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Validation of Viral Inactivation by a Heat Inactivation Effluent Discharge System in a High Containment Research Laboratory Using Viral Surrogates Phi6 and MS2

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By

Michael Ranjan Kshatri

April 23rd, 2018

Abstract

The 2014 outbreak of Ebola increased the need for many types of research involving work with infectious Ebola virus. Due to the high mortality rate and limited treatment options, Ebola can only be handled at the highest level of laboratory containment, biosafety level (BSL) 4. One of the requirements of a BSL4 laboratory is that liquids from sinks, drains, and other liquid disposal systems cannot be discharged directly into the sanitary sewer. One option for such laboratories is an effluent discharge system (EDS) where effluent is held in a tank at high temperature for a period of time to inactivate microbes before being disposed of in the sanitary sewer. These systems must be validated to ensure they can inactivate viruses like Ebola before disposal. Surrogate viruses Phi6 and MS2 were used to serve as models for the behavior of human viruses. These viruses were used to determine effective time and temperature combinations for an EDS. In this project, Phi6 and MS2 were suspended in water with protein added and were exposed to temperatures of 70°C and 90°C at time points 0 seconds, 30 seconds, 1 minute, 5 minutes, 6 minutes, 7 minutes, and 10 minutes to mimic an EDS. An inactivation curve was developed using the double agar layer technique to determine different log reductions over time so that protocols may be established for the EDS. Based on the results, it would be recommended to run the EDS at these temperatures for 10 minutes or longer to ensure complete inactivation.

Validation of viral inactivation by a heat inactivation Effluent Discharge System in a high containment research laboratory using viral surrogates Phi6 and MS2

by

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APPROVAL PAGE

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Acknowledgments

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Introduction

Biosafety Level Four (BSL4) laboratories are both intense and stressful environments, however they are integral to conducting research on dangerous bacteria and viruses. In 2014, the world saw the outbreak of Ebola—a devastating virus that attacked numerous communities all around the world. The hardest hit areas included countries such as Sierra Leone and Liberia. Ebola is an enveloped filovirus that infects humans through bodily fluids such as blood (Bibby et al, 2017). Once the first cases were identified, it became necessary to investigate ways to contain and treat the virus due to its high virulence. Moreover, it was found that Ebola could survive for around eight days in wastewater (de Carvalho et al., 2017). If Ebola could persist in the environment and in wastewater, methods to completely inactivate the virus in these media are needed.

Various experiments have been done to determine effective protocols to inactivate enveloped viruses such as Ebola. Examples include chlorine procedures using surrogates, or viruses used to model Ebola, where viral particles were inactivated within 15 minutes of exposure to chlorine (Kosel et al., 2017). However, it is complex to establish and maintain the kinetics required for efficient inactivation of viruses using chlorine. Tests performed with ultraviolet (UV) radiation have inactivated the virus by damaging the RNA and the bonds between nucleic acids, thus reducing infectivity (Beck et al., 2016). Despite the successful inactivation rates, UV radiation is energy intensive and like chlorine, is difficult to keep the kinetics stable.

Although there are effective treatments, methods like exposure to chlorine and radiation can have drawbacks such as high cost, creating hazardous byproducts, requiring long

contact times, and also needing a large amount of training. With all these methods available, it is necessary to determine what treatment is best to follow. One method that has been explored is the use of an effluent discharge system (EDS) to inactivate viruses so that they may be ready for disposal into the regular wastewater stream. Validating these systems are important, but it is still a question if surrogates are a good way to show they work. As a result, this project was designed to validate an effluent discharge system that will be used at Georgia State University's BSL4 lab. The system was verified by using surrogates Phi6 and MS2 and exposing the viruses to $90^{\circ}C$ at time points 0 seconds, 30 seconds, 1 minute, 5 minutes, and 10 minutes to mimic the conditions of the EDS. Using these surrogates, an optimal time of exposure in the EDS can be determined. With this data, systems similar to the effluent discharge system used could develop protocols to be implemented in other facilities. Training programs could be developed to prepare individuals to work with the EDS; in turn, if another outbreak of Ebola occurs, proper inactivation of the virus can be done using an effluent discharge system to improve treatment in facilities and prevent further infections.

Working with Ebola requires access to a BSL4 laboratory and careful handling. To test new methods like the use of an EDS, surrogates can be used to model the Ebola virus. In this case, MS2 and Phi6 were chosen to model the Ebola virus. The two viruses were chosen due to being able to be handled in a BSL1 or BSL2 laboratories and can easily be propagated and reproduced. In conjunction, Phi6 was chosen due to having an enveloped virus similar to Ebola (de Carvalho et al., 2017). Like Ebola, Phi6 also inserts its membrane protein into the host and can spread its genetic information into the cytoplasm of the host (Laurinavičius et al., 2004). As a result, it can be concluded that what happens to Phi6 during the inactivation experiments can

help us understand what would happen to Ebola. Furthermore, enveloped viruses react differently from non-enveloped viruses due to the lipid bilayer interacting with the surrounding environment and being more susceptible to disruptors such as heat and detergents (Yinyin et al., 2016). With that, it was also necessary to use a non-enveloped virus, in this case MS2, for these trials to determine how such viruses like MS2 would respond to the exposure to heat.

In terms of its similarity to Ebola, MS2 was chosen as a surrogate due to its genome also being composed of single-stranded RNA. Combined with Phi6, it is possible to use these surrogates to predict the rate of inactivation of Ebola if it was in the EDS.

Literature Review

2.1 Inactivation of Bacteriophages with Chlorine Solutions

When examining the literature, there were numerous inactivation studies done on

viruses Phi6 and MS2 ranging from the use of UV radiation, ozone solutions, gamma irradiation,

hydrodynamic cavitation, hot bubble columns, exposure to nanoparticles, and even creating

solutions with different fatty acids. Of the examined studies, one of the most widely

investigated techniques involved the use of chlorine compounds. Gallandat and Lantagne

(2017) investigated surface disinfection using MS2, Phi6, M13, and PR772 to model Ebola. Each

of these viruses were exposed to 0.1% and 0.5% sodium hypochlorite (NaOCl) for 1, 5, and 10

minutes on stainless steel. It was hypothesized that the higher concentration of NaOCl would

have the higher rate of inactivation and Phi6 would be more susceptible to the solution

(Gallandat and Lantagne, 2017). After exposure, the virus samples were then plated using the

double agar layer procedure. For MS2, there was a 3.4 log reduction after ten minutes to the

0.5% concentration of NaOCl while Phi6 was not detected at all after ten minutes. A reason that

Phi6 was seen to be completely inactivated was due to the fact that enveloped viruses like Phi6

and Ebola are extremely susceptible to chlorine (Gallandat and Lantagne, 2017). When chlorine

interacts with the lipid bilayer, it disrupts the hydrogen bonds that exist between the nucleic

acids.

In another experiment done by Gallandat, Lantagne, and Wolfe (2017), four different

chlorine types (Sodium hypochlorite, sodium dichloroisocyanurate, high-test hypochlorite, and

generated hypochlorite) were used to measure disinfection of Phi6 on three different surface

types (stainless steel, heavy-duty tarp, and nitrile). After exposure, virus samples were plated

using the double agar layer technique. In all tests, less than 8 PFUs/cm^2 were seen after

exposure to compounds containing 0.5% chlorine (Gallandat, Wolfe, Lantagne, 2017). An issue

seen in these experiments was that if the surface was wiped to remove any remaining virus and

solution, there was a possibility for the spread of infection. Although chlorine was effective in

this experiment and in the test done with MS2 in the previously described study, chlorine

generates harmful mutagenic byproducts (Kosel et al., 2017). If the virus of interest is

inactivated but poses another threat to a researcher or healthcare employee, it can be

troublesome to properly dispose of the biohazard waste. Moreover, if concentrations of

chlorine are made inaccurately, then inactivation times can vary and thus may not fully

inactivate the viruses in waste.

Moreover, two studies done by Wolfe et al. (2017) and Wolfe et al. (2016) looked at if

washing one's hands with soap or with chlorine was most effective at inactivating Phi6.

Individuals were recruited to see how they would feel after washing their hands with various

solutions. After washing, the liquid runoff was obtained in a collection bag and then plated

using the double agar layer technique. With soap, Phi6 saw an average log reduction of 2.76

without a soil load on one's hands in comparison to an average of 3.2 with a soil load (Wolfe et

al., 2017). Chlorine was seen to inactivate Phi6 completely after a long period of exposure. Even

though tap water showed some log reduction, it is not completely effective to inactivate

viruses. Furthermore, the new waste created will still have active viral particles and could lead

to further infections—washing one's hands will remove the virus from the individual's hands

but will create a new environmental hazard. In Wolfe et al. (2016), participants did report that

chlorine was effective, but that the communities felt uncomfortable using the chlorine

solutions. In conjunction, soap is always available but in rural communities or in arid areas, water may not always be available to use. Lastly, it was reported in Wolfe et al. (2016) that a chlorine sanitizer would also be difficult to distribute to communities. In this study, it is important to note that the best method to inactivate viruses and keep communities safe may not be culturally acceptable to those using the technique.

2.2 Ultraviolet and Radiation Inactivation Procedures

Another method investigated in the literature involved UV-induced inactivation using MS2 as a surrogate virus. In many water treatment plants, especially those in the United States, UV irradiation is widely used to disinfect the water supply and make it safe for consumption. In one study, it was hypothesized that the rates of RNA damage would mimic the loss of viral activity due to exposure to UV light. UV irradiation would be an effective inactivation technique due to the UV light breaking bonds and creating lesions in the virus's RNA code, hence rendering the virus unable to infect the host (Beck et al., 2016). In Beck et al. (2016), MS2 was suspended in 1X PBS and was exposed to UV light. After the UV light was shot through the virus sample, it was found that the nucleic acids were indeed mutated and that the capsid surrounding the core was damaged, thus rendering the virus inactive.

Continuing with UV methods, Rattanakul and Oguma (2017) examined how UV and chlorine could inactivate the MS2 virus. To see what worked best to inactive the virus, the team examined inactivation through UV irradiation, chlorination, and a combination of the two. Afterwards, the MS2 sample was then incubated and grown using the double agar layer technique. Along with that, RT-qPCR assays, virus attachment assays, and virus genome penetration assays were performed to determine what percent of virus was inactivated

(Rattanakul and Oguma, 2017). Among the three techniques, UV and chlorination treatment showed the highest amount of virus inactivation at 86%. Despite this being an effective method, it is not entirely efficient due to requiring a myriad of different procedures to inactive the virus. Overall, the technique is time-consuming and involves careful monitoring to ensure that enough of the virus is inactivated. Even then though, based on the results there is not a complete inactivation of MS2 using the techniques described.

While UV radiation and chlorine are ways to inactivate viral particles, Wu et al. (2018) examined the ability of reactive oxygen species (ROS) and di-valent cations in different acidic and basic environments to inactivate MS2. MS2 was placed in a solution of cations and exposed to sunlight through direct or indirect endogenous inactivation or indirect exogenous inactivation (Wu et al., 2018). Once the virus was exposed, then the double agar layer assay was used to count plaque forming units. A slightly more acidic pH of 4 was found to cause higher inactivation rates while pH values of 5 to 8 reduced the rate of inactivation (Wu et a., 2018). As a result, it is possible to conclude that the environment surrounding the virus has an effect on the effectiveness of inactivating the virus. In comparison to inactivating viruses with chlorine, UV irradiation was found to produce less toxic byproducts (Wu et al., 2018). Although UV irradiation may be somewhat safer for biohazard disposal, this process is quite expensive due to the high costs of maintaining the technology used and the amount of training required to run the whole procedure. Not every healthcare facility or community is going to have the resources necessary to carry out this inactivation method.

Majiya et al. (2018) looks at another protocol in photodynamic inactivation (PDI) of bacteriophage MS2. In this study, the team actually uses PDI to target the A-protein and render

it ineffective to inactivate the virus. To combine with the solution, tetrakis porphyrin tetra p-toluenesulfonate (TMPyP) was added to allow smooth reactions for the singlet oxidation (Majiya et al., 2018). The team describes how singlet oxidation diminishes the infectivity of the virus by damaging the A-protein and altering the alpha-helix and beta-sheet domain that makes up MS2. Photodynamic Inactivation was done here by combining TMPyP with 0.1 μM to 50μM in solution. Afterwards, the double agar layer assay was performed to see the titers of MS2 obtained. Along with that, virus samples pre-PDI and post-PDI were analyzed using SDS-PAGE and immunoblotted to determine how much of the virus was inactivated. When 0.2 μM of solution was used, there was a 9.5 log reduction PFU/mL within 60 seconds when the solution was illuminated at 32 milliwatts per centimeter (Majiya et al., 2018). At other time points, there was a log reduction of 1.5 PFU/mL in 10 seconds and 4.0 log reductions within 30 seconds. Based on these results, the method proposed by Majiya et al. (2018) is extremely effective at inactivating MS2. However, the use of this procedure requires a highly sophisticated lab and numerous complex solutions and light resources to conduct. Even though this is effective, it may not be practical in all communities or healthcare facilities that may be used to handle an outbreak of Ebola.

Another method that has been explored involves a natural circulation solar thermal system in Manfrida, Petela, and Rossi (2017). In third world countries, solar-powered systems are being developed and used for the disinfection of water. The system described by Manfrida, Petela, and Rossi is driven by buoyancy. Water gets heated and then is sent to a discharge pipe to be reused and cleaned again. To run the apparatus, reverse osmosis and ultrafiltrification is done through high-pressure pumps. The heat from the sun will then be used to heat the water

inside the collector. This system described by the team is quite practical due to the use of natural resources and water. However, reverse osmosis makes this highly expensive due to needing water that is extremely pure and also requiring intense maintenance for the membranes that will be used for reverse osmosis. Despite the high cost though, this system could be developed further and implemented into communities that could use this for healthcare research and viral treatment.

2.3 Inactivation Methods Involving Titanium Oxide or Ozone

Besides the use of UV radiation and chlorine in treating water waste, titanium oxide is also widely used for wastewater treatment. In Syngouna and Chrysikopoulos (2017), inactivation tests were done with MS2 at room temperature using titanium oxides in the presence and absence of quartz sand along with and without ambient light. Three sets of experiments were done in PBS and one in deionized water. MS2 inactivation was carried out in 50-mL glass reactor tubes by diluting MS2 stock solution in the container; PBS, quartz sand, and light (other trials used sand and no light or no sand and light) were then added and run for three different concentrations of MS2. When the titanium oxides started to interact with the different environments, hydroxyl free radicals and hydrogen peroxide were generated (Syngouna and Chrysikopoulos, 2017). At the end of the trials, titanium nanoparticles were determined to inactivate MS2 with or without light. However, the rates of inactivation varied greatly and would require more research to see if this method is capable of consistently inactivating all of the virus titer present.

Along with titanium oxide, chlorine, and UV light, ozone is used in water treatment plants to remove any pathogens. One study looked at how ozone could be used to inactivate

MS2 due to ozone's strong oxidizing capacity and ability to disrupt viral structure (Cai et al.,

2014). Cai et al. (2014) had starting titers of 5.0 x 10^{10} and 3.0 x 10^{11} and were placed in water

samples. The experiments were done in 2000 mL batch reactors and were temperature

controlled with a water bath. Virus sample was then plated using the double agar layer assay.

Results showed that when 0.15 mg/L of ozone were used, MS2 titers had a 1.75 log reduction

over 60 seconds. When a higher concentration of ozone was used though, such as one trial

using 1.5 mg/L, there was an 8-log reduction of MS2 within 10 seconds. At lower temperatures,

the team reports that inactivation rates are lowered while higher temperatures increased the

rate of inactivation (Cai et al., 2014). Overall though, in this study ozone was shown to

effectively inactivate ozone through the use of a high concentration of ozone at a high enough

temperature to remove infectivity of the virus.

2.4 Inactivation Method through Fatty Acids

An interesting procedure that has been studied to inactivate viruses like Phi6 involves

the use of fatty acids. Sands (1977) hypothesized that oleic acid (18:1) and palmitoleic acid

(16:1) would completely inactivate Phi6. Treatment with either fatty acid was done with 50

µg/mL at 25 or zero degrees Celsius for 30 minutes. Viral solutions were then plated using the

double agar layer technique. Results showed that less than 1% of initial titer was present after

30 minutes. Ultracentrifugation with oleic acid eliminated most of Phi6 and was shown to be

the most effective fatty acid. In another study, Sands et al. (1978) looked at dicaprylin and used

similar methods to see how the long-chain monogylceride would interact with Phi6. Similar

results were obtained due to the fatty acid inactivating the virus by altering the structure of the

bacteriophage's surface (Sands et al., 1978). Although interesting, thirty minutes is quite long to

inactivate the virus if such a method is being used in a fast-paced environment like a hospital or clinic.

2.5 Inactivation by Hydrodynamic Cavitation and a Hot Bubble Column Evaporator

When investigating the literature, two articles found used water as a way of inactivating viruses, albeit not using an effluent discharge system. Kosel et al. (2017) looked at how hydrodynamic cavitation could be used to inactivate MS2. Cavitation is defined as the "appearance of vapor cavities inside an initially homogenous liquid medium and occur is the pressure is lowered below vapor pressure" (Kosel et al., 2017, p. 465). The medium in this case was created with water and some UV and hydrogen peroxide that worked in reducing pressure using the Venturi effect. After the media went through the device, hydrodynamic cavitation was found to cause damage to the capsid protein, A-protein, and damaged the host receptors that allow MS2 to bind to the host (Kosel et al., 2017). Although effective at diminishing the infectivity of MS2, it is an extremely complicated process that involves careful monitoring of vapor pressure, maintaining a stable temperature, and ensuring that all the liquid that goes through is properly disinfected and disposed.

Lastly, a study conducted by Garrido, Pashley, and Ninham (2017) looked at inactivating MS2 through the use of a hot bubble column evaporator (HBCE). The way this apparatus works is by heating up the column to 150°C. Once heat is supplied, room temperature air is pumped through the silica gel and then an electric heater stabilizes the temperature inside the column. Charge is also stabilized by using NaCl and allows water bubble to form. When the inner environment is stabilized, the hot air bubbles collide with viral particles and inactivate MS2 (Garrido, Pashley, and Ninham, 2017). At these conditions, the team found that MS2 is

inactivated within minutes by plating the residual liquid using the double agar layer assay.

Similar to the hydrodynamic cavitation study, this method is effective, but quite complex, time-consuming, and not entirely practical. Furthermore, it is quite costly and involves controlling numerous variables to allow the inactivation process to take place.

Methodology

        Bacteriophage MS2 and hosts *Pseudomonas syringae* and F. amp *Escherichia coli* were

obtained from stocks created previously in the laboratory. Bacteriophage Phi6 was propagated

in September 2017. To create the stock, one milliliter of P. *syringae* was placed in a flask with

100 milliliters of tryptic soy broth (TSB). The solution was then incubated at 22°C for 20 hours.

Following the incubation period, 0.5 mL of Phi6 was then added to the flask and incubated at

22°C for 20 hours to allow the virus to infect the host. Afterwards, the infected host was

removed. Sloppy agar was made from 30 grams of tryptic soy agar (TSA), one liter of distilled

water, and 3.75 grams of bacto agar. The mixture was then autoclaved on the liquid cycle for 45

minutes. To grow the virus to be propagated, 50 mL of TSA sloppy agar, 5 mL of host, and 5 mL

of ϕ6 were plated on 100 mL agar plates and incubated at room temperature.

        After the incubation period, the sloppy agar layer was removed and placed into 10

individual 50 mL centrifuge tubes. Two thirty-minute centrifuge cycles were done at 4°C. The

first cycle was run at 2500 rpm while the second was run at 3500 rpm so that all of the virus

supernatant could be collected. With this process, the initial titer of Phi6 came out to be 1.0 x

10^{10} PFU. The virus supernatant was then placed into 1 mL cryotubes and stored at -80°C for

future use.

        The EDS in Petit Science Center is designed where viral effluent from the Ebola BSL4 lab

is filtered down into the EDS where the biohazard is then exposed to the high temperatures. To

validate the EDS, surrogates MS2 and Phi6 were used to model the inactivation curve. At the

beginning of each trial, 1 mL of propagated *P. syringae* was placed in 50 mL of TSB. The host

was then incubated at 22°C for 20 hours. In another flask, a drop of F. amp E. coli was placed in

50 mL TSB and incubated at 37°C at 100 rpm for 20 hours.

After the incubation period, 5 mL aliquots of *P. syringae* were put into sterile 15 mL

conical tubes. 0.5 mL of the F. amp *E. coli* overnight culture was put into 50 mL fresh TSB and

incubated for two hours so that the host could be in its exponential phase when performing the

double agar layer plaque assay. To prepare the virus sample that would be exposed to the EDS,

10 mL of 1X Phosphate Buffered Saline was placed into a 15 mL conical tube along with 120 μL

of fetal bovine serum (FBS) and 100μL of Phi6. This solution was made to mimic the effluent

flowing from the laboratory plumbing into the EDS, which would normally consist of water with

a small amount of protein content due to very large dilution effects. Prior to adding in MS2, the

virus titer was diluted by one so that the MS2 titer being added was also 1.0×10^{10} PFU. Upon

mixing the solution both viruses would have a starting titer of 1.0×10^8. A separate tube

containing only the 1X PBS and FBS was made to serve as a time and temperature control.

From the virus solution, 1 mL was placed into 6 separate 1.5 mL centrifuge test tubes.

The virus samples were then put in the EDS well, which is a cylindrical container connected to

the EDS main chamber (Figure 2.1) that is used to validate the system and exposed to water at

90°C. The initial trial tested time points 0 seconds, 5 minutes, and 10 minutes (the normal cycle

of the EDS is 93°C for 60 minutes). The following three trials then looked at time points 0

seconds, 30 seconds, 1 minute, and 5 minutes. A 10-fold dilution series was then performed on

the virus samples for each time point to be plated using the double agar layer technique. 5 mL

of top agar (0.75%), 1 mL of P. syringae (for Phi6) or 1 mL (for MS2), and the mL dilution of virus

sample were plated on 1.5% bottom agar. Plates containing Phi6 were incubated at 22°C for 24

hours while plates containing MS2 were incubated at 35°C for 24 hours. For the trials with time points 0 seconds, 30 seconds, 1 minute, and 5 minutes, only dilutions 5, 4, 3, 2, and 1 were plated for 0 seconds and 5 minutes while all dilutions were plated for time points 30 seconds and 1 minute.
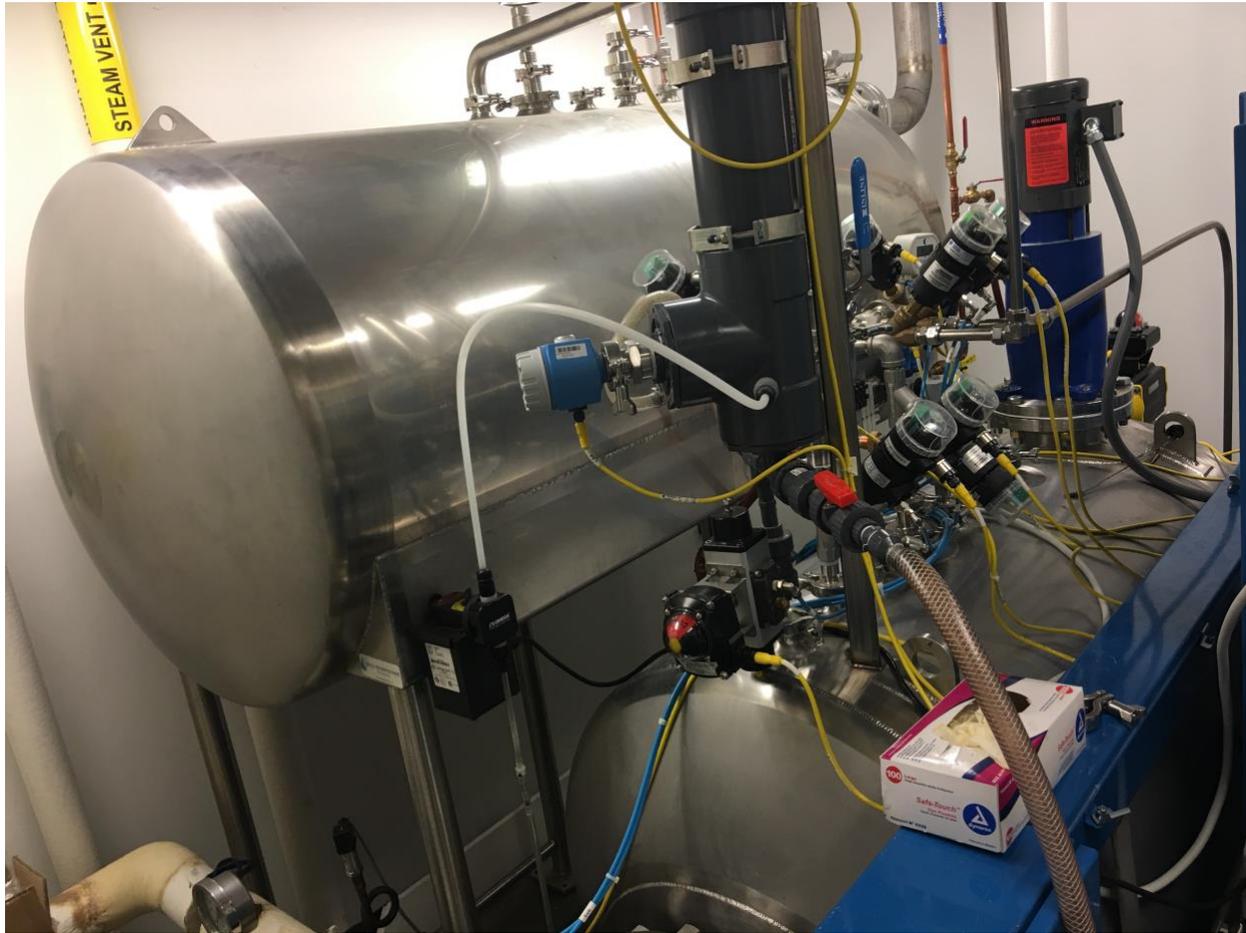
Figure 2.1 Effluent Discharge System

To test the inactivation rate for viruses exposed to water at 70°C, a similar procedure was used. However, these trials were performed using the 8.5 Quart Temperature Controlled Sous Vide circulating water bath (Chef Series Stainless Steel, manufactured by Dash), because the temperature on the EDS cannot be easily changed for experimental purposes. Prior to each trial, the water was brought to the designated temperature and held constant while the test

tubes were placed into the water bath. The first initial trial tested 0 seconds, 30 seconds, 1 minute, and 5 minutes. Two trials were then conducted at 0 seconds, 1 minute, 5 minutes, and 6 minutes. A final trial was performed at 0 seconds, 5 minutes, 6 minutes, and 7 minutes to determine if the 8-log reduction occurred at 7 minutes. Once the viruses were exposed to the water for the desired contact time, 10-fold dilution series were performed on each time point using the same double agar layer technique done for the 90°C tests.

3.1 Data Analysis

After incubation, plaque forming units (PFU) were measured in PFU/mL. Average virus concentration and log reduction were calculated using Microsoft Excel. Inactivation was expressed as $\log_{10}(N_t/N_o)$, where N_o is the virus titer in PFU/mL at time 0 and N_t is the virus titer in PFU/mL at time t. To obtain the slipe of the inactivation curve, $\log_{10}(N_t/N_o)$ was plotted against time and linear regression was done using GraphPad Prism Version 7.00.

Results

Experiments were conducted to evaluate the inactivation kinetics of an EDS run at two possible temperatures—70°C and 90°C. The time points evaluated at 70°C were 0 seconds, 30 seconds, 1 minute, 5 minutes, 6 minutes, and 7 minutes. As the amount of time viruses were exposed to the temperature increased, the rate of inactivation followed a linear trend. As a result, a linear regression was performed to determine the rate of inactivation for both viruses seen in Figure 1. The slopes of lines for both viruses represent the rate of inactivation. Based on the data, Phi6 had a faster rate of inactivation at 1.19 $\log_{10}$ per minute (95% CI -1.604, -0.7831) compared to MS2 at 1.135 $\log_{10}$ per minute (95% CI -1.387, -0.8824). Using their respective slopes, it was possible to construct table 4.1 to determine the amount of time it takes to obtain different log reductions. At this temperature, both MS2 and Phi6 reached an 8-log reduction after 7 minutes, as expected at high temperature. Using the linear regression, it was confirmed that MS2 and Phi6 inactivation rates are statistically different with a p-value less than 0.05.
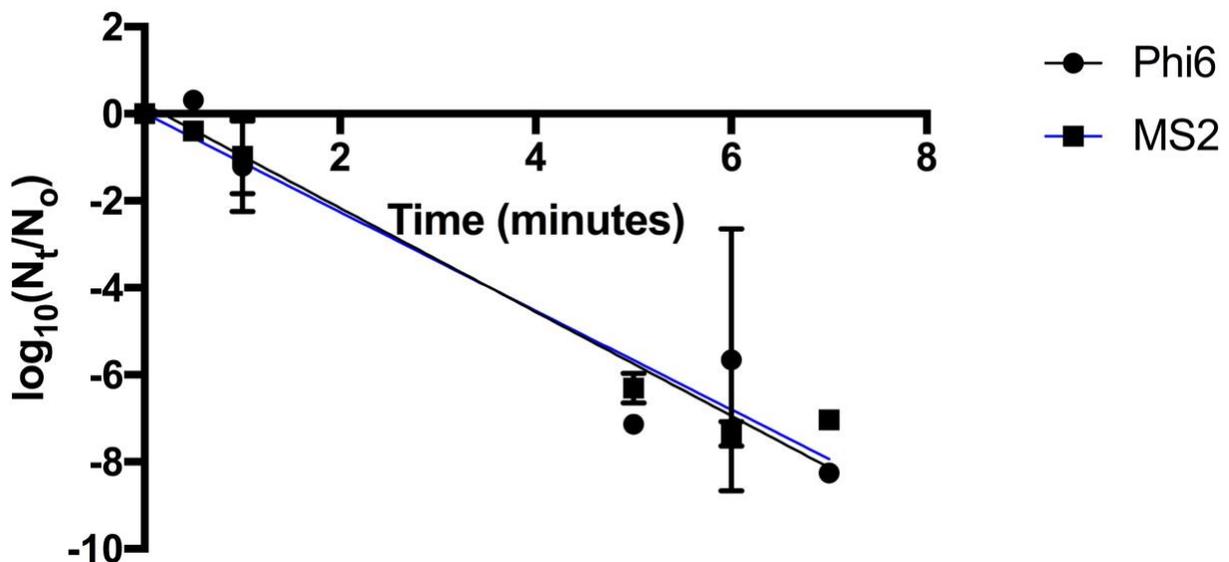
Figure 4.1 Inactivation of Phi6 and MS2 vs time at 70°C

Table 4.1 Time required to achieve specific log reductions for MS2 and Phi6 at 70°C

	1-log	2-log	3-log	4-log	5-log	6-log	7-log	8-log
Phi6	1.00 minute	1.86 minutes	2.70 minutes	3.53 minutes	4.37 minutes	5.20 minutes	6.05 minutes	6.89 minutes
MS2	0.89 minutes	1.77 minutes	2.65 minutes	3.53 minutes	4.41 minutes	5.30 minutes	6.18 minutes	7.06 minutes

At 90°C, time points tested included 0 seconds, 30 seconds, 1 minute, 5 minutes, and 10 minutes. In comparison to the 70°C exposure, the rate of inactivation was much faster. Viruses declined at a relatively steep rate within the first minute of exposure as seen in Figure 4.2, which are the results of one preliminary trial to determine what the kinetics might look like. A linear regression was performed on the data between 1 minute and 10 minutes to determine the rate of inactivation. Based on the data, Phi6 was inactivated at 0.6731 $\log_{10}$ per minute (95% CI -2.269, 0.9229) while MS2 had a rate of inactivation at 0.6363 $\log_{10}$ per minute (95% CI -2.029, 0.7565). However, there was one outlier in trial 1 at the 10 minutes that was likely a random contaminant. In trial 1, 5 minutes and 10 minutes were done to see when the detection limit of the experiment was reached. Since 10 minutes inactivated all virus, subsequent trials were done using 0 seconds, 30 seconds, 1 minute, and 5 minutes to determine when exactly the 8-log reduction occurred as seen in Table 4.2. Based on the linear regression though, the MS2 and Phi6 inactivation rates did not appear statistically different by having a p-value at 0.2419.
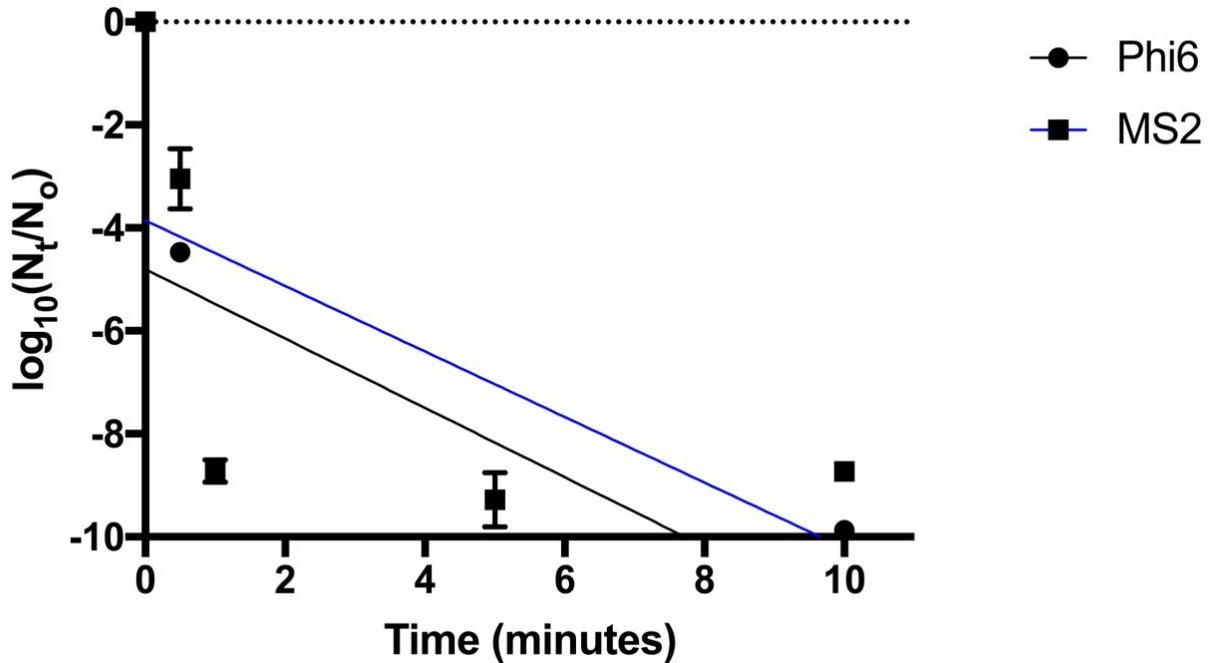
Figure 4.2 Inactivation of Phi6 and MS2 vs time at 90°C

Table 4.2 Time required to achieve specific log reductions for MS2 and Phi6 at 90°C

	1-log	2-log	3-log	4-log	5-log	6-log	7-log	8-log
Phi6	-5.64 minutes	-4.15 minutes	-2.67 minutes	-1.19 minutes	0.29 minutes	1.78 minutes	3.27 minutes	4.75 minutes
MS2	-4.48 minutes	-2.91 minutes	-1.34 minutes	0.24 minutes	1.89 minutes	3.38 minutes	4.49 minutes	6.52 minutes

Because of the possible outlier in the first trial, and because the result at 10 minutes was potentially a contaminant, three replicates of the experiment were graphed using time points 0 seconds, 30 seconds, 1 minute, and 5 minutes. Linear regression and showed a rate of inactivation of 1.349 $\log_{10}$ per minute for Phi6 (95% CI -4.988, 2.29) and 1.224 $\log_{10}$ per minute (95% CI -4.461, 2.012) for MS2, faster than the rates at 70°C. In this case, 5 minutes was sufficient to achieve an 8-log reduction for Phi6 while MS2 required slightly more than 5 minutes seen in Table 4.3. Overall, it makes very little difference in the overall inactivation

kinetics because they are so rapid at this high temperature. Similar to the 90ºC experiments,

Phi6 and MS2 inactivation rates were not statistically different with a p-value at 0.2518.
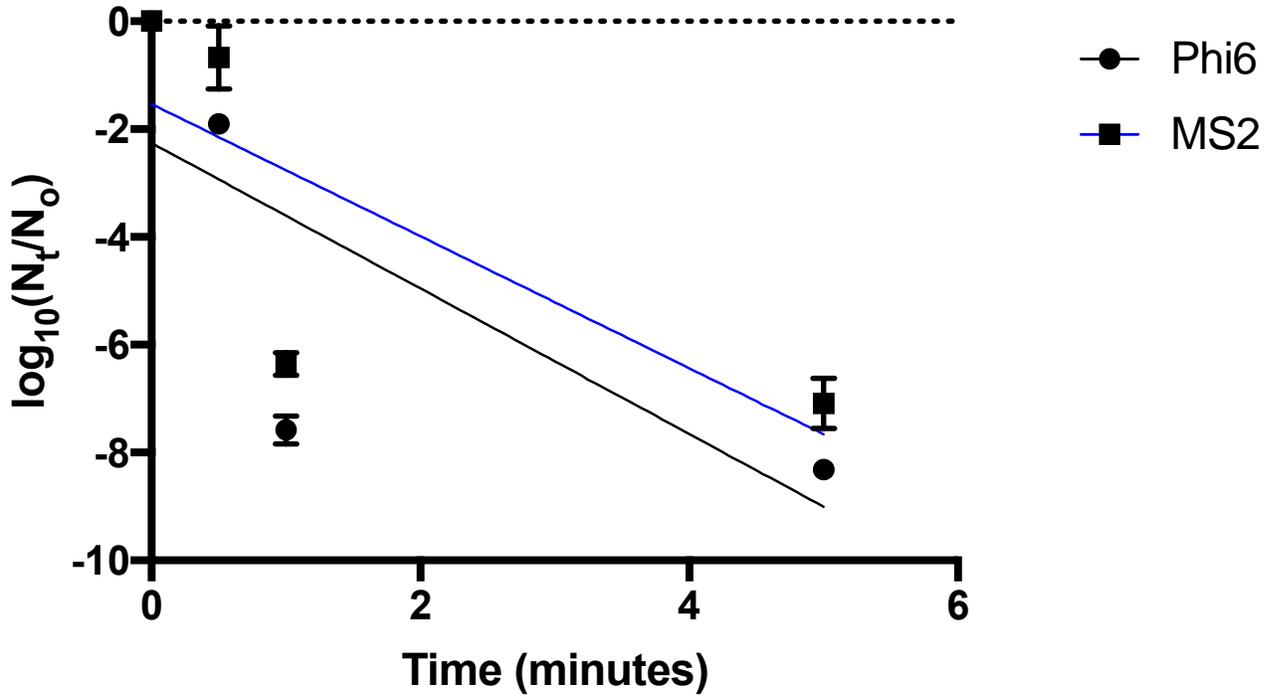
Figure 4.3 Inactivation of Phi6 and MS2 vs time at 90ºC excluding trial 1 outlier

Table 4.3 Time required to achieve specific log reductions for MS2 and Phi6 at 90ºC excluding

trial 1 outlier

	1-log	2-log	3-log	4-log	5-log	6-log	7-log	8-log
Phi6	-0.93 minutes	-0.19 minutes	0.55 minutes	1.29 minutes	2.03 minutes	2.77 minutes	3.52 minutes	4.26 minutes
MS2	-0.44 minutes	0.38 minutes	1.19 minutes	2.01 minutes	2.83 minutes	3.65 minutes	4.46 minutes	5.28 minutes

Discussion

At 70°C, inactivation for both viruses followed a linear trend with Phi6 reaching 8 log inactivation in all three trials prior to 7 minutes while MS2 required 7.06 minutes to each 8 log inactivation. At 90°C, both viruses were inactivated quickly, with Phi6 reaching 8 log reduction in less than 5 minutes while MS2 required 6.52 minutes. However, when comparing the rates of reaction at the different temperatures, it was seen that the 70°C experiments had the higher rate of inactivation. This is counterintuitive to other studies showing that the persistence of Phi6 and MS2 is impaired at a higher temperature such as in the inactivation study with ozone (Cai et al., 2014). When examining the data, the first trial at 90°C likely had a contaminant at 10 minutes; when it is not included, the rate of inactivation was faster at 90°C than 70°C as expected.

When the data for both viruses are compared, it is consistently seen that MS2 required a slightly longer contact time, but not very long. A reason as to why MS2 may had been able to survive the designated time is due to its robustness. Robustness is defined as the variance in phenotype that occurs as a response to the environment and to promote survivability of the species (McBride, Ogbunugafor, and Turner, 2008). In the case of Phi6 and MS2, it is possible for RNA viruses to be altered through directional selection (McBride, Ogbunugafor, and Turner, 2008). Seen in the results, MS2 was able to survive longer in the EDS conditions and would not reach the 8-log inactivation until later. As a result, it would be recommended to run the EDS for about ten minutes to ensure that all infectious particles are completely inactivated.

Based on the results of these experiments, an EDS is an effective method of inactivating viruses and could be a protocol implemented in other laboratories, medical facilities, and clinics

in other countries to prevent another outbreak and reduce the risk of infection. During the outbreak, there was an incident in Dallas where an Ebola patient was admitted to the hospital to receive treatment. The hospital was not prepared to handle the hazardous waste generated from working with Ebola due to not having an on-site autoclave or biocontainment unit (Cummings et al., 2016). Furthermore, there was no incinerator present and there was no clear way to know if it was safe to handle biohazard waste. An EDS protocol to inactivate viral particles such as this one could be implemented for hospitals like the one in Dallas. Moreover, healthcare facilities in rural and suburban areas could also utilize an EDS due to the only main source of inactivation being water. Unlike chlorine solutions, there is no risk for the generation of mutagenic byproducts (Kosel et al., 2017). Once the viruses are exposed to the hot temperatures for the designated time, the effluent simply needs to be disposed of and fresh water can be added to the system for the next run. In comparison to the methods where photodynamic inactivation or disinfection through titanium oxide is performed, it is not a necessity to use expensive products or filters to inactivate infectious particles. For laboratories that run EDS systems, validation of their viral inactivation ability is required both for safety and for being in compliance with legal regulations. To do this validation with Ebola or other BSL4 viruses is not really practical or workable. This work also shows that validation to ensure that an EDS is working properly can be quickly and easily performed with surrogate viruses, making frequent validations practical and simple to make sure EDS setups are working as they should/

5.1 Limitations

For this study, one limitation is that only distilled water was used to validate the EDS. Other media sources that could be tested include tap water, sewer water, and blood to

investigate how the rate of inactivation for MS2 and Phi6 would be affected when subjected to either 70°C and 90°C.

5.2 Future Directions

To further test the efficiency of the effluent discharge system, different temperatures and time points could be chosen to mimic the system. At 90°C, more time points could be tested such as 15 seconds, 45 seconds, 2 minutes, and 3 minutes to construct a more linear curve and determine when exactly the rate of inactivation begins to plateau. In areas where it may not be possible to generate enough energy to consistently hold the water at a constant 70°C or 90°C, temperatures such as 50°C and 60°C could be performed at multiple different time points. With that in mind, future studies could be performed using these parameters to determine the rate of inactivation at these temperatures and see if the EDS is still effective at completely inactivating the surrogates used in conjunction with the double agar layer technique. Moreover, other pathogens could be subjected to similar conditions in the EDS to determine if the system can inactivate other viruses or bacteria rather than just Phi6, MS2, and Ebola.

5.3 Conclusion

The 2014 outbreak of Ebola increased the need for many types of research involving work with the infectious Ebola virus. However, Ebola is highly virulent, and research can only be conducted in a BSL4 laboratory. Liquids used in that type of lab cannot go into regular drains or sinks and must be properly sterilized before disposal. After seeing the destruction caused by the Ebola virus, it is important to have an effective protocol established for disposing of the virus and finding new treatments that can prevent future outbreaks and infections.

For this project, surrogates Phi6 and MS2 were used to validate an EDS that could be used in laboratories to conduct research and ensure that there is no risk of infection to the environment or individuals conducting research. Virus samples were exposed to two different temperatures, 70°C and 90°C to mimic conditions of the EDS. It was hypothesized that within 10 minutes there would be a complete inactivation of both viruses. After exposing the viruses to these temperatures, a 10-fold dilution series was performed using the double agar layer technique to determine the rate of inactivation. Based on the results, utilizing an EDS would be effective at eliminating viral particles and making it safe to dispose of the effluent without having the risk of infection. To further explore the capabilities of the EDS, more tests need to be done at different temperatures, time points, and in other media. However, as the need for research on novel and dangerous viruses increases, an EDS is bound to be an effective method at improving workplace safety and making it possible to carry out this research.

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