	1.168	NA

ND: no data
SD: standard deviation

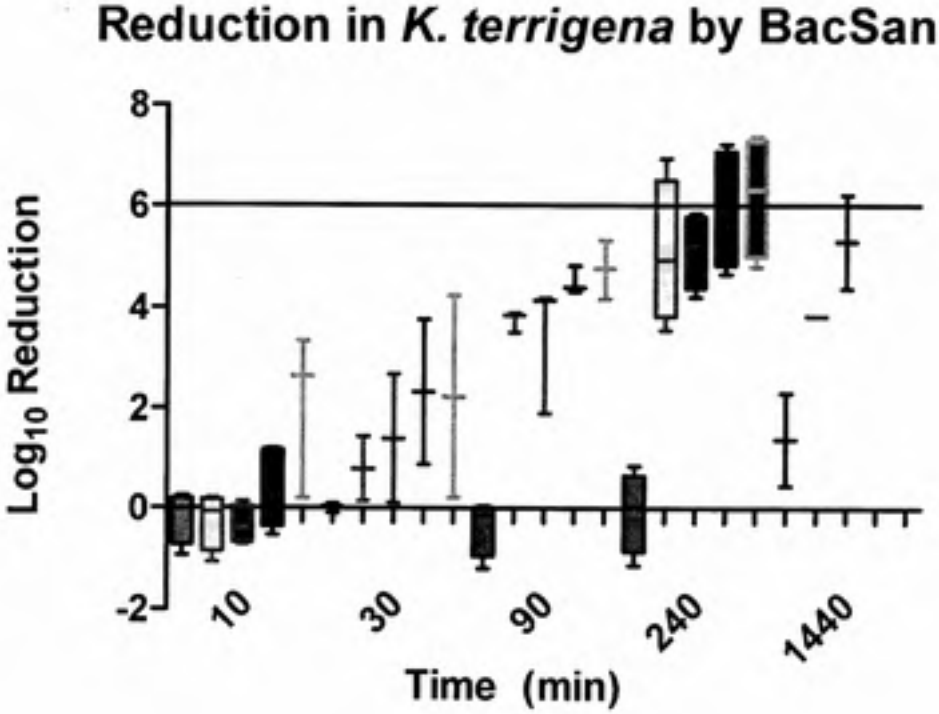
Table 14. Log_{10} *K. terrigena* reduction over time with 1:35,000 concentration of BacSan

28.6 µL (1:35,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	3.337	ND	4.772	5.609	ND
2	ND	ND	ND	4.805	ND
3	0.205	0.221	4.176	**7.012**	**>8.543**
4	2.635	4.237	5.325	**7.376**	ND
Mean	2.059	2.229	4.758	**6.201**	**8.543**
SD	1.644	2.840	0.575	1.202	NA

ND: no data
SD: standard deviation

Figure 5 summarizes the $\log_{10}$ reductions of *K. terrigena* reached by each concentration of BacSan over time. The orange bars with orange outline represent $\log_{10}$ reductions in *K. terrigena* in the beaker with 0 µL BacSan, the yellow bars with green outline represent 10 µL (1:100,000), bright blue with light blue outline 13.3 µL (1:75,000), pink with purple outline 20 µL (1:50,000) and yellow outline 28.6 µL (1:35,000). See Appendix B for individual graphs of the efficacy of each concentration of BacSan against *K. terrigena*. A horizontal line at a $\log_{10}$ reduction of 6 signifies the US EPA, WHO, and NSF-International performance criterion for bacteria.

Figure 5. Log_{10} *K. terrigena* reductions over time with 0 uL, 10 uL, 13.3 uL, 20 uL and 28.6 uL BacSan



5.3. MS-2

Five trials were conducted to determine the $\log_{10}$ reductions in MS-2 reached by various concentrations of BacSan over time, up to 1440 minutes (24 hours). The curve of natural virus die-off served as a standard for comparison against those beakers with added BacSan. Table 15 presents the natural $\log_{10}$ reductions in MS-2 concentration over time. Overall, the natural $\log_{10}$ reduction of MS-2 over 10, 30, 90, 240 and 1440 minutes remained within 0.253-$\log_{10}$ of zero, on average.

Table 15. M S-2 reduction over time with 0 uL BacSan

0 µL BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	-0.020	0.000	-0.009	-0.206	-0.406
2	-0.058	0.026	0.082	-0.111	0.143
3	0.027	0.093	-0.045	-0.330	ND
4	-0.015	-0.006	0.077	-0.083	1.021
5	-0.064	-0.206	-0.263	-0.412	ND
Mean	-0.026	-0.019	-0.032	-0.228	0.253
SD	0.037	0.112	0.140	0.141	0.720

ND: no data
SD: standard deviation

Tables 16, 17, 18 and 19 present $\log_{10}$ reductions in MS-2 concentration over time for those beakers containing 10 µL (1:100,000), 13.3 µL (1:75,000), 20 µL (1:50,000), and 28.6 µL (1:35,000) of BacSan, respectively. On average over the five trials, MS-2 reductions came within less than 0.153-$\log_{10}$ of the 4-$\log_{10}$ reduction level at 1440 minutes (24 hours) with 13.3 µL (1:75,000) BacSan. At the BacSan volume of 20 µL (1:50,000) and 28.6 µL (1:35,000), the 4-$\log_{10}$ reduction was met or exceeded at 1440 minutes (24 hours).

Table 16. MS-2 reduction over time with 1:100,000 concentration of BacSan

10 µL (1:100,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.055	0.085	0.389	0.532	1.690
2	0.008	0.184	0.303	0.211	**2.461**
3	0.139	0.218	0.440	0.264	ND
4	0.004	-0.031	0.129	0.099	2.841
5	0.139	-0.119	-0.076	0.042	ND
Mean	0.069	0.067	0.237	0.230	**2.331**
SD	0.067	0.142	0.211	0.191	0.586

ND: no data
SD: standard deviation

Table 17. MS-2 reduction over time with 1:75,000 concentration of BacSan

13.3 µL (1:75,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.049	-0.005	0.402	0.835	**>2.925**
2	-0.082	0.166	0.321	0.877	**>3.461**
3	0.218	1.654	0.726	0.353	ND
4	0.000	0.122	0.154	0.617	**5.155**
5	ND	0.057	-0.100	0.257	ND
Mean	0.046	0.399	0.301	0.588	**>3.847**
SD	0.127	0.705	0.306	0.278	1.164

ND: no data
SD: standard deviation

Table 18. MS-2 reduction over time with 1:50,000 concentration of BacSan

20 µL (1:50,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.118	0.098	0.361	1.426	**>3.925**
2	0.006	0.122	0.666	1.258	**>4.461**
3	0.478	>1.699	0.526	0.686	ND
4	-0.020	0.177	0.434	1.120	8.021
5	0.059	0.041	0.255	0.856	ND
Mean	0.128	>0.427	0.448	1.069	**>5.469**
SD	0.203	0.713	0.157	0.299	2.226

ND: no data
SD: standard deviation

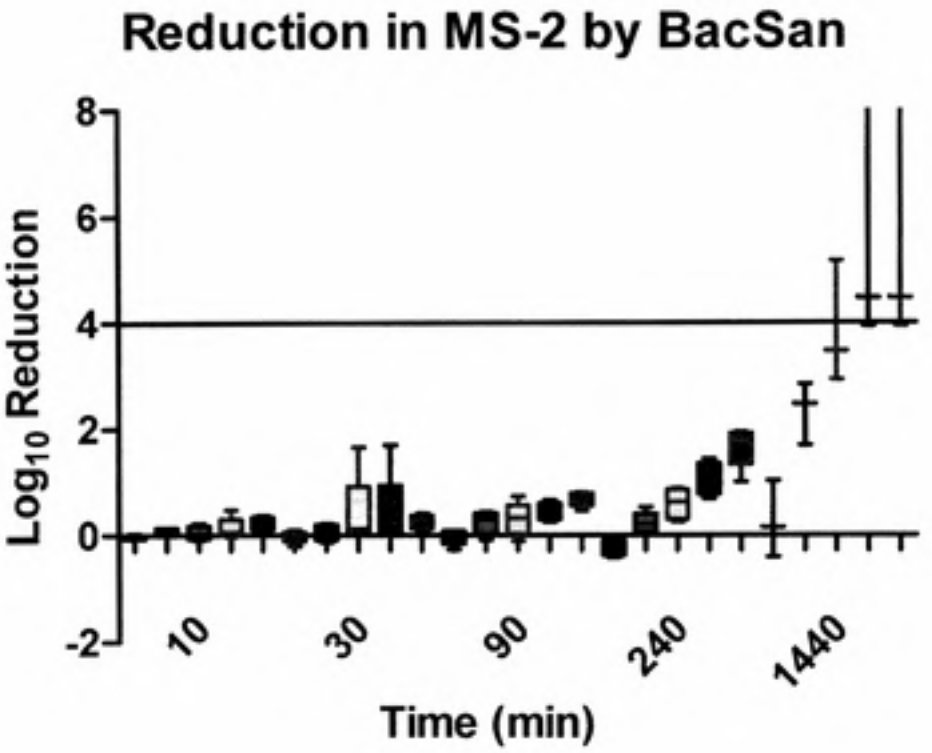
Table 19. MS-2 reduction over time with 1:35,000 concentration of BacSan

28.6 µL (1:35,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.240	0.061	0.457	1.779	>3.925
2	0.058	0.331	0.802	1.942	**>4.461**
3	0.373	0.416	0.654	0.996	ND
4	0.171	0.330	0.775	1.899	**>8.368**
5	0.302	0.190	0.664	1.666	ND
Mean	0.229	0.266	0.670	1.656	**>5.585**
SD	0.121	0.140	0.136	0.385	2.425

ND: no data
SD: standard deviation

Figure 6 summarizes the $\log_{10}$ reductions of MS-2 reached by each concentration of BacSan over time. The bars with bright blue outline represent $\log_{10}$ reductions in MS-2 in the beaker with 0 µL BacSan, the orange bars with red outline represent 10 µL (1:100,000), yellow with green outline 13.3 µL (1:75,000), green with purple outline 20 µL (1:50,000) and bright blue with light blue outline 28.6 µL (1:35,000). See Appendix C for individual graphs of the efficacy of each concentration of BacSan against MS-2. A horizontal line at a $\log_{10}$ reduction of 4 signifies the US EPA, WHO, and NSF-International performance criterion for viruses.

Figure 6. Log_{10} MS-2 reductions over time with 0 uL, 10 uL, 13.3 uL, 20 uL and 28.6 uL BacSan



5.4. Poliovirus-1

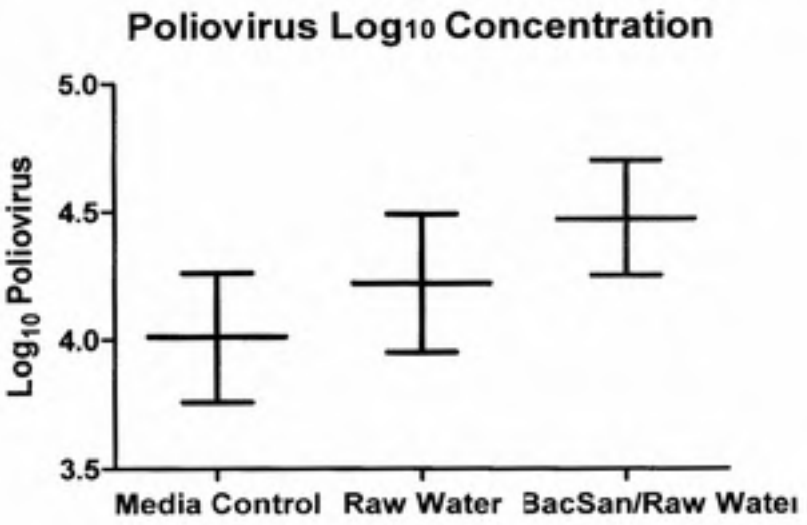
5.4.1. Interference Assay

An interference assay was completed to determine how the presence of the disinfectant and/or raw water effects (or not) the infectivity assay for poliovirus-1 on buffalo green monkey kidney (BGMK) cells. The MPN of cell cultures infected with the same amount of polio but exposed to either media, raw water, or a 1:35,000 concentration of BacSan in raw water, are outlined in Table 19.

Table 20. Log_{10} concentration of poliovirus on cells exposed to media, raw water, or BacSan/raw water

	Log_{10} Concentration	95% Confidence Interval	
Media	4.01	3.76	4.26
Raw Water	4.22	3.95	4.49
BacSan/Raw Water	4.47	4.25	4.70

Figure 7. Log_{10} and 95% confidence intervals for cell culture plates exposed to either media, raw water, or BacSan/raw water and equal amounts of Poliovirus-1



5.4.2. Cytotoxicity Assay

A cytotoxicity assay was completed to determine whether or not the presence of BacSan at a 1:35,000 concentration in raw water damages the cell culture relative to those cells exposed to raw water without BacSan. After 48 hours of incubation, no damage was visually detectable, with use of a dissecting microscope, on cells exposed to either condition.

5.4.3. Poliovirus BacSan Assay

Two trials were conducted to determine the $\log_{10}$ reductions in Poliovirus-1 reached by various concentrations of BacSan over time, up to 1440 minutes. The curve of natural bacterial die-off served as a standard for comparison against those beakers with added BacSan. Table 21 presents the natural $\log_{10}$ reductions in Poliovirus-1 concentration over time. Reductions remained, on average, within 0.544-$\log_{10}$ over times 10, 30, 90, and 240 minutes. Reductions reached 1.097-$\log_{10}$ after 24 hours, according to one trial.

Table 21. Polivirus-1 reduction over time with 0 uL BacSan

0 µL BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.388	0.388	0.000	0.388	1.097
2	0.434	0.610	0.648	0.700	ND
Mean	0.411	0.499	0.324	0.544	1.097
SD	0.033	0.157	0.458	0.221	

ND: no data
SD: standard deviation

Tables 22, 23, 24 and 25 present $\log_{10}$ reductions in poliovirus-1 concentration over time for those beakers containing 10 µL (1:100,000), 13.3 µL (1:75,000), 20 µL (1:50,000), and 28.6 µL (1:35,000) of BacSan, respectively. On average over the two trials, poliorvirus-1 reductions did not reach the 4-$\log_{10}$ performance criterion for viruses after 1440 minutes with any level of BacSan.

Table 22. Polivirus-1 reduction over time with 1:100,000 concentration of BacSan

10 µL (1:100,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.188	0.388	0.045	0.346	0.405
2	0.210	0.651	0.743	0.651	ND
Mean	0.199	0.520	0.394	0.498	0.405
SD	0.016	0.186	0.494	0.216	

ND: no data
SD: standard deviation

Table 23. Polivirus-1 reduction over time with 1:75,000 concentration of BacSan

13.3 µL (1:75,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.296	0.296	0.000	0.388	0.651
2	0.434	0.545	0.651	0.743	ND
Mean	0.365	0.420	0.326	0.566	0.651
SD	0.098	0.176	0.461	0.251	

ND: no data
SD: standard deviation

Table 24. Polivirus-1 reduction over time with 1:50,000 concentration of BacSan

20 µL (1:50,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.388	0.296	0.188	0.473	0.743
2	0.434	ND	0.925	0.827	ND
Mean	0.411	0.296	0.557	0.650	0.743
SD	0.033		0.521	0.250	

ND: no data
SD: standard deviation

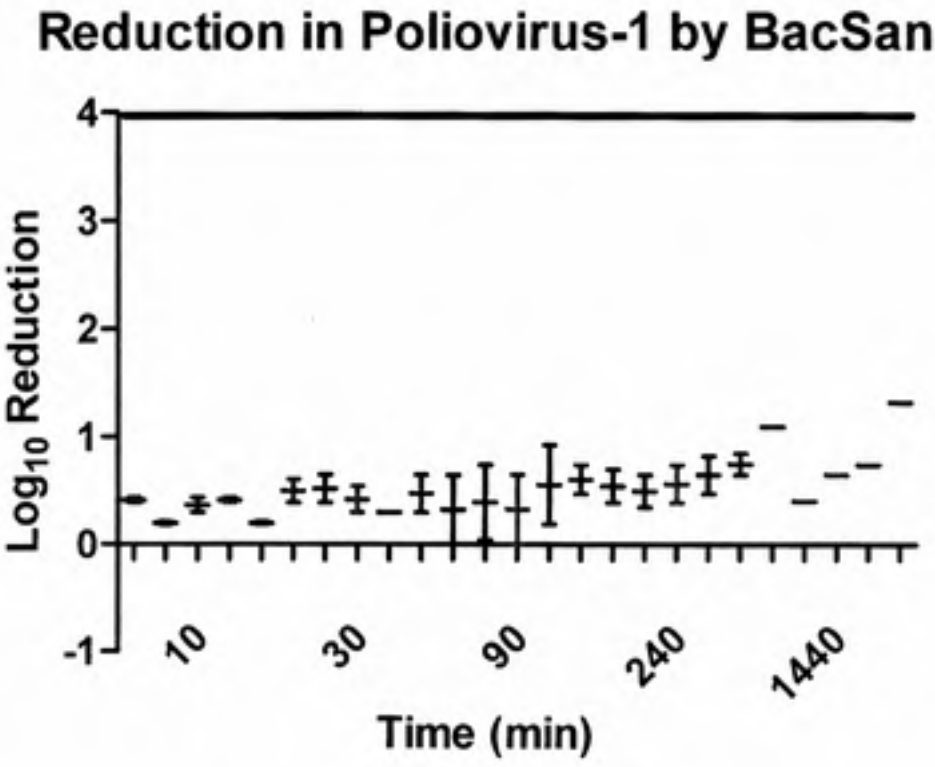
Table 25. Polivirus-1 reduction over time with 1:35,000 concentration of BacSan

28.6 µL (1:35,000) BacSan per Liter					
N	Time (min)				
	10	30	90	240	1440
1	0.188	0.296	0.473	0.851	1.321
2	0.210	0.651	0.743	0.651	ND
Mean	0.199	0.474	0.608	0.751	1.321
SD	0.016	0.251	0.191	0.141	

ND: no data
SD: standard deviation

Figure 8 summarizes the $\log_{10}$ reductions of Poliovirus-1 reached by each concentration of BacSan over time. The bars with dull blue outline represent $\log_{10}$ reductions in poliovirus in the beaker with 0 µL BacSan, the bright blue lines represent 10 µL (1:100,000), red 13.3 µL (1:75,000), green 20 µL (1:50,000) and purple 28.6 µL (1:35,000). See Appendix C for individual graphs of the efficacy of each concentration of BacSan against Poliovirus-1. A horizontal line at a $\log_{10}$ reduction of 4 signifies the US EPA, WHO, and NSF-International performance criterion for viruses.

Figure 8. $\log_{10}$ poliovirus-1 reductions over time with 0 uL, 10 uL, 13.3 uL, 20 uL and 28.6 uL BacSan



5.4.4. Antisera Deactivation Assay

A 1:100 concentration of undiluted antisera successfully deactivated poliovirus-1.

5.5. Rotavirus

No results were obtained for rotavirus, as no CPE was observed on experimental MA-104 cell cultures over time. See section 6.2.2.

5.6. BacSan risk assessment

5.6.1. Risk assessment

Below is an analysis of the amounts of chemical constituents BacSan contributes to water at the various concentrations of the disinfectant tested in this report, compared to the US EPA Regulations and WHO Guidelines for Drinking Water Quality. Concentrations in excess of US EPA Regulations and/or WHO Guidelines are highlighted in yellow.

Table 26. Chemicals in BacSan and US EPA, WHO guidelines concerning them

	Concentration in 1 L Treated Water (mg/L)				US EPA Regulations	WHO Guidelines
	10 µL (1:100,000)	13.3 µL (1:75,000)	20 µL (1:50,000)	28.6 µL (1:35,000)	(mg/L)	(mg/L)
Silver	0.000335	0.000670	0.001005	0.001340	0.1	0.1
Copper	6.365000	12.730000	19.095000	25.460000	1.3	2
Gold	0.000001	0.000003	0.000004	0.000005	ND	ND
Aluminum	0.422100	0.844200	1.266300	1.688400	0.05 - 2	0.2
Calcium	0.001541	0.003082	0.004623	0.006164	ND	ND
Chromium	0.002546	0.005092	0.007638	0.010184	0.1	0.05
Iron	0.011390	0.022780	0.034170	0.045560	0.3	2
Magnesium	0.001072	0.002144	0.003216	0.004288	ND	ND
Manganese	0.000670	0.001340	0.002010	0.002680	0.05	0.4
Sodium	0.000509	0.001018	0.001528	0.002037	ND	200
Nickel	0.001943	0.003886	0.005829	0.007772	ND	0.07
Zinc	2.613000	5.226000	7.839000	10.452000	5	3
Nitrates	18.760000	37.520000	56.280000	75.040000	10	50
Chloride	4.087000	8.174000	12.261000	16.348000	250	250

ND: no data (not regulated)

The levels of copper, aluminum, zinc and nitrates contributed by BacSan to water are in excess of exposure levels recommended by the US EPA and WHO at even a 1:100,000 concentration of BacSan, except in the case of zinc, where levels are excessive at concentrations of 1:75,000 and upward.

5.6.2. Analysis of BacSan Residual

The concentration of copper in BacSan was found to be 73,833 mg/L by analyzing its concentration with use of a HACH Copper Pocket Colorimetric II Test Kit, as shown in Table 26. The expected concentration, according to the manufacturer's specifications, was 95,000 mg/L.

Table 27. Expected and measured copper concentrations in undilute BacSan[1]

	Expected Concentration (mg/L)	HACH[2] Reading (mg/L) Trials		
		1	2	3
10^-5 Dilution	0.95	0.63	0.79	0.75
10^-6 Dilution	0.095	0.07	0.19	0.19
Undilute BacSan Concentration (mg/L)	95,000	63,700	80,900	76,900
Weighted Average	95,000	73,833		

[1]Calculated using 95000 mg/L copper concentration in undiluted BacSan
[2]HACH Copper Pocket Colorimetric II Test Kit (detection range 0.04 – 5 mg/L)
AMDL: above method detection limit (5 mg/L)
BMDL: below method detection limit (0.04 mg/L)
ND: no data (cannot be calculated)

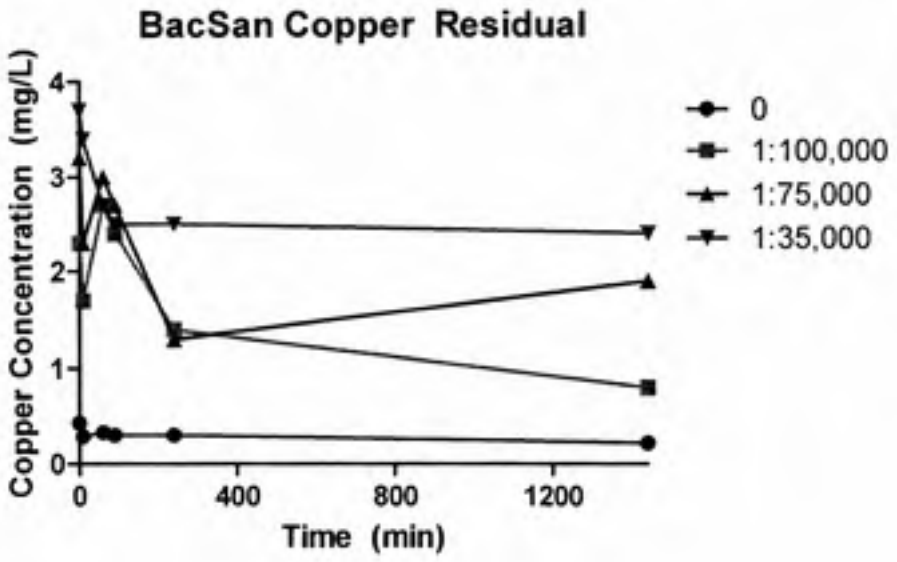
BacSan's residual was analyzed by determining if the copper concentration in water changed over time after the addition of BacSan. Copper concentrations for each BacSan concentration at each timepoint are illustrated in Table 27 and Figure 12. The copper concentrations in water with added BacSan varied within ≤ 0.72 standard deviations over 1440 minutes.

Table 28. Measured copper concentrations[1] in water with added BacSan over time

BacSan Concentration	Time (minutes) 0	10	60	90	240	1440	Standard Deviation	% Reduction over 1440 min.
0	0.43	0.29	0.33	0.30	0.30	0.22	0.07	48.84%
1:100,000	2.3	1.7	2.7	2.4	1.4	0.8	0.71	65.22%
1:75,000	3.2	2.3	3.0	2.7	1.3	1.9	0.72	40.63%
1:35,000	3.7	3.4	2.7	2.5	2.5	2.4	0.55	35.14%

[1]HACH Copper Pocket Colorimetric II Test Kit (detection range 0.04 – 5 mg/L)

Figure 9. Copper concentration in water with added BacSan over time



6. Discussion

The purpose of this study was to determine at what concentration and over what length of contact time BacSan meets US EPA, WHO, and NSF-International performance criteria in reducing the concentrations of two indicator bacteria, *E. coli* and *K. terrigena*, the indicator bacteriophage MS-2, and the pathogenic viruses Poliovirus-1 and Rotavirus SA-11. The concentrations tested were 1:100,000; 1:75,000; 1:50,000; and 1:35,000, and the time points at which microbial concentrations were assayed were at 10, 30, 90, 240

and 1440 minutes after inoculation with BacSan. A risk assessment was also conducted to determine if BacSan has the potential to cause negative aesthetic effects or harm to human health when present at concentrations necessary to achieve essential reductions in microbial concentration. The residual of BacSan in water was tested to determine to what extent BacSan was present at various contact times; this served as means of assessing changes over time in exposure to the elements in BacSan by consumers of treated water and the potential of BacSan to provide protection from recontamination.

6.1. Indicator Microorganisms

This research demonstrates that a reduction of $\geq$6.8-$\log_{10}$ ($\geq$99.9999%) was achieved (24 hours, 13.3 µl BacSan/L) for *E. coli* and for *K. terrigena*, a reduction of 8.5-$\log_{10}$ (99.9999997%) was achieved (24 hours, 20 µl BacSan/L). For MS-2 coliphage, a reduction of >5.5-$\log_{10}$ (>99.9997%) was achieved (24 hours, 20 µl BacSan/L). BacSan thus meets the performance criterion, set by the US EPA, WHO, and NSF-International, for two indicator bacteria, *E. coli* and *K. terrigena*, and the indicator bacteriophage, MS-2, when exposed to least a 1:35,000 concentration of BacSan over 24 hours. Indicator microorganisms such as these are believed to be accurate surrogates for their pathogenic counterparts and as such, this data suggests that BacSan is effective against both bacteria and viruses after 1440 minutes of contact time at a concentration of 1:50,000.

However, because the mechanism of microbial inactivation differs for every disinfectant, the reliability of indicators as accurate surrogates can vary depending on the product. Ozone, for example, has shown the same effectiveness against MS-2 as against poliovirus, indicating that in the case of this disinfectant, MS-2 is an accurate surrogate for poliovirus (Shin et. al 2002). Copper, silver, and chloride, however, were found to be

approximately 10 times more effective against MS-2 than poliovirus, indicating that in the case of these disinfectants, MS-2 is *not* an accurate surrogate for poliovirus (Yahya 1992). As such, it was important to explore the generalizability of the MS-2 results for BacSan by studying the disinfectant's efficacy against pathogenic viruses.

6.2. Pathogenic Viruses

6.2.1. Poliovirus-1

This research shows that BacSan's effectiveness against the pathogenic virus, Poliovirus-1, is not sufficient to meet performance criteria at concentrations up to 1:35,000 and contact time up to 1440 minutes (24 hours). BacSan reduced poliovirus by 1.321-$\log_{10}$ (92.89%), or 1.621-$\log_{10}$ (95.59%) when corrected for interference (as described below), at the highest concentration of BacSan at 1440 minutes. BacSan would need to be more than 100 times more effective against poliovirus in order to the meet US EPA, WHO, and NSF-International performance criteria of 4-$\log_{10}$ reduction.

The beaker with the largest concentration of BacSan performed only 0.224-$\log_{10}$ better than that without BacSan at 1440 minutes. The three lesser concentrations of BacSan, 1:100,000; 1:75,000; and 1:50,000, performed worse than the beaker without BacSan at 24 hours. The beaker without BacSan on average resulted in 0.692, 0.446, and 0.354 greater $\log_{10}$ reductions, respectively, at this contact time. Such results would indicate that BacSan is having a negligible, if not positive, effect on poliovirus concentrations over time.

A possible explanation for these results stems from data from the interference assay. This assay indicates that the exposure of BGMK cells to BacSan may increase their susceptibility to poliovirus infection. This observation may be explained by the

mechanism by which BacSan is believed to work. The active ingredients in BacSan, including copper and silver, are thought to deactivate bacterial cells in part by disrupting cell membranes (Silvestry-Rodriguez 2007). It is possible that BacSan damaged the cultured BGMK cells in a similar fashion and thus increased their susceptibility to infection by Poliovirus-1.

In the interference assay, the $\log_{10}$ concentration of poliovirus on cells exposed to the maximum concentration of BacSan in raw water was found to be 0.30-$\log_{10}$ higher than those exposed to raw water without BacSan; these differences were witnessed in spite of the fact that the two groups were each exposed to the same amount of virus. This data implies that the reductions in poliovirus found in this study may be underestimated due to interference; the ability to determine the extent of interference, however, is limited by the fact that only one replicate of the interference assay was completed and for only one concentration (1:35,000) of BacSan. It is unlikely, however, that more interference assays would find that interference fully compensates for the >2-$\log_{10}$ difference between the reductions currently shown (1.321-$\log_{10}$) at 1:35,000 and 1440 minutes and those required by the US EPA, WHO and NSF-International (4-$\log_{10}$). For the sake of illustration, in Figure 10, the reductions of poliovirus at each concentration of BacSan at 1440 minutes were corrected for the interference found at a 1:35,000 concentration of BacSan in one trial. In this case, as mentioned above, the $\log_{10}$ reduction at 1440 minutes at a concentration of 1:35,000 would be 1.621-$\log_{10}$.

Figure 10. Poliovirus reduction at 1440 minutes and corrected for interference

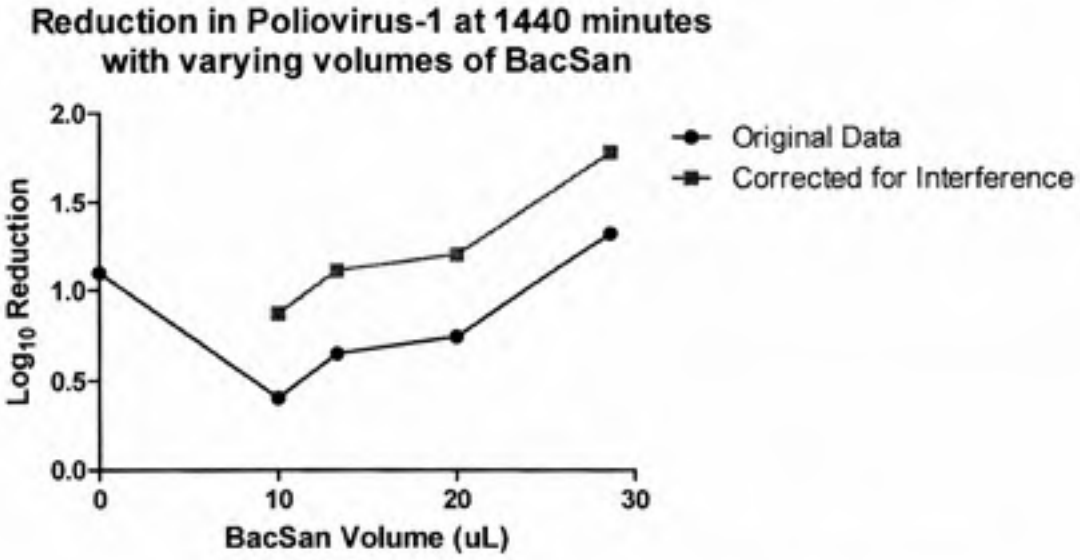


Table 29. Log_{10} poliovirus reductions corrected for interference found at 1:35,000 concentration of BacSan at 1440 minutes

	Original Data	Corrected for Interference
1:100,000	0.405	0.870
1:75,000	0.651	1.110
1:50,000	0.743	1.200
1:25,000	1.321	1.780

On another level, this interference assay could shed some light as to the potential mechanism by which BacSan works in deactivating microbes. BacSan caused damage to the mammalian cells that was not visible through visual inspection (as determined by the cytotoxicity assay), but which increased the susceptibility of the cells to infection. This could potentially support the idea that BacSan works in part by damaging cell membranes. Further exploring how this occurred and to what other cells it occurs could prove useful in understanding the mechanism of disinfection and thus contribute to the creation of new and better heavy metal-based disinfectants.

6.2.2. Rotavirus SA-11

Two assays were completed to determine BacSan's efficacy in reducing the concentration of rotavirus in raw water. In neither of these assays were results obtained. Rotavirus was co-spiked with poliovirus in raw water, and though the poliovirus assays on BGMK cells were successful, the MA-104 cells on which water samples were plated produced no cytopathogenic effects (CPE) from rotavirus. As there were three additional steps in the process between the drawing of water samples and the plating of dilutions on cell monolayers relative to the poliovirus assay, there were more levels on which the experiment needed to be optimized. The water samples at each timepoint and for each concentration of BacSan were first incubated with trypsin to digest the viral coat and they were then incubated with antisera to deactivate the poliovirus in the sample. The MA-104 cells were then washed to rid them of serum prior to dispensing the water samples onto the cells. Since the success of every step was essential in order for this assay to work, there were multiple areas in which the experiment needed to be optimized for this particular application, and such optimization was not achieved at the time of this study's completion.

6.3. Risk Assessment

The levels of copper, aluminum, zinc and nitrates contributed by BacSan to water at a 1:35,000 concentration are in excess of exposure levels recommended by the US EPA and WHO. As a concentration of 1:35,000 of BacSan in water is required for the disinfectant to meet reduction standards for MS-2, the amount of potentially harmful ingredients contributed at this BacSan concentration is of greatest interest.

As little as 10 µL BacSan per liter of water contributes levels of aluminum in excess of those recommended by the US EPA and WHO. The amount of aluminum added to water treated with the maximum volume of BacSan (28.6 µL, or 1:35,000 concentration) results in a concentration of aluminum that is ~1.7 times higher than the US EPA recommends, and 8.4 times higher than the WHO recommends. Aluminum is regulated only as a secondary contaminant by the US EPA because adverse health effects of exposure to the chemical are unknown. Current research is exploring the possibility of exposure to aluminum in drinking water leading to Alzheimer's disease, but no clear conclusions have been drawn. There are, however, possible adverse aesthetic effects relating to the smell, taste, or color of water containing concentrations of aluminum above the regulated level.

The amount of zinc added to water treated with a 1:35,000 concentration of BacSan (28.6 µL) results in a concentration of zinc ~2 times higher than the US EPA recommends, and 3.5 times higher than the WHO recommends, though as little as 13.3 µL (1:75,000) of BacSan leads to zinc levels in excess of these recommendations. Zinc, like aluminum, is only regulated as secondary contaminant by the US EPA due to the fact that it can cause adverse aesthetic effects at high concentrations. Zinc can cause adverse health effects, but only at a level much higher than would feasibly occur in drinking water (EPA 2008).

The amount of copper added to water treated with a 1:35,000 concentration of BacSan (28.6 µL) results in a concentration of copper ~20 times higher than the US EPA recommends, and ~13 times higher than the WHO recommends; as little as 10 µL of added BacSan (1:100,000) adds almost 6 times the US EPA's recommended level of

copper and 2 times the WHO's recommendations. Acute exposure to copper above safe levels can lead to nausea, vomiting, diarrhea, and upset stomach. Long-term exposure to high levels of copper can cause kidney or liver damage. Infants under the age of one year are particularly susceptible to these negative health effects; repeated exposure to copper above safe levels can lead to infantile kidney disease.

The amount of nitrates added to water treated with a 1:35,000 concentration of BacSan results in a concentration of nitrates 7.5 times higher than the US EPA recommends, and 1.5 times higher than the WHO recommends. Just 10μL of BacSan (1:100,000) adds 2 times the US EPA's recommendations, though BacSan does not contribute levels of nitrates in excess of WHO recommendations until 20 μL of BacSan is added (1:50,000). Excessive levels of nitrates can cause serious illness and sometimes death. In infants, high levels of nitrates convert to nitrite in the body and decrease the capacity of the child's blood to carry oxygen, leading to what can be an acute condition that can lead to death in a period of days (EPA 2008). A lifetime exposure to levels of nitrates above the recommended level can cause diuresis, increase starchy deposits and hemorrhaging of the spleen (ibid.).

The levels of zinc and aluminum in BacSan may make treated water taste or appear less desirable; this could make it more difficult to convince potential users to treat their water with BacSan. However, this is less worrisome than the levels of copper and nitrates in the disinfectant, as these can lead to serious health conditions when ingested.

It was found that the concentration of copper in BacSan was lower than that expected per the manufacturer's specifications (73,833 mg/L versus 95,000 mg/L). Since the same batch of BacSan was used over the 11-month course of this study, and the

copper concentration was measured at the end of the study period, this could indicate that over time, the concentration of copper – and thus other active BacSan ingredients – decreased. As BacSan may have lost efficacy over time as a result, this could bring into question the poliovirus data, which could be underestimated by the fact that it utilized 9 and 10 month-old BacSan; the other trials used 1 to 5 month-old BacSan.

Otherwise, if the copper concentration – and thus other active BacSan ingredients – did not change over the life of the product, or not to the extent that this discrepancy would be reached, this could indicate that quality control could be improved on the part of the manufacturer in order to guarantee that the intended concentrations of each of the ingredients is actualized.

BacSan's residual was explored by determining if the concentration of metals added by BacSan to water changes over time. Copper was measured as an indicator of the residual. It was found that the concentration of copper in water treated with BacSan remained within ≤ 0.72 standard deviations over 1440 minutes (24 hours). The copper concentration in the water samples with 1:100,000; 1:75,000; and 1:35,000 concentrations of BacSan decreased by 34.78%, 59.38%, and 64.86%, respectively, over this time period. If this reduction were generalized to the other BacSan ingredients, and taking into account the actual, rather than the reported, concentrations of ingredients – also by generalizing the copper results – the concentrations of ingredients at 24 hours at the highest and second highest tested concentrations of BacSan would be as shown in Tables 30 and 31, respectively. Concentrations in excess of levels considered benign by the US EPA and the WHO are highlighted yellow.

Table 30. Concentration of BacSan ingredients of interest at 24 hours, 1:50,000 (mg/L)

	Expected 1:50,000 (mg/L)	Actual[1] 1:50,000 (mg/L)	1440 minutes[2] 1:50,000 (mg/L)	US EPA regulations	WHO guidelines
Copper	19.095	14.9	5.24		
Aluminum	1.2663	0.984	0.346	0.05 – 2.00	0.2
Zinc	7.839	6.09	2.14	5	3
Nitrates	56.28	43.7	15.4	10	50

[1]Based on 77.7% of expected, as found in the case of copper
[2]Based on reduction by 35.14%, minimum reduction found over 24 hours

Table 31. Concentration of BacSan ingredients of interest at 24 hours, 1:35,000 (mg/L)

	Expected 1:35,000 (mg/L)	Actual[1] 1:35,000 (mg/L)	1440 minutes[2] 1:35,000 (mg/L)	US EPA regulations	WHO guidelines
Copper	25.46	19.8	12.8	1.3	2
Aluminum	1.6884	1.31	0.851	0.05 – 2.00	0.2
Zinc	10.452	8.12	5.27	5	3
Nitrates	75.04	58.3	37.2	10	50

[1]Based on 77.7% of expected, as found in the case of copper
[2]Based on reduction by 35.14%, minimum reduction found over 24 hours

This indicates that BacSan may retain a relatively strong residual (~65% effectiveness) over 1440 minutes (24 hours) and thus may protect against recontamination. It also, however, indicates that the concentration of the harmful constituents of BacSan are not settling out of treated water over this amount of time to the extent that would be required to remove possible harmful effects.

Because of the risks posed by the concentrations of nitrates and copper in water treated with BacSan, this disinfectant may not be a good choice for use in treating water meant for ingestion by children, the elderly, or other individuals who are more highly susceptible to the risks posed by these constituents.

The turbidity of the water collected from University Lake on March 17, 2008 was up to 14 times higher than that collected at the other three sources and dates (7.33 NTU, 5.19 NTU, and 1.54 NTU versus 20.91 NTU). This may have caused variation in results; it is unknown how turbidity effects the efficacy of BacSan, however.

7. Conclusions and Recommendations for Further Research

BacSan is a point-of-supply disinfectant that performs very well against some bacteria and viruses. It did not perform adequately against a key pathogenic virus when examined here. Further, BacSan may pose potential risks to users due its composition. The following conclusions can be made:

1. BacSan meets and/or exceeds the 6-$\log_{10}$ performance criteria, as set by the US EPA, WHO, and NSF-International, for the indicator bacteria *E. coli* and *K. terrigena*.
    a. For *E. coli*, the minimum concentration of BacSan to meet this standard was 1:75,000 at 1440 minutes (24 hours).
    b. For *K. terrigena*, the minimum concentration of BacSan to meet this standard was 1:50,000 at 1440 minutes (24 hours), or 1:35,000 at 240 minutes (4 hours).
2. BacSan exceeds the 4-$\log_{10}$ performance criteria, as set by the US EPA, WHO, and NSF-International, for the indicator bacteriophage MS-2 at a concentration of 1:50,000 in raw water at a contact time of 24 hours.
3. BacSan achieves a 1.321-$\log_{10}$ reduction in the pathogenic virus Poliovirus-1 at a concentration of 1:35,000 and after a contact time of 24

hours. This falls short of meeting the 4-$\log_{10}$ reduction standard set by the US EPA, WHO and NSF-international.

4. BacSan contributes concentrations of copper and nitrates above those considered safe for human consumption by the US EPA and the WHO
    a. Copper is in excess of US EPA and WHO recommendations at a concentration $\geq$1:100,000 of BacSan
    b. Nitrates are in excess of US EPA and WHO recommendations at a concentration $\geq$1:100,000 and $\geq$1:50,000 of BacSan, respectively.
5. BacSan contributes concentrations of aluminum and zinc at levels that may cause negative aesthetic effects (taste, color, smell) at concentrations of BacSan $\geq$ 1:100,000 and 1:75,000, respectively.

The following research is recommended to improve and further explore the efficacy of BacSan:

1. More trials with Poliovirus-1 should be completed to provide further support for the evidence presented in this study.
2. More trials to determine the extent of interference with regard to Poliovirus-1 assays at various concentrations of BacSan should be completed such that any systematic error cause by interference can be accounted for.
3. A study to determine at what dilution of BacSan in polio media the disinfectant is neutralized should be completed to further validate the poliovirus results presented in this study.
4. A study to determine how turbidity effects the efficacy of BacSan should be completed.

5. The rotavirus assay should be optimized such that the efficacy of BacSan against this virus can be determined; rotavirus is one of the most important pathogenic viruses.
6. The efficacy of BacSan against protozoans and helminthes should be explored, as these are important sources of waterborne disease in the developing world.
7. Further work with the composition of BacSan to optimize its efficacy and decrease its potential for deleterious health effects is recommended in order to capitalize on the promising potential of this product for drinking water disinfection. The synergies of heavy metals as disinfectants (Rodriguez et al 2007), as well as their increased abilities in the presence of small amounts of chlorine (Landeen et al 1989; Yahya et al 1990), should be further explored, particularly such that the nitrate and copper concentrations in BacSan can be reduced without compromising and perhaps increasing, the efficacy of the product.
8. The concentration of copper in numerous "batches" of BacSan should be measured initially and over an extended period of time to better understand the quality control of the products' manufacture, as well as the manner in which the product ages.

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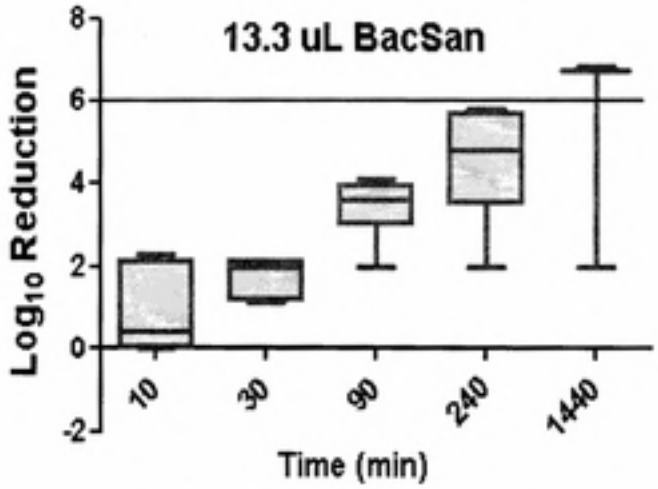
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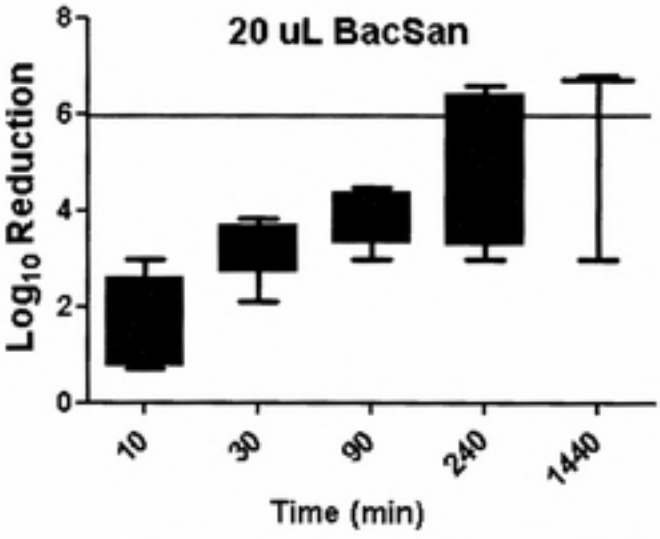
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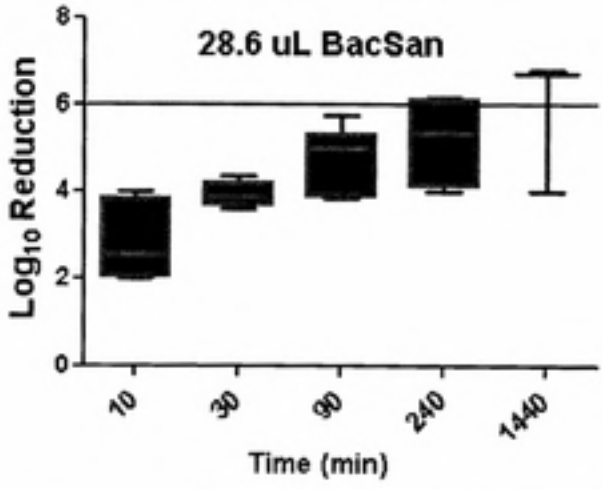
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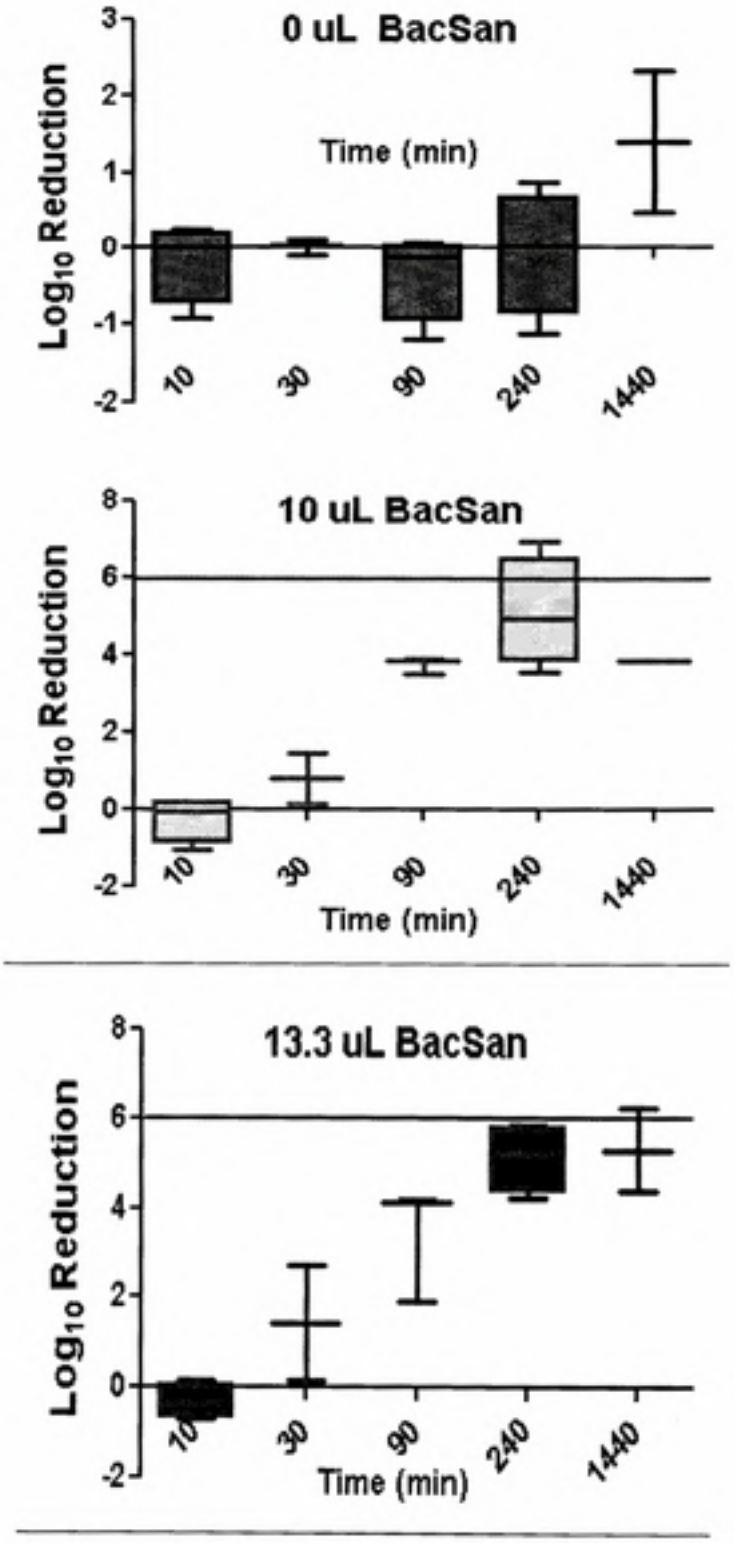
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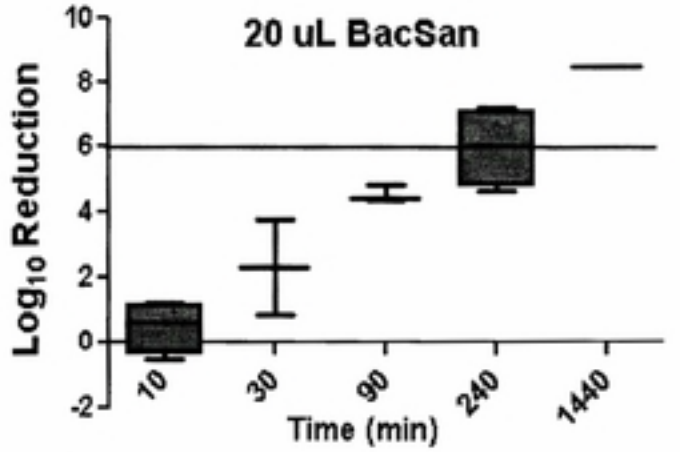

13.3 uL BacSan
Log10 Reduction
8
6
4
2
0
-2
10
30
90
240
1440
Time (min)

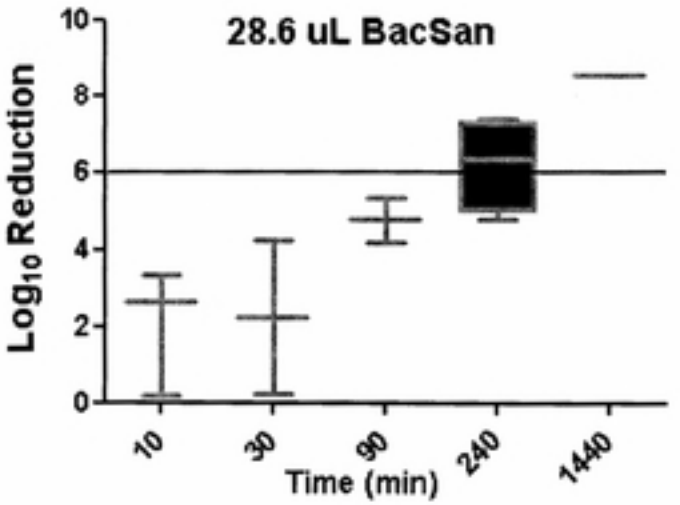

20 uL BacSan
Log10 Reduction
8
6
4
2
0
10
30
90
240
1440
Time (min)


28.6 uL BacSan
Log10 Reduction
8
6
4
2
0
10
30
90
240
1440
Time (min)

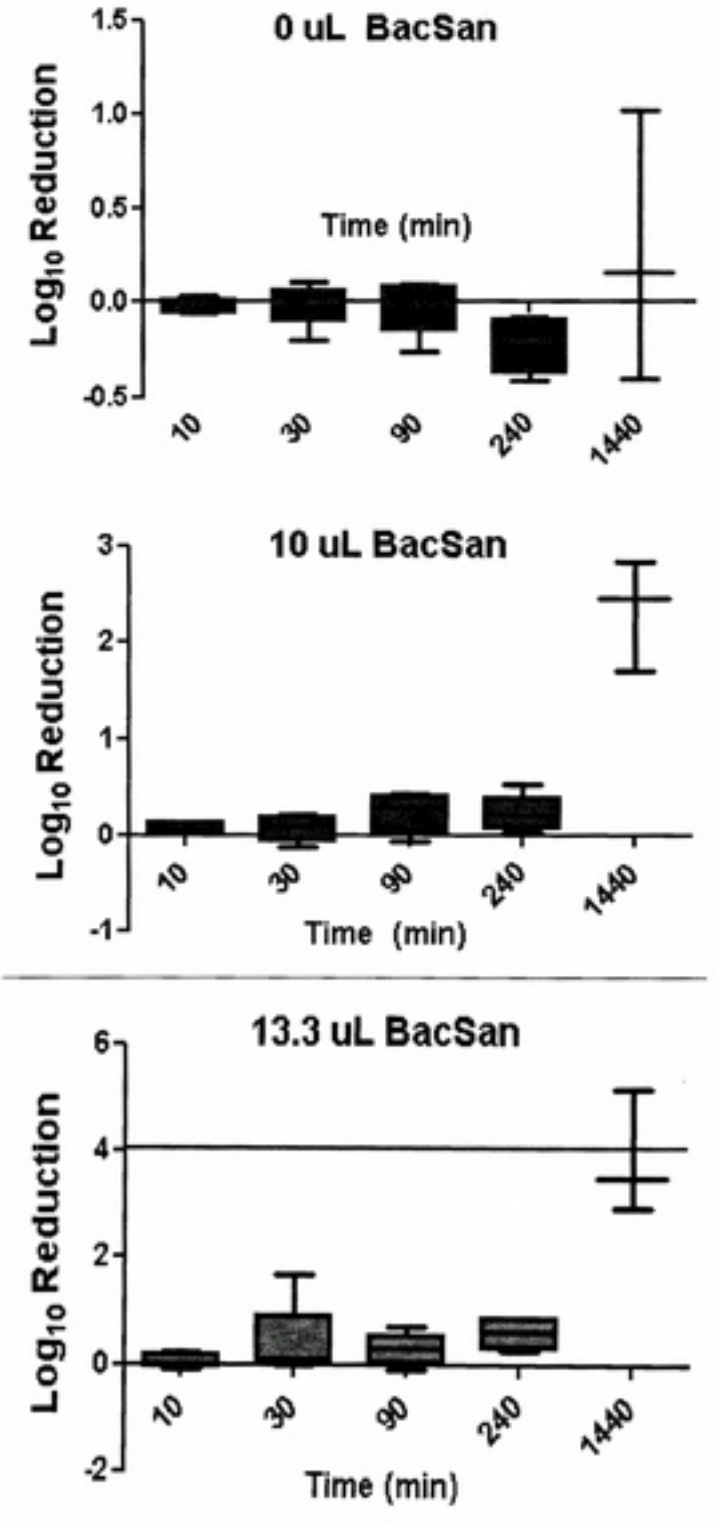
APPENDIX B. *K. terrigena* reductions graphed per volume of BacSan added to water (N=4)

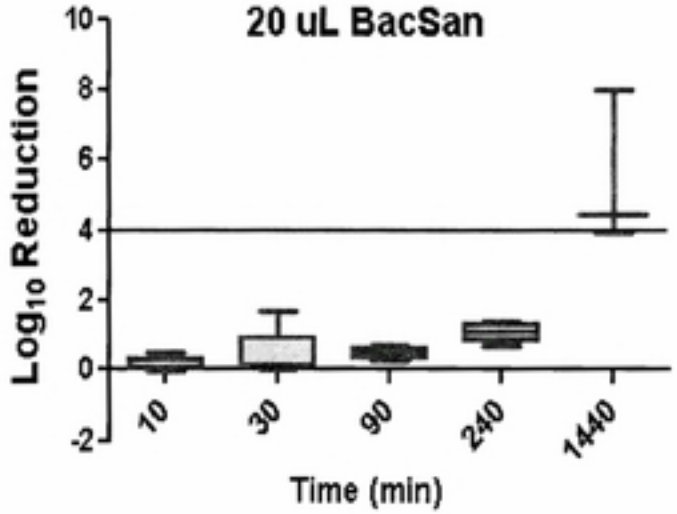


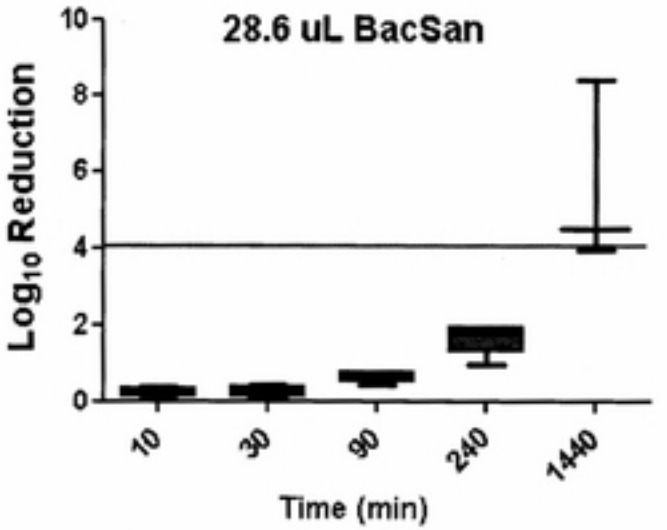

20 uL BacSan
Log10 Reduction
10
8
6
4
2
0
-2
10
30
90
240
1440
Time (min)

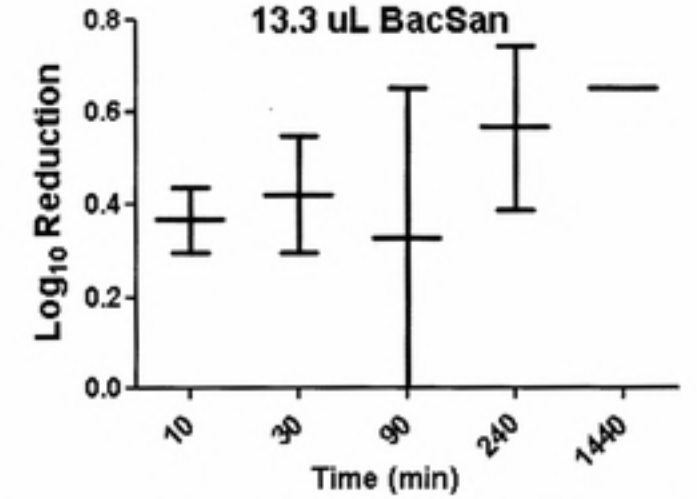

28.6 uL BacSan
Log10 Reduction
10
8
6
4
2
0
10
30
90
240
1440
Time (min)

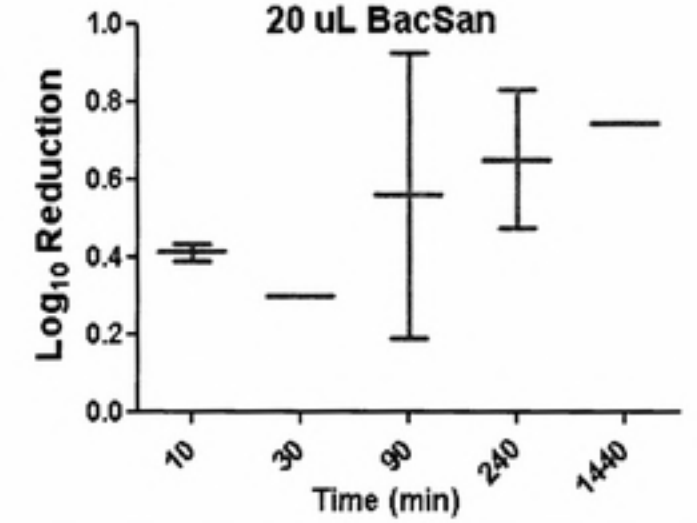
APPENDIX C. MS-2 reductions graphed per volume of BacSan added to water (N=5)

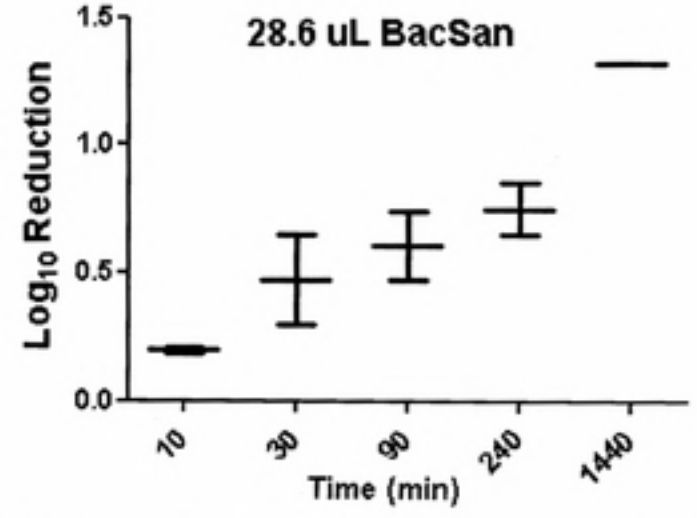



20 uL BacSan
Log10 Reduction
10
8
6
4
2
0
-2
10
30
90
240
1440
Time (min)


28.6 uL BacSan
Log10 Reduction
10
8
6
4
2
0
10
30
90
240
1440
Time (min)


13.3 uL BacSan
Log10 Reduction
0.8
0.6
0.4
0.2
0.0
10
30
90
240
1440
Time (min)


20 uL BacSan
Log10 Reduction
1.0
0.8
0.6
0.4
0.2
0.0
10
30
90
240
1440
Time (min)


28.6 uL BacSan
Log10 Reduction
1.5
1.0
0.5
0.0
10
30
90
240
1440
Time (min)