Survival and Sodium Hypochlorite Disinfection of Potential Ebola Virus Surrogates MS2, PhiX-174, and Phi6 in a Conservative Matrix of Raw Hospital Sewage and Human Fecal Waste

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ABSTRACT

Joseph Hale Strasser: Survival and Sodium Hypochlorite Disinfection of Potential Ebola Virus Surrogates MS2, PhiX-174, and Phi6 in a Conservative Matrix of Raw Hospital Sewage and Human Fecal Waste

(Under the direction of Dr. Mark Sobsey)

Between March 2014 and January 2016, West Africa experienced the largest Ebola virus disease (EVD) outbreak in history. For the duration of the epidemic, a number of instances occurred where infected individuals were transported outside of West Africa to be treated in hospitals in more developed nations (i.e. United States). Fecal wastes produced from Ebola cases in health care facilities in the United States can be discharged directly into local sewage systems without pre-treatment in accordance to guidance from the US Centers for Disease Control and Prevention (CDC). There is a need for understanding how Ebola virus may persist in these wastes and to develop protocols for appropriate in situ disinfection pre-treatment of Ebola fecal wastes. Potential Ebola virus surrogates, MS2 coliphage, PhiX-174 coliphage, and Phi6 bacteriophage, were tested for survival at 22 and 37 ^{O}C and disinfection with sodium hypochlorite in a conservative matrix of raw hospital sewage amended with human fecal waste. Phi6 required approximately nine days for a 5 $\log_{10}$ (99.999%) target reduction at the ambient condition (22 ^{O}C) and, due to the presence of an outer lipid envelope layer, may conservatively represent the survivability of Ebola virus in a comparable fecal waste matrix. Target 5 $\log_{10}$ reductions of MS2 and PhiX-174 at 22 ^{O}C required a predicted six and twelve weeks, respectively. All surrogate viruses were inactivated more rapidly at 37 ^{O}C than at 22 ^{O}C. Surrogates Phi6 and PhiX-174 were disinfected with sodium hypochlorite to a target 5 $\log_{10}$ (99.999%) reduction in 10 minutes at initial free chlorine concentrations of 2,800 and 3,500 mg/L, respectively. Free chlorine was rapidly consumed in the organic rich matrix, and therefore, high initial sodium hypochlorite concentrations were required to achieve significant reductions in viral titer. MS2 was extremely resistant to free chlorine disinfection and target reductions were not achieved with even the highest tested initial free chlorine concentrations. Disinfection kinetics were adequately described using the Selleck model, accounting for a first-order disinfectant decay rate. Enveloped surrogate persistence in raw sewage and fecal waste indicates precautionary pre-treatment with a high concentration of free chlorine may greatly reduce risk. Due to its nearly universal availability in health care settings, sodium hypochlorite (liquid bleach) is an appropriate chemical disinfectant for inactivating Ebola virus in a fecal waste matrix, but is not highly recommended because of the significant concentrations and volumes required to overcome the oxidizable organic content of such a matrix.

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INTRODUCTION

Between March 2014 and January 2016, West Africa experienced the largest Ebola virus disease (EVD) outbreak in history. EVD causes severe viral hemorrhagic fever in infected individuals. The epidemic resulted in 28,646 cases of EVD and 11,323 deaths, with most of these deaths occurring in Guinea, Liberia, and Sierra Leone {WHO 2016}. As the epidemic became a health crisis and difficult to control during the second half of 2014, the World Health Organization declared a public health emergency of international concern. This significant outbreak was caused by the species *Zaire ebolavirus*, a virus belonging to the Filoviridae family, a group of enveloped, filamentous viruses containing a negative-sense single-stranded RNA genome {CDC 2014}. The infectious dose of Ebola virus is believed to be <10 infectious virus particles {Jelden et al. 2015}.

For the duration of the epidemic, a number of instances occurred where unknowingly infected individuals left West Africa and then became ill or infected individuals were transported outside of West Africa to be treated in hospitals in more developed nations (i.e. United States). These individuals posed a great risk of spreading the virus to others and were treated in health care facilities under quarantine conditions, making it possible to contain the viruses they shed from bodily fluids.

The exact concentration of infective Ebola virus in different bodily fluids is unknown, but non-human primate Ebola virus organ infectivity titers have exhibited high viral concentrations approaching 10^7 and 10^8 PFU mL^{-1} and viral loads in human blood, stool, and urine have shown genome copy concentrations up to 10^8 mL^{-1}, 10^7 mL^{-1}, and $10^{5.5}$ mL^{-1}, respectively {Geisbert,

Hensley et al. 2003; Wolf et al. 2015}. Infected individuals may produce up to nine liters of liquid waste in a single day, mostly in the form of watery diarrhea {Lowe et al. 2014}.

Currently, fecal wastes produced from Ebola cases in health care facilities in the United States can be discharged directly into local sewage systems without pre-treatment in accordance to guidance from the US Centers for Disease Control and Prevention (CDC) {CDC US 2014}. It is assumed, but not well documented, that dilution and conventional wastewater treatment will result in removal and inactivation of infectious Ebola virus, resulting in negligible risk. However, there is concern that Ebola may persist in these wastes. Bibby et al. (2015) found recoverable infectious Ebola virus for a period of at least 8 days in virus-seeded, irradiated sewage and a study by Cook et al. (2015) showed 15 days were required for a 4 $\log_{10}$ reduction of Ebola virus in a dried organic load {Bibby et al. 2015; Cook et al. 2015}. Given this possible persistence, there is concern that Ebola virus in local sewage systems could pose EVD risk to sewer workers and others (during sewer overflow or servicing events) who might become exposed to feces and sewage that contains shed Ebola virus. Thus, there is a need to determine the survivability of infectious Ebola virus in fecal wastes and sewage and the effectiveness of chemical disinfection pre-treatment to inactivate it in fecal wastes and sewage.

A number of disinfectants are recommended to inactivate Ebola in healthcare settings, including sodium hypochlorite (liquid bleach) {CDC US 2014}. However, disinfectants have only recently been studied for their ability to inactivate Ebola virus in sewage, and have not been studied at all in raw sewage or raw human fecal waste. Appropriate in situ disinfection pre-treatment of sewage and fecal waste produced by Ebola patients will reduce risks associated with direct discharge into local sewage systems.

OBJECTIVE

The purpose of this research was to quantify the survival and inactivation by sodium hypochlorite disinfection (and model the inactivation kinetics) of Ebola virus surrogates MS2, PhiX-174, and Phi6 in a conservative matrix consisting of raw hospital sewage amended with human fecal waste. Ultimately, this research will contribute to the development of a protocol for the in situ disinfection pre-treatment of Ebola virus to achieve a target 5 $\log_{10}$ (99.999%) virus reduction within 10 minutes in the fecal waste of infected individuals being treated in hospitals and health care settings in the United States and other developed and developing nations.

BACKGROUND AND REVIEW OF THE LITERATURE

Ebola Virus

The 2014 West African Ebola virus disease (EVD) outbreak was caused by the genera *Ebolavirus* (EBOV), and more specifically viruses belonging to the species *Zaire ebolavirus*. The epidemic resulted in 28,646 cases of EVD and 11,323 deaths. Most of these deaths occurred in Guinea, Liberia, and Sierra Leone {WHO 2016}. As of January 14th, 2016, the outbreak was considered to be over but with further flare-ups possible.

Ebola virus belongs to the family Filoviridae, a group of enveloped, filamentous viruses containing a negative-sense single-stranded RNA genome {CDC 2014}. *Marburgvirus* and *Ebolavirus* are the only two members of this family and are known to cause severe hemorrhagic fever in humans and non-human primates {CDC 2014}. Infections during periods of outbreak are typically a result of direct contact with bodily fluids of infected individuals or cadavers {WHO 1978}. Probable routes of exposure to Ebola virus include host mucosal surfaces, skin abrasions, and parenteral introduction {Feldmann and Geisbert 2011}. The infectious dose of Ebola virus is believed to be <10 infectious viral particles {Jelden et al. 2015}.

Studies of non-human primates indicate that, upon entering the host, the preferred replication sites of Ebola virus are likely monocytes, macrophages, and dendritic cells, which facilitate dissemination of the virus to the lymph nodes, liver, and spleen {Geisbert et al. 2003}. The exact concentration of infective Ebola virus in different bodily fluids is unknown, but non-human primate Ebola virus organ infectivity titers have exhibited high infectious virus concentrations approaching 10^7 and 10^8 mL^{-1} and viral loads in human blood, stool, and urine have shown

genome copy concentrations up to 10^8 mL^{-1}, 10^7 mL^{-1}, and $10^{5.5}$ mL^{-1}, respectively {Geisbert, Hensley et al. 2003; Wolf et al. 2015}. Infected individuals may produce up to nine liters of liquid waste in a single day, mostly in the form of watery diarrhea which may contain blood {Lowe et al. 2014}. It is this diarrhea, water, and various other body fluid mixture deposited into hospital toilets that is the matrix of concern to this report, and in which surrogate virus survival and disinfection tests will occur to ensure safe disposal to wastewater conveyances and treatment facilities.

Virus Survival and Persistence

A number of possible physical, chemical, and biological factors may influence the fate and persistence of viruses in sewage and fecal waste. Important physical factors are heat or thermal effects, aggregation, and adsorption or embedding {Sobsey 2005}.

Elevated temperatures are known to inactivate virus infectivity by inducing protein denaturation and possibly by disrupting enzymatic activity required for infection of host cells {Melnick and Gerba 1980}. In a survival study of a highly persistent enveloped animal virus, Aujeszky's disease virus (pseudorabies virus), in swine fecal waste under anaerobic storage conditions, virus inactivation rates increased with increasing temperature. Aujeszky's disease virus required two weeks for 4 $\log_{10}$ inactivation at 20 ^{O}C (near ambient) but required only 5 hours for 4 $\log_{10}$ inactivation at 35 ^{O}C (mesophilic conditions) and 10 minutes for 4 $\log_{10}$ inactivation at 55 ^{O}C (thermophilic conditions) {Bøtner 1991}.

Aggregation or clumping of viral particles protects them from inactivation by chemical or biological agents {Sobsey 2005}. It is very unlikely that all virions will be single particles and aggregates can consist of several hundred particles {Melnick and Gerba 1980}. In a study of poliovirus aggregate halogen disinfection in water, Young and Sharp (1977) observed a continuously decreasing reaction rate of bromine inactivation for aggregated viral particles {Young and Sharp 1977}. Viral particles can also readily adsorb to or embed themselves in particulate material. Studies have shown that a virus that is adsorbed generally has a better chance of survival than one that is freely suspended {Gerba 1975}. A small batch virus survival study by Sobsey et al. (1980) showed that reovirus persisted longer in solutions containing soil loads than control solutions that were soil-free, due to adsorption effects {Sobsey et al. 1980}. A recent study of Ebola virus persistence in irradiated wastewater by Bibby et al. (2015) displayed an apparent rapid viral decrease (4 $\log_{10}$ reduction within the first day), followed by a slight recovery and subsequent steady inactivation. This initial rapid decrease was attributed to possible viral particle aggregation or adsorption to wastewater particles {Bibby et al. 2015}.

Chemical properties of a sewage or fecal waste matrix of particular interest to virus survival and persistence are pH and ammonia content {Sobsey 2005}.

Viruses tend to survive best near neutral pH and worst at pH extremes. Ajariyakhajorn et al. (1997) found that both pseudorabies virus (PRV) and porcine reproductive and respiratory syndrome virus (PRRSV) survived in swine slurry longest at pH 7.0, compared to pH 4.0 and 10.0, with PRV and PRRSV persisting for 8 and 14 days, respectively {Ajariyakhajorn et al. 1997}.

Ammonia (NH_3) has virucidal activity and, when present in higher concentrations at a pH > 8, can inactivate viruses in a fecal waste matrix {Sobsey 2005}. A study by Vinnerås et al. concerning chemical disinfection of separated fecal matter showed that 30 g of ammonia per kilogram of feces at pH 9.3 displayed a slow initial reduction but had significant effects on *S. typhimurium* 28b phage survival over a period of 21 days (1 $\log_{10}$ greater reduction than control) and 50 days (7 $\log_{10}$ greater reduction than control) {Vinnerås et al. 2003}.

Microbial activity (production of proteolytic enzymes) and predation (engulfment, ingestion) are noteworthy biological components affecting persistence of viruses in sewage and feces. Antiviral bacterial activity was demonstrated in poliovirus persistence studies performed by Deng and Cliver (1992). These field experiments were conducted in raw mixed septic tank effluent and swine manure slurry compared to an autoclaved mixed waste. At 25 ^{O}C in raw mixed waste, poliovirus experienced a 90% reduction in virus titer in 6.8 days compared to 11.2 days for the autoclaved mixed waste, which was a statistically significant difference. At 37 ^{O}C, the 90% reductions were 1.3 and 3.9 days in raw mixed waste and autoclaved mixed waste, respectively {Deng and Cliver 1992}.

Pathogenic virus survival often exhibits first-order inactivation kinetics and is typically modeled using linear regression analysis. Stallknecht et al. (1990) modeled viral decay rates of four avian influenza viruses in water using linear regression analysis and trendlines adequately explained results in all cases. Casanova et al. (2009) found that inactivation of swine transmissible gastroenteritis virus (TGEV) and mouse hepatitis virus (MHV) in water and pasteurized sewage followed first-order kinetics and was appropriately modeled using regression analysis. Additionally, Ebola virus survival kinetics has been primarily modeled using first-order

(exponential decay) linear regression. Fischer et al. (2015), used linear regression modeling to describe virus inactivation over time when Ebola virus persistence was tested in a number of dry and liquid conditions in simulated outbreak environments. Bibby et al. (2015) tested the persistence of Ebola virus in sterilized wastewater and used linear regression analysis to model persistence over a period of 8 days. Sagripanti et al. (2010) also found that linear regression modeling adequately described the inactivation kinetics of Ebola virus in solutions dried on different surfaces.

Ebola Virus Persistence in Biological Fluids and Waste Matrices

Recent studies on the persistence of Ebola virus in biological fluids and environmental media have indicated a noteworthy survivability of the enveloped virus. In a study by Chughtai et al. (2016) of particular human body fluids, Ebola virus was isolated from semen, aqueous humor, urine, and breast milk 82, 63, 26, and 15 days after onset of illness, respectively. Viral RNA was isolated from these fluids for an even longer period {Chughtai et al. 2016}.

In media of distinct importance to the research conducted in this report (water, feces, sewage), Ebola virus persists similarly. In a study by Cook et al., Ebola virus (Makona variant) was suspended in a simulated organic load (meant to mimic residual bodily fluids) and then dried on a number of surfaces to quantify persistence. Based on a model projection, roughly fifteen-days would be required to achieve a 4 $\log_{10}$ reduction (99.99%) {Cook et al. 2015}. In another study by Bibby et al, Ebola virus spiked into gamma-irradiated wastewater matrix showed persistence (recoverable active viruses) for at least 8 days {Bibby et al. 2015}. In a related study of filovirus (*Zaire ebolavirus, marburgvirus)* survival in tissue culture media at room temperature by Piercy

et al, Ebola virus required twenty-six days for a 4 $\log_{10}$ reduction in virus titer {Piercy et al. 2010}. These few studies give apparently different quantitative results but indicate a minimum of 8 days are required for an appropriate reduction and maximum survival periods could be on the order of several weeks. It is important to consider the physical, chemical, and microbiological differences between a simulated organic load matrix, a biologically inert sewage matrix (pasteurized by high-energy irradiation), a tissue culture medium matrix, and a non-pasteurized fecal waste and hospital sewage matrix utilized in the experiments of this report.

Surrogate Viruses

Due to the designation of Ebola virus as a biosafety level-4 pathogen (BSL-4), experimentation with the virus is exceedingly difficult. In order to perform representative tests concerning the survival and disinfection of Ebola virus, surrogate viruses were used instead. An ideal surrogate would be at least as persistent as Ebola virus, more (but not exceptionally more) resistant to disinfection processes, and relatively simple to work with and recover/enumerate. MS2 coliphage, PhiX-174 coliphage, and Phi6 bacteriophage were the surrogates considered and used to test survival and disinfection in this report.

MS2 bacteriophage (Leviviridae) is a single-stranded RNA virus that infects *Escherichia coli*, making the virus a 'coliphage' {Abedon and Calendar 2005}. MS2 only infects *E. coli* carrying an F (fertility) plasmid that produces a pilus used in bacterial conjugation. The bacteriophage attaches to this pilus before entering the host cells {Davis et al. 1961}. An MS2 virion is non-enveloped and has an icosahedral protein capsid {Golmohammadi et al. 1993}. MS2 has been previously used as a surrogate for norovirus and other human viral pathogens in survival and

disinfection studies and has shown resistance to chlorine disinfection {Sobsey et al. 1988; Shin and Sobsey 1998; Gray et al. 1993; Dawson et al. 2005}.

PhiX-174 bacteriophage (Microviridae) is a non-enveloped virus that also infects *E. coli* and has a circular single-stranded DNA genome, which was the first DNA-based genome sequenced {Sanger et al. 1977}. PhiX-174 infects by binding to lipopolysaccharides on the bacterial host cell surface (somatic coliphage) then injecting the viral genome through the membrane {Jazwinski and Kornberg 1975}. The virus has commonly been used as a blood-borne pathogen surrogate for Hepatitis B and C (HBV, HCV) and Human Immunodeficiency Virus (HIV) in barrier penetration studies based on its size, morphology, and environmental stability similarities {Ball et al. 2012}. PhiX-174 has also been reported to survive longer than other bacteriophages and human enteric viruses in groundwater (Charles et al, 2009).

Phi6 bacteriophage (Cystoviridae) is a double-stranded RNA virus that infects the plant bacterium *Pseudomonas syringae* {Vidaver et al. 1973}. The virus has been suggested and tested as a surrogate for pathogenic enveloped viruses, like Ebola virus and avian influenza, and survival studies have been conducted in water, on PPE, and in pasteurized human sewage {Casanova and Weaver 2015}. Phi6 bacteriophage may more accurately represent Ebola virus survival and disinfection compared to the previously mentioned coliphages (MS2 and PhiX-174) due to the presence of an outer lipid envelope layer. Phi6 and also Phi8, of the same bacteriophage family, were reported to at survive at least as long as avian influenza virus H5N1 in fresh water and were somewhat persistent when subjected to chlorine disinfection (Adcock et al 2009).

Candidate Disinfectant: Free Chlorine

Free chlorine, typically used in the form of sodium hypochlorite (liquid bleach), has long been used in water and wastewater treatment to inactivate harmful pathogens due to its availability, ease-of-use, affordability, acceptability, and high disinfection efficacy. Despite the extensive use of chlorine, the exact mechanism by which the compound inactivates bacteria and viruses is not clearly understood. Possible modes of action include oxidation of sulfhydryl groups on certain amino acids in structural proteins and enzymes, loss of intracellular contents from cells, decreased adenosine triphosphate (ATP) production, breaks in DNA and RNA, depressed DNA and RNA synthesis and function, and several others {Rutala and Weber 2008}. Most vegetative bacteria have a low tolerance to chlorine and are inactivated relatively easily. Viruses, however, are moderately resistant to the disinfection actions of chlorine {CDC 2012}. Additionally, non-enveloped (naked protein coat) viruses (ex: norovirus) tend to be more resistant than lipid-surrounded enveloped viruses (ex: influenza) {Maillard 2001}. Electron microscopy has shown that chlorine can cause complete degeneration of enveloped viral particles and destruction of nucleic acids {Shirai et al. 1999}.

Several parameters influence the virucidal activity of free chlorine. Classic factors such as concentration, contact time, temperature, and pH all affect the efficacy of free chlorine disinfection. Ideal conditions for disinfection are slightly acidic pH (approximately pH 6) combined with elevated temperature {Northern Territory Government 2013}. The chemical speciation of chorine is pH dependent, with hypochlorous acid as the major species and hypochlorite ion as the minor species at pH less than 7 and vice-versa at pH greater than 7.

Hypochlorous acid is the stronger disinfectant of the two species and greater $\log_{10}$ reductions are realized in lower pH experimental conditions {LeChevallier and Au 2004}.

Composition of the disinfection matrix is another particularly important factor. Although free chlorine is effective at treating low-turbidity drinking water, raw wastewater and fecal waste are rich in oxidizable organics and requires significantly higher concentrations of free chlorine in order to reach acceptable pathogen reductions {Maillard 2001}. A disinfection matrix rich in organics will rapidly consume free chlorine (formation of organochlorides/chloramines) so excess amounts must be added to maintain a desired free chlorine residual. The free chlorine concentration at which the residual is achieved is known as a chlorination breakpoint. Upon reaching 'breakpoint chlorination,' the free chlorine residual will increase predictably as more chlorine is added {Bowman and Mealy 2007}. In addition to consuming free chlorine, suspended organic material may also facilitate the aggregation and protection of viral particles from inactivation {Maillard 2001}.

Typically, Ct (the product of disinfection concentration, C, and contact time, t) values are the standard for determining the efficacy of a candidate disinfectant against a target organism. However, these values are not always transferable across organisms or test conditions {Haas et al. 1996}. The majority of disinfection kinetic models, including the classic Chick-Watson linear model (Ct), do not consider the decay of the disinfectant over time and may not predict Ct values for organisms that are not disinfected according to a first-order decay rate {Haas and Joffe 1994}. A study by Venczel et al., showed significantly higher Ct 99.99% values for MS2 bacteriophage reduction were required when free chlorine disinfection experiments were performed in water amended with oxidizable organic material in the form of humic acid

(consumption of free chlorine residual) compared to disinfection experiments in demand-free test water {Venczel et al. 2004}.

Under circumstances when inactivation kinetics does not conform well to first-order models due to disinfectant demand conditions, models that include a term for the first-order decay of disinfectant concentration over time should be considered. According to Haas (1979), assuming that the disappearance of a disinfectant is governed by irreversible first-order kinetics, which must be independent from the rate of organism inactivation, the Selleck rate law can include such a term {Haas 1979}. The model was initially derived to describe the inactivation of bacteria in wastewater with chlorine disinfectant, which is directly applicable to the disinfection experiments in this report. The Selleck model is an empirical model and is therefore applicable to any chemical disinfectant, organism, and test matrix {Gyürék and Finch 1998}.

Free Chlorine and Ebola Virus

According to the US CDC, Ebola virus will be inactivated relatively easily using many common chemical agents including household bleach solution when used according to the label instructions. In a figure published on the CDC 'Ebola virus in medical waste' webpage, enveloped viruses are presented as the most susceptible microorganisms to disinfection and sterilization (even more susceptible than vegetative bacteria like *S. aureus*) {CDC 2015}. Current literature depicts differing results of the performance of chlorine against Ebola virus.

In a 2015 systematic review of the efficacy of chlorine solution for hand hygiene and glove disinfection conducted by Hopman et al, limited and low quality evidence was found concerning

the ability of chlorine to inactivate Ebola virus {Hopman et al. 2015}. However, in a recently published study by Cook et al, 0.5% and 1% sodium hypochlorite solutions sufficiently inactivated the Ebola virus (Makona variant) in a dry, simulated organic soil load within a five-minute contact time. This simulated organic load composition was based on the ASTM International Quantitative Carrier Testing 2 international standard and included 106.25 µL of Ebola virus stock, 12.5 µL 5% BSA (bovine serum albumin), 17.5 µL 5% tryptone, and 50 µL 0.4% mucin. Ten microliters of this virus-soil load mixture was deposited on a stainless steel carrier disk and was then treated with 50 µL of 0.01%, 0.1%, 0.5%, and 1% sodium hypochlorite solution. No significant reduction in viral titer occurred for 0.01% and only a partial reduction occurred for 0.1% (about 3 $\log_{10}$ reduction after 10-minute contact time) {Cook et al. 2015}. It must be noted that these experiments required a significant volume of disinfectant (5:1 disinfectant solution to virus matrix (v/v)) to achieve desired reductions of Ebola virus. Another recent study by Bibby et al. (2017) studied the disinfection of Ebola virus using sodium hypochlorite in sterilized municipal wastewater. Their research showed that 5 and 10 mg/L of initial free chlorine resulted in free chlorine residuals of 0.52 and 1.11 mg/L respectively and was sufficient to disinfect Ebola virus to the detection limit (4.2 $\log_{10}$) within 20 seconds of contact time. For a lower initial free chlorine concentration, 1 mg/L, a 3.5 $\log_{10}$ reduction was recorded at 20 seconds, but further inactivation was not evident due to the rapid decay of the free chlorine residual. The municipal sewage matrix used for this disinfection study had a COD concentration of 54.7 mg/L, TOC concentration of 31.6 mg/L, TSS concentration of 129 mg/L, and ammonia concentration of 32.5 mg/L. Such sewage may not be adequately representative of fecal waste matrices and strong sewage that could come from patients in healthcare settings.

Rationale for this Study

While a number of studies have investigated the survival and disinfection of viruses in various sewage and organic matrices, there is little or no research concerning survival of viral pathogens and surrogates in raw hospital sewage amended with human fecal waste. Researchers have attempted to determine what the persistence of Ebola virus might be in sewage, but these matrices are either heat pasteurized or irradiated prior to introducing Ebola or surrogate viruses and do not represent the biologically active conditions of raw sewage and feces. Additionally, despite free chlorine being a suggested disinfectant for inactivation of Ebola virus in health care settings, there have been no studies concerning sodium hypochlorite disinfection in a matrix that conservatively reflects fecal wastes associated with patients suffering from EVD. These voids in available research are the basis for the content of this report.

METHODS

Surrogate Viruses

The potential Ebola surrogate viruses used in survival and disinfection experiments in this report were Phi6 bacteriophage (capable of infecting *Pseudomonas syringae*), MS2 coliphage (capable of infecting F_{amp} *E. coli*), and PhiX-174 coliphage (capable of infecting CN13 *E. coli*). These surrogate viruses were chosen because of their previous use as surrogates for human viral pathogens like norovirus, hepatitis B and C, HIV, avian influenza, etc. Surrogates were also chosen based on their acceptance in BSL-2 laboratories and their ease of recovery and enumeration. MS2 coliphage stock was prepared from American Type Culture Collection (ATCC) seed stock (coliphage propagation protocol included in appendix). PhiX-174 coliphage stock was also prepared from ATCC seed stock (coliphage propagation protocol included in appendix). Phi6 bacteriophage stock was prepared from Georgia State University seed stock and was propagated with the sloppy agar method described by Casanova and Waka (2013). Viral stocks were stored at -80 ^{O}C in small aliquots to reduce freeze-thaw inactivation. Aliquots were removed from frozen storage approximately 0.5 hours before beginning experiments and viruses were spiked directly from thawed stock.

Fecal Waste Matrix Collection and Preparation

The representative matrix utilized in survival and disinfection experiments consisted of 25% human fecal matter from bowel movements of humans and 75% raw hospital sewage (w/w). Fresh fecal samples were collected in sealable plastic collection containers from anonymous volunteers in the UNC Environmental Virology Laboratories (25% of the total matrix weight).

Hospital sewage was collected from manhole access points to the sewage system at three sampling sites at UNC Hospitals. Three equal volumes from each site were combined to form a representative hospital sample (75% of the total matrix weight, i.e. one quarter of the final matrix from each hospital sewage collection site). At each collection site, sewage was collected directly as grab samples in sterile 500 mL polypropylene bottles and kept chilled in a cooler with ice during transport back to the laboratory. The sewage in the sewer pipeline was not treated, and came directly from one of the following hospital departments:

1. Gastroenterology + Medical Intensive Care Unit
2. Burn + Labs + Intensive Care Unit
3. Emergency Room + Neurology

On the day of collection, fecal and hospital samples were mixed vigorously and the resulting matrix was stored in a walk-in refrigerator at 4 ^{O}C (± 1 ^{O}C) in 20 or 35 mL aliquots. Disinfection experiments were performed in 20 mL sample volumes of the matrix and survival experiments were performed in 35 mL sample volumes of the matrix. The matrix aliquots were not pasteurized prior to survival or disinfection experiments to most appropriately represent actual waste conditions. The initial pH of this matrix was tested using an electronic glass pH electrode (matrix pH 6.7 ± 0.2). New matrices were produced with the same procedures approximately every 2 weeks to ensure "freshness" by avoiding changes in composition or biological activity that could occur during prolonged storage.

Matrix Analysis

A sample of raw hospital sewage and human fecal waste matrix was analyzed at an Environmental Protection Agency certified analysis facility (Microbac Laboratories, Marietta,

OH). The report parameters included biochemical oxygen demand (BOD), total suspended solids (TSS), total organic carbon (TOC), chemical oxygen demand (COD), and ammonia content. The pH was measured at the UNC Environmental Virology Lab using an electronic glass pH electrode. Constituent measurements are summarized in Table 1.

Constituent	Measured Value
pH	6.7 ± 0.2
BOD (mg/L)	19,600
COD (mg/L)	63,500
TSS (mg/L)	73,200
TOC (mg/L)	13,600
Ammonia (mg/L)	216

Table 1*: Measured constituent values from matrix analysis. All constituents except pH were measured at Microbac Laboratories, Marietta, OH.*

Candidate Disinfectant

Sodium hypochlorite (free chlorine) used in the following experiments was a 7.85% (78,500 mg free Cl/L) household-bleach (commercially available as 'Harris Teeter – Your Home' brand sodium hypochlorite solution). Approximate concentrations of 0 mg/L, 500 mg/L (0.05%), 1000 mg/L (0.1%), 1500 mg/L (0.15%), 2000 mg/L (0.2%), 2500 mg/L (0.25%), 3000 mg/L (0.3%), or 4000 mg/L (0.4%) free chlorine were delivered to 20 mL volumes of fecal waste matrix containing surrogate viruses. Initial concentration of stock bleach disinfectant was confirmed prior to use with a Hach Pocket Colorimeter II apparatus using a standard N,N-diethyl-*p*-phenylenediamine (DPD) colorimetric method according to the instructions of the colorimeter manufacturer.

Breakpoint Chlorination

Breakpoint chlorination experiments were performed in the same matrix as batch disinfection experiments. A total residual chlorine test was conducted in 30 mL of undiluted matrix. Liquid bleach was added step-wise from 250 mg/L to 10,000 mg/L initial free chlorine. After each chlorine addition, the matrix was shaken vigorously for 35 seconds to ensure sufficient contact between the chemical disinfectant and the matrix. The matrix was then diluted 1,000 fold (in distilled water) to reduce turbidity and to get the concentration into the detectable range of the instrument when testing using a Hach Pocket Colorimeter II. The measured total Cl was recorded and adjusted for dilution (Table A1). Adjusted residual total chlorine concentrations were plotted versus initial free chlorine concentrations to determine a likely breakpoint (Figure A1). Linear regression analysis was performed from where residual total chlorine values began to significantly increase ($R^2 = 0.99$). The predicted breakpoint for this test was approximately 2,300 mg/L.

In another experiment to determine breakpoint chlorination, a residual free chlorine test was conducted. A 1:100 dilution of the fecal waste matrix was dosed with stepwise additions of free Cl from about 2 mg/L to over 100 mg/L. After dosing, the matrix was shaken vigorously for 35 seconds to ensure sufficient contact between the chemical disinfectant and the matrix. The mixture was then allowed to sit for a total contact time of 10 minutes. An additional 10-fold dilution was required to reduce turbidity when testing with a Hach Pocket Colorimeter II apparatus. The measured free Cl was recorded and adjusted for dilution (Table A2). The initial free Cl values were also adjusted to reflect the initial values required in an undiluted matrix. Dilution adjusted residual free chlorine concentrations were plotted versus initial free chlorine

concentrations to determine a likely breakpoint (Figure A2). Linear regression analysis was performed from where residual free chlorine values began to significantly increase ($R^2 = 0.997$). The predicted breakpoint for this test was approximately 2,500 mg/L.

Chlorine Neutralization

Appropriate volumes of stock 1000 mg/L sodium thiosulfate were used to neutralize chlorine for all neutralization and disinfection experiments. A chlorine neutralization experiment was performed for an initial chlorine concentration of 500 mg/L in 20 mL of distilled water. Tests were run in a sterile 50-mL polypropylene, conical bottom, screw-capped tube (Falcon). Distilled water has little or no chlorine demand, therefore if neutralization was achieved, no subsequent microbial inactivation was assumed. Neutralization tests were confirmed with a Hach Pocket Colorimeter II apparatus utilizing free chlorine DPD test packets. Four separate neutralization concentrations were tested for their neutralization ability: 0.5x (250 mg/L sodium thiosulfate), 1.0x (500 mg/L), 1.5x (750 mg/L), and 2.0x (1000 mg/L). The appropriate volume of household bleach solution was added to 20 mL of distilled water and shaken thoroughly. The mixture was allowed to sit for two minutes prior to adding the sodium thiosulfate test concentration. After adding the neutralizer, the mixture was shaken vigorously to ensure contact between sodium thiosulfate and free chlorine prior to determining free chlorine concentration. The 0.5x and 1.0x tests did not adequately neutralize the available free chlorine. Subsequent tests with 1.5x and 2.0x neutralizer concentrations provided positive neutralization results. Seemingly, a minimum of 1.5x (750 mg/L) concentration of neutralizer compared to free chlorine concentration was required to neutralize the distilled water matrix used in these test experiments. Using this empirical value, minimum 1.5X neutralization volumes were multiplied by a factor of four (to

6X concentration) to ensure complete free chlorine neutralization during disinfection experiments. Neutralization volumes were based on residual chlorine concentration values obtained in breakpoint experiments. Control experiments where only excess sodium thiosulfate was added were conducted to ensure there were no quantifiable effects of sodium thiosulfate on the inactivation of the surrogate organisms of interest.

Batch Disinfection Experiments

At the start of disinfection experiments, viral stocks were spiked into a 20 mL matrix aliquot in a sterile 50-mL polypropylene, conical bottom, screw-capped tube (Falcon) to a target concentration of approximately 10^7 to 10^8 PFU/mL. A baseline sample of 2 mL was taken following spiking to assess the initial concentration to determine $\log_{10}$ reductions at subsequent time points. This sample was filtered using a 0.22-µm filter (Millipore EX) and a 3 mL disposable syringe (BD).

Following baseline, the matrix was dosed with sodium hypochlorite disinfectant to a desired initial free chlorine concentration, a timer was started, and the matrix was shaken well for approximately 35 seconds to mix the disinfectant evenly throughout the matrix.

Small 8 mL polypropylene screw-cap tubes (Sarstedt) were prepared before disinfectant dosing for each time point and were filled with an appropriate amount of neutralizer (1000 mg/L stock sodium thiosulfate) to cease disinfection at the time of sampling. The neutralization volumes required were determined by the methods described previously. Control tests were conducted to ensure that the shaking technique used had no quantifiable effect on the inactivation of surrogate viruses.

Time points were taken at 1, 3, 10, and 30 minutes following disinfectant dosing (5-minute time points were taken for a few initial trials). At each time point, 5 mL of matrix was poured into the neutralization vials (pre-filled with the appropriate neutralizer) and the vials were shaken vigorously for approximately 1-minute to ensure neutralization. After the 30-minute period, each of these neutralized samples was filtered using the same method as the baseline sample. This filtrate was then diluted (if necessary) to the appropriate dilution in 1.5 mL polypropylene centrifuge tubes (Eppendorf) using stock 1X phosphate saline buffer (PBS) as the diluent. The preparation of PBS stock is described in the appendix. The viral concentrations of these neutralized and diluted samples were subsequently determined using a double agar layer plaque assay enumeration method (DAL). The DAL method utilized was a modified version of the EPA 1602 method (Section 11.3).

Survival Experiments

Somewhat similar to previously described disinfection methodologies, at the beginning of survival experiments, viral stocks were spiked into a 35 mL matrix aliquot in a sterile 50-mL polypropylene, conical bottom, screw-capped tube (Falcon) to a target concentration of approximately 10^7 to 10^8 PFU/mL. A baseline sample was taken following spiking to assess the initial virus concentration in order to later determine $\log_{10}$ reductions based on remaining virus concentrations at subsequent time points. All samples were collected and filtered using a 0.22-µm filter and a 3 mL disposable syringe (BD). Sealed 50 mL tubes were then placed in the dark at either ambient temperature (22 ºC) or mesophilic temperature (37 ºC incubator room) for the duration of the survival experiment. Samples were taken every 24 hours for several days

following baseline. After a period of several days, samples were taken intermittently at longer time intervals until a maximum of 18 days. At least two replicate samples ($n \geq 2$) were taken for each sampling day of interest.

At each time point, samples were filtered as previously described and this filtrate was then diluted (if necessary) to the appropriate dilution as previously described. The virus concentration of these diluted samples was subsequently determined using a double agar layer enumeration method (DAL) that was a modification the EPA 1602 method (Section 11.3).

Quantification of Results

Plates for DAL plaque assay ('bottom agar' plates) were prepared several days before the experiments. Polystyrene, 100 mm diameter plates were filled with approximately 10-12 mL of 1.5 times (1.5X) standard concentration of tryptic soy agar (TSA) (60 g/L). TSA powder was mixed with the appropriate amount of distilled water and then autoclaved. Plates for *P. syringae* (Phi6 host) contained only 1.5X TSA and 4M $MgCl_2$ stock was added as 0.25% of the total agar volume. Plates for *E. coli* host were made the same way but appropriate antibiotic stocks (Streptomycin and Ampicillin for *E. coli* F_{amp}; Nalidixic acid for *E. coli* CN13) were added as 1% of the total agar volume after agar cooled to approximately 50-60 ^{O}C prior to plating the agar. Streptomycin-ampicillin antibiotic stock was prepared by adding 0.15 g ampicillin sodium salt and 0.15 g streptomycin sulfate to 100 mL of deionized water and this solution was filtered using a 0.22 μm filter and stored frozen in small volumes. Nalidixic acid antibiotic stock was prepared by adding 1.0 g nalidixic acid sodium salt to 100 mL of deionized water and this solution was filtered using a 0.22 μm filter and stored in small volumes. After adding the TSA mixture, plates

were allowed to cool, uncovered, to room temperature. Plates were then inverted and stored at 4 ^{O}C up to six weeks in sealed bags to prevent contamination.

Top (semisolid) agar for DAL was made the day of the experiment. An appropriate number of 20 mL glass test tubes were covered with aluminum foil and autoclaved along with 0.7X TSA (28 g/L). Following autoclaving, the top agar and tubes were placed in a 45-50 ^{O}C water bath. After agar had reached the water bath temperature, 4M $MgCl_2$ was added as 0.25% of the total agar volume and appropriate antibiotic stock was added as 1% of the total agar volume to top agar and 5 mL of the agar was dispensed into each test tube.

Host bacteria for DAL were prepared from stationary phase hosts produced from frozen stocks. Log phase *E. coli* hosts were prepared from overnight cultures that were stored at 4 ^{O}C following exponential growth. Log phase hosts were grown in 50 mL of tryptic soy broth (TSB) growth media with 1% of the total media volume as stationary phase host stock and 1% of the total media volume as appropriate antibiotic stock. This mixture was then incubated for 1.5 to 2 hours to reach logarithmic phase of growth. *P. syringae* host does not require a log phase for infection and was prepared 18-24 hours prior to running experiments using 50 mL of TSB and 1 mL of frozen host stock prepared previously by propagation in TSB. There was no antibiotic used for *P. syringae* growth.

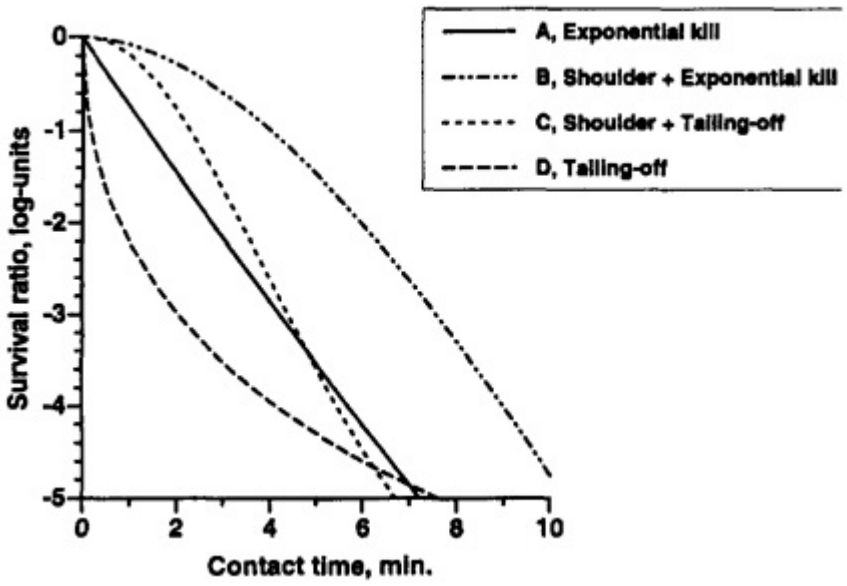
DAL was performed by adding approximately 850 µL of appropriate bacterial host to the top agar test tube containing 5 mL of agar. Next, 100 µL of sample was added to the top agar. The contents of the test tube were swirled to mix contents thoroughly and then the agar mixture was poured carefully (to minimize the formation of bubbles) on to the appropriate 'bottom agar'

plate. Plates were manipulated to spread agar uniformly across all parts of the plate and were allowed to cool (uncovered) to room temperature. Dilutions were typically plated in duplicate. Plates were then inverted. *P. syringae* host plates were placed in a 24 ^{O}C incubator (*P. syringae* host does not grow above 25 ^{O}C) and *E. coli* host plates were placed in a 37 ^{O}C incubator room. The plates were incubated overnight to allow host bacteria lawn to form and for viral plaques to develop. After 18-24 hours of incubation, the plaques were counted and plaque-forming units (PFU) per milliliter concentration were determined by averaging countable plates, accounting for sample volume and sample dilution. The lower detection limit for PFU was <1 per 100 μL (<10 per mL). For reporting purposes, undiluted counts lower than the detection limit were halved and calculations were conducted using the value 5 PFU/mL for non-detectable counts. This assumption was made to allow non-detectable counts to be included in average and standard error calculations. DAL plates at the upper detection limit were marked as too numerous to count (TNTC) and further dilutions were reviewed to determine viral concentration. Data was collected in a lab notebook and then entered into Microsoft Excel spreadsheets.

Virus inactivation for survival and disinfection experiments was calculated by a $\log_{10} (N_t / N_0)$ method where N_t is the viral concentration at time t and N_0 is the viral concentration at time 0. Inactivation was displayed as negative $\log_{10} (N_t / N_0)$ values over a period of minutes for disinfection experiments and days for survival experiments. Virus inactivation values were averaged for each time point and standard error was calculated and included on plots of inactivation as error bars.

Disinfection Kinetics Modeling

Due to poor 'goodness-of fit' using conventional first-order disinfection kinetic models like Chick-Watson, the kinetics of free chlorine disinfection for each surrogate virus was modeled using the Selleck model of disinfection, which shows strong tailing-off of viral concentration over time. This tailing off is most accurately represented by curve labeled 'D' in the following figure from Gyürék and Finch (1998).



The Selleck model of disinfection, under disinfectant demand conditions, includes a parameter (k') that accounts for the decay in concentration of the disinfectant being utilized. The Selleck rate law accounting for first order free chlorine decay and its integrated form are expressed below:

$$\frac{dS}{dt} = -\frac{nC_o \exp(-k't)}{k} S^{(n+1)/n}$$

$$S = \left[1 + \frac{C_o}{kk'}(1 - e^{-k't})\right]^{-n}$$

$$\log\left(\frac{N}{N_o}\right) = -n\,log\left[1 + \frac{C_o}{kk'}(1 - e^{-k't})\right]$$

A demand condition refers to conditions that lead to the irreversible consumption of the disinfecting-ability of a chosen chemical disinfectant. The matrix used in the experiments contained a considerable amount of oxidizable organic material, which led to the reduction of free chlorine to organochlorides and assorted chloramines. These reduced chlorine compounds maintain disinfecting capabilities, but are only minimally effective when compared to the disinfecting ability of free chlorine. In this case, the k' constant can be determined empirically by measuring free chlorine concentration decay over time. Initial free chlorine values were chosen (20,000; 25,000; and 30,000 mg/L) and appropriate volumes of liquid bleach were dosed into a 20 mL volume of matrix. Concentration of free chlorine was recorded in mg/L at 1.5, 4, 10, and 30-minute time points using a Hach Pocket Colorimeter II apparatus. Exceedingly high initial free chlorine concentrations were needed to overcome a high upper detection limit (20 mg/L) of the analytical method so the decrease in concentration could be measured over time. This detection limit was a product of the dilution necessary to reduce the significant turbidity of the matrix when measuring free chlorine concentration with the pocket colorimeter. This limitation required an assumption that free chlorine consumption occurs proportionally in a similar manner at initial free chlorine concentrations more relevant to the disinfection concentrations used in batch disinfection experiments (500 mg/L (0.05%) to 4000 mg/L (0.4%)).

Three separate experiments with varying initial free chlorine values (2.0%; 2.5%; and 3.0%) were combined on the same figure (Figure A3). To determine k' in the context of the Selleck equation, the natural log of the free chlorine concentration at time t (C_t) divided by the initial free

chlorine concentration (C_0) was plotted versus time. Linear regression analysis (y-intercept set at 0) provided assumed average k' values (negative slope) for each initial free chlorine value. Associated R^2 values were low but using empirically derived k' values proved to be highly predictive of inactivation kinetics. To determine the k' values for initial free chlorine concentrations of relevance to this report, observed k' values were plotted versus initial free chlorine concentration (Figure A4) and linear regression analysis was performed ($R^2 = 0.93$). Disinfectant decay constants were predicted via extrapolation to initial free chlorine values used in disinfection experiments. These k' values are summarized in Table A3. Using these empirically derived k' values in the Selleck equation in JMP statistical software produced low mean squared error and the generated curves were highly predictive of disinfection kinetics.

RESULTS

Survival

Ebola virus surrogates MS2 (Figure 1), PhiX-174 (Figure 2), and Phi6 (Figure 3) were spiked into a matrix of 75% hospital sewage and 25% human fecal waste and tested for persistence over a maximum of 18 days. Survival experiments were performed at both 22 ^{O}C (ambient conditions) and 37 ^{O}C (mesophilic conditions). These tests were conducted to determine baseline survivability of each surrogate in a conservative matrix to indicate the possible persistence of the Ebola virus in a similar matrix in health care facilities or sewage collection at different temperatures indicative of temperate and tropical climates.

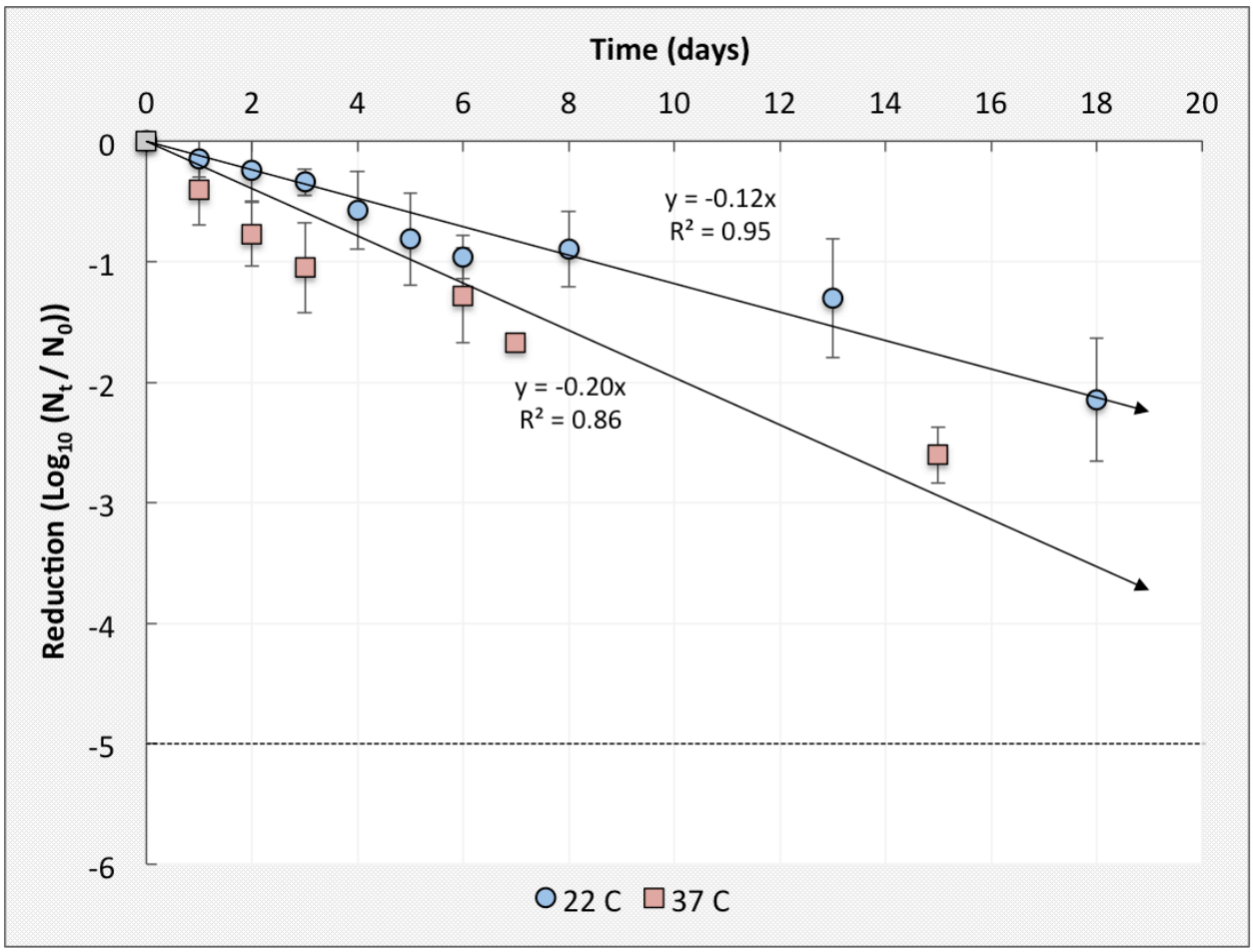


Figure 1*: Survival of surrogate MS2 coliphage (average t_0 titer: 8.2 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and human fecal waste at 22 ^{O}C (ambient) (blue circles) and 37 ^{O}C (mesophilic) (red squares). Bars show the standard error for each time point (n ≥ 2 for each point). Linear trendlines are shown with corresponding R^2 values. The dashed line represents a target 99.999% reduction in viral titer.*

The reduction of MS2 titer in a matrix of raw hospital sewage and human fecal waste over 18 days at 22 ^{O}C and 15 days at 37 ^{O}C is summarized in Figure 1. Observed viral inactivation values are expressed as data points with corresponding error bars. Predicted viral inactivation rates obtained from regression analysis are shown as a solid line. There was increasing inactivation of MS2 at both temperature conditions and reductions in viral titer followed first-order kinetics. MS2, and each of the other potential Ebola virus surrogates, Phi6 and PhiX-174, experienced a

consistently more rapid decline in infectivity at 37 ^{O}C than at 22 ^{O}C. Linear regression analysis predicted 5 $\log_{10}$ (99.999%) reductions of MS2 at approximately 42 and 25 days at 22 ^{O}C (R^2 = 0.95) and 37 ^{O}C (R^2 = 0.86) respectively. Ninety percent (1 $\log_{10}$) inactivation of MS2 required 8.3 days at ambient (22 ^{O}C) temperature and only 5.0 days at mesophilic (37 ^{O}C) temperature. Average daily inactivation was 0.12 $\log_{10}$ at 22 ^{O}C and 0.20 $\log_{10}$ at 37 ^{O}C.

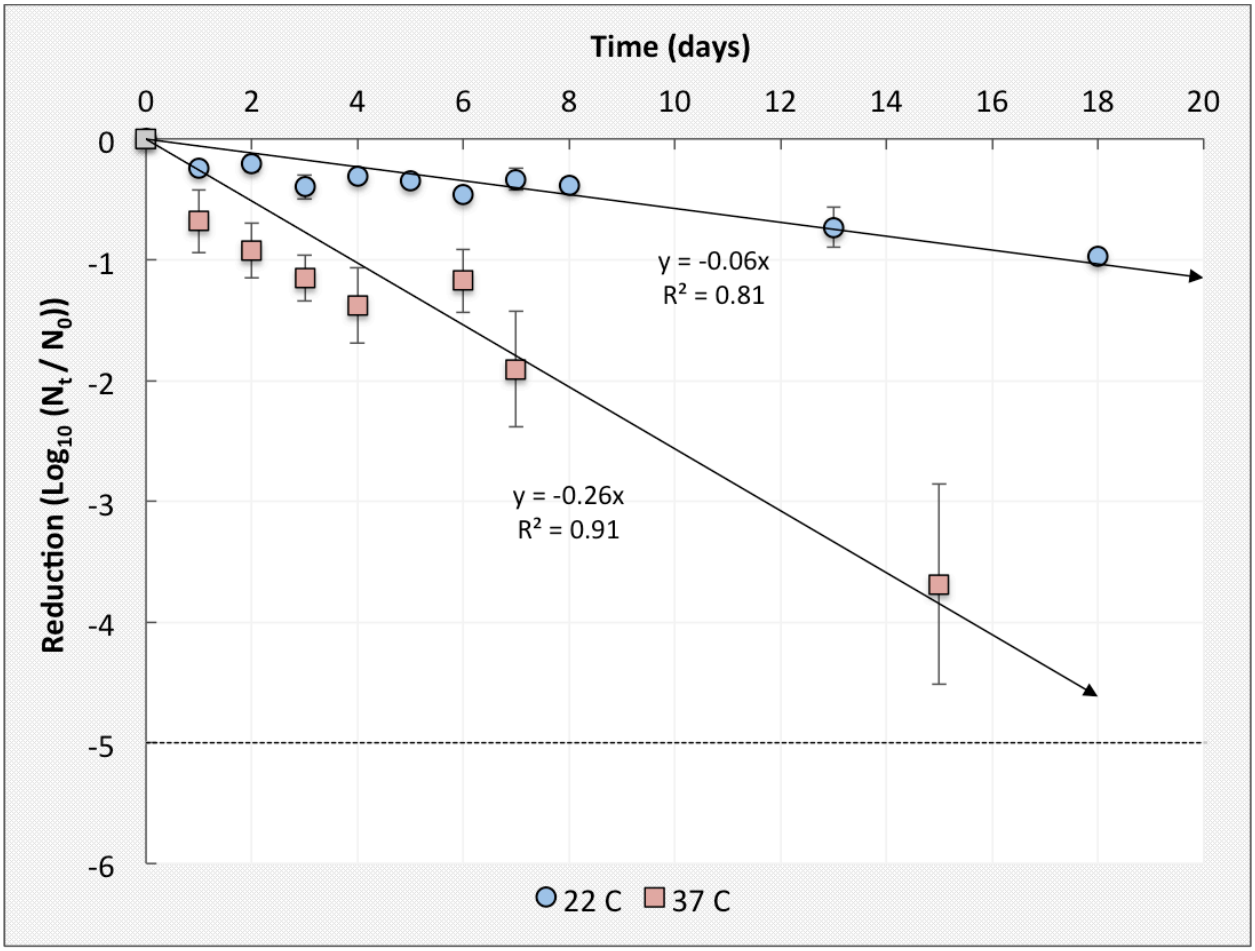


Figure 2*: Survival of surrogate PhiX-174 coliphage (average t_0 titer: 7.0 $\log_{10}$ PFU/mL) expressed as $\log_{10}$ (N_t/N_0) over time (days) in a matrix of raw hospital sewage and human fecal waste at 22 ^{O}C (ambient) (blue circles) and 37 ^{O}C (mesophilic) (red squares). Bars show the standard error for each time point (n ≥ 2 for each point). Linear trendlines are shown with corresponding R^2 values. The dashed line represents a target 99.999% reduction in viral titer.*

The reduction of PhiX-174 titer in a matrix of raw hospital sewage and human fecal waste over 18 days at 22 ^{O}C and 15 days at 37 ^{O}C is summarized in Figure 2. Observed viral inactivation values are expressed as data points with corresponding error bars. Predicted viral inactivation rates obtained from regression analysis are shown as a solid line. There was a progressive inactivation of PhiX-174 over time at both temperature conditions and reduction in viral titer followed first-order kinetics. Ninety percent (1 $\log_{10}$) inactivation of PhiX-174 required 17 days at ambient (22 ^{O}C) temperature and only 3.9 days at mesophilic (37 ^{O}C) temperature. Linear regression analysis predicted 5 $\log_{10}$ (99.999%) reductions at approximately 83 and 19 days for 22 ^{O}C (R^2 = 0.81) and 37 ^{O}C (R^2 = 0.91) respectively. Average daily inactivation was 0.06 $\log_{10}$ at 22 ^{O}C and 0.26 $\log_{10}$ at 37 ^{O}C.

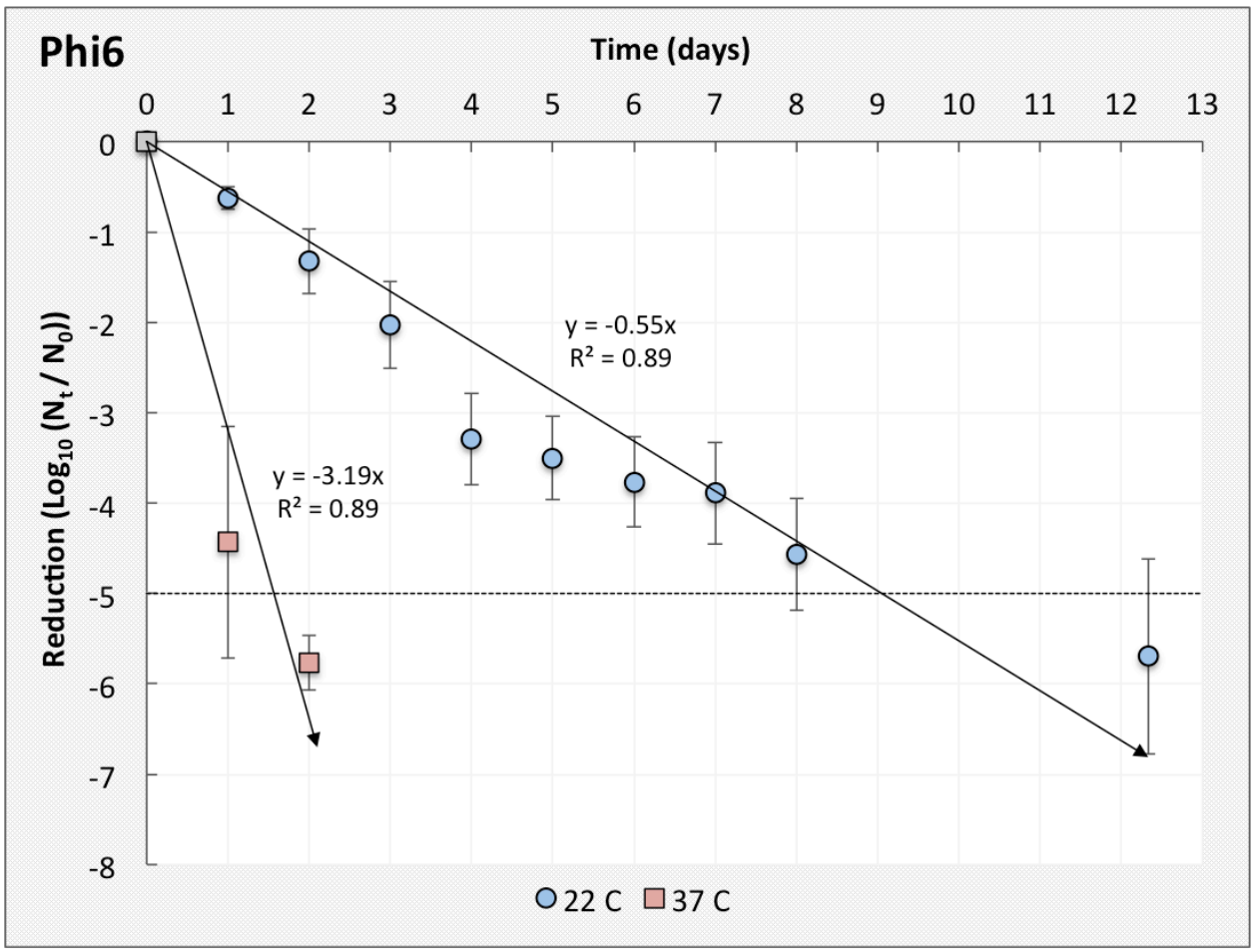


***Figure 3**: Survival of surrogate Phi6 bacteriophage (average t_0 titer: 7.8 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and human fecal waste at 22 ^{O}C (ambient) (blue circles) and 37 ^{O}C (mesophilic) (red squares). Bars show the standard error for each time point ($n \geq 2$ for each point). Linear trendlines are shown with corresponding R^2 values. The dashed line represents a target 99.999% reduction in viral titer.*

The reduction of Phi6 titer in a matrix of raw hospital sewage and human fecal waste over 18 days at 22 ^{O}C and 15 days at 37 ^{O}C is summarized in Figure 3. Observed viral inactivation values are expressed as data points with corresponding error bars. Predicted viral inactivation rates obtained from regression analysis are shown as a solid line. There was a progressive inactivation of Phi6 at both temperature conditions and reduction in viral titer followed first-order kinetics. Linear regression analysis predicted 5 log_{10} (99.999%) reductions at approximately 9 and 1.5

days for 22 ^{O}C ($R^2 = 0.89$) and 37 ^{O}C ($R^2 = 0.89$) respectively. Ninety percent inactivation of Phi6 required only 1.82 days at ambient (22 ºC) temperature and a mere 0.31 days (about 7 hours) at mesophilic (37 ºC) temperature. Average daily inactivation was 0.55 $\log_{10}$ at 22 ^{O}C and 3.2 $\log_{10}$ at 37 ^{O}C. The limit of detection (<10 PFU/mL) was reached within one day 3 out of 4 trials for survival experiments at mesophilic (37 ^{O}C) temperature. The limit of detection was 10 PFU/mL so maximum reductions were approximately 6 $\log_{10}$.

Results from regression analysis for the reduction of each surrogate virus at each temperature condition is summarized in Table 2. Time in days to achieve 90%, 99%, 99.9%, 99.99%, and 99.999% (1, 2, 3, 4, and 5 $\log_{10}$) are shown below for MS2, PhiX-174, and Phi6 at both 22 ^{O}C and 37 ^{O}C for comparison. Phi6 was inactivated most rapidly at both temperature conditions with 99.999% or 5 $\log_{10}$ reduction achieved in 9.1 and 1.6 days at 22 and 37 ^{O}C, respectively. MS2 and PhiX-174 showed considerable persistence at the ambient temperature condition, with MS2 requiring a predicted 6 weeks and PhiX-174 requiring a predicted 12 weeks for achieving the target 5 $\log_{10}$ reduction.

Reduction	**22 ^{O}C (ambient)**			**37 ^{O}C (mesophilic)**		
($\log_{10}(N_t/N_0)$)	MS2	PhiX-174	Phi6	MS2	PhiX-174	Phi6
-1 (90%)	8.3	17	1.8	5.0	3.9	0.31
-2 (99%)	17	33	3.6	10	7.7	0.63
-3 (99.9%)	25	50	5.5	15	12	0.94
-4 (99.99%)	33	67	7.3	20	15	1.3
-5 (99.999%)	42	83	9.1	25	19	1.6

Table 2*: Predicted times (in days) based on linear regression modeling for decimal reductions of MS2, PhiX-174, and Phi6 in a matrix of hospital sewage and fecal waste at 22 ^{O}C and 37 ^{O}C.*

Disinfection

Ebola virus surrogates MS2, PhiX-174, and Phi6 were spiked into a matrix of 75% hospital sewage and 25% human fecal waste and disinfected with free chlorine (liquid bleach – sodium hypochlorite). Disinfection experiments tested varying initial free chlorine concentrations for their ability to inactivate surrogate viruses at 1, 3, 10, and 30-minute time points. These tests were performed to evaluate inactivation and resistance for each virus surrogate in a conservative matrix to depict the possible inactivation of the Ebola virus in a similar high strength fecal waste matrix in health care facilities or sewage collection systems. This conservative matrix made it particularly difficult to achieve desired disinfection targets and is therefore a probable 'worst case scenario' matrix to perform chemical disinfection. Double agar layer viral titer (plaque) assays were conducted to determine the extent of virus inactivation at each time point. Figures 4, 5, and 6 show average reductions at the 10-minute time point (target in situ disinfectant contact time) for varying initial free chlorine concentrations.

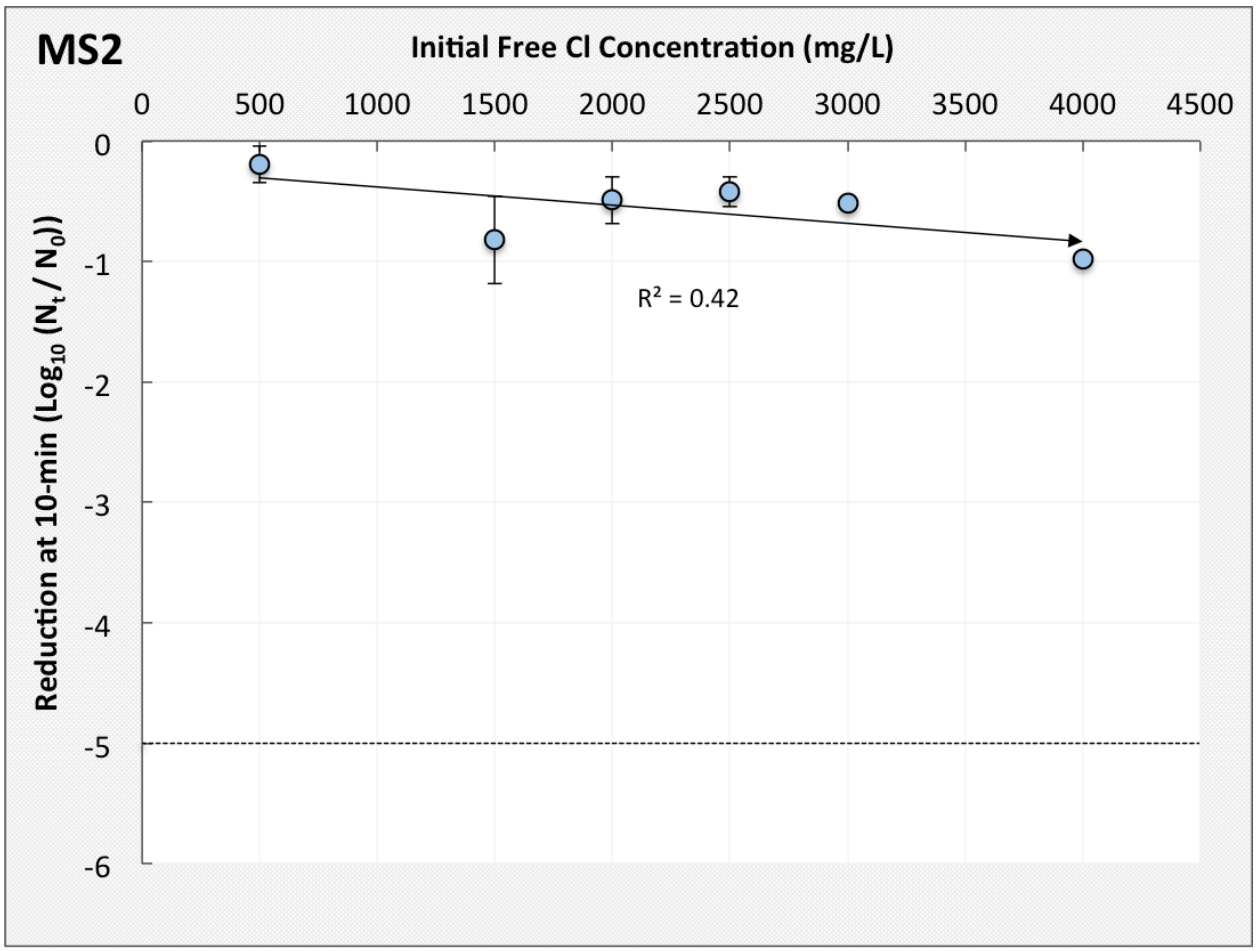


Figure 4*: Free chlorine disinfection of surrogate MS2 coliphage after 10 minutes contact time (average t_0 titer: 8.3 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) vs. varying initial free chlorine concentrations (mg/L) in a matrix of raw hospital sewage and human fecal waste. Bars show the standard error for each time point (n ≥ 2 for each point except 4000 mg/L). Linear trendline is shown with corresponding R^2 value. The dashed line represents a target 99.999% reduction in viral titer.*

The free chlorine disinfection of MS2 coliphage in a matrix of raw hospital sewage and human fecal waste after 10 minutes of contact time for varying initial free chlorine concentrations is summarized in Figure 4. Observed viral inactivation values are expressed as data points with corresponding standard error bars. Predicted viral inactivation obtained from regression analysis is shown as a solid line. MS2 showed considerable resistance to disinfection and there was a modest negative correlation between initial free chlorine concentration and log_{10} reductions (R^2 =

0.42). Regression analysis predicted approximately 24,000-mg/L (2.4%) initial free chlorine concentration would be required to achieve a 5 $\log_{10}$ (99.999%) reduction within 10 minutes.

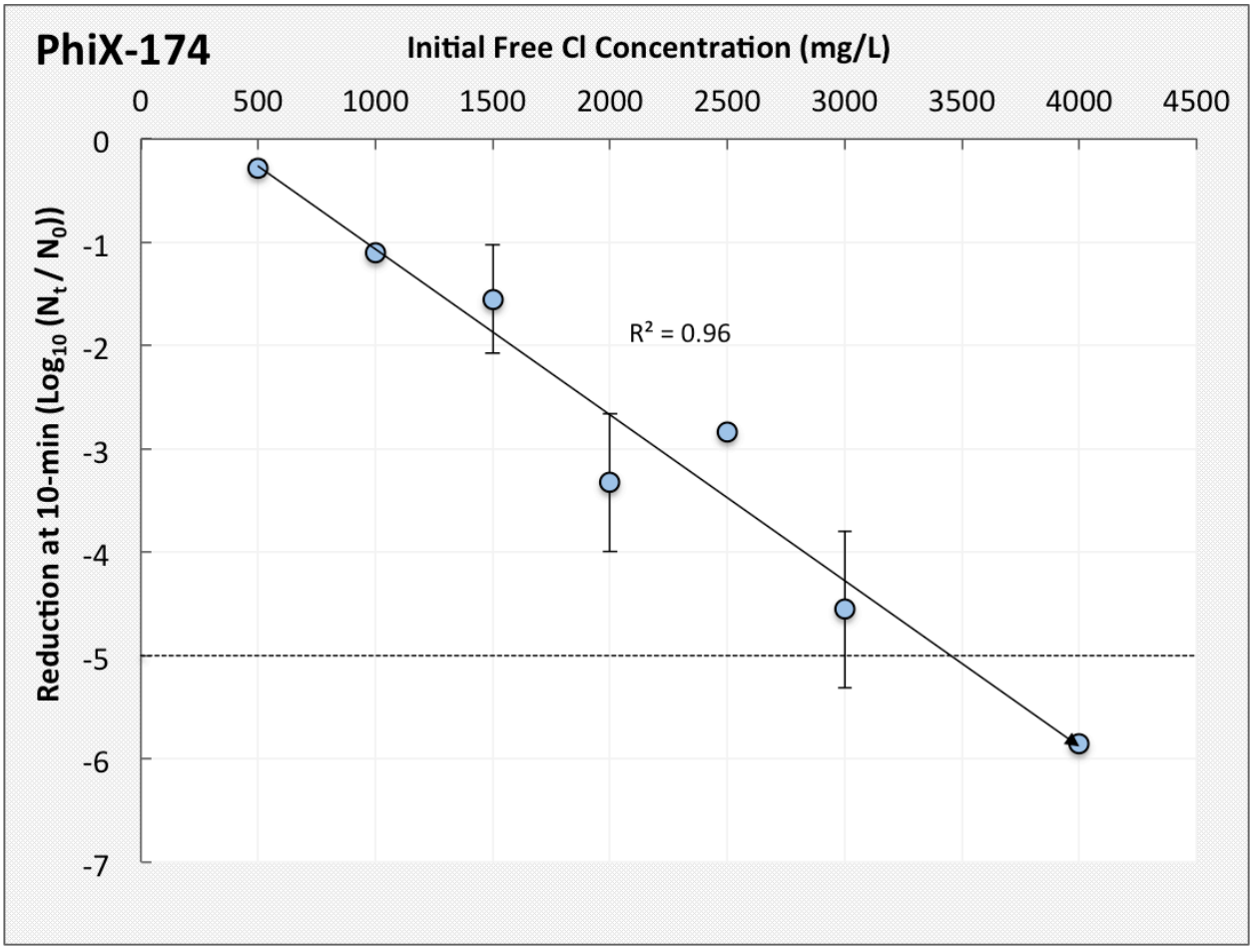


Figure 5*: Free chlorine disinfection of surrogate PhiX-174 coliphage after 10 minutes contact time (average t_0 titer: 7.2 $\log_{10}$ PFU/mL) expressed as $\log_{10}$ (N_t/N_0) vs. varying initial free chlorine concentrations (mg/L) in a matrix of raw hospital sewage and human fecal waste. Bars show the standard error for each time point ($n \geq 2$ for each point except 4000 mg/L). Linear trendline is shown with corresponding R^2 value. The dashed line represents a target 99.999% reduction in viral titer.*

The free chlorine disinfection of PhiX-174 coliphage in the high strength matrix of raw hospital sewage and human fecal waste after 10 minutes of contact time for varying initial free chlorine concentrations is summarized in Figure 5. Observed viral inactivation values are expressed as

data points with corresponding standard error bars. The predicted viral inactivation rate obtained from regression analysis is shown as a solid line. PhiX-174 disinfection displayed a strong negative correlation between initial free chlorine concentration and $\log_{10}$ reductions ($R^2 = 0.96$). Regression analysis predicted approximately 3,500 mg/L (0.35%) initial free chlorine concentration would be required to achieve a 5 $\log_{10}$ (99.999%) reduction within 10 minutes.

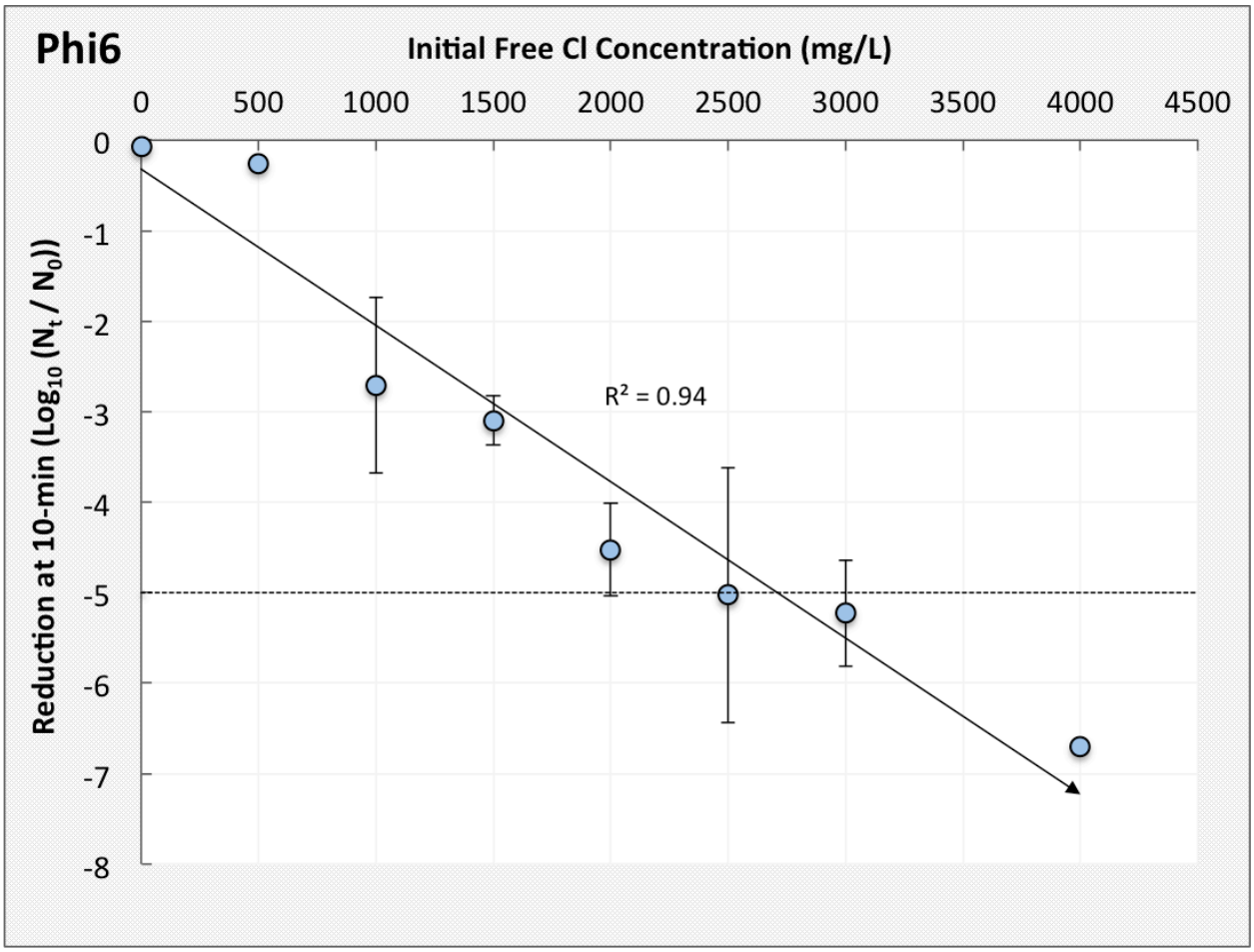


***Figure 6**: Free chlorine disinfection of surrogate Phi6 bacteriophage after 10 minutes contact time (average t_0 titer: 7.9 $\log_{10}$ PFU/mL) expressed as $\log_{10}$ (N_t/N_0) vs. varying initial free chlorine concentrations (mg/L) in a matrix of raw hospital sewage and human fecal waste. Bars show the standard error for each time point ($n \geq 2$ for each point except 4000 mg/L). Linear trendline is shown with corresponding R^2 value. The dashed line represents a target 99.999% reduction in viral titer.*

The free chlorine disinfection of Phi6 bacteriophage in the high strength matrix of raw hospital sewage and human fecal waste after 10 minutes of contact time for varying initial free chlorine concentrations is summarized in Figure 6. Observed viral inactivation values are expressed as data points with corresponding standard error bars. The predicted viral inactivation rate obtained from regression analysis is shown as a solid line. Phi6 disinfection displayed a strong negative correlation between initial free chlorine concentration and $\log_{10}$ reductions ($R^2 = 0.94$). Regression analysis predicted approximately 2,800 mg/L (0.28%) initial free chlorine would be required to achieve a 5 $\log_{10}$ (99.999%) reduction within 10 minutes.

Surrogate	Free Cl Required for 1 $\log_{10}$ Reduction (mg/L)	Free Cl Required for 5 $\log_{10}$ Reduction (mg/L)	*Estimated Bleach Volume Needed for 5 $\log_{10}$ Reduction in a 7.5 L Toilet Bowl
MS2	3800	24,000	2.3 L
PhiX-174	960	3,500	0.33 L
Phi6	400	2,800	0.27 L

***Table 3**: Comparison of initial free chlorine concentrations (mg/L) required for 1 and 5 $\log_{10}$ reductions of each surrogate virus and the volume of bleach required to achieve a 5 $\log_{10}$ reduction in 10 minutes in actual-scale conditions. *Actual amount (L) of 7.85% bleach needed for 5 $\log_{10}$ inactivation of surrogate virus in a 7.5 L toilet bowl.*

Results from regression analysis for the disinfection of each surrogate virus is summarized in Table 3. Initial free chlorine concentrations required to achieve 90% and 99.999% are shown above for MS2, PhiX-174, and Phi6 for comparison. An additional column is included to provide a volume of 7.85% bleach that would be required to achieve a 5 $\log_{10}$ reduction of each surrogate virus in a comparable matrix in a 7.5 L toilet bowl. Phi6 was most readily disinfected by free chlorine and PhiX-174 was slightly more resistant, with 5 $\log_{10}$ reductions of each estimated to require 0.27 and 0.33 L of bleach, respectively. MS2 proved exceedingly difficult to disinfect in

this matrix and, in a 'real world' scenario, would require over 2 L of liquid bleach for target virus disinfection in a 7.5 L toilet.

Disinfection Kinetics

The following figures (7, 8, and 9) show the free chlorine disinfection of surrogate viruses at 1, 3, 10, and 30-minute time points and their best-fit inactivation kinetic modeling curves. As described in the methods section, disinfection was modeled using the Selleck Model equation, which accounts for disinfectant demand conditions using a k' value (a decay constant accounting for consumption of free chlorine).

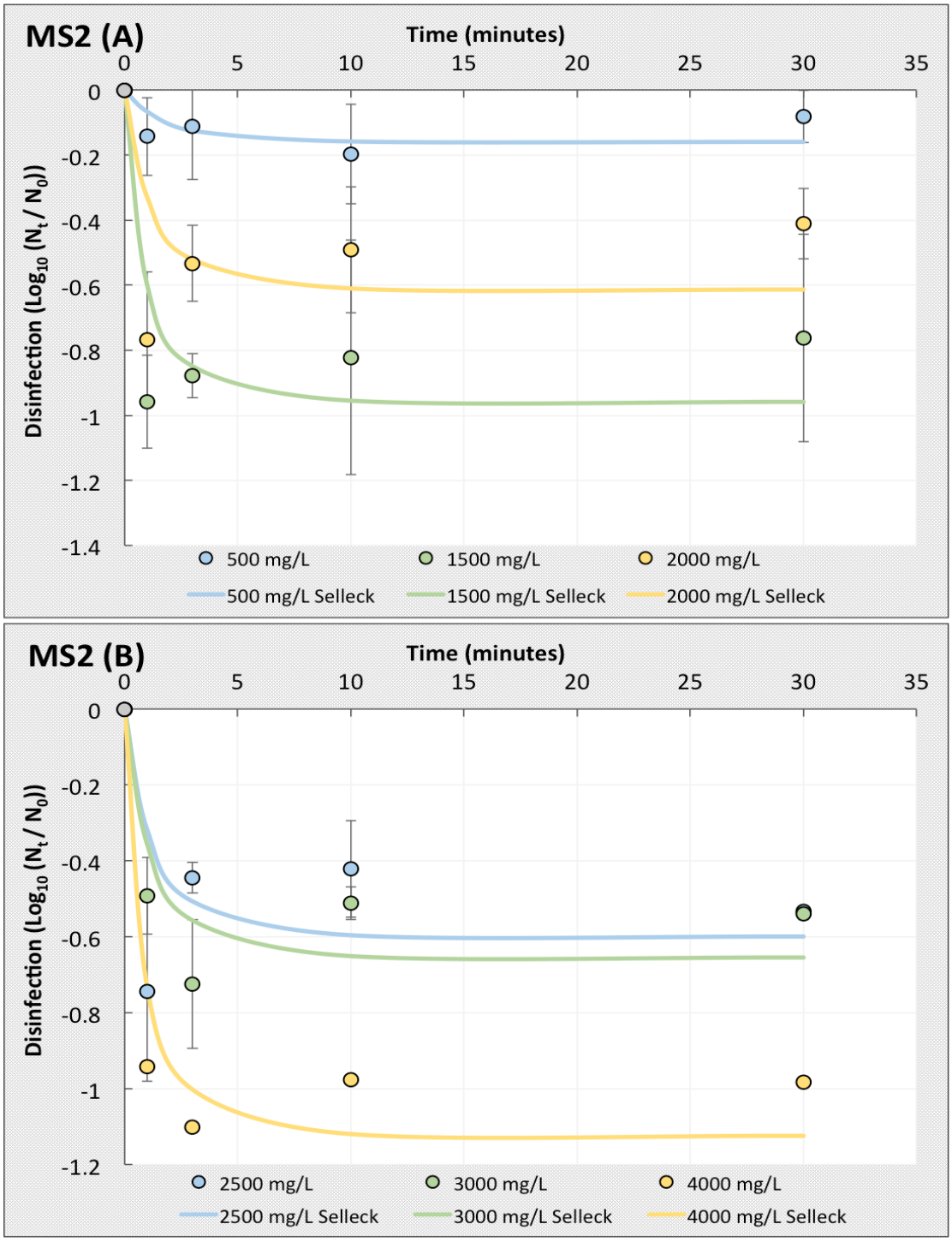


Figure 7*: Free chlorine disinfection of surrogate MS2 coliphage (average t_0 titer: 8.3 $\log_{10}$ PFU/mL) expressed as $\log_{10}$ (N_t/N_0) at 1, 3, 10, and 30-minute time points with varying initial free chlorine concentrations of* ***(A)*** *500, 1500, 2000 mg/L and* ***(B)*** *2500, 3000, 4000 mg/L in a matrix of raw hospital sewage and human fecal waste. Disinfection kinetics predicted using Selleck Model under disinfectant demand conditions are shown for each initial free chlorine concentration (smooth curves). Bars show the standard error for each time point (n ≥ 2 for each data series except 4000 mg/L).*

MS2 Selleck Model of Disinfection Kinetics				
Initial Free Cl (mg/L)	Predicted Reduction at 10 min ($\log_{10}(N_t/N_0)$)	Virus Inactivation Rate Constant (k)	Free Cl Decay Rate Constant (k')	Mean Squared Error (MSE)
500	-0.16	2409.89	-0.4675	< 0.01
1500	-0.96	402.28	-0.4605	0.05
2000	-0.61	1408.68	-0.4570	0.06
2500	-0.60	1852.70	-0.4535	0.05
3000	-0.65	1896.54	-0.4500	0.02
4000	-1.1	732.72	-0.4430	0.02

Table 4*: Selleck model of disinfection kinetics with predicted reductions and model parameter values for free chlorine disinfection of MS2 coliphage in a matrix of raw hospital sewage and human fecal waste. Mean squared error (MSE) values included.*

The kinetics and Selleck model estimation of free chlorine disinfection of MS2 is described in Figure 7. Parameters of MS2 disinfection kinetics modeling are shown in Table 4. The extent of disinfection was not always consistent with initial free chlorine concentration but similar inactivation trends were seen in most curves. Seemingly, most disinfection occurred within the first three minutes of contact time. This rapid initial decrease was followed by a slight recovery in viral titer by the 30-minute time point. Disinfection was characterized by a strong tailing-off from T_0 viral titer over time, eventually approaching 0 slope. Free chlorine decay rate constants were very similar at all chlorine concentrations dosed, ranging from -0.44 to -0.47.

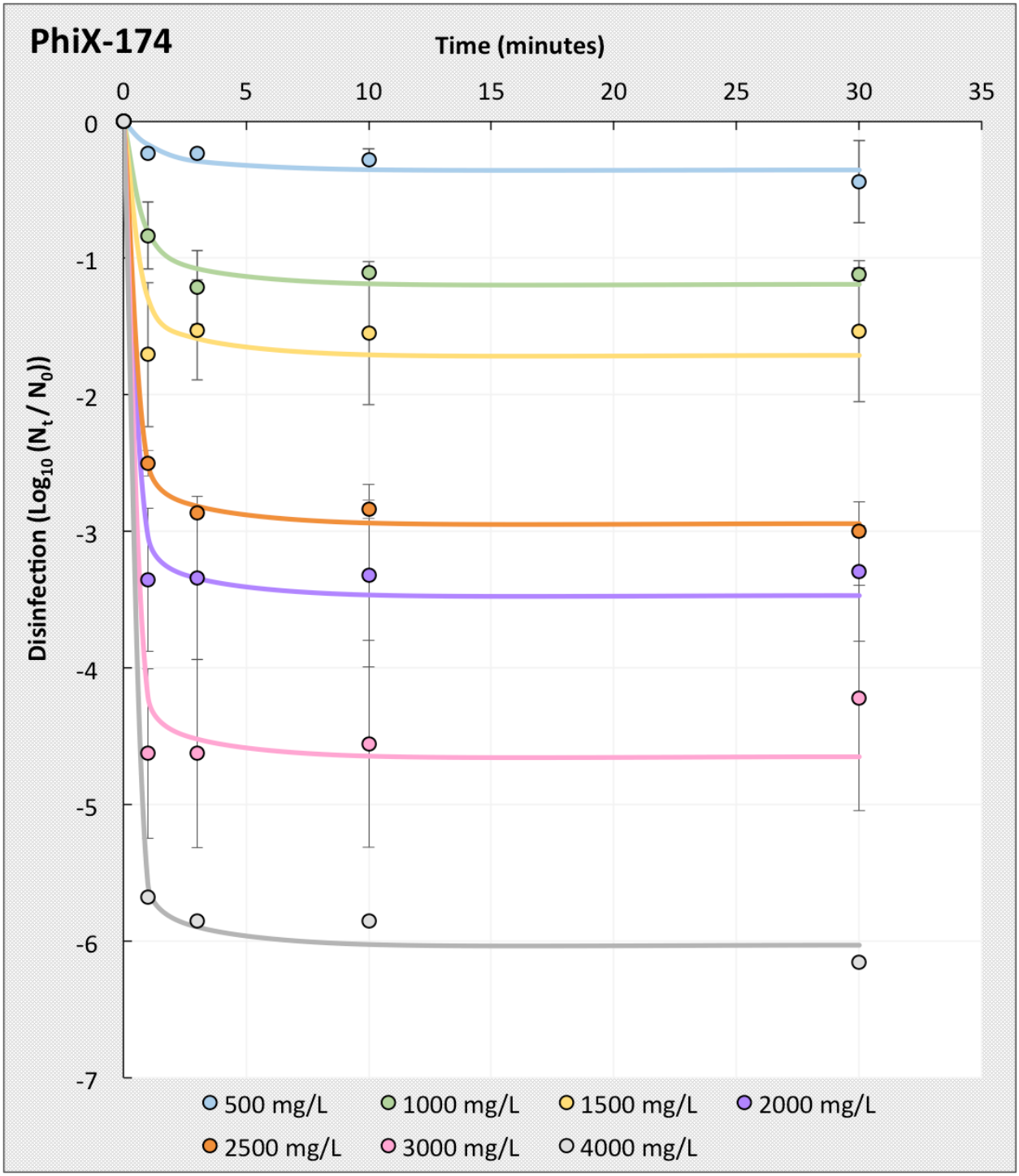


***Figure 8**: Free chlorine disinfection of surrogate PhiX-174 coliphage (average t_0 titer: 7.2 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) at 1, 3, 10, and 30-minute time points with varying initial free chlorine concentrations of 500, 1000, 1500, 2000, 2500, 3000, and 4000 mg/L in a matrix of raw hospital sewage and human fecal waste. Disinfection kinetics predicted using Selleck Model under disinfectant demand conditions are shown for each initial free chlorine concentration (smooth curves). Bars show the standard error for each time point ($n \geq 2$ for each data series except 4000 mg/L).*

PhiX-174 Selleck Model of Disinfection Kinetics				
Initial Free Cl (mg/L)	Predicted Reduction at 10 min ($\log_{10}(N_t/N_0)$)	Virus Inactivation Rate Constant (k)	Free Cl Decay Rate Constant (k')	Mean Squared Error (MSE)
500	-0.35	848.04	-0.4675	< 0.01
1000	-1.2	148.26	-0.4640	< 0.01
1500	-1.7	65.54	-0.4605	0.06
2000	-3.5	1.48	-0.4570	0.04
2500	-2.9	6.29	-0.4535	< 0.01
3000	-4.6	0.15	-0.4500	0.09
4000	-6.0	0.01	-0.4430	0.01

Table 5*: Selleck model of disinfection kinetics with predicted reductions and model parameter values for free chlorine disinfection of PhiX-174 coliphage in a matrix of raw hospital sewage and human fecal waste. Mean squared error (MSE) values included.*

The kinetics and Selleck model estimation of free chlorine disinfection of PhiX-174 is described in Figure 8. Parameters of PhiX-174 disinfection kinetics modeling are shown in Table 5. The extent of disinfection was strongly correlated with initial free chlorine concentration. Similar inactivation trends were seen in most curves, with most disinfection seeming to occur within the first three minutes of contact time, followed by strong tailing-off from T_0 viral titer and eventually approaching 0 slope. As noted previously, free chlorine decay rate constants were very similar at all chlorine concentrations dosed, ranging from -0.44 to -0.47.

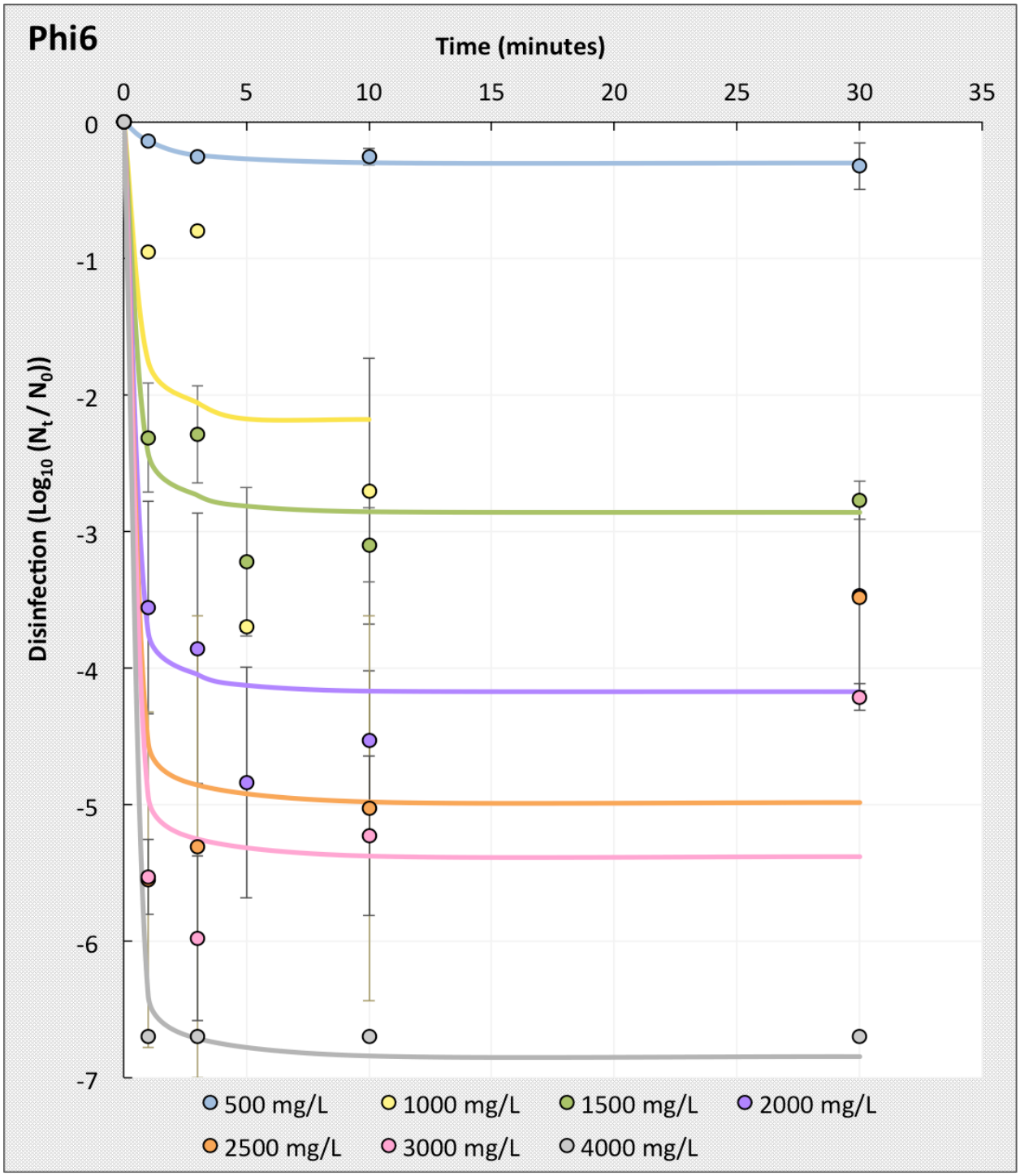


***Figure 9**: Free chlorine disinfection of surrogate Phi6 bacteriophage (average t_0 titer: 7.9 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) at 1, 3, 10, and 30-minute time points (1000, 1500, and 200 mg/L also have 5-minute time points) with varying initial free chlorine concentrations of 500, 1000 (no 30-minute time point), 1500, 2000, 2500, 3000, and 4000 mg/L in a matrix of raw hospital sewage and human fecal waste. Disinfection kinetics predicted using Selleck Model under disinfectant demand conditions are shown for each initial free chlorine concentration*

(smooth curves). Bars show the standard error for each time point (n ≥ 2 for each data series except 4000 mg/L).

Phi6 Selleck Model of Disinfection Kinetics				
Initial Free Cl (mg/L)	Predicted Reduction at 10 min ($\log_{10}(N_t/N_0)$)	Virus Inactivation Rate Constant (k)	Free Cl Decay Rate Constant (k')	Mean Squared Error (MSE)
500	-0.25	1080.41	-0.4675	< 0.01
1000	-2.7	14.40	-0.4640	1.20
1500	-3.1	4.52	-0.4605	0.09
2000	-4.5	0.29	-0.4570	0.24
2500	-5.0	0.06	-0.4535	0.87
3000	-5.2	0.03	-0.4500	0.57
4000	-6.7	0.001	-0.4430	0.03

***Table 6**: Selleck model of disinfection kinetics predicted reductions and parameter values for free chlorine disinfection of Phi6 bacteriophage in a matrix of raw hospital sewage and human fecal waste. Mean squared error (MSE) values included.*

The kinetics and Selleck model estimation of free chlorine disinfection of Phi6 is described in Figure 9. Parameters of Phi6 disinfection kinetics modeling are shown in Table 6. As with the other viruses tested, the extent of disinfection was strongly correlated with initial free chlorine concentrations, but some curves showed great variability in the extent of viral inactivation over time. Again, most disinfection seemed to occur within the first three minutes of contact time. After a rapid initial decrease in virus concentration, a tailing-off occurred that approached 0 slope over time. In some cases, there was a modest recovery in virus concentration by the 30-minute time point. As noted previously, free chlorine decay rate constants were very similar at all concentrations dosed, ranging from -0.44 to -0.47.

DISCUSSION

Survival

In this study, three surrogate viruses – two non-enveloped coliphages (MS2, PhiX-174) and one enveloped bacteriophage (Phi6) – were tested for their persistence in a matrix of 75% raw hospital sewage and 25% human fecal waste (w/w). Survival experiments were conducted at two temperatures, 22 ^{O}C (ambient) and 37 ^{O}C (mesophilic), to model temperature conditions in temperate and tropical climates, respectively. Previous studies have shown the survival of both potential surrogate viruses, like Phi6 {Casanova and Weaver 2015}, and the actual Ebola virus {Bibby et al. 2015}, but these studies were conducted in pasteurized or irradiated sewage. To date, there are no published studies of Ebola or potential surrogate virus survival in raw sewage amended with human fecal waste. This survival study was meant to indicate the possible persistence of Ebola virus in such a matrix, which may occur in a hospital toilet or sewage collection system in which water availability or use is low.

Survival kinetics were modeled using linear regression, as has been previously suggested for viral {Stallknecht et al. 1990; Casanova et al. 2009} and Ebola virus persistence {Sagripanti et al. 2010; Fischer et al. 2015; Bibby et al. 2015}. The most comparable study of Ebola virus survival occurred in irradiated hospital sewage and indicated that approximately five days were required for a 5 $\log_{10}$ (99.999%) reduction in infectivity at ambient temperature conditions {Bibby et al. 2015}. The results of this report show that enveloped surrogate bacteriophage, Phi6, experienced a similar inactivation under ambient conditions but was slightly more persistent, requiring at least eight days for a 5 $\log_{10}$ reduction. It should be noted, however, that

there were non-trivial differences in matrix constituents. The study by Bibby et al. used microbially inert sewage (gamma irradiated) that contained 600 times less TSS, 500 times less COD, 100 times less ammonia, and 350 times less TOC than the raw sewage plus fecal waste matrix of this study. Non-enveloped surrogate coliphages, MS2 and PhiX-174, showed considerable persistence compared to their enveloped counterpart (predicted 5 $\log_{10}$ reductions at 42 and 83 days respectively) and would be considered exceedingly conservative surrogates for Ebola virus survival in a comparable fecal waste matrix.

Temperature was a significant predictor of viral reduction. Each potential surrogate virus was inactivated more rapidly at 37 ^{O}C than at 22 ^{O}C. Linear regression predicted 5 $\log_{10}$ reductions would be achieved roughly 1.7, 4.4, and 5.7 times faster at the mesophilic temperature condition of 37 ^{O}C than at the ambient temperature condition of 22 ^{O}C for MS2, PhiX-174, and Phi6, respectively (Table 2). A number of previous studies indicate a similar trend of increased virus inactivation at higher temperature, including Bøtner's study of the survival of the enveloped Aujeszky's disease virus survival in pig slurry at different temperatures {Bøtner 1991}. Casanova and Weaver (2015) showed that Phi6 inactivation in pasteurized sewage occurred more rapidly at 30 ^{O}C than 22 ^{O}C. A study of filovirus survival by Piercy et al. (2010) reported that *Zaire ebolavirus* experienced a greater reduction in virus infectivity at ambient temperature conditions compared to 4 ^{O}C over a period of 26 days in guinea pig sera and tissue culture media.

Comparison studies of virus survival in water and survival in more contaminated media (sewage and feces) have indicated that greater viral reduction occurs in contaminated media. Casanova et al. (2009) studied survival of surrogate mammalian coronaviruses (enveloped) in water and pasteurized settled sewage and found greater inactivation in pasteurized sewage than either

surface water or buffered water. A number of researchers have suggested factors leading to greater virus reduction in more contaminated media. Ward et al. (1986) suggested pH extremes, other microorganisms, and proteolytic enzyme activity as possible factors contributing to greater virus inactivation in natural waters and wastewaters. The results of this survival study were likely unaffected by pH, which was within a neutral range (6.7 ± 0.2). Persistence of surrogates may have been lessened by microbial and proteolytic enzyme activity, considering a raw sewage and fecal waste matrix was used. These factors may have had greater effects at mesophilic conditions where enteric and other microbes would thrive and enzymatic reactions would occur at an increased rate. Rates of inactivation were also potentially increased by the virucidal capabilities of ammonia, which was present in substantial concentration (216 mg/L). However, components of contaminated media that may enhance virus survival are suspended organic material and aggregation of viral particles or adsorption of viruses to wastewater particles. Studies have shown that a virus that is adsorbed generally has a better chance of survival than one that is freely suspended {Gerba 1975}. Viral association with particles, including other viral particles, has been recognized to provide protection from inactivation {Hoff et al. 1986}.

Due to the presence of an outer lipid envelope layer, the surrogate bacteriophage Phi6 may conservatively reflect the survivability of Ebola virus (enveloped) in a similar fecal waste matrix with comparable physical and chemical constituents and could be utilized in future risk assessment modeling. The persistence of this enveloped surrogate indicates the need for in situ pre-treatment protocols for the disinfection of Ebola waste in developed country health care settings as well as developing world settings, like West Africa.

Disinfection

Ebola virus has been previously studied for its response to free chlorine disinfection in a dried simulated organic load (Cook et al. 2015) and pasteurized municipal sewage (Bibby et al. 2017), but never in raw sewage amended with notable concentrations of human fecal waste. In this study, the virus disinfection ability of free chlorine (sodium hypochlorite) was tested on potential Ebola surrogate viruses MS2, PhiX-174, and Phi6 in a conservative matrix of 75% raw hospital sewage and 25% human fecal waste. Considerable initial free chlorine concentrations were required to achieve the desired 5 $\log_{10}$ reduction target in viral titer. This result was not unexpected, based on the matrix analysis report, which indicated very high BOD, COD, TSS, and TOC concentrations compared to typical community raw sewage. Free chlorine is a strong oxidizing agent and disinfection ability was rapidly diminished in this matrix. Cook et al. (2015) indicated that sodium hypochlorite solutions (0.5 and 1.0%) were successful in inactivating Ebola virus after 5 minutes of contact time, but it must be noted that these experiments required a significant volume of disinfectant (5:1 disinfectant solution to virus matrix (v/v)) ratio to achieve desired reductions of Ebola virus. Bibby et al. (2017) reported that, in pasteurized settled sewage, only 5 mg/L initial free chlorine was required to reach the detection limit of 4.2 $\log_{10}$ within 20 seconds. For a lower initial free chlorine concentration, 1 mg/L, a 3.5 $\log_{10}$ reduction was recorded at 20 seconds, but further inactivation was not evident due to the rapid decay of the free chlorine residual. When comparing the results of these two studies to surrogates like Phi6 and PhiX-174 utilized in this study, data seems to indicate that the oxidizable organic concentration of the disinfection matrix is a more influential obstacle to Ebola virus disinfection than the actual chlorine resistance of the virus. This condition would be the case when attempting disinfection in

a hospital toilet containing Ebola waste, which would contain high concentrations of organics and oxidizable matter.

Generally, similar results were seen for Phi6 and PhiX-174 disinfection. Both bacteriophage surrogates showed strong correlations between initial free chlorine concentration and viral reduction at 10 minutes ($R^2 \geq 0.94$). Linear regression predicted 5 $\log_{10}$ reductions would be reached at initial free chlorine concentrations of 3,500 mg/L (0.35%) for PhiX-174 and 2,800 mg/L (0.28%) for Phi6.

MS2 showed extensive resistance to free chlorine disinfection and only a weak relationship was seen between initial free chlorine concentration and viral reduction at 10 minutes. An estimated 24,000 mg/L would be needed for a 5 $\log_{10}$ (99.999%) reduction in MS2 titer. Application of this initial concentration to a 'real life' situation corresponds to a liquid bleach (7.85%) volume of 2.3 L for a 7.5 L toilet containing the same conservative fecal waste matrix.

A consistent trend seen across initial free chlorine concentrations was that most disinfection occurred within the first 1-3 minutes of contact time. Selleck disinfection kinetics was able to describe this extreme 'tailing-off' phenomenon with low mean squared error values. As was described by Bibby et al (2017), the free chlorine residual would rapidly decay and very little inactivation would occur beyond initial sodium hypochlorite dosing. Some disinfection runs experienced a phenomenon where a slight to moderate recovery of viral titer occurred by the 30-minute time point. This recovery is not attributed to viral amplification, but to either viral aggregation or adsorption. Aggregation of viral particles to one another would result in particles being detected as single infectious units. Adsorption to suspended material may protect viral

particles from initial inactivation and subsequent disassociation may occur from possible changing physical and chemical conditions at each time point.

Phi6 bacteriophage and PhiX-174 coliphage may conservatively represent the sodium hypochlorite disinfection of Ebola virus in a similar matrix on a realistic scale. As an enveloped virus, the mechanism by which Phi6 is inactivated by sodium hypochlorite could be representative of Ebola or another lipid enveloped pathogenic virus disinfection with free chlorine. Due to its nearly universal availability in health care settings, sodium hypochlorite (liquid bleach) is an appropriate chemical disinfectant for inactivating Ebola virus in a fecal waste matrix, but is not highly recommended because of the significant concentrations and volumes required to overcome the oxidizable organic content of such a matrix thereby posing potential risks of insufficient inactivation.

Limitations

There are a number of limitations to the research presented in this report. One substantial limitation is variation in the fecal waste matrix used in survival and disinfection experiments. To ensure freshness and consistent microbial activity of the matrix for survival experiments, a new mixture was produced about every 2 weeks. The matrices were produced with the same procedure and composition each time, but there is inherent variability in constituents between different hospital sewage samples and different fecal samples. Identifying an effective chlorine dose is a source of uncertainty when disinfecting with free chlorine because of varying amounts of oxidizable organic matter (different free chlorine breakpoints). Some variability would be

expected in results from experiments with different matrices, but results were shown to be sufficiently consistent to quantify performance targets based on chlorine dose and contact time.

Another limitation of the experiments was the assumption that DAL plates with no detectable viral plaques for undiluted samples would have PFU counts half the limit of detection (LOD = 10 PFU/mL). This assumption was made to allow non-detectable counts to be included in average and standard error calculations. It is possible that microbial reduction performance is greater than reported based on lower limits of detection.

A limitation of the disinfection kinetics modeling was the assumption made to find free chlorine decay constants for each initial concentration. One such assumption was that free chlorine decay occurred in a linear fashion although poor correlations were found as shown by Figure A3. Another assumption was that free chlorine residual decay occurred in the same manner at lower initial concentrations as the high initial concentrations that were needed to overcome a high residual free chlorine detection limit resulting from the degree of dilution required to reduce turbidity for pocket colorimeter testing. However, predicted k' values were very similar and resulted in in modest mean squared error values when modeling free chlorine disinfection kinetics.

Future Work

A number of additional investigations are underway to further the research in this report. An added enveloped surrogate, swine transmissible gastroenteritis virus (TGEV), is being tested for survival and free chlorine disinfection using the same experimental methods described in this

report. TGEV is a mammalian coronavirus that has been used as a surrogate for severe acute respiratory syndrome (SARS) coronavirus in survival studies {Casanova et al. 2009}. In addition to sodium hypochlorite, a number of other chemical disinfectants are being tested for their ability to achieve target viral reductions of each surrogate virus in the conservative fecal waste matrix. These disinfectants include sodium hydroxide (lime with pH adjustment), two quaternary ammonium compounds (benzalkonium chloride and cetylpyridinium chloride), peracetic acid, and possibly a phenolic compound or an anionic detergent. For promising disinfectants, protocols will be developed to achieve 5 $\log_{10}$ reductions within 10 minutes of contact time. These protocols will then be tested using 'actual scale' conditions (bedpan or toilet).

Next, successful protocols will be tested on Ebola ΔVP30 virus at the University of Wisconsin. As described by Halfman et al. (2008), Ebola ΔVP30 virus is a biologically contained version of the functional Ebola virus that is presumably not infectious to humans or non-human primates. The only difference between the wild-type infectious Ebola virus and the biologically contained virus is the lack of the viral protein 30 gene (VP30). This gene encodes a transcription factor that is essential to viral replication within the host. The mutant ΔVP30 virus can only infect a Vero (green monkey epithelial cells) cell line (VeroVP30) that expresses the VP30 gene on behalf of the mutant virus. This 'lock and key' model allows for the study of a morphologically indistinguishable version of the Ebola virus in Biosafety Level 3 (BSL-3) laboratories, rather than BSL-4 laboratories required for wild-type Ebola virus research {Halfman et al. 2008}. Upon the development of protocols from the findings from these current and further proposed studies, recommendations can be made to the US CDC, WHO, and health care facilities concerning disinfection and disposal of fecal waste from Ebola patients.

CONCLUSIONS

- Surrogate virus survival in representative worst-case matrix of 75% raw hospital sewage and 25% human fecal waste followed first-order (exponential) kinetics and viral reductions were greater at 37 ^{O}C than at 22 ^{O}C for all tested surrogate viruses.
- Due to the presence of an outer lipid envelope layer, the surrogate bacteriophage Phi6 may conservatively represent the survivability of Ebola virus (enveloped) in a similar fecal waste matrix with comparable physical and chemical constituents and the results for its survival in fecal wastes over time could be utilized in risk assessment modeling.
- MS2 and PhiX-174 are likely too conservative as surrogates for Ebola virus survival in raw hospital sewage and human fecal waste because they are non-enveloped viruses that survived considerably longer in the fecal waste matrix tested.
- The persistence of enveloped surrogate virus Phi6 for several days in the fecal waste matrix tested indicates the need for an in situ pre-treatment protocol for the disinfection of Ebola waste in developed and developing nation health care settings.
- Ebola surrogate viruses Phi6 and PhiX-174 can be disinfected to a target 5 $\log_{10}$ (99.999%) reduction if high, but manageable, initial free chlorine concentrations are attained: however, free chlorine is rapidly consumed in a conservative fecal waste matrix rich in oxidizable organics.
- The surrogate fecal indicator virus MS2 is extremely resistant to chlorine disinfection in the fecal waste matrix tested and is therefore a far too conservative surrogate for Ebola virus sodium hypochlorite disinfection.

- The Selleck model of disinfection kinetics, under disinfectant demand conditions, suitably described surrogate virus inactivation while providing low mean squared error indicative of reproducible disinfection results.
- Due to its nearly universal availability in health care settings, sodium hypochlorite (liquid bleach) is an appropriate chemical disinfectant for inactivating Ebola virus in a fecal waste matrix, however, it is not preferred because of the need for significant concentrations and volumes required to overcome the oxidizable organic content of such a matrix thereby posing potential risks of insufficient inactivation.

REFERENCES

Abedon, S. T. & Calendar, R. (2005). The Bacteriophages: Chapter 15. City, State: Oxford University Press, 2nd edition.

Adcock, N. J.; Rice, E. W.; Sivaganesan, M.; Brown, J. D.; Stallknecht, D. E.; Swayne, D. E. (2009) The use of bacteriophages of the family Cystoviridae as surrogates for H5N1 highly pathogenic avian influenza viruses in persistence and inactivation studies. *J Environ Sci Health A Tox Hazard Subst Environ Eng, 44*(13), 1362-1366.

Ajariyakhajorn, C.; Goyal, S. M.; Robinson, R. A.; Johnston, L. J.; Clanton, C. A. (1997). The survival of Salmonella anatum, pseudorabies virus and porcine reproductive and respiratory syndrome virus in swine slurry. *New Microbiology, 20*(4), 365-369.

Ball, A. R.; Malia, S. A.; Desai, L. (2012). A Simple Solution for Medical Device Viral Ingress Testing of Human Immuno Virus and Hepatitis B Virus. *Toxikon Corporation,* Poster #518.

Bibby, K.; Fischer, R. J.; Casson, L. W.; Stachler, E.; Haas, C. N.; Munster, V. J. (2015). Persistence of Ebola Virus in Sterilized Wastewater. *Environmental Science and Technology Letters, 2*, 245-249.

Bibby, K.; Fischer, R. J.; Casson, L. W.; Carvalho, N.; Haas, C. N.; Munster, V. J. (2017). Disinfection of Ebola Virus in Sterilized Municipal Wastewater. *PLoS Neglected Tropical Diseases, 11*(2).

Bøtner, A. (1991) Survival of Aujeszky's disease virus in slurry at various temperatures. *Veterinary Microbiology, 29*(3-4), 225-235.

Bowman, G. & Mealy, R. (2007). The Fundamentals of Chlorine Chemistry and Disinfection. *Wisconsin Department of Natural Resources*. (http://dnr.wi.gov/regulations/labcert/documents/training/cl2chemistry.pdf). (accessed September 28, 2016).

Casanova, L.; Rutala, W. A.; Weber, D. J.; Sobsey, M. D. (2009). Survival of surrogate coronaviruses in water. *Water Research, 43*, 1893-1898.

Casanova, L. & Waka, B. (2013). Survival of a Surrogate Virus on N95 Respirator Material. *Infection Control and Hospital Epidemiology, 34*(12), 1334-1335.

Casanova, L. & Weaver, S. (2015). Inactivation of an Enveloped Surrogate Virus in Human Sewage. *Environmental Science and Technology: Letters, 2*, 76-78.

CDC (Center for Disease Control and Prevention) (2012). The Safe Water System: Effect of Chlorination on Inactivating Selected Pathogens (http://www.cdc.gov/safewater/effectiveness-on-pathogens.html) (accessed September 28, 2016).

CDC US (2014). Interim Guidance for Environmental Infection Control in Hospitals for Ebola Virus (https://www.cdc.gov/vhf/ebola/healthcare-us/cleaning/hospitals.html)

CDC (2014). Viral Hemorrhagic Fevers: Filoviridae (http://www.cdc.gov/vhf/virus-families/filoviridae.html) (accessed August 18, 2016)

CDC (2015). Information on the Survivability of the Ebola Virus in Medical Waste (http://www.cdc.gov/vhf/ebola/healthcare-us/cleaning/ebola-virus-survivability.html#n4) (accessed September 29, 2016)

Charles, K. J.; Shore, J.; Sellwood, J.; Laverick, M.; Hart, A.; Pedley, S. (2009) Assessment of the stability of human viruses and coliphage in groundwater by PCR and infectivity methods. *Journal of Applied Microbiology, 106*(6), 1827-1837.

Chughtai, A. A.; Barnes, M.; Macintyre, C. R. (2016). Persistence of Ebola virus in various body fluids during convalescence: evidence and implications for disease transmission and control. *Epidemiology and Infection, 144*(8), 1652-1660.

Cook, B. W.; Cutts, T. A.; Nikiforuk, A. M.; Poliquin, P. G.; Court, D. A.; Strong, J. E.; Theriault, S. S. (2015). Evaluating environmental persistence and disinfection of the Ebola virus Makona variant. *Viruses, 7*(4), 1975-1986.

Davis, J. E.; Strauss, J. H.; Sinsheimer, R. L. (1961). Bacteriophage MS2: another RNA phage. *Science*, *134*(3488), 1427.

Dawson, D. J.; Paish, A.; Staffell, L. M.; Seymour, I. J.; Appleton, H. (2005). Survival of viruses on fresh produce, using MS2 as a surrogate for norovirus. *Journal of Applied Microbiology, 98*(1), 203-209.

Deng, M. Y. & Cliver, D. O. (1992). Inactivation of poliovirus type 1 in mixed human and swine wastes and by bacteria from swine manure. *Applied Environmental Microbiology, 72*(6), 2016-2021.

Feldmann, H. & Geisbert, T. W. (2011). Ebola haemorrhagic fever. *Lancet*, *377*(9768), 849–862.

Fischer, R.; Judson, S.; Miazgowicz, K.; Bushmaker, T.; Prescott, J.; Munster, V. J. (2015). Ebola Virus Stability on Surfaces and in Fluids in Simulated Outbreak Environments.

Emerging Infectious Diseases, 21(7), 1243-1246.

Geisbert, T. W.; Hensley, L. E.; Larsen, T.; Young, H. A.; Reed, D. S.; Geisbert, J. B.; Davis, K. J. (2003). Pathogenesis of Ebola Hemorrhagic Fever in Cynomolgus Macaques: Evidence that Dendritic Cells Are Early and Sustained Targets of Infection. *The American Journal of Pathology*, *163*(6), 2347–2370.

Geisbert, T. W.; Young, H. A.; Jahrling, P. B.; Davis, K. J.; Larsen, T.; Kagan, E.; Hensley, L. E. (2003). Pathogenesis of Ebola Hemorrhagic Fever in Primate Models : Evidence that Hemorrhage Is Not a Direct Effect of Virus-Induced Cytolysis of Endothelial Cells. *The American Journal of Pathology*, *163*(6), 2371–2382.

Gerba, C.P. (1975). Fate of Wastewater Bacteria and Viruses in Soil. *Journal of the Irrigation and Drainage Division, Proceedings of the American Society of Civil Engineers, 101*(IR3), 157-174.

Golmohammadi, R.; Valegård, K.; Fridborg, K.; Liljas, L. (1993). The refined structure of bacteriophage MS2 at 2·8 Å resolution. *Journal of Molecular Biology 234*(3), 620–639.

Gray, M.; De Leon. R.; Sobsey, M. D. (1993) Survival of hepatitis A virus (HAV), poliovirus and F specific coliphages in disposable diapers and landfill leachates. *Water Science and Technology, 27*(3-4), 429-432.

Gyürék, L. L. & Finch, G. R. (1998). Modeling Water Treatment Chemical Disinfection Kinetics. *Journal of Environmental Engineering, 124*(9), 783-793.

Haas, C. N. (1979). Discussion of 'Kinetics of bacterial deactivation with chlorine' by Robert E. Selleck.' *Journal of Environmental Engineering, 105*(6), 1198-1199.

Haas, C. N. & Joffe, J. (1994). Disinfection under dynamic conditions: modification of Hom's model for decay. *Environmental Science and Technology, 28*(7), 1367-1369.

Haas, C. N.; Joffe, J.; Anmangandla, U.; Jacangelo, J. G.; Heath, M. (1996). Water Quality and Disinfection Kinetics. *American Water Works Association, 88*(3), 95-103.

Halfman, P.; Kim, J. H.; Ebihara, H.; Noda, T.; Neumann, G.; Feldmann, H.; Kawaoka, Y. (2008). Generation of biologically contained Ebola viruses. *PNAS, 105*(4), 1129-1133.

Hoff, J. C. & Akin, E. W. (1986). Microbial resistance to disinfectants: mechanisms and significance. *Environmental Health Perspectives, 69*, 7.

Hopman, J.; Kubilay, Z.; Allen, T.; Edrees, H.; Pittet, D.; Allegranzi, B. (2015). Efficacy of chlorine solutions used for hand hygiene and gloves disinfection in Ebola settings: a systematic review. *Antimicrobial Resistance and Infection Control, 4*(1), O13.

Jazwinski, S. M. & Kornberg, A. (1975). DNA replication in vitro starting with an intact phiX174 phage. *PNAS, 72*(10), 3863-3867.

Jelden, K. C.; Gibbs, S. G.; Smith, P. W.; Schwedhelm, M. M.; Iwen, P. C.; Beam, E. L.; Hayes, A. K.; Marion, N.; Kratochvil, C. J.; Boulter, K. C. (2015). Nebraska Biocontainment Unit patient discharge and environmental decontamination after Ebola care. *American Journal of Infection Control*, *43*(3), 203–205.

LeChevallier, M. W. & Au, K. K. (2004). Water treatment and pathogen control: Process efficiency in achieving safe drinking water. *WHO/IWA Publishing,* ISBN 9241562552.

Lowe, J.; Gibbs, S.; Schwedhelm, S.; Nguyen, J.; Smith, P. (2014). Nebraska Biocontainment Unit perspective on disposal of Ebola. *American Journal of Infection Control, xxx*, 1-2.

Maillard, J. (2001). Virus susceptibility to biocides: An understanding. *Reviews in Medical Microbiology, 12*(2), 63-74.

Melnick, J. L. & Gerba, C. P. (1980). The ecology of enteroviruses in natural waters. *Critical Reviews in Environmental Control, 10,* 65-93.

Northern Territory Government (2013). Environmental Health Fact Sheet: The Effect of pH on Disinfection in Aquatic Facilities (http://www.health.nt.gov.au/library/scripts/objectifyMedia.aspx?file=pdf/86/67.pdf&siteID=1&str_title=pH%20and%20Disinfection.pdf). (accessed September 28, 2016).

Piercy, T. J.; Smither, S. J.; Steward, J. A.; Eastaugh, L.; Lever, M.S. (2010). The survival of filoviruses in liquids, on solid substrates and in a dynamic aerosol. *Journal of Applied Microbiology, 109*(5), 1531-1539.

Rutala, W. A. & Weber, D. J. (2008). CDC Guideline for Disinfection and Sterilization in Healthcare Facilities. 39-42.

Sagripanti, J. L.; Rom, A. M.; Holland, L. E. (2010). Persistence in darkness of virulent alphaviruses, Ebola virus, and Lassa virus deposited on solid surfaces. *Arch Virol, 155*, 2035-2039.

Sanger, F.; Air, G. M.; Barrell, B. G.; Brown, N. L.; Coulson, A. R.; Fiddes, J. C.; Hutchison, C. A.; Slocombe, P. M.; Smith, M. (1977). Nucleotide sequence of bacteriophage ΦX174 DNA. *Nature*, *265*(5596), 687–95.

Shin, G. A. & Sobsey, M. D. (1998) Reduction of Norwalk Virus, Poliovirus 1 and Coliphage MS2 by Free Chlorine, Chlorine Dioxide and Ozone Disinfection of Water. *In: Proc. 1998 Water Quality Technology Conference, Amer. Water Works Assoc.*, Nov. 1-4, Denver, CO, and * *Wat. Sci. Tech., 38*(12), 151-154.

Shirai, J.; Kanno, T.; Tsuchiya, Y.; Mitsubayashi, S.; Seki, R. (1999). Effects of Chlorine, Iodine, and Quaternary Ammonium Compound Disinfectants on Several Exotic Disease Viruses. *J. Vet. Med. Sci. 62*(1), 85-92.

Sobsey, M. D. (2005). Viruses in Animal Manures. *Contemporary Perspectives on Infectious Disease Agents in Sewage Sludge and Manure: Compost Science and Utilization,* 107-118.

Sobsey, M. D.; Dean, C. H.; Knuckles, M. E.; Wagner, R. A. (1980). Interactions and survival of enteric viruses in soil materials. *Applied Environmental Microbiology, 40*(1), 92-101.

Sobsey, M. D.; Fuji, T.; Shields, P. A. (1988) Inactivation of hepatitis A virus and model viruses in water by free chlorine and monochloramine, In: Proceedings of the IAWPRC Specialized Conference on Water and Wastewater Microbiology, Newport Beach, CA., *Wat. Sci. Tech., 20*(11, 12), 385-391.

Stallknecht, D. E.; Shane, S. M.; Kearny, M. T.; Zwank, P. J. (1990). Persistence of Avian Influenza Viruses in Water. *Avian Diseases, 34*(2), 406-411.

Vidaver, A. K.; Koski, R. K.; Van Etten, J. L. (1973). Bacteriophage ϕ: A lipid-containing virus of Pseudomonas phaseolicola. *Journal of Virology, 11*, 799-805.

Vinnerås, B.; Holmqvist, A.; Bagge, E.; Albihn, A.; Jonsson, H. (2003). The potential for disinfection of separated faecal matter by urea and by peracetic acid for hygienic nutrient recycling. *Bioresource Technology, 89*(2), 155-161.

Ward, R. L.; Knowlton, D. R.; Winston, P. E. (1986). Mechanism of inactivation of enteric viruses in fresh water. *Applied Environmental Microbiology, 52*, 450-459.

WHO (World Health Organization) (1978). Ebola haemorrhagic fever in Sudan, 1976. *Bulletin of the World Health Organization*, *56*(2), 247–270.

WHO (2016). Ebola Situation Report (http://apps.who.int/ebola/current-situation/ebola-situation-report-30-march-2016) (accessed August 17, 2016).

Wolf, T.; Kann, G.; Becker, S.; Stephan, C.; Brodt, H. R.; de Leuw, P.; Grünewald, T.; Vogl, T.; Kempf, V. A. J.; Keppler, O. T.; Zacharowski, K. (2015). Severe Ebola virus disease with

vascular leakage and multiorgan failure: treatment of a patient in intensive care. *Lancet*, *385* (9976), 1428–1435.

Young, D. C.; Sharp, D. G. (1977). Poliovirus aggregates and their survival in water. *Applied Environmental Microbiology 33*(1), 168-177.

APPENDIX

Appendix A. Methods: Surrogate Viruses

Coliphage Virus Stock Propagation

General Procedure:
Day 1: Grow overnight (stationary phase) host, prepare stock solutions
Day 2: Prepare Enrichment culture
Day 3: Centrifuge and aliquot

Important Notes:
Stocks can be propagated from either 1)frozen stocks stored in the -80 or 2) puling coliphage plaques from either SAL or DAL plates.

***E. coli* Host**	**Detectable Coliphages**	**Antibiotics***
Famp	F+ male specific	Streptomycin-ampicillin
CN13	Somatic coliphages	Nalidixic Acid

100x antibiotic stock solutions and their preparation
15g ampicillin sodium salt + 0.15g streptomycin sulfate into 100 mL DI water; sterile filter with 0.22μm filter; aliquot in to small volumes; store frozen.
1g nalidixic acid sodium salt into 100mL DI water; sterile filter with 0.22 μm filter; aliquot into small volumes; store frozen.

Propagation from Frozen Stock

Materials:
1/2X Tryptic Soy Broth
1X Tryptic Soy Broth
100X Antibiotic (appropriate for host *E. coli* strain)
125mL sterile shaker flask
125mL sterile glass bottle
E. coli host strain (appropriate for desired coliphage virus)
4M Magnesium Chloride ($MgCl_2$)
Frozen coliphage virus stock (minimum 20uL)
Chloroform
Polystyrene pipettes (5mL, 10mL, 25mL)
Centrifuge

Day 1

Prepare all materials, stock solutions, and start overnight culture.

Overnight culture (stationary phase) prep
Prepare a sterile 125mL shaker flask with:
25mL 1X TSB (or more if necessary)
0.25mL appropriate antibiotic
Using a sterile wooden applicator stick and a frozen *E. coli* host culture (log phase), scrape a small amount of the frozen material into the TSB.

Day 2
Prepare a 125mL sterile glass bottle with:
100mL 1/2 X TSB
1.25 mL 4M $MgCl_2$
5mL appropriate log phase host
1mL 100x appropriate antibiotic
20uL stock coliphage virus

Incubate overnight at 37 ^{O}C with or without shaking.

Day 3
1. Pour entire contents of incubated culture into a 250mL centrifuge tube and add 5mL of chloroform to tube.
Be careful, Chloroform is hazardous and must be added in the fume hood in the pathogen lab. Also it must be discarded in hazardous waste.
2. Shake 250mL tube well.
3. Balance tube with another centrifuge tube rack before placing into the centrifuge
Do not place only one tube into the centrifuge, you will break it
4. Centrifuge at 3000 RPM for 30min at 4C
5. Remove from centrifuge and aliquot supernatant (minimum 200-300uL)

Appendix B. Methods: Survival and Disinfection Experiments

PBS Stock Preparation
1. Start with 800 mL of distilled water
2. Add 8 g of NaCl
3. Add 0.2 g of KCl
4. Add 1.44 g of Na_2HPO_4
5. Add 0.24 g of KH_2PO_4
6. Adjust the pH to 7.4 with HCl
7. Add distilled water to a total volume of 1 liter

Appendix C. Methods: Breakpoint Chlorination

Initial Free Chlorine (mg/L)	Adjusted Residual Total Chlorine (mg/L)
0	0
250	0
500	0
750	0
1000	0
1500	10
2000	20
2500	70
3000	120
3500	160
4000	240
4500	310
10000	1330

Table A1*: Breakpoint chlorination of undiluted raw hospital sewage and fecal waste matrix expressed as adjusted residual total chlorine (mg/L) versus initial free chlorine concentration (mg/L).*

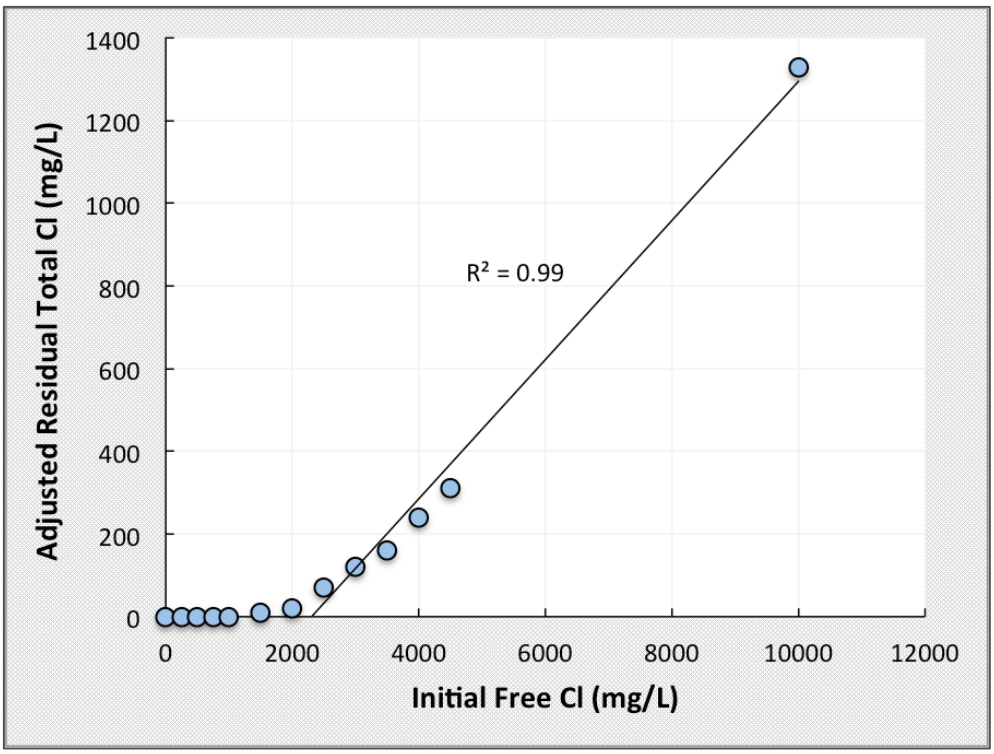


Figure A1*: Breakpoint chlorination of undiluted raw hospital sewage and fecal waste matrix expressed as adjusted residual total chlorine (mg/L) versus initial free chlorine concentration (mg/L). Linear trendline begins at point of significant increase in residual total chlorine and corresponding R^2 value is shown.*

Initial Free Chlorine (mg/L)	Adjusted Residual Free Chlorine (mg/L)
228	0
570	0.09
1140	0.11
2280	0.15
3420	1.7
4560	4.1
5700	6.9
6840	9.8
7980	11.8
11400	19.9
22800	55
34200	81

Table A2*: Breakpoint chlorination of 1:100 dilution of raw hospital sewage and fecal waste matrix expressed as adjusted residual free chlorine (mg/L) versus initial free chlorine concentration (mg/L).*

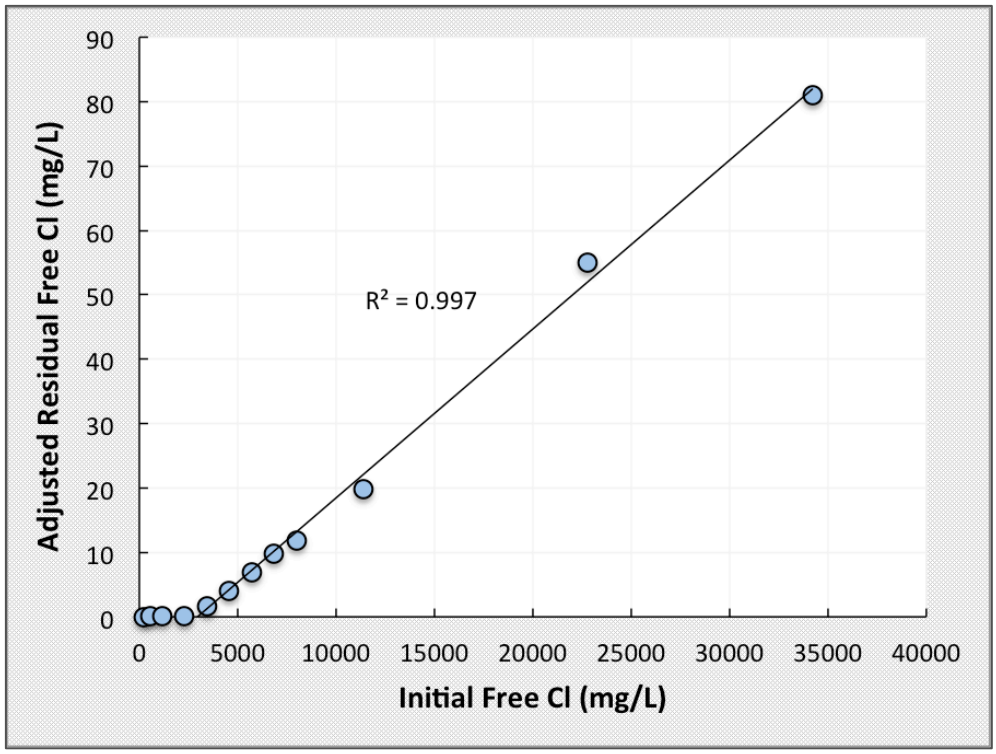


Figure A2*: Breakpoint chlorination of 1:100 dilution of raw hospital sewage and fecal waste matrix expressed as adjusted residual free chlorine (mg/L) versus initial free chlorine concentration (mg/L). Linear trendline begins at point of significant increase in residual free chlorine and corresponding* R^2 *value is shown.*

Appendix D. Methods: Selleck Disinfection Kinetics

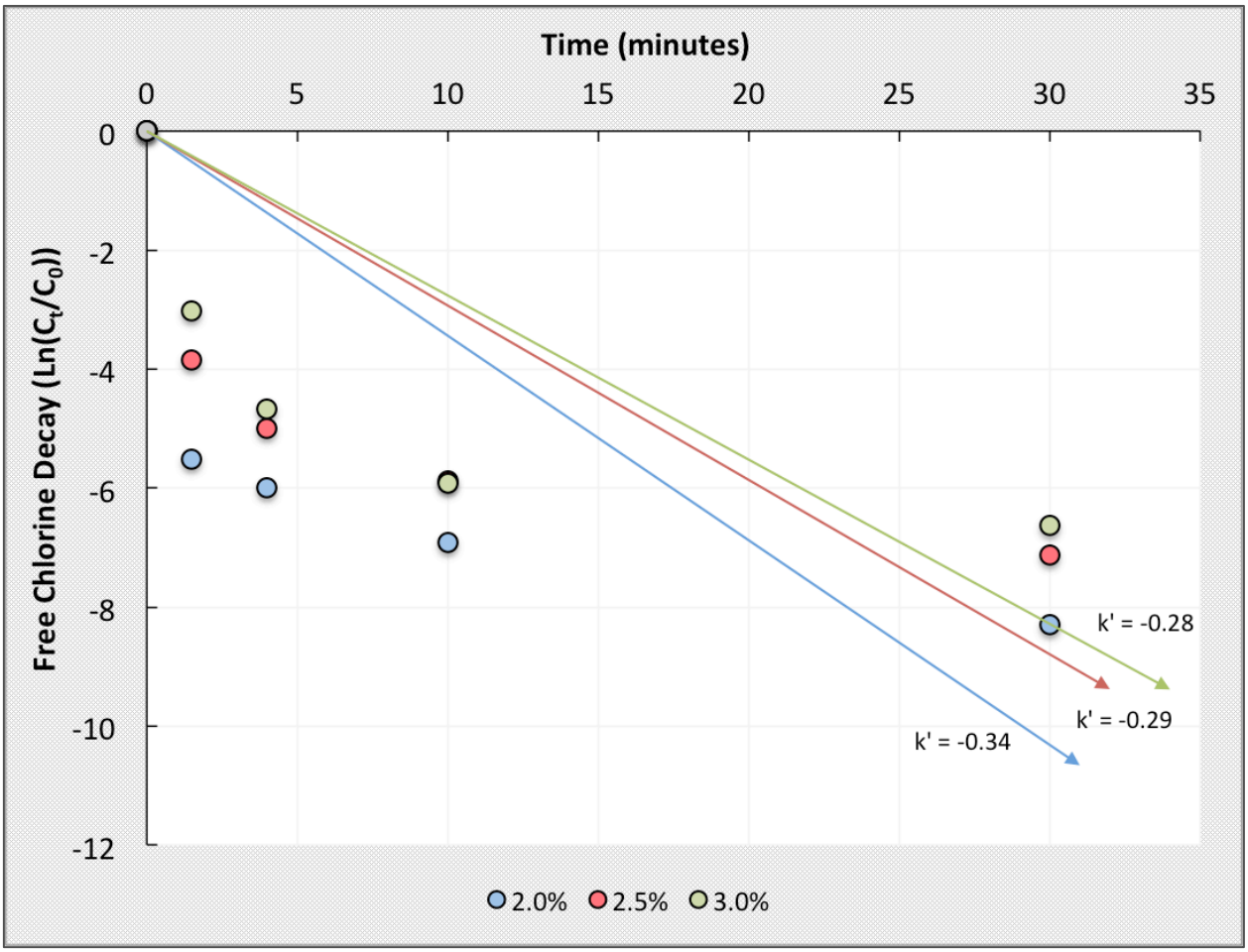


***Figure A3**: Free chlorine decay expressed as the natural log of C_t divided by C_0 at 1.5, 4, 10, and 30-minute time points in a matrix of raw hospital sewage and human fecal waste. Linear trendlines are shown with corresponding k' value. R^2 values were 0.56, 0.27, and 0.16 for 2.0%, 2.5%, and 3.0%, respectively.*

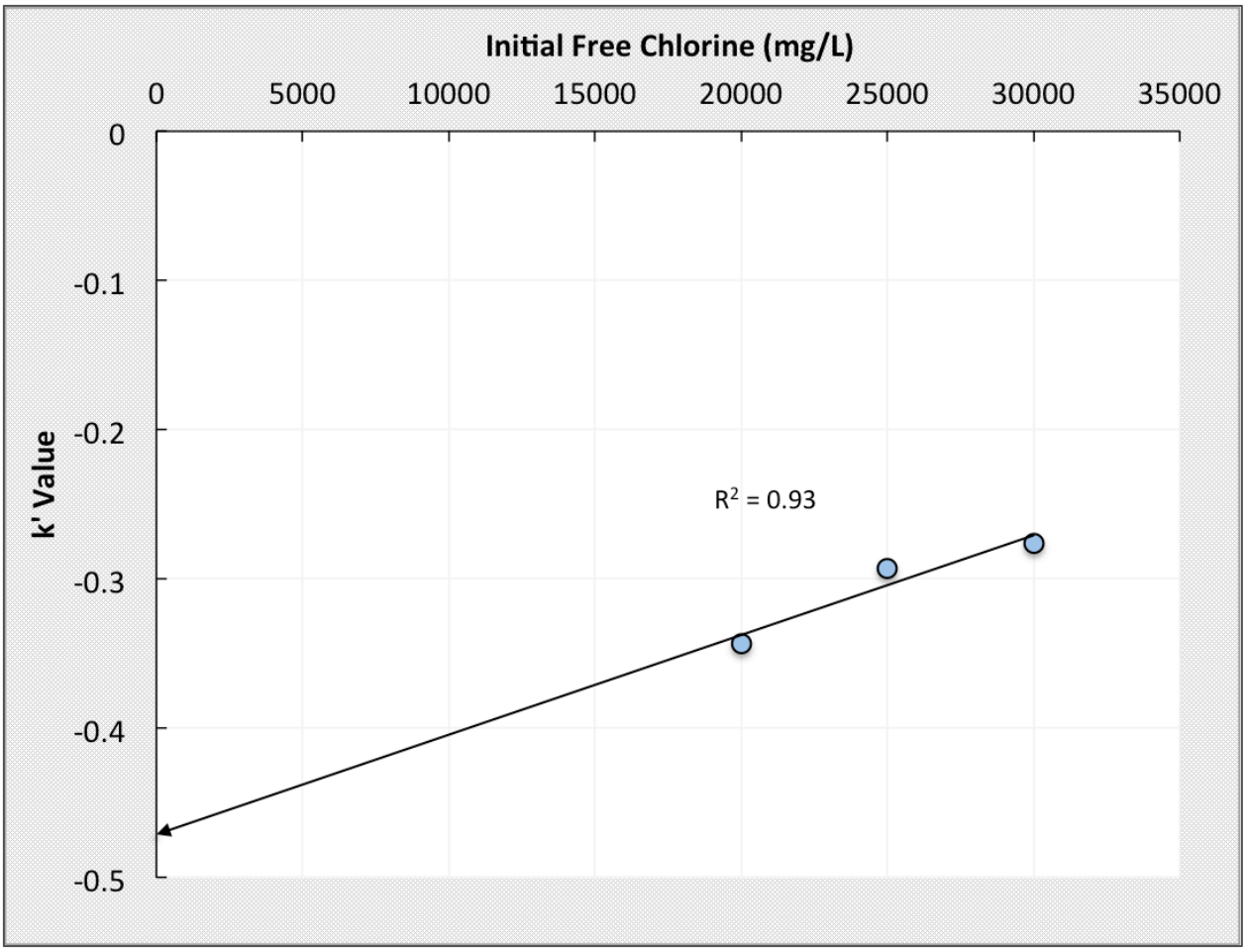


Figure A4*: Observed k' values compared to initial free chlorine concentration. Regression analysis allowed for extrapolation of k' values at free chlorine concentrations of interest to this report (R^2 = 0.93).*

Initial Free Cl (mg/L)	Predicted k'
500	-0.4675
1000	-0.4640
1500	-0.4605
2000	-0.4570
2500	-0.4535
3000	-0.4500
4000	-0.4430

Table A3*: Predicted k' values compared to initial free chlorine concentration determined by extrapolation from linear regression.*

Appendix E. Methods: Background Microbial Testing

Tests were performed on the matrix to determine the background levels of bacterial indicators and phages of interest. Enterococci and thermotolerant coliform concentrations were detected in hospital sewage samples by standard membrane filtration (MF) methods. A composite hospital sewage sample was diluted to an expected contamination level using PBS as diluent. A 10 mL volume of each desired dilution was vacuum filtered through a 0.45 µm pore size, 47 mm diameter cellulose ester membrane filter. Filters were then placed on selective agar media by gently rolling the filters to exclude air bubbles underneath. Each dilution was plated in duplicate.

Appendix F. Results: Survival

Time (days)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error
0	0	-	-
1	-0.15	0.34	0.15
2	-0.24	0.52	0.26
3	-0.34	0.16	0.11
4	-0.58	0.46	0.32
5	-0.81	0.54	0.38
6	-0.96	0.31	0.18
8	-0.90	0.44	0.31
13	-1.31	0.69	0.49
18	-2.14	0.72	0.51

Table A5*: Survival of MS2 coliphage (average t_0 titer: 8.2 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and fecal waste at 22 ^{O}C.*

Time (days)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error
0	0	-	-
1	-0.40	0.50	0.29

2	-0.77	0.46	0.26
3	-1.05	0.53	0.37
6	-1.28	0.55	0.39
7	-1.67	0.08	0.06
15	-2.60	0.33	0.23

***Table A6**: Survival of MS2 coliphage (average t_0 titer: 8.2 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and fecal waste at 37 ^{O}C.*

Time (days)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error
0	0	-	-
1	-0.24	0.12	0.05
2	-0.20	0.14	0.07
3	-0.40	0.14	0.10
4	-0.31	0.03	0.02
5	-0.35	0.02	0.01
6	-0.46	0.04	0.03
7	-0.33	0.16	0.09
8	-0.39	0.08	0.06
13	-0.73	0.23	0.17
18	-0.97	0.03	0.02

***Table A7**: Survival of PhiX-174 coliphage (average t_0 titer: 7.0 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and fecal waste at 22 ^{O}C.*

Time (days)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error
0	0	-	-
1	-0.68	0.52	0.26
2	-0.92	0.45	0.23
3	-1.15	0.33	0.19
4	-1.38	0.44	0.31
6	-1.17	0.37	0.26
7	-1.91	0.68	0.48
15	-3.69	1.17	0.83

***Table A8**: Survival of PhiX-174 coliphage (average t_0 titer: 7.0 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and fecal waste at 37 ^{O}C.*

Time (days)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error

0	0	-	-
1	-0.62	0.33	0.12
2	-1.32	0.88	0.36
3	-2.03	1.08	0.48
4	-3.29	0.87	0.50
5	-3.51	0.80	0.46
6	-3.77	0.99	0.50
7	-3.89	1.37	0.56
8	-4.57	0.87	0.62
12.33	-5.69	1.87	1.08

***Table A9**: Survival of Phi6 bacteriophage (average t_0 titer: 7.8 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and fecal waste at 22 ^{O}C.*

Time (days)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error
0	0	-	-
1	-4.43	2.56	1.28
2	-5.76	0.61	0.30

***Table A10**: Survival of Phi6 bacteriophage (average t_0 titer: 7.8 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) over time (days) in a matrix of raw hospital sewage and fecal waste at 37 ^{O}C.*

Appendix G. Results: Disinfection

Initial Free Cl (mg/L)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error
500	-0.20	0.22	0.15
1500	-0.82	0.51	0.36
2000	-0.49	0.43	0.19
2500	-0.42	0.18	0.13
3000	-0.51	0.06	0.04
4000	-0.98	-	-

***Table A11**: Free chlorine disinfection of MS2 coliphage after 10 minutes of contact time (average t_0 titer: 8.3 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) vs. varying initial free chlorine concentrations (mg/L) in a matrix of raw hospital sewage and human fecal waste.*

Initial Free Cl (mg/L)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error

500	-0.28	0.11	0.08
1000	-1.10	0.02	0.02
1500	-1.55	0.74	0.52
2000	-3.23	1.50	0.67
2500	-2.84	0.10	0.07
3000	-4.55	1.31	0.76
4000	-5.85	-	-

Table A12*: Free chlorine disinfection of PhiX-174 coliphage after 10 minutes of contact time (average t_0 titer: 7.2 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) vs. varying initial free chlorine concentrations (mg/L) in a matrix of raw hospital sewage and human fecal waste.*

Initial Free Cl (mg/L)	Avg. $Log_{10}(N_t/N_0)$	Std. Dev.	Std. Error
0	-0.07	0.03	0.02
500	-0.25	0.10	0.06
1000	-2.70	1.68	0.97
1500	-3.10	0.55	0.27
2000	-4.53	1.45	0.51
2500	-5.03	1.99	1.41
3000	-5.23	1.17	0.59
4000	-6.70	-	-

Table A13*: Free chlorine disinfection of Phi6 bacteriophage after 10 minutes of contact time (average t_0 titer: 7.9 log_{10} PFU/mL) expressed as log_{10} (N_t/N_0) vs. varying initial free chlorine concentrations (mg/L) in a matrix of raw hospital sewage and human fecal waste.*

Appendix H. Practicum Reflection: OWASA

Between January 30th and February 8th, 2017, I completed a 62.5-hour practicum experience at Orange Water and Sewer Authority. 'OWASA' is a local government entity that provides water and wastewater services to the towns of Carrboro and Chapel Hill (includes UNC Chapel Hill). Most of my time at OWASA was spent at the administrative building on Jones Ferry road, adjacent to the water treatment plant. The preceptor for this experience was Adam Haggerty, asset management and facilities engineer.

During a smaller portion of my time at OWASA, I toured several different departments to get an overall feel for the function of a utility. On my first day, I was allowed to sit in on a capital improvement projects meeting where an independent consulting firm was present to facilitate prioritizing infrastructure improvements that will be made over the next five years. Representatives from several departments were in attendance to place a 0-10 value on proposed improvements, with the highest scoring improvements being prioritized for accelerated completion. In parallel, I completed a mock exercise where I also rated each of the capital improvements and compared my prioritization to department representatives. This

meeting provided me with insight to the difficult budgetary and temporal decisions that must be made when conducting business with a substantial number of assets.

On the following day, I toured the water treatment plant and shadowed an operator conducting a routine check of operations at the plant. I asked the operator questions about the water treatment system OWASA utilizes and how it compares to treatment systems I have learned about in courses I have taken, such as Dr. Mark Sobsey's environmental microbiology course. I was also able to speak with the scientists working at the regulatory compliance lab at the water treatment plant. Later in the day, I accompanied the water treatment plant laboratory supervisor, Katie Harrold, on a short trip to OWASA's largest water resource, Cane Creek Reservoir. At the reservoir, Katie sampled disks that are used to measure the concentration of toxins produced by freshwater cyanobacteria found in the lake.

During another shadowing experience, I rode along with Nick Rogers, assistant manager of distribution and collection systems, to develop a better understanding of operations and maintenance for the utility. I accompanied Nick to several job sites including the wastewater treatment plant and some routine repairs on Rosemary Street in downtown Chapel Hill. We also investigated a possible main break on the north side of town that turned out to be a simple storm drain blockage.

I was also able to spend time on a survey crew with a few members of the engineering department who use advanced surveying equipment to map all points of interest and interface the data with geographic information system (GIS) software. The past few years, this team has been working to map the entire OWASA infrastructure for in depth understanding of the cartography of all connections and assets.

A considerable goal of my practicum experience was to utilize and develop proficiency with SQL software to analyze data entered into a 'MP2' database, which is used to record all maintenance work orders scheduled, completed, or otherwise. By writing SQL code to select and filter appropriate date entries, I managed to complete reports on several different parameters of interest to the asset management department. One report focused on the mean time between failure (MTBF) of all recorded equipment, including all vehicles, pumps, generators, filters, etc. The engineering department will use this report to identify frequently failing equipment over an adjustable time period. This information can be cross-referenced with preventative and corrective maintenance costs to flag equipment or other assets for retirement, assuming maintenance costs are greater than the replacement cost.

Other reports completed include: mean time to repair (MTTR) - a measure of the average time required to fix a piece of equipment, scheduled compliance (%) - a measure of the successful completion of a maintenance activity, efficiency (%) - a measure of the actual hours on work orders compared to the total hours available, productivity (%) - a measure of overall labor performance taking into account both efficiency and utilization, preventative maintenance (%) - the ratio of preventative maintenance hours worked to total maintenance work order hours worked, scheduled utilization (%) - the ratio of scheduled maintenance activity to available maintenance hours, and overtime (%) - a measure of the maintenance work hours paid at premium rates compared to total maintenance work hours.

These SQL reports will provide OWASA the ability to critically review their labor resources as well as the viability of their equipment. The code written for these parameters can be used for any time period and will be utilized in future reviews of labor and infrastructure assets. For example, several older vehicles had short MTBF periods as well as considerable MTTR values and will likely be tagged for retirement upon intermittent evaluation.

A significant event in OWASA's history occurred during my practicum experience as the utility was unable to provide water to its customers over about a 24-hour period from February 3rd to February 4th. A shutdown of the water treatment plant resulted from a fluoride overfeed error made by a WTP operator. The over fluoridated water was contained to the water treatment plant and was not distributed to OWASA customers. In emergency incidences, the utility can receive water from Durham via two interconnections on the east side of Chapel Hill. These interconnections were quickly opened to ensure ample water for the service area. Unfortunately, a 12-inch diameter water main break occurred near the interconnection site with Durham the next morning. This break likely occurred in response to unusually high pressure that the pipe does not experience under normal distribution from OWASA's water treatment plant. From this point, the distribution system lost pressure and water levels in storage tanks around the service area began to rapidly fall. OWASA issued an emergency do-not-use and do-not-drink advisory to its customers at this point. The main break was isolated and the operations and maintenance crew began repair work. Meanwhile, the water treatment plant flushed its system to remove over-fluoridated water and pumped the water directly to the wastewater treatment plant. Later that same evening, a rarely used interconnect with Chatham County was activated to ensure reserve levels did not fall below critical levels. The next day, the water main break was repaired and the water treatment plant began distributing water once again. The do-not-use and do-not-drink advisories were lifted at this point and water service returned to normal.

On the Friday the service interruption occurred, members of every department scrambled to correct failures, notify customers, and support the water treatment plant staff and operations and maintenance crew. Considering the nature and timing of the failures, it was highly impressive to see the collaborative work of the utility to return service after only a one-day outage.

Overall, my time spent at OWASA was productive for both the utility and me. I developed technical skills in Microsoft SQL software and learned aspects of the entire water and wastewater services system. The experience provided me with the opportunity to see both a local government and business perspective of public health and environmental services, other than my typical laboratory setting.