EVALUATION OF VIRAL INACTIVATION AND SURVIVAL IN THREE UNIQUE ENVIRONMENTS, THROUGH THE USE OF MS2 COLIPHAGE AS A SURROGATE

By

Hannah P. Sassi

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Hannah P. Sassi, titled “Evaluation of Viral Inactivation and Survival in Three Unique Environments, Through the Use of MS2 as a Surrogate” and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

____________________________________ Date: 04/18/16
Charles P. Gerba

____________________________________ Date: 04/18/16
Kelly A. Reynolds

____________________________________ Date: 04/18/16
Ian L. Pepper

____________________________________ Date: 04/18/16
Channah M. Rock

____________________________________ Date: 04/18/16
Sadhana Ravishankar
Final approval and acceptance of this dissertation
is contingent upon the candidate’s submission of the final copies of the dissertation to the
Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend
that it be accepted as fulfilling the dissertation requirement.

________________________________________________ Date: 04/18/16

Dissertation Director: Charles P. Gerba

________________________________________________ Date: 04/18/16

Dissertation Director: Kelly A. Reynolds
STATEMENT BY AUTHOR

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SIGNED: HANNAH P SASSI
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DEDICATION

I want to dedicate this work to my nephews, Carson and Harrison, as an example of the love and support you will receive from our family in all of your adventures. I can’t wait to see where you will go!
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ABSTRACT

Surrogate organisms have been used to study highly pathogenic organisms, or organisms that cannot be cultured in the laboratory. Surrogates are selected based on multiple similarities to the pathogen, such as morphology, genome size and structure, and environmental characteristics. This dissertation utilized MS2 coliphage as a surrogate for norovirus and Ebola virus in three environments. MS2 is an icosahedral, single-stranded RNA bacteriophage. It is a male-specific coliphage that infects the bacteria *Escherichia coli*. Its properties, such as morphology and survival in the environment, have been likened to those of many enteric viruses. Because of this, it has been used as a surrogate for pathogenic enteric viruses for disinfection testing on surfaces, in water and in food; modeling the movement and survival of pathogens in different environments; and transfer properties from surfaces. This dissertation utilized MS2 as a surrogate in three different studies. In the first, MS2 is used as a surrogate for human enteric viruses in irrigation canals to predict the re-suspension of pathogenic viruses from bed sediment into overlying irrigation water using a flume to re-create field conditions in the laboratory. MS2 re-suspension into the overlying water was characterized at varying flow rates and velocities using two sediment types. Its overall re-suspension was not statistically significantly different \( (p>0.05) \) between flow rates. The additional studies in this dissertation used MS2 as a surrogate for Ebola virus in human waste. Ebola virus is a BSL-4 organism that is spread through direct contact with bodily fluids. It is found in bodily fluids in concentrations between \( 10^{5.5} \) and \( 10^{8} \) genome copies per milliliter. In the first study using MS2 as a surrogate for Ebola virus, efficacies of four disinfectants were tested using \( 10^{12} \) PFU of MS2 in one liter containing 2.25\% (w/v) organic matter at three contact times (1, 15 and 30 minutes). The purpose of this study was
to assess the disinfectants on reducing virus in waste before toilet flushing. Peracetic acid and quaternary ammonium formulation were found to reduce the concentration of MS2 in the toilet bowl the fastest (within one minute) with the greatest reduction (2.26 and 1.99 log_{10}), when compared with the other disinfectants. Reductions observed from hydrogen peroxide were significantly less than those from peracetic acid and quaternary ammonium ($p<0.05$). The contamination of restroom surfaces by MS2 was also evaluated after toilet flushing with and without disinfectant treatment. All four disinfectants were found to significantly reduce the viral concentrations on fomites after 15 minutes of contact ($p<0.05$). Despite disinfectant use, three sites were contaminated in 100% of trials (N=18). These were the toilet bowl rim, the toilet seat top and underside. The final study evaluated the inactivation of MS2 and several other viruses by thermophilic and mesophilic anaerobic digestion. Little information is available on the influence of the wastewater treatment process, specifically anaerobic digestion, on emerging viruses, such as Ebola virus. It is important to evaluate this process due to the environmental disposal and discharge of wastewater and solids into the environment. All viruses were recoverable after mesophilic digestion (reductions from 1.8-6.6 log_{10} per mL), except the lipid-containing bacteriophage Φ6. Thermophilic digestion inactivated all viruses significantly ($p=0.0011$) more than mesophilic digestion. The reductions by thermophilic digestion ranged from 2.8-7.1 log_{10} per mL. The inactivation between the initial concentration and both digestion types was statistically significant ($p=0.007$).
INTRODUCTION

1. Explanation of the Problem

Surrogates are frequently utilized to determine environmental effects on organisms that are difficult or unsafe to use in environmental research. The use of surrogates is often debated due to the difficulty in validating them. Despite this, surrogates are still necessary to assess risks associated with certain pathogens. To improve surrogate selection, standard criteria were developed. The criteria include practical, biological and environmental similarities between the comparable organisms (Sinclair et al, 2012). This dissertation uses MS2 as a surrogate to investigate survival, fate and transport of viruses in three environmental studies: re-suspension from irrigation canal sediments, reduction in human waste from recommended disinfection practices, and reduction during mesophilic and thermophilic anaerobic digestion.

Norovirus is a major cause of foodborne illness (58%) in the United States, with a majority of these outbreaks occurring in fresh produce (33%) (Hall et al, 2004). Norovirus cannot be cultured in the laboratory, making it necessary to use a surrogate (Wyer et al, 2010). Many enteric viruses and bacteria have been observed to settle in higher concentrations in aquatic sediments than the overlying water. This settling also extends their viability in aquatic environments (Burton et al, 1987; LaBelle & Gerba, 1980). Sediments and waters in irrigation canals in the Yuma Valley have tested positive for Salmonella and Escherichia coli (Obergh, 2015; Carpenter, 2007). During the winter months, this area supplies at least 90% of fresh produce to the US (Yuma Chamber of Commerce). The objective of this portion of the study was to characterize the re-suspension of sediment-borne bacteria and viruses into overlying irrigation water quality, through the use of non-pathogenic surrogates (E. coli ATCC 25922 and MS2
The data from this study will aid in evaluating potential risks from sediment-borne pathogens to human health and food safety.

Ebola virus is an RNA, filamentous filovirus with a high documented case-fatality rate (90%). Its importance in the United States drastically increased when the first imported case was observed in 2014 (Fauci, 2014). Due to the recent outbreak in West Africa, disinfection practices and recommendations were compiled for healthcare practitioners by the Centers for Disease Control and Prevention and the World Health Organization (CDC 2015; WHO, 2014).

Environmental control of Ebola virus is extremely important because it is shed in high numbers in bodily fluids ($10^5$-$10^8$) (Bibby et al., 2015). This results in a high number of virus excreted per patient, per day, into the environment. Because the CDC recommended no treatment of bodily fluids before flushing down the toilet, follow up disinfection recommendations were supplied by the US Army Institute for Public Health (2015). The disinfection of waste before flushing is important because some pathogens have the ability to aerosolize (Gerba, 1975) and spread to surfaces in the restroom (Best et al., 2012; Barker & Jones, 2005). It is also important because waste is further treated at wastewater treatment centers all over the US. Untreated waste entering the wastewater treatment facility could pose threats to wastewater worker health and safety; and incomplete inactivation during the treatment process could lead to environmental disposal of infectious materials. The survival of Ebola virus in wastewater has been scarcely researched (Bibby et al., 2015; Casanova & Weaver, 2015). Because of this, its survival during wastewater treatment is also unknown. This objective of this portion of the study was to validate recommendations by the US Army Institute of Public Health for disinfecting Ebola-contaminated waste before flushing; and to assess the influence of anaerobic digestion processes on Ebola
surrogates, if untreated waste enters into the wastewater treatment process. MS2 was utilized as a surrogate in all experiments.

2. Specific Aims and Hypotheses

This dissertation had three specific aims and hypotheses that were investigated in three separate studies. They are represented in the appendix by three separate manuscripts:

2.1 Specific Aim 1: Assess the prevalence of enteric viruses and bacteria in bed sediments and re-suspension into the overlying irrigation water. To achieve this aim, field measurements and characteristics of canals in Yuma, Arizona were collected. This data included water depth, canal width, turbidity and temperature of water, and sediment size characterizations. This information was used to create a bench-scale model of an irrigation canal using a closed system flume and two types of sediment (clay and sand). The flow rates of water were measured at 0.41, 0.73, and 1.46 L/s. MS2 was used as a surrogate for enteric viruses and Escherichia coli 25922 was used as a surrogate for E. coli O:157:H7. It was hypothesized that a significant proportion (p<0.05) of the starting inoculum (10^9), for both organisms, would not re-suspend from the sediment into the overlying water (Appendix A).

2.2 Specific Aim 2: Evaluate the efficacy of four disinfectants (chlorine bleach, quaternary ammonium, hydrogen peroxide and peracetic acid) on the reduction of virus concentration in surrogate human waste (1) in the toilet bowl before flushing and (2) on surfaces in the restroom after flushing. To achieve this aim, human infectious waste was simulated using tryptic soy broth inoculated with 10^{12} PFU MS2 coliphage. Samples were taken directly from the
toilet bowl before addition of treatment; and at 1, 15 and 30 minutes after addition of the treatment to determine the impact of the disinfectant on the viral concentration in the toilet bowl. To test the deposition of aerosolized viral particles on restroom surfaces, inoculated broth was added to the toilet and flushed. Eight surfaces around the toilet were then sampled. Impact of the disinfectants was tested by adding one cup of the disinfectant after the broth and virus, and flushing. The same surfaces sampled after flushing. This procedure also tested the effects of 15- and 30-minute contact times. The hypotheses of this aim were that the disinfectants would significantly reduce ($p<0.05$) the concentration of virus in the toilet bowl and on surfaces, when compared to concentrations without treatment (Appendix B).

2.3 Specific Aim 3: Determine the impact of mesophilic and thermophilic anaerobic digestion on the inactivation of five viruses, to estimate the survival and inactivation of emerging viruses during the wastewater treatment process. The five viruses represented varying phenotypic and genotypic characteristics. This aim was achieved by creating miniature anaerobic digestion tubes and recreating mesophilic and thermophilic digestion conditions. It was hypothesized that thermophilic digestion would have a significantly greater ($p<0.05$) impact on inactivating the viruses than mesophilic digestion; and that significantly less ($p<0.05$) reduction would be observed in the non-enveloped mammalian viruses (Appendix C).
3. Dissertation Format

This dissertation contains three manuscripts that were prepared for submission to peer-reviewed journals. The manuscript contained in Appendix A is entitled “Potential for Viral and Bacterial Re-suspension from Sediments into Overlying Water in Irrigation Canals”. This manuscript was prepared for the Journal of Food Protection and aligns with Specific Aim 1. Appendix B contains the manuscript “Disinfectant Use to Reduce an Ebola Surrogate in Toilet Flush Aerosols”. This manuscript was prepared for the American Journal of Infection Control and aligns with Specific Aim 2. Lastly, Appendix C contains the manuscript “Survival of Viruses during Thermophilic and Mesophilic Anaerobic Digestion: Assessing the Potential for Survival of Emerging Viruses”. This manuscript was prepared for the Journal of Residual Sciences and Technology and aligns with Specific Aim 3. In addition to these manuscripts, a literature review investigating MS2 as a surrogate for pathogenic viruses is provided. A brief summary of methods, results and interpretation of results is also provided for each specific aim after the literature review.
4. Review of the Literature: MS2 Coliphage as a Surrogate in Environmental Research

4.1. Introduction

MS2 was first isolated by Alvin J. Clark in the 1960s. The exact isolation of the bacteriophage is unclear in the literature, however, it was noted that its ability to only infected F\(^+\) strains of *Escherichia coli* made it distinct from other coliphages (Strauss & Sinsheimer, 1963). Over a decade later, the full genome was sequenced. The genome consists of three viral polypeptides, in total the genome is 3.5 kb in size (Fiers *et al*., 1976). For many years, MS2 and other bacteriophages were used to study and understand viral replication and the fundamentals of transcription (Fiers *et al*., 1976). MS2 is a single-stranded RNA bacteriophage that infects the F-pilus. It is characterized as icosahedral in shape and approximately 26-34 nm in diameter. It belongs to the family *Leviviridae* and genus *Levivirus* (Bozkurt *et al*., 2015; Calendar, 1988).

In the environment, MS2 is most frequently isolated in feces and sewage, and it can replicate readily in the mammalian gastrointestinal tract (Calendar, 1988). Because of this, and its similarity in size, shape and nucleic acid type, MS2 has been suggested as a surrogate for enteric viruses such as hepatitis A virus, enteroviruses and human noroviruses (Bozkurt *et al*, 2015). It has been especially useful as a surrogate for non-culturable noroviruses (Wyer *et al*, 2010). MS2 has also been used as a surrogate for pandemic strains of influenza and other respiratory viruses because of its ability to aerosolize and survive similarly to respiratory viruses (Fisher & Shaffer, 2010; Balazy *et al*, 2009; Eninger *et al*, 2008).

Surrogates are useful in environmental research to learn more about the transport, persistence and survival of high risk pathogens (Sinclair *et al*, 2012). Obtaining such information is essential for application in quantitative microbial risk assessment. When selecting a surrogate,
there are three categories of organism characteristics that have to be considered: practical attributes, biological attributes and environmental attributes (Sinclair et al, 2012). These attributes are important because they determine the suitability of surrogates to the virus they are representing. It also allows discrepancies between viruses and their surrogates to be identified and adjusted for when determining risk model parameters.

The practical attributes of MS2 (Table 1) in surrogate selection are one of the reasons that it is so widely used in a variety of settings and studies. It is not a risk to human health because of its inability to infect humans (Bozkurt et al, 2015). It is also easy to use in any microbiological laboratory because its ability to be propagated in high numbers (Pepper and Gentry, 2015), clarity and simplicity of viability assays. Assaying and propagating the virus have been well-established in literature (Calendar et al, 1988; Sifuentes et al, 2014; Sassi et al, 2015; Reynolds et al, 2015; USEPA, 2001) and quantitative results are obtained within 18-24 hours. In some instances, MS2 plaques can be observed after only 4 hours, because of *Escherichia coli*’s rapid growth time (Kropinski et al, 2009).

The shape and size of MS2 is similar to that of many human viruses (Table 2). Environmental attributes of MS2, as compared to respiratory and enteric viruses, are explored through review of research on the use of MS2 surrogates in the environment. This review will explore the uses of MS2 as a surrogate for disinfection; environmental survival and fate and transport; and transfer and tracer studies.
Table 1: MS2 surrogate characteristics

<table>
<thead>
<tr>
<th>Practical</th>
<th>Biological</th>
<th>Environmental</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Available in high numbers</td>
<td>Non-enveloped</td>
<td>Viable in high and low RH</td>
<td></td>
</tr>
<tr>
<td>Rapid reproduction</td>
<td>ssRNA; 3.5 kb genome</td>
<td>UV resistant at certain wave lengths</td>
<td></td>
</tr>
<tr>
<td>Viability assay results in 18-24 h</td>
<td>Hydrophobic</td>
<td>Released in feces</td>
<td></td>
</tr>
<tr>
<td>Easy to isolate from environment</td>
<td>Isoelectric point: 2.2-4.0</td>
<td>Increased survival in high organic content</td>
<td></td>
</tr>
</tbody>
</table>

Table 2: Comparison of MS2 characteristics with other viruses it has been used as a surrogate for

<table>
<thead>
<tr>
<th>Virus Characteristics of MS2 Comparison to Human Viruses</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Virus</th>
<th>Family</th>
<th>Genus</th>
<th>Genome type and size</th>
<th>Capsid Shape and Size</th>
<th>Isoelectric Point</th>
<th>Host</th>
<th>Host Receptor</th>
<th>Transmission</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2</td>
<td>Levirida</td>
<td>(+)</td>
<td>ssRNA; non-enveloped</td>
<td>Icosahedral; 26-34 nm</td>
<td>2.2-4.0</td>
<td>F+ E. coli</td>
<td>F-pilus</td>
<td>Environmental</td>
<td>Bozkurt et al, 2015</td>
</tr>
<tr>
<td>Levivirus</td>
<td>3.5 kb</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Virus Family</td>
<td>Genus</td>
<td>(+) ssRNA; dsRNA; 7.5 kb</td>
<td>Non-enveloped</td>
<td>Icosahedral; Spherical; Wheel-shaped</td>
<td>Length (kb)</td>
<td>Host(s)</td>
<td>Receptor(s)</td>
<td>Transmission</td>
<td>References</td>
</tr>
<tr>
<td>---------------------</td>
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<td>-------------</td>
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<td>------------------------------------------------</td>
</tr>
<tr>
<td>Noroviruses</td>
<td>Caliciviridae; Norovirus</td>
<td>(+) ssRNA; 7.5 kb</td>
<td>Non-enveloped</td>
<td>Icosahedral; 27-38 nm</td>
<td>5.5-6.0</td>
<td>Human; B cells; heparin sulfate</td>
<td>HBGA; B cells; heparin sulfate</td>
<td>Fecal-oral</td>
<td>Bozkurt et al, 2015; Feng et al, 2011</td>
</tr>
<tr>
<td>Polioviruses</td>
<td>Picornaviridae; Enterovirus</td>
<td>(+) ssRNA; 7.5 kb</td>
<td>Non-enveloped</td>
<td>Icosahedral; ~30 nm</td>
<td>3.8-8.3</td>
<td>Human</td>
<td>CD155</td>
<td>Fecal-oral</td>
<td>Michen &amp; Graule, 2010; Strauss &amp; Strauss, 2002</td>
</tr>
<tr>
<td>Influenza A Viruses</td>
<td>Orthomyxovirus; Influenzavirus A</td>
<td>(-) ssRNA; ~14 kb</td>
<td>Enveloped; Spiked</td>
<td>Spherical; 80-120 nm</td>
<td>4.5-7.0</td>
<td>Human; Avian; Mammal</td>
<td>Sialic acid-containing molecules</td>
<td>Droplet/aerosol</td>
<td>Bouvier &amp; Palese, 2008; Strauss &amp; Strauss, 2002; Michen &amp; Graule, 2010; Garcia-Sastre, 2010</td>
</tr>
<tr>
<td>Rotaviruses</td>
<td>Reoviridae; Enterovirus</td>
<td>dsRNA; 7.5 kb</td>
<td>Non-enveloped</td>
<td>Wheel-shaped; 30 nm</td>
<td>4.4-4.5; 6.6-7.5</td>
<td>Human</td>
<td>PVR (CD55)</td>
<td>Fecal-oral</td>
<td>Bozkurt et al, 2015; NIH, 2010</td>
</tr>
<tr>
<td>Virus Family</td>
<td>Genus</td>
<td>Type</td>
<td>Structure</td>
<td>Size</td>
<td>Host</td>
<td>Receptors</td>
<td>Route of Transmission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
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<td>------</td>
<td>-----------</td>
<td>----------------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Picornaviridae; Hepatovirus</td>
<td>(+) ssRNA; Non-enveloped</td>
<td>Icosahedral; 27-32 nm</td>
<td>2.8</td>
<td>Human</td>
<td>HSPGs</td>
<td>Fecal-oral</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Adenoviridae; Mastadenovirus | dsDNA; Non-enveloped; Spiked | Icosahedral; 70-100 nm | 9.7 | Human | CD46; CAR | Fecal-oral |
| *Adenoviruses* | | | | | | | Bozkurt et al, 2015; Strauss & Strauss, 2002 |
4.2 Disinfection

*Chlorine and chlorine-compounds*

Chlorine and chlorine-compounds have been used for disinfection of municipal water in the United States for over a century (Fair et al., 1948). However, it has little effect on some microorganisms such as protozoan oocysts; and, enteric viruses are generally more resistant than bacteria (Sobsey, 1989). MS2 has been useful for the assessment and evaluation of these disinfectants in laboratory and full-scale pilot studies. Its similar characteristics to many pathogenic viruses make it ideal for use in evaluation of disinfectants on viruses (Table 2). Its usefulness has been proven when being evaluated side-by-side with human norovirus and poliovirus, when assessing the effectiveness of monochloramine (Shin & Sobsey, 1998). A dose of 2 mg/L was evaluated against three viruses, MS2, poliovirus 1 and human norovirus. Results showed that norovirus was more sensitive to monochloramine than MS2 and poliovirus. A 1\log_{10} reduction was observed for norovirus, but there was no detectable reduction seen for MS2 and poliovirus (Shin & Sobsey, 1998). This study determined that MS2 is more resistant to monochloramine than norovirus, but similar to poliovirus. MS2 and poliovirus have shown similar resistance to free chlorine and chlorine dioxide, also (Sobsey, 1989; Tree et al., 2004). Similarly, MS2 was determined to be a good model for inactivation of hepatitis A virus by monochloramine (Sobsey et al., 1988).

Due to the fact that MS2 has shown similar resistance to chlorine-compound inactivation as poliovirus, it is important to compare it with other viruses. MS2 was found to be more resistant to free chlorine than hepatitis A virus, poliovirus 2, Coxsackievirus B5, reovirus and simian rotavirus (Grabow et al., 1985). When these viruses were exposed to free chlorine levels
of 0.1-0.2 mg/L, MS2 was recovered in concentrations of 1.5-2.0 log\text{_{10}} greater than all other viruses. Because of this, MS2 can be a suitable surrogate for disinfection using free chlorine for many enteric viruses (Grabow et al, 1985). Casteel et al also investigated the efficacy of free chlorine at 20 and 200 mg/L concentrations at inactivating MS2 and hepatitis A virus on produce (2008). At all free chlorine levels tested inactivation of MS2 and hepatitis A on produce was similar. Hepatitis A was slightly more difficult to reduce at longer contact times; however, it was judged to not likely be significantly different (Casteel et al, 2008). Other studies have found that hepatitis A may be more resistant to free chlorine than MS2 at pH levels above 7.0 (Sobsey et al, 1988; Grabow et al, 1983).

As mentioned previously, MS2 is commonly used as a surrogate for human norovirus in disinfection studies. There are also two other commonly used surrogates for norovirus: feline calicivirus (EPA-approved) and murine norovirus. These viruses have been compared in disinfection testing in multiple studies. Murine norovirus is suspected to be most similar to human noroviruses, though, because of their genetic and morphologic similarities (Bae & Schwab, 2008). The efficacy of household bleach (0.6% sodium hypochlorite) on surfaces was evaluated using these three viruses (D’Souza & Su 2010). Household bleach reduced MS2 and felicine calicivirus by >6 log\text{_{10}}. In comparison, murine norovirus was more difficult to inactivate (2.5 log\text{_{10}}). Murine norovirus was considered to be the most representative of human noroviruses in this study (D’Souza & Su, 2010). In another study, these same viruses and human norovirus GII.4 were evaluated using sodium hypochlorite as a surface disinfectant, when present in fecal matter (Park & Sobsey, 2011). Infectivity assays for the culturable viruses showed similar reductions between murine norovirus and MS2 ($p=0.8703$), but significantly less reduction for feline calicivirus ($p<0.05$). When RNA concentrations for all four viruses were evaluated, MS2 was reduced the
least \((0.8 \log_{10})\). Human norovirus and feline calicivirus RNA concentrations were inactivated by \(1.4 \log_{10}\). None of the differences between these viruses was statistically significant \((p>0.05)\). The consistency between all three virus inactivation rates provide a good estimate for the infectivity reduction of human norovirus. Results from this study, and similar ones, showed that feline calicivirus was the least resistant norovirus surrogate for disinfectants (Park & Sobsey, 2011; D’Souza & Su, 2010; Havelaar & Nieuwstad, 1985). When compared with results from D’Souza & Su, this study suggested that organic material played a role in preventing the inactivation of MS2 by sodium hypochlorite (D’Souza & Su, 2010; Park & Sobsey, 2011).

Free chlorine inactivation in chlorine demand-free water testing has established MS2 as a suitable surrogate for norovirus, based on CT values (Shin & Sobsey, 2008). It is important to note that chlorination causes an initial reduction \((\sim 1 \log_{10})\) in MS2 infectivity within the first few minutes of contact and then little additional reduction \((< 1 \log_{10})\) in the following 30 minutes. This was observed in laboratory studies using wastewater (Tree et al., 2003) and chlorine demand-free water (Shin & Sobsey, 2008). When the inactivation rates of MS2 were compared to those for human norovirus and poliovirus, they were all similar (Shin & Sobsey, 2008). The effects of chlorine and chlorine-compounds on MS2 have been extensively studied (Table 2). MS2 has been considered to be an effective surrogate in chlorination studies for norovirus, poliovirus and hepatitis A virus.

**Ultraviolet light**

In addition to chlorine and chlorine-compounds, MS2 resistance to UV disinfection has also been observed when compared with multiple viruses and other organisms. MS2 inactivation by UV light has been extensively researched using various wavelengths of UV light, treatment
regimes and organisms (Table 3). This research is useful for full-scale treatment plants who employ UV light treatment as a means to disinfect effluent waters before environmental discharge (Tree et al, 2005). MS2 was found to be more sensitive to UV inactivation when compared to feline calicivirus. At 100 and 130 mWs/cm², the reductions for feline calicivirus were approximately 3 log₁₀. MS2 was not recoverable at either dose. This study also evaluated hepatitis A and poliovirus 1. The reductions for poliovirus 1 were similar to that of MS2; however, hepatitis A was more resistant than MS2. The results from this study were unique to the rest of the studies presented; likely because there was no mono-dispersion of the viruses before treatment (Nuanualsuwan et al, 2002). When MS2 and feline calicivirus were compared in primary and secondary wastewaters, MS2 was resistant to UV treatments (Tree et al, 2005). This again demonstrated that MS2 was more resistant to disinfection in the presence of organic matter.

MS2 was less resistant than Bacillus subtilis spores at certain wavelengths in sterile waters. When exposed to lower wave lengths, MS2 was three times more sensitive than the spores; but, when it was exposed to higher wavelengths (254 nm), MS2 was more resistant (Mamane-Gravetz et al, 2005). Multiple viruses and bacteriophages have been evaluated when exposed to 254 nm doses of UV. Meng and Gerba (1996) found that adenoviruses were more resistant than all other test viruses (poliovirus 1, MS2 and PRD-1) in comparative evaluations of UV inactivation. The dose required to inactivate MS2 was only half of that needed for the adenoviruses; however, it was almost 2-3 times greater than that needed for poliovirus 1 and PRD-1. This suggests that MS2 is not a suitable surrogate for the inactivation of adenoviruses by UV treatments (Meng and Gerba, 1996). When the k-values for a wide range of pathogens was assessed (Figure 1), MS2 was noted to have similar inactivation rates as Clostridium perfringens.
and *B. subtilis* spores. It persisted more readily than five other viruses assessed (Hijnen *et al.*, 2005).

**Figure 1**: *k*-values reported for pathogenic microorganisms from UV exposure.

![Figure 1](image-url)

*Source: Hijnen *et al.*, 2005*

As an assumed norovirus surrogate, MS2 has been used to measure the effects of treatment regimens consisting of ultraviolet (UV) and a chemical disinfectant. When MS2 was treated with both UV and hydrogen peroxide, there was an increase in reduction over UV treatment by itself (Koivunen & Heinonen-Tanksi, 2005; Sherchan *et al.*, 2014). Similar results were observed when high doses of peracetic acid were combined with UV treatment. Reductions were observed to be 1.20-2.58 log$_{10}$ when the treatments were combined. This is compared with only 0.79-1.40 log$_{10}$ reduction when only UV was used (Koivunen & Heinonen-Tanksi, 2005). A
combination of treatments has been extensively evaluated using free chlorine and monochloramine with UV. It is important to note that in this study, it was determined that simultaneous exposure to both UV and chlorine/monochloramine resulted in greater reductions in MS2 concentrations than when the exposures were done sequentially (i.e. chlorine/monochloramine exposure followed by UV) (Shang et al, 2007). These studies did not utilize a side-by-side comparison to pathogenic viruses. MS2 in these studies was assumed to be inactivated similarly to norovirus. This assumption is valid, due to the proven similarities of MS2 to norovirus from both chlorine and UV testing (Park & Sobsey, 2011).

Table 3: UV treatment experiments using MS2 as a surrogate for comparison of survival with other organisms

<table>
<thead>
<tr>
<th>Treatment Dose/Fluence; Test Medium</th>
<th>Comparable Organisms</th>
<th>Conclusions MS2 on Surrogate use</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-125 mW s/cm2; sterile water</td>
<td>hepatitis A, poliovirus, feline calicivirus</td>
<td>Comparable to poliovirus, not to hepatitis A or norovirus</td>
<td>Nuanualsuwan et al, 2002</td>
</tr>
<tr>
<td>10-30 mW s/cm2; primary and secondary wastewater</td>
<td>poliovirus, feline calicivirus</td>
<td>Comparable to poliovirus; more resistant than feline calicivirus; suggested for norovirus</td>
<td>Tree et al, 2005</td>
</tr>
<tr>
<td>0.038-1.644 W/m2; sterile waters</td>
<td>Bacillus subtilis spores</td>
<td>MS2 more sensitive at lower fluences; spores more sensitive at 0.886 W/m2 fluence</td>
<td>Mamane-Gravetz et al, 2005</td>
</tr>
<tr>
<td>9-120 mW s/cm2; liquid suspension</td>
<td>adenoviruses, poliovirus, PRD-1 bacteriophage</td>
<td>Adenoviruses more resistant to UV; MS2 not a good surrogate for adenoviruses; similar to poliovirus</td>
<td>Meng and Gerba, 1996</td>
</tr>
<tr>
<td>0-30 mW s/cm2; buffered water</td>
<td>hepatitis A, Coxsackievirus, rotavirus, PhiX174</td>
<td>MS2 most resistant of test viruses; suitable surrogate for most enteric viruses</td>
<td>Battigelli et al, 1993</td>
</tr>
</tbody>
</table>
Other chemical disinfectants

Biocides, such as glutaraldehyde, have varying effects on MS2, when compared with other norovirus surrogates. One and two percent glutaraldehyde, one, two and five percent trisodium phosphate and 70% ethanol were evaluated on the efficacy of reduction for MS2, feline calicivirus and murine norovirus (D’Souza & Su, 2010). Most of the treatments affected all three viruses to a similar degree. Ethanol was highly ineffective in reducing all three viruses. Conversely, trisodium phosphate (5%) was the most effective treatment for all three viruses, reducing them by >6 log₁₀. MS2 was observed to be almost twice as resistant to glutaraldehyde than both other test viruses (D’Souza & Su, 2010). Greater resistance to biocides has been observed by MS2 when compared to K coliphage (Maillard et al, 2004).

Due to similar inactivation times observed between poliovirus and MS2, they have been used to evaluate the efficacy of hand scrubs on skin. The scrubs tested included 70% ethanol, 7.5% povidone-iodine surgical scrub and bar soap (Davies et al, 1993). Of the three treatments, 7.5% povidone-iodine scrub observed the greatest average log-reduction of MS2 (2.80 log₁₀) followed by bar soap (2.29 log₁₀) and then 70% ethanol (1.09 log₁₀). Poliovirus was more resistant to inactivation by ethanol (0.42 log₁₀ reduction), but similar to the reductions from soap and water (2.10 log₁₀). This study aided in establishing MS2 as a viable surrogate for enteric viruses in hand hygiene protocols (Davies et al, 1993; Sickbert-Bennett et al, 2005).

The effect of iodine on MS2 was used to predict how it would impact survival of waterborne enteric viruses. Iodine has been historically used for municipal water treatment. In comparison with other bacteriophages (ΦX174, PRD-1 and GA), MS2 was more susceptible to iodine treatment. It was inactivated by >99.999% within ten minutes at a concentration of 1 mg/L of iodine, ΦX-174 also had a similar inactivation (>99.99%). PRD-1 and GA were the only
phages recoverable after 60 minutes (Brion et al, 2004). MS2’s sensitivity to iodine was confirmed in two types of water: simulated wastewater from showers (SSW) and phosphate buffered demand free water (PBDFW). This study did not test a comparative virus, however, but rather assumed the likeness to enteroviruses, based off of previous disinfection studies (Brion & Silverstein, 1999). Within 18 seconds MS2 was reduced by >99% in both test waters (Brion & Silverstein, 1999). This study also observed an inc MS2 by rease1 to 2 log\textsubscript{10} after iodine residuals were gone. Regrowth was only observed when organic matter was present (beef extract). This increase in titer was believed to be due to reversible conformational changes in the capsid of the virus, affecting proteins involved in the attachment of the host bacterium. Therefore, it may not be a suitable surrogate for iodine testing (Brion & Silverstein, 1999).

MS2 has also been evaluated as a surrogate for testing ozone disinfection. Ozone is commonly used in European countries for drinking water disinfection (Battigelli et al, 1991). When tested with poliovirus 3, MS2 reductions were significantly greater than poliovirus reductions. An average difference of 1.6 log\textsubscript{10} was observed (Finch & Fairbairn, 1991). However, when MS2 was treated in water with norovirus and poliovirus 1, results for all viruses were observed to be similar within the first 10 seconds (3-3.5 log\textsubscript{10} reduction) (Shin & Sobsey, 1998). Infectivity results for poliovirus and MS2 also suggested that these viruses show likened sensitivity to ozone. This study highlights the difficulties of validating a norovirus surrogate without an infectivity assay (Shin & Sobsey, 1998). MS2 has been recommended as a surrogate for hepatitis A virus for ozone disinfection (Hall & Sobsey, 1993). The influence of other factors, such as temperature, pH, dissolved organic carbon (DOC), total organic carbon (TOC) and biological oxygen demand (BOD), on ozone disinfection of MS2 was investigated. Increasing concentrations of these was correlated to a higher ozone demand, resulting in less inactivation of viruses (Helmer
& Finch, 1993; Janex et al, 2000). These factors should be taken into account when selecting a surrogate for ozone disinfection because they can influence the effectiveness of ozone treatment (Helmer & Finch, 1993; Janex et al, 2000).

Filtration

Filtration is another commonly used practice for water disinfection, especially for viruses. Micro- and ultra-filtration with coagulants were found to significantly enhance the removal MS2, when compared with filtration methods without coagulants (Fiksdal & Leiknes, 2006). However, this study did not compare the efficacy of this method on MS2 with other viruses. When norovirus-like particles (NLP) and MS2 were filtered using a coagulation rapid sand filtration method, the removal of MS2 was consistently greater than NLP removal. In this filtration process, MS2 was not considered to be a good surrogate for norovirus (Shiraski et al, 2010). This conclusion was validated when recombinant norovirus particles were compared with MS2 in quartz sand filtration (Redman et al, 1997). In a high-rate filtration experiment, MS2 was also determined to not be a good surrogate for poliovirus during filtration (Nasser et al, 1995). This study did, however, validate MS2 as a surrogate for hepatitis A virus during filtration. The removal of MS2 was consistently less than hepatitis A. Both viruses were also affected by the presence of humic acid and turbidity (Nasser et al, 1995).

Summarization

MS2 has been used as a surrogate for multiple viruses and in multiple disinfection studies. In many of these experiments, the suitability of MS2 as a surrogate has been determined for specific viruses (Table 4).
Table 4: Recommendations for use of MS2 as a surrogate for enteric viruses, based on disinfectant type.

<table>
<thead>
<tr>
<th>Disinfection Type</th>
<th>Norovirus</th>
<th>Poliovirus/Enterovirus</th>
<th>Hepatitis A</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine</td>
<td>Recommended</td>
<td>Recommended</td>
<td>Recommended at low/neutral pH</td>
<td>Shin &amp; Sobsey, 1998; 2008; Sobsey, 1989; Grabow et al, 1985; 1983; Sobsey et al, 1988</td>
</tr>
<tr>
<td>UV*</td>
<td>Recommended, when high levels of organic matter present</td>
<td>Recommended</td>
<td>Not recommended</td>
<td>Nuanualsuwan et al, 2002; Tree et al, 2005; Hijnen et al, 2005;</td>
</tr>
<tr>
<td>Iodine</td>
<td>More research needed; likely not recommended</td>
<td>More research needed</td>
<td>More research needed</td>
<td>Brion et al, 2004; Brion &amp; Silverstein, 1999</td>
</tr>
<tr>
<td>Biocides</td>
<td>Recommended</td>
<td>Recommended</td>
<td>More research needed</td>
<td>D'Souza &amp; Su, 2010; Davies et al, 1993</td>
</tr>
<tr>
<td>Ozone</td>
<td>Recommended</td>
<td>Recommended</td>
<td>Recommended</td>
<td></td>
</tr>
</tbody>
</table>

*Denotes MS2 is not a suitable surrogate for adenoviruses (not included in table)
4.3 Transport and Survival

Water

Due to its ability to be propagated in high (>10^8) numbers, MS2 has been used extensively as a tracer in the environment and in different types of waters including groundwater, surface water and municipal water. However, because of MS2’s low isoelectric point, it is considered to be a very conservative estimate for movement of most enteric viruses, such as enteroviruses and noroviruses, out of groundwater reservoirs. This also allows it to be a good surrogate for these viruses because it leeches faster and more readily due to its low isoelectric point, making it difficult to adsorb to saturated soils (Attinti et al, 2009; Jin et al, 2000). In The Netherlands, MS2 was inoculated into anoxic water and injected 12 m underground into an injection well. The water was monitored for a period of 163 days for the movement of virus out of the water column and through the saturated soil surrounding it (Van der Wielen et al, 2008). The reduction of MS2 was measured by inactivation, dispersion, dilution and adsorption after the observation period. Results from this study were applied to a risk assessment to determine the appropriate microbial protection zone to protect ground waters from contaminant leeching through saturated soils (Van der Wielen et al, 2008). A similar study used MS2 to gather information on the movement of viruses in aquifers. The virus was inoculated into the water and was sampled for 47 hours at varying intervals. In addition to water samples, the saturated soil around the wellbore was sampled. From this study, a transport coefficient created for the transport of viruses through water and soil columns consisting of mainly sand and gravel (Woessner et al, 2001).

MS2 has been used to assess the survival of viruses in water (Hodgson et al, 2004; Allwood et al, 2003; Bae & Schwab, 2008). Its survival in natural waters was found to be similar to feline calicivirus (Allwood et al, 2003), poliovirus (Callahan et al, 1995), Coxsackievirus (Gordon &
Toze, 2003), but less than adenoviruses (Orgorzaly et al, 2010) and murine norovirus (Bae & Schwab, 2008). There are numerous factors that affect the survival of enteric viruses in water, such as pH, microbial flora and temperature (John & Rose, 2005). Temperature has a significant impact on survival of viruses in different types of water. Bae and Schwab determined that MS2 reduction was significantly different ($p<0.05$) in surface waters incubated at 4 °C than ground waters incubated at 4 °C (Bae & Schwab, 2008). On average, the surface water samples used in this study had higher pH, turbidity, and conductivity than the ground water samples used. This study also suggested murine norovirus as a more suitable norovirus surrogate than MS2 for survival in ground and surface water (Bae & Schwab, 2008). Turbidity and presence of organic matter in water has been linked to greater inactivation at temperatures above 20 °C for viruses. In addition, temperature had a greater affect on inactivation of MS2 than poliovirus (John & Rose, 2005). However, other factors, such as pH, presence of natural microflora, hardness and turbidity effected the two viruses similarly (John & Rose, 2005).

Decay rates in natural water caused by sunlight were found to be very similar between human adenoviruses and coliphages. MS2 was deemed a conservative estimate for poliovirus 3 and adenovirus 2 for decay from natural sunlight in various water sources. It is noted that F-specific coliphages, such as MS2, are found in lower concentrations in wastewater than somatic coliphages. However, when treatment efficacies were compared, F-specific and somatic coliphages were removed at similar rates by sunlight in wastewaters (USEPA, 2015).

In a unique study, the transport and survival of MS2 was studied in recreational streams in Southwest Wales, UK (Wyer et al, 2010). Approximately $10^{17}$ PFU was released into the midpoint of a stream during a rainfall event. The water was monitored and sampled regularly for 44 h following the event. The first detection of MS2 ranged from 0.62 h to 3.13 h after inoculation.
Maximum concentrations recovered ranged from 1.0E+01 to 9.0E+04 PFU per mL (Wyer et al., 2010). In addition to transport in streams, the transport of viruses using MS2 has been simulated in a municipal drinking water system. In this study, MS2, a salt tracer and a computational fluid dynamics model were utilized for dispersion comparisons. Due to its strong correlation with the salt tracer and computational fluid dynamics model coefficients, MS2 was determined to be a sufficient biological tracer for viruses in water distribution systems. However, because this study was performed in a municipal water setting, it was not possible to compare the dispersion of MS2 to other enteric viruses due to safety concerns (Sinclair et al., 2009).

Table 5: Recommendations for use of MS2 as a surrogate for enteric viruses, based on water type.

| Recommendations for MS2 as a Surrogate for Survival and Transport in Water |
|-----------------------------|-----------------|-----------------|-----------------|-----------------|
| Water Type                  | Norovirus       | Poliovirus/Enterovirus | Adenoviruses    | References      |
| Ground                      | Recommended     | Recommended      | Not recommended | Bae & Schwab, 2008; |
|                             |                 |                  |                 | Orgorzaly et al, 2010; |
|                             |                 |                  |                 | Attini et al, 2009 |
| Surface                     | More research needed | Recommended      | Not recommended | John & Rose, 2005; |
|                             |                 |                  |                 | Callahan et al, 1995 |
| Waste                       | Recommended     | Recommended      | Recommended     | USEPA, 2015      |
| Drinking/Tap                | More research needed | More research needed | More research needed | Sinclair et al, 2009; |
|                             |                 |                  |                 | Allwood et al, 2003 |
Norovirus is the most common cause of foodborne gastroenteritis in the United States. It is attributed to at least 58% of outbreaks each year (Hall et al, 2012). Due to MS2’s likeness to many enteric viruses, including norovirus, it has also been employed in numerous food studies, including survival on food products, during processing and storage, and during washing. Norovirus outbreaks have been associated with multiple food items. The two commodities with the highest norovirus incidence are leafy greens and mollusks (Hall et al, 2012). Norovirus was also attributed to food handlers in 53% of outbreaks between 1998 and 2008 (Hall et al, 2012). MS2 survival on meat was tested using pork chops to compare survival at two different storage temperature (Brandsma et al, 2012). First chops were sealed and incubated at 2 °C. Then, a subset of pork chops was removed from incubation on a weekly basis and stored at 4 °C. This simulated use of pork chops in the home and in retail storage facilities. At the end of the study (7 weeks), the results from plaque assays and PCR were compared to assess the overall survival of MS2 on the pork chops. The maximum reduction of virus on the pork chop when they were vacuum packed was 1.1 log_{10} PFU/cm². This slightly increased when the chops were exposed to oxygen and kept at 4 °C (1.2 log_{10} PFU/cm²). This study showed that enteric viruses on meat will likely persist for many weeks during storage (Brandsma et al, 2012).

MS2 survival on fresh fruit and vegetable surfaces has been studied extensively (Allwood et al, 2004; Dawson et al, 2005; Lee et al, 2015). Likewise, mechanisms to remove MS2 from fresh fruits and vegetables have been studied. Common mechanisms include chlorine wash water (Dawson et al, 2005; Casteel et al, 2009), temperature control (Allwood et al, 2004), and sunlight (Carratala et al, 2013). MS2 transfer from food contact surfaces to cleaning cloths and from
cleaning cloths to food contact surfaces was also assessed (Gibson et al, 2012). These cloths consisted of two cellulose and cotton blended cloths; one terry cloth (100% cotton); one microfiber; and one unwoven cloth (viscose/polyester blend). The microfiber cloth was able to remove the most virus on both surfaces (~4 log₁₀). The other cloths performed similarly for both surfaces (~2.5-3 log₁₀ reduction). The transfer from cloths to surfaces was done using a virus cocktail containing MS2, PRD1 and feline calicivirus. On stainless steel surfaces, the terry cloth transferred the most virus (~3 log₁₀) and on the acrylic-based surface, the nonwoven cloth transferred approximately 2.5 log₁₀ virus. This study is important because the spread of viruses in food preparation facilities has been documented (Gibson et al, 2012).

As a surrogate for enteric viruses in food products, MS2 has also been utilized in liquid products such as juices and milk (Horm & D’Souza, 2011). To evaluate the survival, MS2, feline calicivirus and murine norovirus were inoculated into orange juice, skim milk, and pomegranate juice. Juice mixtures were also evaluated (1:1 v/v orange and pomegranate juice; 1:1 v/v orange juice and pomegranate polyphenol). When MS2 was incubated in orange juice, no reduction was seen until day 21 (0.12 log₁₀). Reduction in milk was observed starting 2 days of incubation and after 21 days of incubation an overall reduction of 0.04 log₁₀ was observed. The same reductions for murine norovirus were observed in orange juice and milk. Pomegranate juice provided the greatest overall log-reduction after 21 days for MS2 (0.15 log₁₀). However, when pomegranate juice was mixed with phosphate buffered saline (PBS), an ever greater reduction as observed (1.84 log₁₀). Murine norovirus was reduced to a greater extent in these juice types. The reductions seen were 2.02 and 1.08 log₁₀ for pomegranate juice and PBS, respectively (Horm & D’Souza, 2011). This is likely due to the face that pomegranates have been observed to naturally inhibit many
pathogens (Al-Zoreky, 2009). This study suggests that in juice and beverages, MS2 is a better surrogate for norovirus than murine norovirus, due to its longer survival.

Mollusks are a common vehicle of norovirus (Su et al, 2009; Davis et al, 2012; Hall et al, 2004). They also naturally produce chitosan, which has inherent antimicrobial properties (Davis et al, 2012). Murine norovirus and feline calicivirus were found to be more resistant to chitosan, than MS2 (Davis et al, 2012; Su et al, 2009). The antimicrobial effects against surrogates also varied by type of chitosan (i.e. water-soluble vs. oligosaccharide). This study suggested that murine norovirus may be the most conservative of the three for evaluating chitosan effects on norovirus. The efficacy of chitosan and MS2 as a representative for other enteric viruses should be further evaluated (Su et al, 2009).

Fomites and Aerosols

MS2 has been utilized as a surrogate for many survival studies on surfaces. One fomite that has been of particular interest for MS2 survival is respirator filters (Fisher & Shaffer, 2010; Rengasamy et al, 2010; Chan et al, 2010). This is, in part, due to the 2009 influenza pandemic. During this time, it was suggested that filtering facepiece respirators could be reused, to off-set high inventory demands (Fisher & Shaffer, 2010). One study evaluated the survival of MS2 on the respirator coupons for up to 10 days. MS2 was applied to the coupons using a nebulizer to simulate aerosol setting; and using a pipette to simulate large droplet settling. MS2 was recoverable on all coupons on the tenth and final sample day. At least 10% of the starting concentration was recoverable after incubation for 4 days for both deposition methods (Fisher and Shaffer, 2010). Another study evaluated the survival of MS2 on the filter of an N95 respirator using antimicrobial technologies (Rengasamy et al, 2010). In this study, MS2 was aerosolized using a nebulizer and
droplets were loaded onto respirator coupons (face velocity: 13.2 cm./s). The survival of MS2 was not significantly different than the control respirator coupons with no antimicrobial treatment at the lower test temperature; however, the survival was significantly reduced on the antimicrobial respirator at the higher test temperature and relative humidity (Rengasamy et al., 2010). Due to the fact that influenza strains are spread through aerosol and droplets (Rengasamy et al., 2010), MS2 was useful because of its ability to be aerosolized and similar size and structure as some respiratory viruses. Its survival on respirators can be considered a conservative estimate for influenza because of the morphological differences between the two viruses (i.e., presence or lack of an envelope) (Bozkurt et al., 2015)

**Fomite Transfer**

Fomites have been identified as a major mode of transmission for many viruses (Julian et al., 2010). Because of this, MS2 has been utilized as a surrogate for a wide range of pathogenic viruses to identify the transfer between fomites and hands (Lopez et al., 2013) and movement between fomites (Casanova et al., 2009; Julian et al., 2010). As a surrogate for fomite transfer, MS2 has been used to model spread of Severe Acute Respiratory Syndrome (SARS) from personal protective equipment (PPE) to hands and clothes. The study assumed MS2 to be a conservative estimate for the survival and movement of SARS (Casanova et al., 2008). In this study, volunteers were given PPE to wear. The PPE was then inoculated with $10^4$ PFU of MS2 and volunteers were instructed to remove the items, following PPE removal protocols. After removal, participants’ hands, face, and clothing were sampled. Virus was detected on 100% of sampled for shirts and 75% for pants that were worn under PPE gowns. MS2 was also detected on the skin on 90% of right hands and 70% of left hands (Casanova et al., 2008). Similarly,
researchers investigated the impact of “double gloving” on the spread of MS2 from gloves to skin and clothing (Casanova et al, 2012). In this study, two trials were performed: one with single-gloved participants and one with double-gloved participants. The participants were instructed to remove their PPE according to Centers for Disease Control and Prevention (CDC) protocols. Double-gloved participants were instructed to remove the outer gloves and dispose of them before removing any other piece of PPE. After removal, the participants’ hands and faces were sampled. When outer gloves were removed, 94% of participants transferred virus onto their inner gloves and 23% transferred virus onto their ungloved hands. However, when only a single glove was worn, 78% of participants transferred virus to their ungloved hands. The use of double-gloving was shown to significantly decrease the amount of MS2 positive hands sampled ($p=0.007$). Other PPE that was sampled (shirt and pants) did not show a statistically significant difference between double and single-gloving. This study successfully utilized MS2 to demonstrate that environmental controls should be put in place to protect healthcare worker health and safety, in addition to PPE (Casanova et al, 2012).

The transfer of MS2 to fingers and fingerpads has also been investigated to understand the role of fomites as a reservoir for human enteric and respiratory pathogens (Lopez et al, 2013; Julian et al, 2010). Lopez et al compared the transfer of MS2 and poliovirus 1 between porous and nonporous surfaces to hands. The porous surfaces transferred much less virus to hands than the nonporous surfaces. The greatest average transfer of MS2 to fingerpads was from acrylic and glass (21.7% and 19.3%). Cotton and polyester transferred the least virus onto hands (0.03% and 0.3%). The transfer of poliovirus was only evaluated from non-porous surfaces. MS2 transfer from non-porous surfaces was ~12% greater than poliovirus transfer. This makes MS2 a suitable surrogate for modeling the transfer of enteroviruses to hands from fomites. The transfer observed for
poliovirus was likened to transfer for hepatitis virus and rotavirus. This suggests MS2 would also
be a suitable surrogate to predict transfer for these organisms (Lopez et al, 2013).

The spread of MS2 in the air after simulated vomiting was tested to determine the spread of
norovirus from aerosolization during vomiting (Tung-Thompson et al, 2015). The surfaces
sampled after each simulated vomiting event determined the amount of virus that settled onto the
surfaces in the chamber (excluding the floor) to be insignificant, compared to the floor ($p>0.05$).
The vomiting incidents were simulated at varying pressures and vomitus viscosity. When vomiting
events with high pressure were simulated, the greatest amount of airborne virus was recovered (7
$\log_{10}$). This study aimed to determine the transmission dynamics of norovirus when it is
aerosolized during vomiting events (Tung-Thompson et al, 2015).

**Tracer Studies in the Built Environment**

The built environment provides an enclosed space for pathogens to spread through direct
contact and fomite transfer. The National Human Activity Patterns Survey estimated that
Americans spend approximately 87% of their times indoors (Klepeis et al, 2001). Because of this,
understanding how viruses are spread throughout the built environment has become important in
preventing illness. To date, MS2 has been published as a tracer in an office (Reynolds et al, 2015),
a long-term care facility (Sassi et al, 2015), a hotel (Sifuentes et al, 2014) and in the home (Tamimi
et al, 2014).

Sifuentes et al (2014) used MS2 as a tracer in a hotel to predict the movement of enteric
viruses, such as norovirus, in the event of an outbreak. In this study, the virus was inoculated onto
a door handle and a faucet handle. The virus was allowed to move throughout the hotel for the
duration of the conference (~8 h). This was then repeated two more times with 1 week intervals
between each trial. At the end of the trials, MS2 was found on 23% of sampled fomites including surfaces in communal rooms, hotel rooms and restrooms. The virus found on surfaces after the implementation of an intervention was significantly reduced ($p<0.05$) than before the intervention. 

ΦX-176 was also evaluated as a tracer throughout the hotel. Its incidence on fomites was similar to MS2 (Sifuentes et al, 2014). In a similar study conducted in a long-term care facility, Sassi et al demonstrated the movement of MS2 throughout the facility to simulate the movement during a norovirus outbreak. In this study, one nursing staff’s hands were inoculated with MS2 and allowed to spread through the facility for four hours. At the end of four hours, fomites throughout the facility and staff members’ hands were sampled. After four hours, 49% of fomites were contaminated with MS2. The reduction of virus on fomites was significantly reduced after the implementation of a hygiene intervention. This study also evaluated the movement of ΦX-176, however, its survival was not consistent enough to evaluate throughout the 8 hour time period (Sassi, unpublished) (Sassi et al, 2015).

The movement of MS2 throughout an office building has also been characterized (Reynolds et al, 2015). In one of the trials a push plate on the entrance to the office was inoculated with MS2 and tracked throughout the office. In another trial, the hands of an office worker were seeded with the virus. For both trials, the inoculation site was seeded again 2 hours later and surfaces and hands around the office were sampled 4 hours after initial inoculations. MS2 found on hands was the same for both inoculum sites (36%). MS2 was detected on a higher percentage of personal and communal fomites when an office workers’ hands were inoculated (56% and 56%) than when the push plate was inoculated (39% and 46%). Reduction on all fomites was significant ($p<0.05$) after the implementation of a hygiene intervention (Reynolds et al, 2015). The movement of MS2 was also used to characterize how pathogens are transferred in the home (Tamimi et al,
In this study, the hands of one adult in a household of at least 4 people (2 adults, 2 or more children) were inoculated with MS2. After eight hours, almost all fomites in the household were positive for MS2 (97.98% and 97.12%); and hands of all household members were also found to be positive (Tamimi et al, 2014).

These studies utilized MS2 as a biological tracer with two main objectives: to characterize the spread of an infectious organism in the environment and to evaluate the impact of a hygiene intervention on the spread and concentrations of the organism. MS2 is an ideal tracer in the built environment because, in addition to movement, its survival in ambient indoor temperature and relative humidity can be assessed. The effect of hygiene products on infectious viruses can also be estimated through the observed inactivation of MS2. MS2 is safe for use with immunocompromised individuals (i.e., in the long-term care facility) because of its inability to infect humans. It is unlikely that enteric viruses will be evaluated in the same way, because of the sensitive nature when working with human subjects. However, based on the transfer data from Lopez et al for poliovirus, we can assume that the MS2 transfer from hand to fomite and fomite to hand in these environmental studies would be similar to what would be observed for enteric viruses.

4.4 Future Uses of MS2

Not only has MS2 been used a surrogate to many viruses, it has been suggested for use as a viral fecal indicator in water environments. In 2012, the EPA began evaluating coliphages (including MS2) as fecal indicators for ambient waters (USEPA, 2015). To do this, they compiled a literature review evaluating current research that either supports or denies coliphage as an indicator for water quality. The result of the literature review recommended that in certain
aquatic environments, coliphages and enteric viruses act and react to conditions very similarly. One aspect of water quality that coliphages were found to be more persistent at varying values than enteric viruses was temperature. Coliphages are very persistent at high temperatures (USEPA, 2015).

The overall conclusion made by the USEPA is that coliphages could be a sufficient indicator of microbial water quality. They have been deemed to be equally as effective as *Escherichia coli* and *Enterococci*; however, there are no new criteria for the use of coliphages as fecal indicators in water. There are some factors that have been discovered that make coliphages less-than-ideal for predicting human fecal contamination. One of those is the discovery that many human male-specific coliphages are also present in mammalian waste (Lin & Ganesh, 2013). The four serogroups of male-specific coliphages typically allow the origin of the waste to be determined, however, the cultural method cannot do this. It must be done by serotyping, genotyping or through PCR. The use of coliphages has been helpful in wastewater though, finding that they typically outnumber pathogenic enteric viruses by 100-fold (Lin & Ganesh, 2013).

The Food and Drug Administration has adopted the use of male-specific coliphage in the National Shellfish Sanitation Program. Coliphage is used for testing criteria in shellfish meat after a closure to determine the microbial water quality. There can be no more than 50 coliphage in 100 g of shellfish after a closure. This is done because viruses can bioaccumulate in filter-feeders, such as shellfish (FDA, 2013). It is likely that more uses for coliphage and MS2 as indicators and surrogates will be proposed because more federal efforts have been put into using male-specific coliphage in federal criteria, in addition to academic and industry research.

From the literature, we can discern that little evident research has been conducted on the suitability of MS2 as a surrogate for emerging infectious diseases. This could be partially due to
the global efforts to research treatments and vaccines, as opposed to environmental aspects of these pathogens (Morens et al, 2004). In addition, many new emerging infectious diseases do not have similar non-pathogenic organanisms in the same family, such as paramyxoviruses and filoviruses (Morens et al, 2004). Some emerging or re-emerging pathogens cannot be cultured, such as norovirus, or are difficult to work with, such as SARS coronavirus (Vega et al, 2004). MS2’s ability to be used in multiple environments and in multiple capacities (i.e., fomite transfer, direct contact, aerosolization) allows it to be used in place of a wide range of organisms.
PRESENT STUDY

The overarching goal of this study was to utilize MS2 as a surrogate for viruses that are either very high risk (Ebola virus) or non-culturable (norovirus); and to predict these viruses’ behavior in three different and unique environments. Also, to utilize MS2 in an environment and methodology that has not been previously published in literature. This chapter provides an overview of the study design and methodologies for each experiment. It also presents key findings related to each specific aim of the dissertation. Detailed analyses of methods, findings and conclusions for each aim are provided in the manuscript and in the appendices. The overarching goal was achieved by identifying data gaps for respective viruses in the subject environments. For Specific Aim 1, the environment was irrigation canals used for watering fresh produce crops in Yuma, Arizona. The environment for Specific Aim 2 was a restroom in a hospital or healthcare setting. The wastewater treatment process was the simulated environment in Specific Aim 3. Using this approach, a surrogate virus, was then utilized to predict the survival, fate, or transport of pathogenic viruses in their respective environment. Three specific aims were developed to identify future research needs and develop recommendations on how to appropriately manage and reduce potential risks posed by viruses in the environments tested. The first aim was to assess the potential impact of sediment-borne enteric viruses and bacteria on irrigation water quality (Appendix A). The second aim was to evaluate the efficacy of disinfectants on reducing the spread of virus onto restrooms surfaces during flushing. Also, to evaluate the efficacy of the same disinfectants on reducing the concentration of virus in the toilet bowl before flushing (Appendix C). The final aim of this dissertation was to determine the impact of two types of anaerobic digestion on the survival of five surrogate viruses (Appendix C).
Methods

The specific aims of this dissertation were achieved through three sets of methodologies. This section will provide a brief summary of the methods utilized to complete each specific aim.

Specific Aim 1: Assessing the impact of sediment-borne pathogens on overlying irrigation water quality

MS2 and *Escherichia coli* 25922 were used as surrogates for common foodborne pathogens found in produce-related outbreaks. In order to simulate an irrigation canal, field measurements (water depth, flow rate, canal width, TSS and sediment sizes) were collected about characteristics from irrigation canals in Yuma, Arizona. They were used to create a laboratory scale representation of irrigation canals in this area. A closed system flume lined with either clay and sand sediments was adjusted to three flow rates (0.41, 0.73 and 1.46 L/s) to represent varying flow rates in the field. The top layer of sediment was inoculated with one of the organisms. Water and sediment samples were taken after the flow rates ran for 30 min. The samples were assayed for either MS2 or *E. coli* 25922. Results were calculated per mL of water and also as the re-suspended proportion of the total inoculum. Each flow rate experiment was completed in duplicate. The significance of the reductions observed in the toilet bowl was evaluated using a multivariate test of means; and the significance of the reductions on restroom surfaces after flushing was evaluated using a two-sample paired t-test.
Specific Aim 2: Evaluating the efficacy of four disinfectants on the viral concentration in the toilet bowl and on restroom surfaces after flushing

In order to investigate this specific aim, high titers of MS2 were achieved ($10^{10-12}$ plaque forming unit/mL). The virus was then added to 1 liter volumes of tryptic soy broth (TSB). TSB was used to represent human waste. This mixture was subsequently added to a commercial toilet bowl. To assess the efficacy of the disinfectant on viral concentrations in the toilet bowl, water samples were taken directly from the bowl at 1, 15 and 30 min after addition of disinfectants. In order evaluate the viral reduction on the surfaces around the toilet, 15 and 30 min contact times were tested with each disinfectant. After the contact time, the toilet was flushed and surface samples were taken. Water samples were also taken after flushing 1, 2 and 3 times. Every experiment was done in duplicate. The results from the experiments were used to determine the significance of the reductions seen when compared to baseline concentrations without treatment. A multivariate test of means was used to evaluate the significance of the viral reductions observed in the toilet bowl after each treatment type and a two-sample, paired t-test was used to evaluate the viral reduction after flushing on restroom surfaces.

Specific Aim 3: Determine the influence of mesophilic and thermophilic digestion on the inactivation of viruses

This aim was investigated through the recreation of anaerobic digesters. Miniature anaerobic digesters (Miles et al, 2011) were created in test tubes with a mixture of 3 mL waste activated sludge and 7 mL primary sludge. The sealed tubes were then flushed with N$_2$ gas and 1 mL of virus was added to triplicate tubes. Five viruses were tested in this specific aim: MS2, Φ6, murine norovirus, poliovirus 1 and adenovirus 4. To assess the influence of mesophilic and
thermophilic digestion on viruses, tubes were incubated at 32 ± 3 °C for 21 days ± 8 hrs or 55 ± 3 °C for 5 days ± 8 hrs. Tubes were also created for a Time 0. This number was used as a baseline to determine the virus reduction after each digestion process. Time 0 tubes were inoculated with virus in triplicate tubes and held for 30 min before extraction. After incubation, viruses were extracted using beef extract and filter sterilized to remove bacteria. The results of the virus assays were used to determine the reduction from both digestion types. The significance of the reductions for each digestion type was evaluated using a Wilcoxon signed-rank test, using Time 0 as a baseline for comparison. Individual viral reduction significance was also assessed using a two-sample t-test.

Results

Specific Aim 1: Assessing the impact of sediment-borne pathogens on overlying irrigation water quality

This specific aim focused on determining the impact of sediment-borne pathogens on overlying water quality and using MS2 and *E. coli* to estimate the re-suspension of enteric pathogens. Results suggest that only a small fraction (0.00-12.00%) of total organisms in sediment are being re-suspended into the overlying water. The re-suspension between both test organisms was not significantly different (*p*>0.05). When re-suspension of *E. coli* in sand sediments was compared with re-suspension of *E. coli* in clay sediments, the difference was not statistically significant (*p*>0.05). However, MS2 re-suspension at the greatest flow rate (1.46 L/s) was statistically significantly greater than MS2 re-suspension at the smallest flow rate (0.41 L/s) (*p*<0.05).
Specific Aim 2: Evaluating the efficacy of four disinfectants on the viral concentration in the toilet bowl and on restroom surfaces after flushing

MS2 was used as a surrogate for Ebola virus to estimate the potential impact of disinfectants on Ebola-contaminated human waste. The results of this study showed that when waste is treated with any of the four test disinfectants for 15 minutes, the concentration of virus deposited on surfaces is significantly reduced \( (p<0.05) \). However, when the 30-minute contact time was evaluated in the two-sample t-test, the hydrogen peroxide was not able to significantly reduce concentrations \( (p= 0.0560) \). Despite the significant reduction in concentrations observed, three surfaces remained contaminated in 100% of trials \( (N=18) \). These surfaces were the toilet bowl rim, the toilet seat top and the underside of the toilet seat. They were also the most heavily contaminated surfaces, on average.

The evaluation of the four disinfectants on reducing the viral load (starting concentration \( \sim 10^7/\text{mL} \)) in the toilet bowl showed that peracetic acid and quaternary ammonium were the fastest acting. Peracetic acid and quaternary ammonium reduced the concentration in the toilet bowl by 2.26 log\(_{10}\) and 1.99 log\(_{10}\) per mL, respectively, in one minute. Hydrogen peroxide was observed to have the least effect on the virus concentration in the toilet bowl at all contact times, reducing the concentration by only 0.06 log\(_{10}\) per mL after 30 minutes. When the treatments were compared to each other using a multivariate test of means, peracetic acid and quaternary ammonium reduced the viral concentration in the toilet bowl significantly more than hydrogen peroxide \( (p<0.05) \). None of these treatments inactivated the concentration to \(<10^3/\text{mL} \). This concentration was demonstrated to produce no recoverable virus on sampled surfaces. The order of efficacy for the test disinfectants on MS2 is as follows: peracetic acid>quaternary ammonium>chlorine bleach>hydrogen peroxide.
Specific Aim 3: Determine the influence of mesophilic and thermophilic digestion on the inactivation of viruses

Determining the impact of anaerobic digestion on five different viruses aided in fulfilling the goals of this dissertation by providing quantified reductions for each virus from mesophilic and thermophilic digestion. The results from this study showed that the bacteriophages tested, MS2 and \( \Phi 6 \), were reduced the greatest from mesophilic digestion (>5.9 \( \log_{10} \) and 6.6 \( \log_{10} \) per mL). The mammalian viruses, poliovirus 1, adenovirus 4 and murine norovirus, were reduced much less (1.8, 2.0 and 2.2 \( \log_{10} \) per mL). At the thermophilic temperature profile, only poliovirus 1 and MS2 were recoverable, likely due to the high initial inoculum (\( 10^{7-8} \)) used for these viruses. The reductions from thermophilic digestion for poliovirus 1 and MS2 were 4.6 and 7.1 \( \log_{10} \) per mL, respectively. \( \Phi 6 \), adenovirus 4 and murine norovirus were reduced by >5.9, >2.8 and >4.1 \( \log_{10} \) per mL, respectively. Both digestion processes were able to significantly reduce the concentrations of virus, however, the reductions observed by thermophilic digestion were significantly greater than mesophilic digestion (\( p=0.0011 \)).

Discussion

The three studies in this dissertation successfully predicted the fate, transport and survival of pathogenic organisms through the use of surrogates. In the first study, the re-suspension of \( E. \) \( coli \) O157:H7 and norovirus from sediments into overlying irrigation water was quantified. These results aided in determining that sediment-borne pathogens are not likely to significantly impact the microbial quality of irrigation water. This determination was made by evaluating the
measurements from irrigation canals in Yuma, Arizona. Sediments in these canals were found to be very large, on average, and gravely. More force is needed to re-suspend pathogens associated with these particles, due to the weight of the particles and the flow and depth of the canals (Wiberg & Smith, 1987). Based on the flow rates and depths of canals measured in the field, the force needed to re-suspend the sediments in the base of the irrigation canals is not likely to be achieved, except in unique disturbances to the canal bed sediments, such as canal dredging, heavy rainfall, or any anthropogenic and natural activities that would re-suspend the sediments in a non-uniform way. This study also concluded that bacteria are no more likely than viruses to be re-suspended into overlying water, when in clay sediment. This was true at all flow rates tested. The three flow rates tested in the lab were used to represent the variation of flow rates measured in the field.

The second study in this dissertation hypothesized that treating infectious waste would significantly reduce the concentration of virus deposited onto surfaces after toilet flushing. The results of this study validated this hypothesis by showing that treating waste before flushing for 15 minutes does significantly reduce viral concentrations on surfaces. Despite this, some surfaces were still positive even with treatment use. This study proved that not all disinfectants are equally effective at reducing virus contamination in the toilet bowl before flushing. Peracetic acid and quaternary ammonium had the greatest impact on virus concentration in the toilet bowl within one and 15 minutes. After 30 minutes, peracetic acid was still able to reduce the concentration by the greatest amount. However, chlorine bleach was more effective than quaternary ammonium after 30 minutes. This study concluded that treating infectious waste before flushing has a significant impact on environmental spread of the virus and should be practiced. This study also showed that it is not sufficient to only disinfect waste before flushing.
None of the tested treatments were able to reduce the virus below its limit of detection in the toilet bowl or on certain surfaces (one PFU/mL or 3 PFU/100cm²).

The final component of this dissertation was able to determine the influence of anaerobic digestion on virus inactivation. Results from this study showed that mesophilic anaerobic digestion was able to significantly reduce all of the tested viruses. However, Φ6 was the only virus reduced below its limit of detection (0.33 PFU/mL). This suggests that lipid-containing viruses, such as Φ6 and Ebola virus, are very sensitive to mesophilic and thermophilic digestion. Reductions observed by the other test viruses also suggest that lipid-containing viruses are more sensitive to anaerobic digestion than non-lipid containing viruses. Due to this, lipid-containing viruses are likely to show greater reductions during the full-scale treatment process than non-lipid containing human viruses, such as poliovirus, norovirus and adenovirus.

Limitations

Each component of this dissertation had evident limitations. These limitations are addressed by recommendations for future work:

Specific Aim 1: Assessing the impact of sediment-borne pathogens on overlying irrigation water quality

- The laboratory-scale recreation of Yuma, Arizona irrigation canals may not have been scaled down to exact characterizations. This was due to the limitations of the equipment sizes. To improve upon this, a field study should be conducted to validate the study’s
findings at the full-scale. This would allow actual canal characteristics to be accurately represented in the study.

- Another limitation to the first specific aim is the use of only MS2 and *E. coli*. Other organisms may re-suspend into the overlying water more readily (or less), such as lipid-containing viruses or gram-positive bacteria. The potential of these organisms to re-suspend into overlying irrigation waters should be properly evaluated.

- The final limitation of this study was the use of only clay and sand sediment types. Although these sediments are smaller than those observed in the field, they might be too conservative to estimate the re-suspension in the irrigation canals. These sediments were used due to size restrictions of the flume. To further validate the findings in this study, the impact of gravel-sized and mixed sediment profiles should be investigated.

Specific Aim 2: Evaluating the efficacy of four disinfectants on the viral concentration in the toilet bowl and on restroom surfaces after flushing

- One limitation to this study was the investigation of virus deposition on surfaces after flushing through the use of only a commercial toilet. This toilet was a valve-type toilet, which typically exert more force during flushing than tank-type toilets. To accurately assess the deposition of virus on surfaces from toilet flushing, a household or non-commercial standard toilet (tank-type toilets) should also be investigated.

- Another limitation to this study was the use of only MS2. Enveloped or lipid-containing viruses might be more sensitive to the disinfection treatments tested in this study. To further evaluate the efficacy of disinfectants on viruses, the study should be completed
using a lipid-containing environmental surrogate virus such as Φ6. This would provide a more robust evaluation of the efficacy of disinfectants on viruses in organic waste.

Specific Aim 3: Determine the influence of mesophilic and thermophilic digestion on the inactivation of viruses

- The main limitation of this study is the use of miniature, individual anaerobic digesters. These digestion tubes provided a very controlled digestion process. Full-scale digestion processes typically have varying environmental components such as microbial populations being introduced, temperature and pH. To compliment this study, an additional study using a full-scale or pilot-scale digestion process could be performed.
- Lastly, the initial inoculums of adenovirus 4 ($10^5$) and murine norovirus ($10^6$) did not allow full reductions to be quantified after thermophilic digestion. Repeating this study with higher initial inoculums would aid in quantifying the reductions observed by thermophilic digestion.

Conclusions and Recommendations

The studies included in this dissertation were designed to predict the fate, transport and survival of viruses that are either highly pathogenic or non-culturable in the laboratory. In doing this, quantitative information was gathered to aid in assessing the impacts of these viruses on human health in their respective environments. From the first study, we were able to conclude that sediment-borne pathogens are not likely to adversely impact irrigation water quality, on a regular basis. This is important because it allows growers to target other potential factors of
contamination through interventions. It also allows them to focus on only sampling water during routine testing. However, because we know from previous studies (Obergh, 2015; Carpenter, 2007) that *E. coli* and *Salmonella* are present in sediments of irrigation canals, we recommend to test waters extensively after disturbances to the bottom sediments in the canals. These disturbances could include canal dredging, heavy rainfall events, or any anthropogenic and natural activities that would non-uniformly re-suspend bottom sediments. If possible, eliminating sediment in irrigations canals all together would be ideal. In doing this, a potential reservoir for foodborne pathogens would also be eliminated. This could be done by limiting access to canals, lining canals with concrete, or continuation of regular dredging before and after produce season.

The second study allowed us to conclude that treating infectious waste before flushing the toilet has a significant impact on reducing environmental spread of pathogens in the restroom. It also proved that, in the presence of high levels of organic matter, the efficacy of disinfectants is greatly reduced because none of the disinfectants were able to completely reduce the virus load in the toilet and on the surfaces. In order to further prevent environmental exposure of healthcare workers to viruses in the restroom, a surface disinfection protocol should be implemented. Also, a thorough human health risk assessment would be instrumental in predicting the impact of treating infectious waste before flushing on the risk of illness from environmental exposure to healthcare workers.

In the final study, we concluded that lipid-containing viruses are likely to be reduced significantly more than non-lipid viruses during anaerobic digestion. This is important because many emerging pathogens include enveloped, lipid-containing viruses such as Ebola virus and pandemic strains of influenza. Knowledge of how our current wastewater treatment practices effect these viruses is helpful in preventing environmental exposures. Also, because thermophilic
anaerobic digestion is not standard practice, it is important that the influence of mesophilic digestion is known for these types of viruses. When possible, thermophilic anaerobic digestion should be practiced to ensure reduction of highly pathogenic viruses before environmental disposal of solids.

Overall, the studies conducted conclude that the use of surrogates is instrumental in predicting the environmental characteristics of less-accessible viruses such as norovirus, Ebola virus or pandemic influenza strains. The studies also provided quantitative values for the effect of different environmental characteristics on different viruses through the use of surrogates. Any of these values could be utilized in human health risk assessments or environmental impact assessments to represent a wide range of organisms that are of concern to public health. The conclusions from the first study could be used to evaluate the risk of produce contamination or produce-related illness due to sediment-borne pathogens in irrigation canals. The second study could be utilized to conduct a situational human health risk assessment for healthcare workers coming into contact with viruses on surfaces in the restroom. It can also be used to investigate alternative treatments for infectious human waste before disposal. Lastly, the results from third study could be used to conduct an environmental assessment for the level of infectious viruses being discharged into the environment in biosolids. These results could also be integrated into a human health risk assessment for illness from viruses through contact with biosolids.
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APPENDIX A: Potential for Viral and Bacterial Re-suspension from Sediments into Overlying Water in Irrigation Canals

Hannah P. Sassi¹, Kang Zhou², Christina M. Morrison¹, Jennifer G. Duan² and Charles P. Gerba¹*

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¹Water and Energy Sustainable Technology Center, Department of Soil, Water and Environmental Science, University of Arizona, 2959 W. Calle Agua Nueva
Tucson, AZ, USA, 85745

²Department of Civil Engineering and Engineering Mathematics, University of Arizona
1209 E. Second Street Rm 206A-1
Tucson, AZ, USA, 85721

* Corresponding Author: Charles P. Gerba, PhD
Telephone: 520-621-6906; Fax: 520-6210
Abstract

Previous studies have identified sediments as a potential reservoir for a wide range of pathogens in aquatic environments due to particle deposition and sorption. Residing in the sediment also allows pathogens to persist longer. *Escherichia coli* and *Salmonella* spp. have been identified in the sediments of man-made canals used for irrigation of produce. This study investigated the impact of re-suspension of enteric bacteria and viruses in sediments on the overlaying water quality. A laboratory flume was used to assess the impact of sediment material and flow rate on re-suspension. The flume was lined with either sand or clay sediments and the top layer was inoculated with MS2 coliphage or *E. coli*. Water was passed over the sediments at 0.41, 0.73, and 1.46 L/s, and water samples were collected to assess the re-suspension of organisms into the water. Results showed between 0-12% of the total inoculum was re-suspended into the overlying water. There was also no statistically significant difference in re-suspension between the bacterium and virus; or in the re-suspension between sand and clay sediments, except at 1.46 L/s (*p*=0.0348). The re-suspension of MS2 from clay sediments was significant when 0.41 and 1.46 L/s were compared (*p*=0.0266). Thus sediments in Yuma, Arizona, irrigation canals are not likely to impact significantly the microbial quality of irrigation water. The exception to this would be during unique events that would disrupt the normal flow of the canal or that would disrupt the bed sediment layer in the canal, such as canal dredging; or events that would increase the flow, such as heavy rainfall.
Introduction

The Centers for Disease Control and Prevention (CDC) estimates that an average of one in six Americans will contract a foodborne illness every year (6). During the years 1998-2008, the CDC estimates there were 13,405 foodborne outbreaks in the United States. Almost half of these outbreaks are due to an unknown food vehicle; however, of the 7,724 outbreaks with an identified food vehicle, fresh produce was responsible for approximately 46% (6). Among the approximate 3,500 fresh produce outbreaks reported between 1998 and 2008, leafy greens and lettuce were responsible for almost 1,150. Fresh produce was also the second most common vehicle for *Escherichia coli* O157:H7 outbreaks between 1982 and 2012 (28, 15). Norovirus, non-typhoidal (NT) *Salmonella*, and *Escherichia coli* O157:H7 are three of the top five illness-inducing pathogens resulting in hospitalizations. When combined, these organisms are estimated to account for over 50% of hospitalizations from foodborne illnesses, based on data from 2011 (6). These three pathogens are also the top three associated with fresh produce outbreaks (9).

Various studies have linked irrigation water to contamination sources for leafy greens and lettuce (30, 1, 16, 12). They have also shown that surface irrigation sources are very vulnerable to contamination from various sources (31). A 2006 outbreak caused by iceberg lettuce from Minnesota was believed to be associated with irrigation water of questionable microbiological quality. An assessment of the farm after the outbreak found 31% positive matches with the same fingerprint as the *E. coli* strain causing the outbreak. These samples came primarily from an adjacent growing field, water-dairy wastewater interfaces, and the nearby dairy farm (12). Again in 2006, there was a multi-state *E. coli* O157:H7 outbreak caused by spinach and linked to feral swine contaminating surface waterways (17).
In 2015, the Food Safety and Modernization Act (FSMA) was officially implemented; and included policies that provide guidelines for agricultural water inspection and quality of water coming into contact with fresh produce (included in Subpart E) \((10)\). Water coming into direct contact with fresh produce during or after harvest should have no detectable \(E. coli\) present in 100 mL samples. For water that will not be in direct contact during or after harvest, the maximum acceptable \(E. coli\) most probable number (MPN) is 235 colony forming units (CFU) per 100 mL for a five sample rolling average \((10)\). Prior to FSMA, the Leafy Greens Management Agreement (LGMA) outlined its own specific guidelines for agricultural water quality. In this agreement, it is recommended that water be tested at least monthly and that the tests should be taken from the most relevant point of use for the water source (i.e., nearest to the outflow for irrigation).

One aspect of water quality that is not addressed is the presence of sediments in irrigation water sources. Sediments are known to provide a reservoir for microorganisms in aquatic environments and can contain high levels of bacteria due mostly to particle sedimentation and sorption \((3)\). In 1970, Geldreich \((11)\) determined that when overlying waters contained less than 200 CFU of fecal coliforms per 100 mL, 23.5\% of sediment samples contained \(Salmonella\). When the fecal coliforms were greater than 200 CFU, the presence of \(Salmonella\) in bottom sediment rose to 68.5\% \((11)\). Despite this, correlations in various water bodies between sediment and overlying water have been reportedly weak in the past \((28)\); these \(r\) values ranged from no correlation to 0.49 \((4, 7, 2)\). The low correlation between sediment concentrations and overlying water concentrations is credited to the lack of re-suspension of sediments in the bodies of water \((26)\). Sediments can also be a problem because they prolong the survival enteric bacteria and viruses in aquatic environments \((3)\). In a 1987 study, survival times for bacteria in sediment were
correlated with particle size, and increased from multiple days to multiple weeks. Survival rates for *Escherichia coli* and *Salmonella* Newport were greatest in sediments with at least 25% clay (3).

Viruses also have an extended survival time when adsorbed to sediments in aquatic environments. In a laboratory study, poliovirus and echovirus survived up to 6 days in sediments, compared to <1 day when suspended in water (21). Viruses also readily adsorb to particulates in water, which increases their likelihood of survival and, in turn, concentrations of viruses are typically much higher in sediments in aquatic environments. Viruses can adsorb to varying types and sizes of sediments, with clays being particularly protective (13, 27). This may be due to adsorption of enzymes and other constituents from the clay surfaces that stabilize the capsid or protect against substances that would inactivate or degrade the virus (13).

Arizona’s Yuma Valley is where 90% of the nation’s winter lettuce is grown (32). Irrigation in this area is supplied primarily by open canals with water sourced from the Colorado River. These canals generally have unrestricted access (e.g., lack of fencing or walls to prevent access) and are vulnerable to contamination from outside sources, such as human and animal encounters. A majority of these canals also remain unlined, including many of the primary and lateral canals. Because of this, the risk of intrusion (animal, human, outside water/environmental sources) is particularly high for growers. Intrusion could cause greater amounts of microbial pathogens to settle into bottom sediments or re-suspension of bottom sediments to release additional pathogens and indicators into the overlaying water.

In a previous study conducted by Carpenter (5) on canals in the Yuma Valley, mean *E. coli* and fecal coliform levels were found to be up to seven and fifteen times greater in sediment than in water for fecal coliforms and *E. coli*, respectively, in comparable volumes. Another study
conducted in Imperial Valley, California, found *Salmonella* gene markers (*invA*) to be up to 2 log\(_{10}\) greater in sediment than in overlying waters of unlined canals (25). Thus, it is important to understand whether or not bottom sediments are influencing the overlying water quality. The objective of this study was to evaluate the effect of varying flow rates and velocities on the re-suspension of *Escherichia coli* and MS2 coliphage into overlying waters using a closed flume system to model open channel irrigation canals. *E. coli* ATCC 25922 was used as a surrogate for *E. coli* O157:H7. MS2 was used as a surrogate for enteric viruses. It is a bacteriophage that is morphologically and genetically similar to many human enteric viruses found in water. MS2 has been used extensively as a model for the survival, fate, and transport of human enteric viruses (30).

**Methods and Materials**

**Laboratory**

An irrigation canal was simulated using a closed-system flume (Figure 1.1) (Hydraulic Design and Product Co.). The flume was 1.6 m in length with a width of 0.15 m, packed with 8-10 cm of sediment (~20.5 kg) for a base layer. The top layer (2-3 cm; ~4.5 kg) was treated with dry heat at 121°C for 15 min to reduce the levels of viable background bacteria that could inhibit the inoculum organisms. This temperature and treatment time were determined to be safe for use without altering physical properties. The stability of clay-like soils has been maintained when treated at 110 °C for 18 h (18). Because only the top layer of sediment was inoculated, the base layer was not heat treated. After heating, the top layer was then transferred to sterile stomacher bags (Seward Laboratory Systems Inc., Davie, FL, USA) and saturated using 1 L of sterile deionized water, inoculated with 2 mL of either *E. coli* (ATCC 25922) or MS2 bacteriophage (Host ATCC 15597). The average concentration of the *E. coli* inoculum was 2E+09 CFU/2 mL
and the average inoculum of the MS2 titer was 4.5E+09 plaque forming unit (PFU)/2 mL. The inoculum and water were hand massaged into sediment in the bags until all sediment was fully saturated. After inoculation, the sediment remained in the stomacher bag and was allowed to dry at room temperature (27 °C) for 1-2 h to facilitate absorption of excess liquid. A baseline sample of approximately 90 g (wet) was taken to ensure attached of the organisms to the sediment. Subsequently, the sediment was added on top of the base layer of sediment in the flume. The top layer was evenly distributed along the flume and loosely packed by hand. Laboratory tests were designed to represent varying flow rates, similar to those measured in the field. However, due to laboratory constraints, an exact scaled-down replication was not possible. Sand and clay were used to represent two sediment types. Gravel sized particles were not used in this study due to the flume size.

After addition of the sediment, the flume holding tank was then filled with 65-gallons (246.05 L) of water. The free chlorine residual (0.02 mg/L) was measured using a Pocket Chlorimeter II (Hach, Loveland, CO, USA) and the pH (7.9) and temperature (25 °C) were measured using a pHTestr30 waterproof probe (Eutech Instruments, Vernon Hills, IL, USA). A volume of 100 mL of 10% sodium thiosulfate solution (Sigma-Aldrich, St. Louis, MO, USA) was added to neutralize the existing low-level of free chlorine. The flow rate (L/s) and velocity (cm/s) were set accordingly by adjusting the lift gate, which controls the rate at which the water discharges back into the holding tank. Each velocity was run for a period of 0.5 h and then water was sampled from the flume stream and from the holding tank. Samples were collected using sterile 250 mL plastic bottles (Thermo Scientific Nalgene, Waltham, MA, USA). In the case of E. coli, one mL of sample was spread plated across 3 plates in volumes of 0.333 mL/plate. In addition, serial 10-fold dilutions were made using sterile 0.01M phosphate buffered saline (PBS) (pH 7.4) (Fisher
Scientific, Waltham, MA, USA). The dilutions were plated in volumes of 100 μL in duplicates. *E. coli* was plated using MacConkey agar (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The plates were then incubated at 37°C for 24 h and *E. coli* colonies (pink) were enumerated.

MS2 was assayed using the double agar overlay method (20). Briefly, 5 mL of sterile top agar was melted and held at 50°C in a water bath. A host of *Escherichia coli* ATCC 15597 was propagated in 125 mL of sterile tryptic soy broth (TSB) (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for approximately 4 hours at 37°C with agitation. After exponential phase was achieved, 0.5 mL of host organism was combined with 1 mL of sample water in the sterile top agar. Serial 10-fold dilutions were also made for MS2 experiments using 0.01M sterile PBS (pH 7.4); for dilutions, a volume of 0.1 mL was used. The tube was gently swirled and then poured onto a sterile tryptic soy agar plate (TSA) (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The plates were allowed to solidify, then they were inverted and incubated for 24 h at 37°C (29). The plaques were enumerated after incubation.

Field

In addition to modeling the irrigation canal system in a flume, field measurements were taken from five canals in the Yuma Valley (Figure 2). These measurements included water depth, flow rate, velocity, and sediment characteristics. The water velocity was measured by acoustic Doppler velocimetry (ADV) at the middle of each cross section in the canal. This was used as the mean velocity to calculate the flow rate of the canal by multiplying the area of cross section. The bed sediments were dried in an oven at a temperature of 110°C for 24 hours and the
size distribution was determined by sieve analysis. A set of sieves with openings of 25.4 mm, 12.7 mm, 8 mm, 4.75 mm, 2 mm, 1 mm, 0.85 mm, 0.425 mm, 0.18 mm, and 0.075 mm was selected for the analysis. The sieves were shaken for 15 min on a mechanical shaker to separate the sediments of different sizes. The sampling bottles containing water and suspended sediments were weighed before filtering; after filtering the water and sediments, the empty bottles were weighed to get the mass of mixture of water and sediments by taking the difference. The density of the mixture was estimated to be the same as water to obtain the volumes of mixture because of the small relative concentration of sediment in each sample. The sediments were retained in the filter paper. The filter paper was then dried in an oven at a temperature of 70 °C for 24 hours. The mass of sediment was obtained by measuring the mass difference of the filter paper with and without the sediment. The suspended sediment concentration was calculated by dividing the sediment mass by the sample volume.

**Statistical Analysis**

The results were analyzed using STATA 14 (StataCorp, College Station, TX, USA). All results were log-transformed to normalize the data set before being used in a parametric analysis. These normalized values were then used in a two-sided, two Sample t-test with equal variances at the 95% confidence level with a null hypothesis of there being no difference in means. For both organisms, each flow rate (Q) was paired with each other and a t-test was run to evaluate if the values per mL were significantly different from each other. *E. coli* in clay was also compared to MS2 in clay, as well as *E. coli* in clay and *E. coli* in sand. The total (flume and tank) re-suspension was also assessed for each organism. The re-suspension observed was transformed
Results and Discussion

Different flow rates were tested to determine if increasing force on the sediment particles would result in a significant increase in the re-suspension of the organisms. The results (Tables 3-5) showed that sediment could re-suspend into the water but probably not at high enough levels to impact the microbial quality of the irrigation water. Results of the t-test showed that when *E. coli* re-suspension (CFU/mL) was compared between sand and clay sediments, the difference was not statistically significant (*p*>0.05) except at the flow rate 1.46 L/s (*p* = 0.0348). Also, there was no significant difference among the three flow rates for *E. coli* re-suspension from sand sediments. However, when the values for *E. coli* re-suspension in clay sediments were compared, the difference between the flow rates 0.73 L/s and 1.46 L/s were significantly different (*p*=0.0126).

In the case of MS2 re-suspension, there was a statistically significant difference only between the smallest and largest flow rates (*p*=0.0266) with clay sediment. The re-suspension of MS2 and *E. coli* from clay sediments were also evaluated using a t-test. There was no statistically significant difference in the re-suspension of MS2 and *E. coli* for each flow rate. This indicates that one organism is no more likely to be re-suspended than the other.

When the re-suspended proportions of total inoculum were used in t-tests, there were no statistically significant differences in means for all tests performed, meaning that all proportions re-suspended were similar with each flow rate. This result suggests that the differences per mL are significant; however, when taken as a proportion of the total concentration in the sediment,
the mean re-suspension is relatively the same overall for each organism and sediment type. This indicates that there is little re-suspension of organisms into the overlying water under the conditions used in this study.

Previous studies of sediment in fresh water environments (lakes, rivers, beaches) have shown bed sediments to be potential reservoirs of *E. coli* and fecal coliforms. It has also been suggested that the numbers of *E. coli* in sediments could potentially be a reliable long-term representation of overall water quality rather than a snapshot of contamination events (27). Pathogens such as *Campylobacter* spp., enteroviruses, and hepatitis A virus have also been detected in various aquatic sediments (26, 22). The role of sediments as a reservoir for both indicator bacteria and pathogens, and given their ability to re-suspend into overlying waters, has the potential to impact the quality of water used for irrigation. However, field measurements, such as flow rate, sediment size, and water depth (Table 6) from canals in Yuma, Arizona, suggest that if pathogens were to be introduced into bottom sediments, they would likely not be a significant risk to the water quality in the canals. The sediment found in the canals in Yuma were composed primarily of gravel (>2.0 mm diameter) and sand (0.06-2.0 mm diameter) particles. These sediment sizes are larger than those that were tested and likely originate from surrounding fields, roads, alleys, and recreational areas (parks and golf courses observed) and likely were transported naturally (wind and rain). Anthropogenic activities around the canals, such as vehicle and machinery operation, have also been observed in this area. Concentrated Animal Feeding Operations (CAFOs) are located in close proximity to produce fields in the Yuma Valley. When these CAFOs were sampled, they were determined to be a potential reservoir for *Salmonella, E. coli* O157 and non-O157 *E. coli* (19). Other observed sources of potential contamination in this area are anthropogenic activity and wildlife.
A combination of large particle sizes found in the canals and relatively low flow rates for the depth and width of the canals, the force needed to re-suspend organisms and sediment would be greater than what is exerted on a regular basis. However, because laboratory parameters may not fully represent an accurate scaled-down simulation of in-field measurements, a field study is needed to validate the assumption that sediments have a low impact on microbial quality of irrigation water. Implementation of sediment sampling may not provide additional guidance on the current water quality of a canal because sediments can harbor pathogens for long periods of time. Additional water testing should be conducted to ensure microbial water in the canals after unique events such as heavy rainfall, canal dredging, or other disturbances to canal flow.

REFERENCES:


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Vegetable Fields in the Southwest Desert. Presentation. *International Association of Food Protection*, Portland, OR, USA.


FIGURES 1-2

Figure 1: Flume water cycling with sample locations
Figure 2: Map of field sites
### TABLES 1-6

*Table 1: Flow rates and correlating velocities*

<table>
<thead>
<tr>
<th>Q (L/s)</th>
<th>1 (cm/s)</th>
<th>2 (cm/s)</th>
<th>3 (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41</td>
<td>5</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>0.73</td>
<td>10</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>1.46</td>
<td>20</td>
<td>30</td>
<td>40</td>
</tr>
</tbody>
</table>

L/s= Liters per second  
cm/s= Centimeters per second

*Table 2: Depth of water (cm) in the flume during experiments at given Q and v.*

<table>
<thead>
<tr>
<th>v (cm/s)</th>
<th>Q= 0.41 Vol. in Flume (L)</th>
<th>Q= 0.73 Vol. in Flume (L)</th>
<th>Q=1.46 Vol. in Flume (L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>5.4 cm 13.19 N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>10</td>
<td>2.7 cm 6.6 4.8 cm 11.72</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>20</td>
<td>1.4 cm 3.42 2.4 cm 5.86</td>
<td>4.8 cm 11.72</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>N/A 1.6 cm 3.91</td>
<td>3.2 cm 7.82</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>N/A N/A 2.4 cm</td>
<td>5.86</td>
<td></td>
</tr>
</tbody>
</table>

N/A=not tested  
L/s= Liters per second  
cm/s= Centimeters per second
### Table 3: PFU/mL re-suspension of MS2 from Clay

<table>
<thead>
<tr>
<th>Q</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41</td>
<td>2.99E+02</td>
<td>3.34E+02</td>
<td>1.10E+01</td>
<td>1.08E+03</td>
</tr>
<tr>
<td>0.73</td>
<td>1.22E+03</td>
<td>1.87E+03</td>
<td>1.50E+01</td>
<td>5.80E+02</td>
</tr>
<tr>
<td>1.46</td>
<td>1.19E+03</td>
<td>1.16E+03</td>
<td>9.20E+01</td>
<td>3.31E+03</td>
</tr>
</tbody>
</table>

*n=9; N=27

Starting inoculum (PFU): 4.57E+09 ± 5.37E+09

### Table 4: CFU/mL re-suspension of E. coli from Clay

<table>
<thead>
<tr>
<th>Q</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41</td>
<td>1.03E+02</td>
<td>1.53E+02</td>
<td>&lt;1</td>
<td>3.20E+02</td>
</tr>
<tr>
<td>0.73</td>
<td>9.60E+02</td>
<td>1.36E+03</td>
<td>&lt;1</td>
<td>3.50E+03</td>
</tr>
<tr>
<td>1.46</td>
<td>1.88E+03</td>
<td>2.60E+03</td>
<td>1.00E+00</td>
<td>8.04E+03</td>
</tr>
</tbody>
</table>

*n=9; N=27

Starting inoculum (CFU): 1.59E+09 ± 1.05E+09
Table 5: CFU/mL re-suspension of E. coli from Sand

<table>
<thead>
<tr>
<th>Q</th>
<th>Mean</th>
<th>SD</th>
<th>Min</th>
<th>Max</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.41</td>
<td>3.90E+02</td>
<td>1.09E+03</td>
<td>&lt;1</td>
<td>3.31E+03</td>
</tr>
<tr>
<td>0.73</td>
<td>1.52E+02</td>
<td>1.98E+02</td>
<td>&lt;1</td>
<td>5.27E+02</td>
</tr>
<tr>
<td>1.46</td>
<td>2.14E+02</td>
<td>5.40E+02</td>
<td>&lt;1</td>
<td>1.64E+03</td>
</tr>
</tbody>
</table>

*n=9; N=27
Starting inoculum (CFU): 2.10E+09 ± 1.28E+09

Table 6: Characteristics of canals and sediment from irrigation canals in Yuma, Arizona

<table>
<thead>
<tr>
<th>Canal</th>
<th>Water Depth (m)</th>
<th>Width (m)</th>
<th>Velocity (cm/s)</th>
<th>Flow Rate (L/s)</th>
<th>D50 (mm)</th>
<th>D84 (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.76</td>
<td>9.33</td>
<td>0.61</td>
<td>39.79</td>
<td>0.36</td>
<td>1.87</td>
</tr>
<tr>
<td>2</td>
<td>0.09</td>
<td>3.35</td>
<td>N/A</td>
<td>N/A</td>
<td>15.9</td>
<td>N/A</td>
</tr>
<tr>
<td>3</td>
<td>0.88</td>
<td>9.33</td>
<td>60.7</td>
<td>4500</td>
<td>11.2</td>
<td>24.5</td>
</tr>
<tr>
<td>4</td>
<td>0.91</td>
<td>2.74</td>
<td>21</td>
<td>349.9</td>
<td>0.17</td>
<td>1.44</td>
</tr>
<tr>
<td>5</td>
<td>0.41</td>
<td>N/A</td>
<td>0.02</td>
<td>N/A</td>
<td>1.45</td>
<td>15.9</td>
</tr>
</tbody>
</table>

N/A = an accurate measurement was not observed or observable due to canal conditions

D50 = Median grain size

D84 = 84th percentile of grain size
APPENDIX B: Disinfectant Use to Reduce an Ebola Surrogate in Toilet Flush Aerosols

Hannah P. Sassi MS¹, Ian L. Pepper PhD¹ and Charles P. Gerba PhD¹*

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Affiliation:

¹Water and Energy Sustainable Technology Center, Department of Soil, Water and Environmental Science, University of Arizona

2959 W. Calle Agua Nueva

Tucson, AZ, USA, 85745

*Corresponding Author:

Charles P. Gerba, PhD

University of Arizona

T: 520-621-6906

F: 520-621-1647

gerba@ag.arizona.edu
Abstract

**Background:** Protocols provided by the Centers for Disease Control do not address treating Ebola human waste before toilet flushing. Past studies have shown that aerosols created during flushing infectious materials results in surface contamination in the restroom; the US Army Institute of Public Health has issued recommended protocols for treating waste before flushing. The goals of this study were to determine the degree of viral contamination of surfaces in restrooms after disposal of organic wastes with and without disinfection, and to determine the efficacy of four disinfectants on viral reduction in the toilet before flushing.

**Methods:** Chlorine bleach, hydrogen peroxide, quaternary ammonium and peracetic acid were tested using MS2 in one liter of typticase soy broth added to a toilet bowl. Virus reduction in the toilet bowl before flushing and virus concentrations on surrounding surfaces after flushing were then determined.

**Results:** After every trial, the toilet bowl rim, toilet seat top, and toilet seat underside were contaminated. All disinfectants significantly reduced concentrations on surfaces when the contact time was 15 min. Hydrogen peroxide resulted in very little reduction of virus in the toilet bowl (<1 log$_{10}$). PAA and the quaternary ammonium formulation also had the greatest log-reductions within the first min of contact.

**Conclusions:** This study showed that viruses were difficult to inactivate in large volumes of organic matter. Environmental surface contamination in the restroom can be minimized if infectious waste is disinfected before flushing.

**Keywords:** Ebola virus; Viral Aerosols; Fomite Transmission; Disinfectants
Introduction

With the Ebola virus (EBV) outbreak in West Africa and the introduction of the disease into the United States for the first time (in humans) in 2014, safe handling and effective disinfection practices of potentially infectious waste have become especially important in the healthcare setting (Fauci 2014; CDC 2016). It has been widely recognized for some time that transmission of infectious diseases in healthcare environments can occur among patients and healthcare workers (HCWs) (Alexander, 1973). The transmission dynamics and highly infectious nature of EBV are extremely important in protecting HCWs in all settings. It is well-established that the primary mode of transmission for EBV is through direct contact with infected bodily fluids. EBV is excreted not only in blood but also in feces, urine, and vomit. When a patient is infected, she can release up to nine liters of stool per day, discharging copious amounts of virus into the environment. The levels of virus in bodily fluids can range from $10^{5.5-10^8}$ EBV genome copies per mL. This is assumed to be well over the suspected median infectious dose of <10 viral particles (Bibby et al., 2015).

Human viruses shed in bodily fluids, such as norovirus, adenovirus, and torque teno virus, can be aerosolized and deposited on hospital surfaces (Bonifait et al., 2015; Verani et al., 2014). This release of virus could result in a heightened risk of environmental contact and transmission for HCWs. In 1979, a Sudanese outbreak of EBV reported that HCWs were up to five times more likely to contract the virus than those who did not practice patient care (Sepkowitz, 1996). Fifteen years later, during the 1995 outbreak of EBV in the Democratic Republic of the Congo, at least 32% of the infected individuals (N=296) were healthcare workers (Sepkowitz, 1996). Since these outbreaks, the Centers for Disease Control and Prevention (CDC) has released multiple guidance documents for hospitals relative to managing EBV patients and
suspected EBV patients. In the most recent document, measures to control environmental spread were provided and outlined (CDC, 2015).

In order to prevent environmental spread of EBV to HCWs, the CDC first suggests the use of proper personal protective equipment (PPE) (CDC, 2015). However, in the past it has been demonstrated that PPE can often act not only as a barrier but also as a vehicle for pathogens. In a 2008 study, the ability of MS2 to spread from gloves to bare hands was characterized. MS2 was found on 90% of ungloved right hands, 70% of ungloved left hands, and on other hospital attire (e.g., scrub pants and shirts) (Casanova et al., 2008). This study was able to demonstrate the relationship of PPE removal with potential infection events. The use of PPE is unavoidable for protecting the health of HCWs in isolation and outbreak scenarios; however, improperly removing PPE, especially gloves, could result in inadvertent pathogen contact through either surface-to-hand or hand-to-hand contact (Casanova et al., 2008). PPE is the final phase in the hierarchy of controls and should not be relied on to prevent worker illness.

Environmental controls, such as elimination through disinfection, should be implemented in addition to PPE (OSHA, 2016).

Use of an EPA-registered disinfectant with claims against non-enveloped viruses (norovirus, poliovirus, adenovirus) is also a specified recommendation to reduce environmental transmission of EBV (CDC, 2015). Environmental fomite transmission of diseases has become one of the most recognized routes of transmission in healthcare settings (Weber & Rutala, 2013). Because of this, environmental disinfection could be one of the most important steps to containing an EBV outbreak in a hospital setting. Currently, flushing human waste contaminated with EBV into a sanitary sewer, without disinfection, is allowed (Bibby et al, 2014). If EBV is being aerosolized during flushing, like many other viruses, the deposition of infectious droplets
onto surfaces could present as an environmental transmission route for HCWs. EBV-Zaire has been demonstrated to survive dried onto glass and plastic surfaces for up to 50 days at lower temperatures (+4°C) (Piercy et al, 2010).

Due to concern over the allowance of untreated infectious waste to be flushed into sanitary sewers, the US Army Institute of Public Health released additional Standard Operating Procedures for treatment of waste in toilets before flushing. Recommendations include adding one cup of 5% or greater sodium hypochlorite or low alcohol quaternary ammonium to toilet bowls and allowing a 15-minute contact time before flushing. Procedures for wiping down surfaces around the toilet are also outlined in this document (US Army Institute of Public Health, 2014).

The main objective of this study was to evaluate the recommendations for in-toilet disinfection of waste before flushing on viral contamination of restroom surfaces. In addition to sodium hypochlorite and quaternary ammonium, two other hospital grade disinfectants were also assessed. The treatments were further evaluated for the reduction of virus deposited onto surfaces around the toilet after flushing. The second objective of this study was to compare the efficacies of four disinfectants on reducing the viral concentration in the toilet bowl before flushing.

**Methods and Materials**

*Inoculation and Sample Collection*

In order to create a baseline for how flushing heavily contaminated organic waste would deposit virus onto commonly touched surfaces around the toilet, 1000 mL volumes of trypticase soy broth (TSB) (BD, Franklin Lakes, NJ, USA) were used to create a replicable and uniform surrogate for human waste. Next, the TSB was inoculated with high titers (average: 1.83E+12 ±
3.8E+12 PFU) of MS2 (ATCC 15597-B1) and added to a commercial valve-type toilet bowl containing 2.8 L of water (American Standard, Piscataway, NJ, USA). The bacteriophage was propagated and assayed as previously described in Sassi et al (2015). After addition of the virus and broth, the toilet was flushed, and surfaces around the toilet (Table 1) were sampled using sponge sticks moistened with 10 mL of letheen broth (3M Brand, St. Paul, MN, USA). An average of 100 cm$^2$ was sampled for each site; however, if the site was smaller than 100 cm$^2$, the entire surface was sampled (i.e., toilet flush handle, 90 cm$^2$). A succession of water samples was also collected after one, two, and three flushes to determine residual virus in the bowl after flushing. For these samples, 9 mL of water was collected from the toilet bowl and transferred to a sterile 15 mL conical tube (BD, Franklin Lakes, NJ, USA) containing 1mL of 10% sodium thiosulfate (Sigma Aldrich, St. Louis, MO, USA) to neutralize any free chlorine in the toilet water.

**Disinfectant Additions**

Four different hospital-grade disinfectants (Table 2) were tested in separate trials to assess efficacy of reducing the viral load deposited on surfaces after flushing. Each treatment was measured out to one cup (~236 mL) and added to the toilet bowl after the TSB and virus. Two contact times, 15 and 30 min, were used for each treatment to assess the reduction in deposition of virus on surfaces. Letheen broth (BD, Franklin Lakes, NJ, USA) and sodium thiosulfate were used to neutralize the treatments. For these trials, the same surfaces were sampled (Table 1). To quantify the reduction of MS2 in the toilet bowl before flushing, three contact times were evaluated. After the addition of organic matter, virus, and treatment, 5 mL samples were collected from the toilet bowl after 1, 15, and 30 min. The samples were then
transferred into sterile 15 mL conical tubes containing either letheen broth or 10% sodium thiosulfate.

**Sample Processing**

After collection, each sponge stick was placed in a sterile plastic bag and eluted using an application of manual pressure. The volume eluted (approx. 4-6 mL) was recorded to calculate a total concentration per sampled surface area. All samples (surface and water) were assayed using the double agar overlay method (Kropinski *et al.*, 2009) in triplicate. Volumes of 1 or 0.1 mL were combined in melted top agar tubes (50°C) with 0.5 mL of host (*E. coli* ATCC 15597) before pouring onto TSA (BD, Franklin Lakes, NJ, USA). When necessary, 10-fold serial dilution of the samples were prepared using 0.01M PBS (pH 7.4) (Sigma Aldrich, St. Louis, MO, USA). Plates were then incubated for 24 h at 37°C and viral plaques enumerated. The concentration per mL of sample was determined for water samples collected from the toilet bowl. The concentration per surface sample was calculated by determining the average concentration per mL of eluent and multiplying by the total volume of eluent collected for the sample. This represents the concentration per 100 cm² for each sample location except the flush handle, which had a concentration per 90 cm². The limit of detection for surface samples was 3 PFU/100 cm².

**Statistical Analysis**

The concentrations per cm² on surfaces after use of disinfectant were compared to the concentrations deposited onto surfaces without treatment using a paired t-test. Values were normalized using a log transformation before being used in a t-test. All t-tests were performed as
paired, two-sided tests with a null hypothesis that the difference in means was equal (H₀: μ₁=μ₂).
The log reductions observed after disinfection treatments in the toilet bowl at 1, 15, and 30 min
contact times were compared using a multivariate test of means for each disinfectant type. All
statistical analyses were performed in STATA 14 (Stata Corp., College Station, TX, USA).

Results

Droplet Deposition on Surfaces

The most heavily contaminated surfaces, on average, were the underside of the toilet seat,
the top side of the toilet seat, and the toilet bowl rim. Virus was detected 100% of the time after
flushing at three locations and they also had the highest concentrations of all surfaces. The least
contaminated surfaces after flushing were the flush handle, the wall behind the toilet (i.e., the
back wall), and the toilet paper dispenser (Table 3 and 4). The flush handle and toilet paper
dispenser were the least frequently contaminated with virus being detected only 17% and 22% of
the time, respectively (Figure 2.1). Virus was only detected in one toilet bowl water sample after
flushing once (1/54). No further flush water samples were found to be positive.

Comparison of Treatments

The results of the paired t-tests showed that there was a significant reduction in
concentration from the baseline (without treatment) with all of the disinfectants, at the 15-minute
contact time (p<0.05). With a 30-minute contact time, all disinfectants except hydrogen peroxide
showed a significant reduction when compared with no disinfectant (Table 5). When 30-minute
contact times were compared with 15-minute contact times, the only treatment that showed a
significant further reduction was chlorine bleach (p= 0.0174).
Comparison of Viral Reduction on Surfaces

Peracetic acid showed the greatest reduction of all treatments for all contact times. The quaternary ammonium treatment produced a $1.99 \log_{10} \text{PFU/mL}$ reduction within one minute of contact; however, the reduction saw only a small increase in virus reduction after 30 minutes. Hydrogen peroxide exhibited the least reduction for all three contact times (Table 4). When these values were analyzed using a multivariate test of means, the only statistically significant differences in average reduction was seen between hydrogen peroxide and quaternary ammonium ($p=0.0016$) and between hydrogen peroxide and peracetic acid ($p=0.0147$). Peracetic acid and quaternary ammonium were able to significantly reduce the concentrations further than hydrogen peroxide.

Discussion

Surface Contamination After Flushing

The deposition of virus after flushing occurred to the greatest extent on locations nearest to the source of the virus (i.e., the toilet bowl). These sites were the toilet bowl rim, the seat top, and the seat bottom. These surfaces were found to be highly contaminated during flushing events in previous studies, in addition to the floor beneath and next to the toilet (Best et al., 2012; Barker and Jones, 2005). Best et al. also found that flushing the toilet with the lid closed significantly reduced the amount of Clostridium difficile spores deposited on surfaces (Best et al., 2012). However, most commercial toilets do not have lids. Instead of closing a toilet lid, targeting frequently contaminated surfaces after flushing with a thorough hygiene protocol is the best way to reduce environmental contact with EBV and other infectious agents excreted in bodily fluids...
in a healthcare setting. The two least contaminated surfaces, the flush handle and the toilet paper dispenser, were the two surfaces that were the furthest away from the source. This suggests that the droplets were not being ejected with enough force to spread viable virus to more distant locations. These surfaces should still be targeted using surface disinfectants, however, due to their incidental and infrequent contamination.

Treating infectious waste with any of the tested disinfectants showed significant reduction in the concentration of MS2 on surfaces, when compared to the baseline with no treatment. The reduction of viral contamination during flushing could be an important control point in reducing environmental contact for HCWs, especially in an outbreak setting. Pathogens in aerosols and suspended droplet nuclei, such as norovirus and \textit{C. difficile}, have been identified in air after flushing (Bonifiat et al, 2015; Best et al, 2012). \textit{Escherichia coli} in droplets has also been captured on gauze over the toilet bowl during flushing. This study also showed that a lower volume of water in the toilet bowl produced an average higher concentration of \textit{E. coli} suspended in droplets than a toilet with a greater volume (Gerba et al, 1975). The toilet tested in the present study had a relatively low volume in the bowl during testing (2.8 L). When an inoculum of $10^6$ PFU was used (data not shown) in the toilet, no virus could be detected on the surrounding surfaces, which demonstrates that the amount of virus being expelled during toilet flushing was less than the assay detection limit (3 PFU/100 cm$^2$). In the study, an average inoculum of $10^{12}$ was used, which resulted in continual contamination of the toilet seat and toilet bowl rim. Elimination and reduction of virus on surfaces minimizes the risk of exposure to staff through contact with fomites.

\textit{Reduction of MS2 in the Toilet Bowl}
The surrogate virus was never inactivated below the limit of detection for all of the disinfectants studied (one PFU/3 mL). This suggests that when present in the high organic matter, such as in bodily fluids, viruses are much more difficult to inactivate; the consequence is that infectious virus is still present in the toilet bowl during flushing. Of all the tested treatments, peracetic acid and quaternary ammonium showed the greatest reduction for the one-minute contact time (2.26 and 1.99 log$_{10}$). It has also been suggested that a higher-than-average (30-60 seconds) contact time is likely unrealistic for a healthcare setting, given the demands of staff availability (CDC, 2008).

**Recommendations**

To reduce environmental contact with EBV and other infectious agents to HCWs, controlling contamination from the toilet is necessary. The concentration of EBV in bodily fluids may be as great as $10^8$/mL (Bibby *et al*, 2015). Results from this study indicate that when high concentrations of virus are present in the toilet bowl, detectable levels of virus on fomites in the restroom can occur in concentrations of $10^1$-$10^5$, even with treatment before flushing. Thus, even a small amount of bodily fluid or fecal material can be expected to contaminate surfaces in the restroom. Treating waste in the toilet before flushing should be practiced in order to reduce significantly the contamination of surfaces in the restroom. For situations where toilets are not readily available, EBV waste should be treated before disposal into the environment. In addition, disinfecting highly contaminated restroom surfaces, such as the toilet bowl and seat, should be practiced regularly and after every flushing event to prevent further environmental spread.

**REFERENCES**


FIGURES 1

Figure 1: Percent positive, by sample location ($N=18$)
### Table 1: Restroom sample locations

<table>
<thead>
<tr>
<th>Sample</th>
<th>Location</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Handle</td>
<td>Toilet flush handle</td>
</tr>
<tr>
<td>2</td>
<td>Toilet Back</td>
<td>Back of toilet, mounting</td>
</tr>
<tr>
<td>3</td>
<td>Back Wall</td>
<td>Wall where toilet is mounted</td>
</tr>
<tr>
<td>4</td>
<td>Floor</td>
<td>Floor underneath toilet</td>
</tr>
<tr>
<td>5</td>
<td>TP Holder</td>
<td>Toilet paper dispenser</td>
</tr>
<tr>
<td>6</td>
<td>Toilet Bowl-In/Rim</td>
<td>Composite of toilet rim and under rim</td>
</tr>
<tr>
<td>7</td>
<td>Toilet Seat Top</td>
<td>Top of the toilet seat</td>
</tr>
<tr>
<td>8</td>
<td>Toilet Seat Under</td>
<td>Under toilet seat (actual seat piece)</td>
</tr>
</tbody>
</table>

### Table 2: List of treatments and percent active ingredient

**Disinfectants**

<table>
<thead>
<tr>
<th>Treatment Type</th>
<th>% Active Ingredient</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleach</td>
<td>5-10: sodium hypochlorite</td>
<td>Clorox (Oakland, CA)</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>0.5-2: hydrogen peroxide</td>
<td>Clorox (Oakland, CA)</td>
</tr>
<tr>
<td></td>
<td>3-5: Alkyl dimethyl benzyl</td>
<td></td>
</tr>
<tr>
<td>Quaternary ammonium</td>
<td>ammonium chloride</td>
<td>Clorox (Oakland, CA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Decon (King of</td>
</tr>
<tr>
<td>Peracetic acid</td>
<td>0.23: peracetic acid</td>
<td>Prussia, PA)</td>
</tr>
</tbody>
</table>
Table 3: Concentrations of virus detected on restroom surfaces after flushing for all trials (N=18)

<table>
<thead>
<tr>
<th>Sample Site</th>
<th>Geometric Mean ± SD (Log10 PFU)</th>
<th>Arithmetic Mean ± SD (PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Flush Handle*</td>
<td>1.65 ± 0.91</td>
<td>2.65E+01 ± 7.64E+01</td>
</tr>
<tr>
<td>2-Toilet Back</td>
<td>2.89 ± 1.04</td>
<td>3.7E+03 ± 7.3E+03</td>
</tr>
<tr>
<td>3-Back Wall</td>
<td>1.63 ± 1.36</td>
<td>1.2E+03 ± 5.3E+03</td>
</tr>
<tr>
<td>4-Floor</td>
<td>3.44 ± 1.08</td>
<td>1.9E+04 ± 4.2E+04</td>
</tr>
<tr>
<td>5-Toilet Paper Dispenser</td>
<td>1.49 ± 1.41</td>
<td>68.5 ± 2.9E+02</td>
</tr>
<tr>
<td>6-Toilet Bowl Rim</td>
<td>3.88 ± 1.59</td>
<td>3.1E+05 ± 9.2E+05</td>
</tr>
<tr>
<td>7-Toilet Seat Top</td>
<td>4.21 ± 1.26</td>
<td>3.4E+05 ± 1.2E+06</td>
</tr>
<tr>
<td>8-Toilet Seat Underside</td>
<td>4.22 ± 1.26</td>
<td>2.5E+05 ± 4.6E+05</td>
</tr>
</tbody>
</table>

*denotes 90cm²
Table 4: Log₁₀ reductions of MS2 per mL by disinfectants in toilet bowl after indicated exposure times

<table>
<thead>
<tr>
<th>Treatment</th>
<th>1 minute</th>
<th>15 minute</th>
<th>30 minute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine Bleach</td>
<td>0.48</td>
<td>1.4</td>
<td>2.83</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td>0.01</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td>Quaternary Ammonium</td>
<td>1.99</td>
<td>1.93</td>
<td>2.22</td>
</tr>
<tr>
<td>Peracetic Acid</td>
<td>2.26</td>
<td>3.37</td>
<td>3.43</td>
</tr>
</tbody>
</table>
APPENDIX C: Survival of Viruses during Thermophilic and Mesophilic Anaerobic Digestion: Assessing the Potential for Survival of Emerging Viruses

Hannah P. Sassi¹, Luisa A. Ikner¹, Sherif Abdel Maksoud¹, Charles P. Gerba¹* and Ian L. Pepper¹

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Affiliation:

¹Water and Energy Sustainable Technology Center, Department of Soil, Water and Environmental Science, University of Arizona

2959 W. Calle Agua Nueva

Tucson, AZ, USA, 85745

*Corresponding Author:

Charles P. Gerba, PhD

University of Arizona

T: 520-621-6906

F: 520-621-1647

gerba@ag.arizona.edu
Abstract

Ebola is shed in high numbers (10^7/mL) in patients’ stool, who may shed up to 9 L/day of waste during their peak illness; little is known of its survival during wastewater treatment. This study evaluated the survival of five surrogate viruses (Φ6, MS2, murine norovirus, poliovirus 1, and adenovirus 4) during mesophilic and thermophilic digestion. Greater inactivation of phages (>5.9-6.6 log_{10}) occurred by mesophilic digestion than animal viruses (1.8-2.2 log_{10}). The results suggest that lipid viruses, like Φ6 and Ebola, are very sensitive to inactivation by mesophilic and thermophilic digestion and are likely to experience greater reduction than non-lipid viruses.

Keywords: Anaerobic digestion; emerging viruses; wastewater; treatment; surrogate; Ebola

Introduction

The recent appearance of Ebola virus (EBV) in the US has led to concerns about the survival of the virus during wastewater treatment, since data on survival of the virus is limited. This virus has the ability to be excreted in high concentrations in bodily fluids including urine, feces, blood and vomit (up to 10^8/mL) (Bibby et al, 2015). However, the survival of EBV in sterile wastewater was recently investigated utilizing surrogates. This study showed that when in sterile wastewater, the concentration of infectious particles was greatly reduced within the first day. Virus inactivation or formation of aggregates with suspended solids in the water are two possible reasons for the rapid reductions (Bibby et al, 2015). Bibby et al (2014) recommended
bacteriophage Φ6 as a potential surrogate for EBV because both contain a lipid and are RNA viruses. Casanova and Weaver (2015) studied the survival of this phage in wastewater and found that at 22 °C the virus was inactivated by 5 log₁₀ in 6 days and 7 log₁₀ in 72 hours.

During the traditional wastewater treatment process, residual solids and semi-solids from the waste stream are referred to as sludge. This historically sludge has resulted from anaerobic digestion, allowing for degradation and reduction of organic matter prior to release into the environment (Gavala et al, 2003). The most widely used type of anaerobic digestion is mesophilic digestion with an optimum temperature range between 32°C and 35°C (Stillwell et al, 2010). Because of the low-energy usage of mesophilic digestion, most wastewater treatment plants employ this method. The Environmental Protection Agency has also approved thermophilic digestion for treatment of sludge to produce Class A biosolids (EPA, 1993). Thermophilic digestion (optimum temperature ~55°C) results in greater pathogen removal; however, it consumes more energy (Nayono, 2010; Stillwell et al, 2010).

Although anaerobic digestion was not initially designed to remove pathogens, it can effectively reduce the levels of a wide range of pathogens including viruses (EPA, 1993). During activated sludge treatment, viruses tend to accumulate in the solid sludge that is produced. Unfortunately, little is known about the survival of emerging viruses by mesophilic and thermophilic digestion processes. The goal of this study was to investigate the removal of several classes of animal viruses to evaluate the survival of EBV surrogates during sludge digestion. The bacteriophage Φ6 was also included as it has been proposed to be a potential surrogate for lipid containing viruses like EBV (Bibby et al, 2015; Casanova, 2015).
Methods

Mammalian Cell and Virus Maintenance

The cell line used to grow and assay the mammalian viruses were PLC (ATCC CRL-8024), RAW 264. 7 (ATCC #TIB-71) and BGM (previously obtained from D. Dahling at the United States Environmental Protection Agency, Cincinnati, OH). Cells were propagated in 225 cm² flasks (Corning®, Corning, NY, USA) for 7 days at 37°C with 5% CO₂ atmosphere. The flasks were then split and used to prepare 24-well cell culture plates (Corning® Costar®, Corning, NY, USA) for assays. RAW cells were maintained and assayed using Dulbecco’s modified Eagle medium (DMEM; MediaTech Inc., Manassas, VA, USA) with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA). The PLC cell line was used as a host for adenovirus type 4 (AD4) using 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT, USA) modified Eagle’s medium (MEM; MediaTech Inc., Manassas, VA, USA) with glucose (Corning, Corning, NY, USA). RAW cells were used for murine norovirus (MNV) using 10% FBS Dulbecco’s MEM (Hyclone Laboratories, Logan, UT, USA) and BGM cells were used for poliovirus type 1 (strain LSc-2ab) (PV1) with 5% calf serum (CS; Hyclone Laboratories, Logan, UT, USA). PV1, MNV, and AD4 were previously propagated and purified using methods described by Soto-Beltran et al 2013 using BGM, RAW and PLC cell lines, respectively. All viruses were stored at -80°C until use.

Bacteriophage Maintenance and Propagation

MS2 (ATCC 15597-B1) was propagated and maintained as previously described by Sassi et al (2015). Φ6 bacteriophage and Pseudomonas syringae (ATCC 10205) were generously
A single colony of *P. syringae* was collected and placed in 10 mL of sterile LB broth (BD, Franklin Lakes, NJ, USA) and grown for 18 h at 26°C with agitation. Then, 0.2 mL of bacteria was combined with 0.1 mL of serially diluted bacteriophage in a sterile top agar tube prepared with LB broth in place of TSB. The tube was then poured over sterile tryptic soy agar (TSA; BD, Franklin Lakes, NJ, USA). Ten-fold serial dilutions were prepared using sterile 0.01M phosphate buffered saline (PBS; Sigma-Aldrich, St. Louis, MO, USA). Plates were incubated at 26°C for 24 h. Following incubation, dilution plates with visible plaques were inverted and 6 mL of PSB was added. Every 30 min, the plates were gently swirled. After 2 h, the plaques were collected using a sterile cell scraper (Corning®, Corning, NY, USA). The PBS and plaques were extracted from the plate and combined in a 50 mL centrifuge tube (BD, Franklin Lakes, NJ, USA). The tube was then centrifuged in tabletop centrifuge at 1,500 x g for 15 min. The supernatant was extracted then passed through a 0.22 μm pore-sized filter (Merck Millipore, Milleriaca, MA).

*Anaerobic Digestion*

To simulate the anaerobic digestion process, micro-scale anaerobic digesters (Miles *et al*, 2011) were created using primary sludge (Total Suspended Solids: 68 mg/L) and waste activated sludge (TSS: 2.75E+04 mg/L) (WAS) from Tres Rios Water Reclamation Facility in Tucson, Arizona. Briefly, 7 mL of primary sludge and 3 mL of WAS were combined in sterile 16x150 mm anaerobic glass tubes (#2048-18150, Bellco Glass, Vineland, NJ, USA). After addition of the sludge, 1 mL of virus was added to triplicate tubes. The tubes were sealed with a 20 mm septum stopper and 20 mm aluminum seal (#2048-11800; #2048-11020, Bellco Glass, Vineyard,
Tubes were then flushed with N₂ gas for 3 minutes at 40 psi using two sterile 18-gauge syringe needles (BD, Franklin Lakes, NJ, USA), one of which was used as an outlet for the air being flushed from the tube.

After preparation, tubes were placed in an anaerobic jar (BBL GasPak 150, BD, Franklin Lakes, NJ, USA) with three catalyst packs (GasPak Pouch, BD, Franklin Lakes, NJ, USA) and sealed. Mesophilic digestion was completed at 32 ± 3°C for 21 days ± 8 hr and thermophilic digestion at 55 ± 3°C for 5 days ± 8 hr. All digestion processes took place with agitation at 120 rpm using a shaking incubator (New Brunswick Scientific, Enfield, CT, USA). A ‘Time 0’ data point was taken by preparing anaerobic tubes in the same way. After the virus was added, the tubes were held for 30 min at room temperature (27°C) and then extracted using the same procedure.

**Virus Extraction**

After digestion, all tubes were centrifuged at 4,500 x g for 15 minutes using a high-speed bench top centrifuge (Allegra, Beckman Coulter, Brea, CA, USA). The supernatant was aspirated into a sterile 15 mL centrifuge tube (BD, Franklin Lakes, NJ, USA). Then, re-suspension of the pellet was performed by vortexing the pellet in 5 mL of 10% beef extract (BD, Franklin Lakes, NJ, USA). It was then agitated using a wrist-action shaker for 30 min. After elution of the virus from the solids, the tubes were centrifuged at 4,500 x g for 15 minutes and the supernatant was collected into a sterile 15 mL centrifuge tube (BD, Franklin Lakes, NJ, USA).

All supernatants were added to a 10 mL syringe (BD, Franklin Lakes, NJ, USA) containing 15 mL of Sephacryl® S-1000 Superfine size-exclusion media (GE Healthcare, Little
Chalfont, UK) and 0.11 g of glass wool (Sigma-Aldrich, St. Louis, MO, USA). The syringes were then centrifuged at 1,000 x g for 8 min. The filtrate from this step was collected in a 50 mL centrifuge tube (BD, Franklin Lakes, NJ, USA) and passed through a 0.22 μm pore-sized filter.

**Bacteriophage Assays**

MS2 and Φ6 bacteriophages were assayed using the double-agar overlay method (Kropinski et al, 2009). For MS2, 0.5 mL of *Escherichia coli* ATCC 15597 was combined with one mL of sample filtrate in a sterile top agar test tube. The mixture was swirled gently and poured over sterile tryptic soy agar (TSA; BD, Franklin Lakes, NJ, USA). The plates were incubated at 37°C for 24 h and plaques were then enumerated. Φ6 was assayed with 0.2 mL of *Pseudomonas syringae* (ATCC 10205) and 1 mL of sample filtrate combined in a sterile top agar tube prepared with LB agar (BD, Franklin Lakes, NJ, USA) and TSA. The tube was gently swirled and poured over sterile TSA. The plates were incubated at 26°C for 24-48 h and then plaques were enumerated. Dilutions were 10-fold serial dilutions prepared with sterile 0.01M PBS (Sigma-Aldrich, St. Louis, MO, USA). All bacteriophage assays were completed in triplicate to yield a limit of detection of one plaque forming unit (PFU)/3 mL.

**Animal Virus Assay**

All TCID<sub>50</sub> assays for mammalian viruses were performed in 24-well cell culture plates. For all viruses, serial 10-fold dilutions were also prepared using the appropriate cell culture media. A volume of 0.2 mL was plated in four replicates for each dilution. For PLC and BGM cells, media was aspirated out of the wells and the monolayer was washed with MEM containing no fetal bovine serum (FBS) two times. The samples were then added to the wells and incubated
for one hour for virus adsorption to the cell monolayer. During this time, plates were placed on an orbital shaker and incubated at 37°C with 5% CO₂ atmosphere. After adsorption, 0.8 mL of 10% FBS MEM with glucose was added to each well for the PLC plates and 0.8 mL of 5% CS MEM was added to each well for the BGM plates. For RAW, the existing media in the wells was aspirated and the virus and media (10% FBS DMEM) were added in one step. There was no adsorption step for this assay. All plates were incubated at 37°C with 5% CO₂ atmosphere.

The cell monolayers were examined for cytopathogenic effect (CPE) for 6 to 9 consecutive days, depending upon the virus, until no new CPE was observed. The TCID₅₀ for each virus was calculated using the Spearman-Kärber formula (Dougherty, 1964). The limit of detection for all viruses was 316 viruses per mL.

Statistical Analysis

A Wilcoxon signed-rank test was used to determine the statistical significance of the log₁₀ reductions for each digestion process. The same test was used to determine if the reduction by thermophilic digestion was statistically different than the reduction observed for mesophilic digestion. The reductions were also compared for each virus using a two-sample t-test to determine if the reduction in concentration was significant, by virus. The statistical analysis for this study was performed using STATA 14 (StataCorp, College Station, TX, USA).

Results and Discussion

Greater inactivation of the phages (>5.9 to 6.6 log₁₀) occurred than the animal viruses (1.8 to 2.2 log₁₀) by mesophilic digestion (Table 1). The reduction of the animal viruses was similar. MS2 and poliovirus were the only recoverable viruses after thermophilic digestion. The
reductions observed (MS2 = 7.1 log_{10}; poliovirus = 4.4 log_{10}) were greater than those observed by mesophilic digestion. These viruses had high initial concentrations (10^8) and this likely contributed to their recovery after thermophilic digestion. All the other viruses were reduced below their detection limit (Table 2). Thermophilic digestion resulted in a statistically significant greater reduction when compared to mesophilic digestion for all the viruses (p=0.0011). Data suggests that lipid viruses, like Φ6, are very sensitive to inactivation by mesophilic and thermophilic digestion.

The reduction of the animal viruses used in this study was comparable to that observed for other enteric viruses naturally present in sewage sludge. In an 18-year study, it was found that mesophilic digestion reduced naturally enteric virus (believed to be largely enteroviruses) numbers in sludge by an average of 94.4% (1.97 log_{10}) after 15 to 20 days (Pepper et al, 2010). At a facility in California, it was found that that mesophilic digestion reduced enteric virus levels by approximately 1.1 log_{10} in 20 days, whereas thermophilic digestion reduced viruses by an average of 3.38 log_{10}, although no virus could be detected in over half of the samples (Berg & Berman, 1980). A similar study in France showed enteric viruses to be reduced by 87.5% (Monpoeho et al, 2004).

In a laboratory study, poliovirus 1 was reduced by 98.8% during mesophilic digestion within 48 hours (Bertucci et al, 1977). The reduction for poliovirus 1 observed after thermophilic digestion in this study was also similar to a previous laboratory-based study (99.8%) (Aitken et al, 2005). In another study where laboratory grown viruses were placed in filter sandwiches and added to an sludge digesters, Coxsackievirus B5 was reduced by 0.475 log_{10}/day and human rotavirus strain Wa by 0.314 log_{10}/day during mesophilic anaerobic digestion (Spillman et al, 1987). At thermophilic temperatures inactivation rates were >8.5 log_{10}/hour.
Overall, based on the literature and the current study, it can be expected that viruses can be reduced by approximately 99% (2 log_{10}) during mesophilic digestion, and greater than 99.99% (>4 log_{10}) by thermophilic digestion. The results also suggest that lipid viruses, like Φ6 are very sensitive to inactivation by mesophilic and thermophilic digestion. If this is representative of the behavior of lipid containing animal viruses like EBV then they are likely to be reduced to a greater degree than the non-lipid human enteric viruses.
Acknowledgements

The authors would like to thank the Tres Rios Water Reclamation Facility for providing primary and waste-activated sludge samples. They would also like to thank Dr. Krista Wigginton and Yinyin Ye (PhD candidate) at the University of Michigan for providing Φ6 and *Pseudomonas syringae* for use in this study.
REFERENCES


# TABLES 1-2

*Table 1: Influence of Mesophilic Digestion on Viruses per mL*

<table>
<thead>
<tr>
<th>Virus</th>
<th>Initial Concentration</th>
<th>Concentration after Digestion</th>
<th>Log10 Reduction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2</td>
<td>6.38E+08 ± 2.83E+08</td>
<td>152 ± 30</td>
<td>6.6 ± 0.11</td>
<td>0.0001</td>
</tr>
<tr>
<td>Adenovirus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenovirus 4</td>
<td>2.13E+05 ± 2.38E+05</td>
<td>917 ± 574</td>
<td>2 ± 0.71</td>
<td>0.0387</td>
</tr>
<tr>
<td>Poliovirus</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Poliovirus 1</td>
<td>3.47E+07 ± 1.20E+07</td>
<td>1.47E+05 ± 1.05E+05</td>
<td>1.8 ± 0.15</td>
<td>0.0012</td>
</tr>
<tr>
<td>F6</td>
<td>1.13E+06 ± 7.69E+05</td>
<td>&lt;0.33</td>
<td>&gt;5.9</td>
<td>0.0029</td>
</tr>
<tr>
<td>Murine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>norovirus</td>
<td>4.06E+06 ± 1.20E+06</td>
<td>1.19E+04 ± 3.54E+03</td>
<td>2.2 ± 0.18</td>
<td>0.0016</td>
</tr>
</tbody>
</table>
Table 2 Influence of Thermophilic Digestion on Viruses per mL

<table>
<thead>
<tr>
<th>Virus</th>
<th>Initial Concentration</th>
<th>Concentration after Digestion</th>
<th>Log_{10} Reduction</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS2</td>
<td>6.38E+08 ± 2.83E+08</td>
<td>53 ± 17</td>
<td>7.1 ± 0.14</td>
<td>0.0001</td>
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<td>Adenovirus</td>
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<td></td>
</tr>
<tr>
<td>4</td>
<td>2.13E+05 ± 2.38E+05</td>
<td>&lt;316</td>
<td>&gt;2.8</td>
<td>0.0399</td>
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<tr>
<td>Poliovirus</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>3.47E+07 ± 1.20E+07</td>
<td>757 ± 237</td>
<td>4.6 ± 0.29</td>
<td>0.0013</td>
</tr>
<tr>
<td>Φ 6</td>
<td>1.13E+06 ± 7.69E+05</td>
<td>&lt;0.33</td>
<td>&gt;5.9</td>
<td>0.0029</td>
</tr>
<tr>
<td>Murine norovirus</td>
<td>4.06E+06 ± 1.20E+06</td>
<td>&lt;316</td>
<td>&gt;4.1</td>
<td>0.0004</td>
</tr>
</tbody>
</table>