THE Water Research FOUNDATION

PROJECT NO.
5029

Environmental Persistence and Disinfection of Lassa Virus and SARS-CoV-2 to Protect Worker and Public Safety

Environmental Persistence and Disinfection of Lassa Virus and SARS-CoV-2 to Protect Worker and Public Safety

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2024

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WRF ISBN: 978-1-60573-682-2
WRF Project Number: 5029

Prepared by University of Notre Dame

Acknowledgments

The authors wish to thank The Water Research Foundation for funding this project work.

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Acronyms and Abbreviations

CPE	Cytopathic effect
CI	Confidence Interval
Ct	disinfection dose (mg/L) x contact time (min)
COVID-19	Coronavirus Disease 2019
DI	Deionized water
DMEM	Dulbecco's modified Eagle's medium
HDPE	High density polyethylene
HIV	Human immunodeficiency syndrome
LASV	Lassa virus
MGD	Million gallons per day
MHV	Mouse hepatitis virus
MLD	Million liters per day
PBS	Phosphate buffered saline
PFU	Plaque forming units
PPE	Personal protective equipment
RNA	Ribonucleic acid
RT	Room temperature
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
T_{90}	Time to 90% reduction in a pathogen of interest
TGEV	Transmissible gastroenteritis virus
$TCID_{50}$	Median tissue culture infectious dose
USEPA	United States Environmental Protection Agency
UV	Ultraviolet
WHO	World Health Organization
WRF	The Water Research Foundation
WW	Wastewater
WWTP	Wastewater treatment plant

Executive Summary

ES.1 Key Findings

- The persistence of Lassa virus (LASV) in wastewater is shorter than that of deionized (DI) water and similar to other enteric viruses.
- The tested LASV strains decay more rapidly on the surfaces of high density polyethylene (HDPE) and stainless steel than in aqueous solution.
- Infectious SARS-CoV-2 inactivates at a faster rate at higher temperatures than at lower temperatures in water and wastewater, and SARS-CoV-2 RNA persists longer than infectious SARS-CoV-2.
- SARS-CoV-2 and LASV are effectively disinfected in wastewater by chlorine and at a more rapid rate than other waterborne viruses of concern.
- Modeling of associated risk shows that the use of bleach pretreatment and personal protective equipment (PPE) result in the lowest risk for a sewer worker inhaling LASV impacted wastewater droplets.

ES.2 Background and Objectives

The overarching goal of this research was to help prepare the wastewater industry for future epidemic or pandemic outbreaks of highly infectious virus. Specifically, the research team assessed the persistence and disinfection of highly pathogenic emerging viruses on the World Health Organization (WHO) watch list: LASV and SARS-CoV-2, the latter being the causative agent of the COVID-19 pandemic. LASV was selected due to its immediate concern for outbreak potential, theoretical transmission via contaminated liquid waste, and physiological and taxonomic diversity that will facilitate extension to future outbreak events.

After the start of the COVID-19 pandemic, additional research was conducted on SARS-CoV-2. SARS-CoV-2 was found to be present in high concentrations of infected individuals, so understanding the persistence and inactivation with commonly used disinfectants in wastewater was critical to assure worker safety. The outcomes of this study look to improve wastewater industry outbreak preparedness and provide detailed guidance on appropriate wastewater management and disinfection practices. In addition to viral exposure and persistence work with LASV, this research effort also resulted in the development of a quantitative exposure model for worker safety and viral release that may be used by wastewater authorities in the event of a virus outbreak to respond in a timely manner to questions regarding worker and public safety.

ES.3 Project Approach

The persistence of LASV strains was assessed in DI water and wastewater and on representative surface materials, stainless steel and Tyvek® (i.e. HDPE). The persistence of viable SARS-CoV-2 and SARS-CoV-2 RNA was analyzed in water and wastewater at two different titers and three different temperatures. Disinfection with multiple sodium hypochlorite concentrations was done in water and wastewater for SARS-CoV-2: hCoV-19/USA/MD-HP01542/2021, B.1.351 and wastewater for LASV strains. Viral quantifications were based on viable virus using the median

infectious tissue culture method ($TCID_{50}$). An exposure and virus release model was developed for Lassa virus to inform workplace safety practices as well as public fears during an emerging virus outbreak. Viral persistence and disinfection experiments were conducted at Biosafety Level 4 (BSL4) at the National Institutes of Health (NIH) Rocky Mountain Laboratories. Data analysis and follow-up experimentation were completed at the University of Notre Dame. In addition to a final report, three journal publications were written, and the project provided training for an environmental engineering graduate student in wastewater disinfection.

ES.4 Results

LASV persistence was analyzed in an aqueous solution and on surfaces. LASV strains both persisted longer in DI water than in wastewater with the time for a 90% reduction (T_{90}) for DI water being 10.0 days (95% CI = 5.4 - 57.6 days) and 6.8 days (95% CI = 4.3 - 15.4 days) for Josiah and Sauerwald, respectively, and for wastewater being 1.8 days (95% CI = 1.4 – 2.4 days) and 1.3 days (95% CI = 1.0 – 1.5 days) for Josiah and Sauerwald, respectively. LASV strains had similar inactivation kinetics on HDPE and stainless steel. The T_{90} for HDPE was 0.5 days (95% CI = 0.45 – 0.66 days) and 1.0 days (95% CI = 0.56 – 4.6 days) for Josiah and Sauerwald, respectively. The T_{90} for stainless steel was 0.4 days (95% CI =0.3 – 0.5 days) and 0.9 days (95% CI = 0.7 – 1.2 days) for Josiah and Sauerwald, respectively.

SARS-CoV-2 persistence was analyzed in water and wastewater at high titer (~10^5 TCID 50 mL ^-1) and low titer (~10^3 TCID 50 mL^-1) and at three different temperatures with isolate nCoV-WA1-2020 (MN985325.1). The T_{90} for SARS-CoV-2 in wastewater for a high titer was 1.6 days (95% CI = 1.4 – 1.8 days) for room temperature, 15 minutes (95% CI = 14 – 17 min) for 50°C, and 2.2 minutes (95% CI = 1.8 – 2.9 min) for 70°C, showing that as temperature increases, infectious SARS-CoV-2 is inactivated at a faster rate. The T_{90} for wastewater with a low titer at room temperature was 2.1 days (95% CI = 1.6 – 3.3 days) and 26 days (95% CI = 9.8 – infinity) for infectious SARS-CoV-2 and SARS-CoV-2 RNA, respectively. In tap water with a high titer and at room temperature, the T_{90} was 2.0 days (95% CI = 1.8 – 2.2 days) for infectious SARS-CoV-2 and 33 days (95% CI = 9.9 – infinity) for SARS-CoV-2 RNA, showing the discrepancy between viable target decay and current molecular measurement methods.

Sodium hypochlorite was used to disinfect both LASV and SARS-CoV-2. The disinfection experiments for LASV showed sodium hypochlorite was effective at inactivating LASV and the Ct values to achieve a 3-log reduction of the Josiah and Sauerwald strains were 0.15 and 0.26 mg-min/L, respectively. Compared to other enveloped viruses, these Ct values are lower for LASV. SARS-CoV-2 was disinfected with sodium hypochlorite in DI water and wastewater. The results showed that less than 1 mg-min/L was needed to obtain a 3-log reduction in infectious SARS-CoV-2 in DI water compared to wastewater, which needed over a 5 mg-min/L Ct value. The Ct value for SARS-CoV-2 was lower than those for other enveloped (H5N1 and Poliovirus 1) and non-enveloped viruses (Human Rotavirus, Coxsackievirus B5, Echovirus E1, and Echovirus E12) of concern under similar conditions.

Modeling of LASV dose via inhalation for a worker on a sewer line showed that a combination of personal protective equipment and bleach was the most effective at reducing risk. A sensitivity analysis of parameters showed the time a worker is in proximity to the contaminated

wastewater had the strongest effect on the final LASV dose, followed by the respirable fraction, the patient excreta production into the sewer, and the inhalation rate of the sewer worker.

ES.5 Benefits

This research project provides critical data on the persistence of LASV in water and wastewater, on surfaces, and with the addition of sodium hypochlorite in wastewater. The persistence of SARS-CoV-2 in water and wastewater was evaluated at different titers and temperatures, and the inactivation was analyzed in wastewater with the addition of different sodium hypochlorite concentrations. A model was also produced to evaluate the risk a sewer worker experiences by working near a wastewater stream containing LASV. This research helps utilities and the overall water sector by providing the necessary Ct values to inactivate LASV and SARS-CoV-2 by 3-$\log_{10}$. By having the Ct values, the water sector can ensure worker and public safety by supplying enough chlorine disinfectant for the correct amount of time into the wastewater stream, which will also reduce the risk of exposure in environmental waters. Utilities and the water sector can use the model to determine the inhaled dose of LASV a worker is likely exposed to based on the user inputs for the number of patients infected with LASV, exposure time to the wastewater stream, and hospital effluent flowrate. The model also provides evidence that exposure to workers can be reduced by using a bleaching pretreatment, such as a chemical toilet or adding disinfectant before entering the municipality, and proper personal protective equipment, including goggles, a protective face mask or shield, waterproof gloves, liquid repellant coveralls, and rubber boots.

ES.6 Related WRF Research

- Understanding the Factors That Affect the Detection and Variability of SARS-CoV-2 in Wastewater (5093)
- Interlaboratory and Methods Assessment of the SARS-CoV-2 Genetic Signal in Wastewater (5089)
- The Use of Next Generation Sequencing (NGS) Technologies and Metagenomics Approaches to Evaluate Water and Wastewater Quality Monitoring and Treatment Technologies (4961)

CHAPTER 1

Lassa Virus and SARS-CoV-2 Environmental Persistence

1.1 Background

1.1.1 Lassa Virus

Lassa virus (LASV) is a single-stranded RNA enveloped virus of the family *Arenaviridae* first described in Nigeria in 1970 (Frame et al. 1970). LASV is the causative agent of Lassa hemorrhagic fever and is endemic in West Africa, causing approximately 300,000 illnesses and 3,000 deaths annually (Geisbert 2018). Notably, there has been a marked increase in LASV infections in recent years (Siddle et al. 2018). The median infectious dose of LASV is low – approximately 15 PFU via inhalation (Stephenson, Larson, and Dominik 1984). Historically, LASV has an overall case-fatality rate of ~1%; however, in a 2016 outbreak in Nigeria the case-fatality rate was 53.9% (WHO 2016) suggesting the potential emergence of a more virulent strain. LASV is primarily spread by direct zoonotic transmission from the multimammate rat (*Mastomys natalensis*) via urine and fomites (Drosten et al. 2002), but both person-to-person and environmental transmission via contact with infectious material, including aerosols, has previously been recognized (Stephenson et al. 1984; Safronetz et al. 2013). There has been an increase in LASV infections over the past decade, which is likely due to an increase in population and urbanization, warmer temperatures, and an increased habitat region for the multimammate rat (Gibb et al. 2017; Klitting et al. 2022). Previous decontamination efforts have focused on fomites (surfaces), and surface disinfection using vapor hydrogen peroxide, thermal inactivation, UV radiation and gamma irradiation have been reported (Otter et al. 2010; Hossain 2022).

1.1.2 SARS-CoV-2

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of coronavirus disease 2019 (COVID-19). SARS-CoV-2 is an enveloped, RNA virus of the *Coronaviridae* family (Buonerba et al. 2021). SARS-CoV-2 RNA is regularly detected in feces of infected individuals and infectious SARS-CoV-2 has been previously isolated from feces, raising concerns about the presence in wastewater and facility worker's safety (Jeong et al. 2020). Furthermore, potentially infectious SARS-CoV-2 in wastewater could lead to surface, marine, or groundwater contamination, however, efforts to detect infectious SARS-CoV-2 in environmental and wastewater samples have been unsuccessful (García-Ávila et al. 2020; Ahmed et al. 2021). Since SARS-CoV-2 RNA is commonly found in stool samples, global wastewater-based surveillance has been used to observe disease trends (Ahmed et al. 2021). The persistence of SARS-CoV-2 in aqueous solutions has been described by the use of surrogate viruses – viruses of similar morphological characteristics. These viruses include bacteriophage Phi6, murine hepatitis virus, feline infectious peritonitis virus, transmissible gastroenteritis virus, and human coronaviruses 229E and OC43 (HCoV-229E and HCoV-OC43) (Silverman and Boehm 2020).

1.1.3 Persistence of Enveloped Viruses

Enveloped viruses, such as Ebola virus, LASV, and SARS-CoV-2, have a lipid outer layer that is necessary for infection, whereas most fecal-oral transmitted viruses, such as norovirus and adenovirus, have a protein outer capsid. As a protein capsid would be expected to be more resistant to environmental stressors than a lipid outer layer, it has been widely assumed that the persistence of enveloped viruses in the environment is negligible. Contrary to this assumption, review of the literature, as shown in Figure 1-1, suggests that the environmental persistence of enveloped virus is highly variable, and it may take days to weeks to achieve a T_{90}, the time for 90% elimination of a specific pathogen, for of many enveloped viruses (Aquino de Carvalho et al. 2017). In addition, the analysis shown in Figure 1-1 also suggests that a single surrogate, the widely used enveloped bacteriophage Phi6, is not adequate to capture the diverse environmental fate of enveloped viral pathogens. Work with the actual virus of concern, as opposed to the surrogate, is essential to determine both the persistence and disinfection in liquid of enveloped viral pathogens (Aquino de Carvalho et al. 2017). Further evaluation of emerging enveloped viruses, such as proposed with LASV and SARS-CoV-2, is essential to direct appropriate medical liquid waste disposal, understand the fate in wastewater and ensure worker safety.

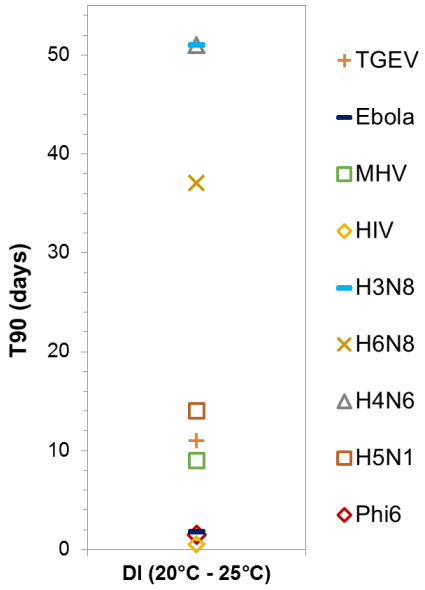

Figure 1-1. T_{90} for Enveloped Viruses in DI Water at 20-25°C.
Bacteriophage Phi6 (Aquino de Carvalho et al. 2017); Ebola (Fischer et al. 2015); HIV (Casson et al. 1992); Avian Influenzas: H3N8, H4N6 (Lebarbenchon et al. 2011), H6N8, H5N1 (Nazir et al. 2010)

1.2 Materials and Methods

LASV and SARS-CoV-2 persistence was determined in water and wastewater. Methodology was adapted from previous Ebola virus persistence work (Bibby et al. 2015; 2017; Fischer et al. 2015). LASV and SARS-CoV-2 cultivation experiments were conducted by NIAID under BSL-4 conditions.

1.2.1 Wastewater Characterization

Wastewater persistence experiments were conducted similarly to as previously described (Bibby et al. 2015). At the project outset, untreated wastewater was collected from an anonymous regional wastewater treatment facility. Following collection, the wastewater was frozen at -80°C to minimize compositional changes prior to analysis for both research tasks described in Chapter 1 and Chapter 2. Wastewater characteristics were determined using EPA Standard Methods, including pH, Chemical Oxygen Demand, Ammonia, Phosphorus, Total Organic Carbon, and Total Suspended Solids. Wastewater samples were shipped to Rocky Mountain Laboratories overnight on ice and stored at -80°C until use.

1.2.2 Lassa Virus Persistence Experiments

1.2.2.1 Water and Wastewater Experiments

LASV stocks of both Josiah and Sauerwald strains were diluted in deionized (DI) water and wastewater to achieve a titer of 10^6 $TCID_{50}$ mL^{-1}. This titer is higher than would be expected under an outbreak scenario and is utilized to elucidate inactivation kinetics. $TCID_{50}$ - an endpoint culture dilution series that is used to determine at what dilution 50% of the infected wells produce cell death – was used to measure viral concentrations. All experiments were completed in triplicate and conducted at 20°C. Spiked wastewater was then distributed into three labeled vials for each aqueous matrix and samples were taken daily for five days. At each time point for each aqueous matrix, including the time zero measurement, 50 µL of aqueous matrix from the bulk wastewater vial was added into 450 µL of Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with heat inactivated fetal bovine serum (FBS, Gibco) and 2%, Pen/Strep (Gibco) to a final concentration of 50 U/mL penicillin and 50 µg/mL streptomycin, and L-glutamine (Gibco) to a final concentration of 2 mM in an appropriately labeled 2 mL screw top vial and frozen at -80°C. Negative controls were 50 µL of non-spiked aqueous matrix.

For each cultivation timepoint, 100 µL from each well of the dilution plate was transferred to a 96 well cell culture plate seeded with Vero cells. The cells were incubated with virus dilutions for one hour then the medium was removed from the two highest concentrations, rinsed two times with PBS and 200 µL of fresh culture medium added. Fresh culture medium (100 µL) was also added to the remaining wells in the plate. The plates were incubated at 37°C for seven days, visually inspected for cytopathic effect (CPE), and scored.

1.2.2.2 Surface Experiments

Solid surface persistence experiments were conducted similarly as previously described (Fischer et al. 2015). Briefly, for each time point, 3 disks (4-cm diameter) of each material were placed individually into wells of a 6-well plate. 10^6 $TCID_{50}$ of the target virus in cell-free medium were evenly distributed on the disks and the plates were allowed to air dry at 20°C. At each timepoint, 450 µL of DMEM (Sigma) modified as described above were placed onto the surface and gently agitated to free surface-bound virus. Viral titers for each surface were determined via culture as described below.

For each cultivation timepoint, 100 µL from each well of the dilution plate was transferred to a 96 well cell culture plate seeded with Vero cells. The cells were incubated with virus dilutions

for one hour then the medium was removed from the two highest concentrations, rinsed two times with PBS and 200 µL of fresh culture medium added. Fresh culture medium (100 µL) was also added to the remaining wells in the plate. The plates were incubated at 37°C for seven days, inspected for CPE, and scored.

1.2.2.3 Statistical Analysis

Statistical analyses and graphing were completed using the monophasic decay equation, which is shown as Equation 1. Points below the limit of detection of 0.8010 log $TCID_{50}$ mL^{-1} were excluded from analysis and a linear trendline was used to estimate the first-order decay rate constant, *k.*

$$C_t = C_0 e^{-kt} \quad \text{(Equation 1-1)}$$

where: C_t = concentration of virus at time, t ($TCID_{50}$ mL^{-1})
C_0 = concentration of virus at time 0 ($TCID_{50}$ mL^{-1})
k = first-order decay rate constant ($days^{-1}$)

1.2.3 SARS-CoV-2 Persistence Experiments

1.2.3.1 Water and Wastewater Experiments

SARS-CoV-2 nCoV-WA1-2020 (MN985325.1) stock virus was diluted to achieve a titer of either ~10^3 $TCID_{50}$ mL^{-1} or ~10^6 $TCID_{50}$ mL^{-1} to observe the inactivation kinetics, however it should be noted these concentrations are increased compared to those of wastewater streams. All experiments were completed in triplicate. Spiked wastewater was distributed into three labeled vials for each concentration and samples were taken daily for seven days. Tests were conducted at 20°C, 50°C, and 70°C in wastewater and at 20°C in tap water. At each time point, including the time zero measurement, 50 µL of sample from the bulk sample vial was added into 450 µL of DMEM (Sigma) supplemented with heat-inactivated fetal bovine serum (FBS, Gibco) and 2%, Pen/Strep (Gibco) to a final concentration of 50 U/mL penicillin and 50 µg/mL streptomycin, and L-glutamine (Gibco) to a final concentration of 2 mM in an appropriately labeled 2 mL screw top vial and frozen at -80°C. Negative controls were 50 µL of non-spiked sample. Culture analysis was performed as described below. For samples exposed to heat via a heat block for 50°C and 70°C, at each time point the triplicate samples were removed and immediately placed in a -80°C freezer until needed for cultivation.

For each cultivation timepoint, 100 µL from each well of the dilution plate will be transferred to a 96 well cell culture plate seeded with Vero cells. The cells were incubated with virus dilutions for one hour then the medium was removed from the two highest concentrations, rinsed two times with PBS and 200 µL of fresh culture medium added. Fresh culture medium (100 µL) was also added to the remaining wells in the plate. The plates were incubated at 37°C, inspected for CPE and scored. All statistical analyses were conducted using GraphPad Prism, and all plotting was completed using Equation 1-1 and GraphPad Prism. Additionally, results were analyzed using the biphasic decay model as shown in Equation 1-2 and the monophasic and biphasic decay models were compared.

$$C_t = C_{f0}e^{-k_1 t} + C_{s0}e^{-k_2 t}$$ (Equation 1-2)

where: C_t = concentration of virus at time, t ($TCID_{50}$ mL^{-1})
C_{f0} = concentration of virus at the start of the fast decay period ($TCID_{50}$ mL^{-1})
C_{s0} = concentration of virus at the start of the slow decay period ($TCID_{50}$ mL^{-1})
k_1 = first-order decay rate constant for the initial and fast decay ($days^{-1}$)
k_2 = first-order decay rate constant for the slow decay ($days^{-1}$)

The stability of the SARS-CoV-2 RNA signal was analyzed by using RT-qPCR after inactivation protocols to remove the samples from a BSL4 laboratory to a BSL2 laboratory. QIAlamp 96 Virus QIAcube HT Kit with a QIAvac 96 vacuum system (Qiagen) was used to extract the RNA from samples. The RT-qPCR assay for SARS-CoV-2 targeted the E gene of SARS-CoV-2 and extracted RNA used QuantiFast Probe RT-PCR + ROX Vial Kit (Qiagen) reagents and a Rotor-Gene Q real-time thermocycler to measure the signal. Standard curves were constructed by using known quantities of *in vitro*-transcribed RNA.

1.3 Lassa Virus Persistence in Water and Wastewater

1.3.1 Results

1.3.1.1 Wastewater Characterization

Primary influent wastewater was aseptically collected from an anonymous wastewater treatment facility. The treatment plant has a conventional activated sludge treatment train and has the capacity to treat 11 million gallons per day. Physiochemical analyses of the wastewater were tested at the University of Notre Dame and the measurements are shown in Table 1-1. The lipid bilayer membrane structure which surrounds the protein capsid in enveloped viruses, like SARS-CoV-2 and LASV, is more susceptible to changes in environmental factors, such as pH and organic matter (Yang et al. 2022).

Table 1-1. Composition of Tested Wastewater for Lassa Virus Experiments.

Analyte	Wastewater Measurement
pH	6.16
Chemical Oxygen Demand (mg/L)	279
Ammonia (mg/L)	29
Nitrate (mg/L)	5.0
Phosphorus (mg/L)	9.2
Total Suspended Solids (mg/L)	134

1.3.1.2 Persistence of Lassa Virus in DI Water and Wastewater

Both LASV strains, Josiah and Sauerwald, were spiked into DI water and samples were collected over a five-day time period. Samples were measured using the $TCID_{50}$ method and the results were transformed using the monophasic decay method. The data is shown in Figure 1-2. The first-order decay rate constant was found to be 0.30 $days^{-1}$ and 0.56 $days^{-1}$ for Josiah and Sauerwald, respectively. Both LASV strains were also spiked into primary influent wastewater, the results were transformed using the monophasic decay method and are also shown in Figure 1-2. The first-order decay rate constant was 1.4 $days^{-1}$ and 2.0 $days^{-1}$ for Josiah and Sauerwald, respectively. Decay rate constants and T_{90}, the time for 90% reduction, for both strains in water and wastewater are summarized in Table 1-2.

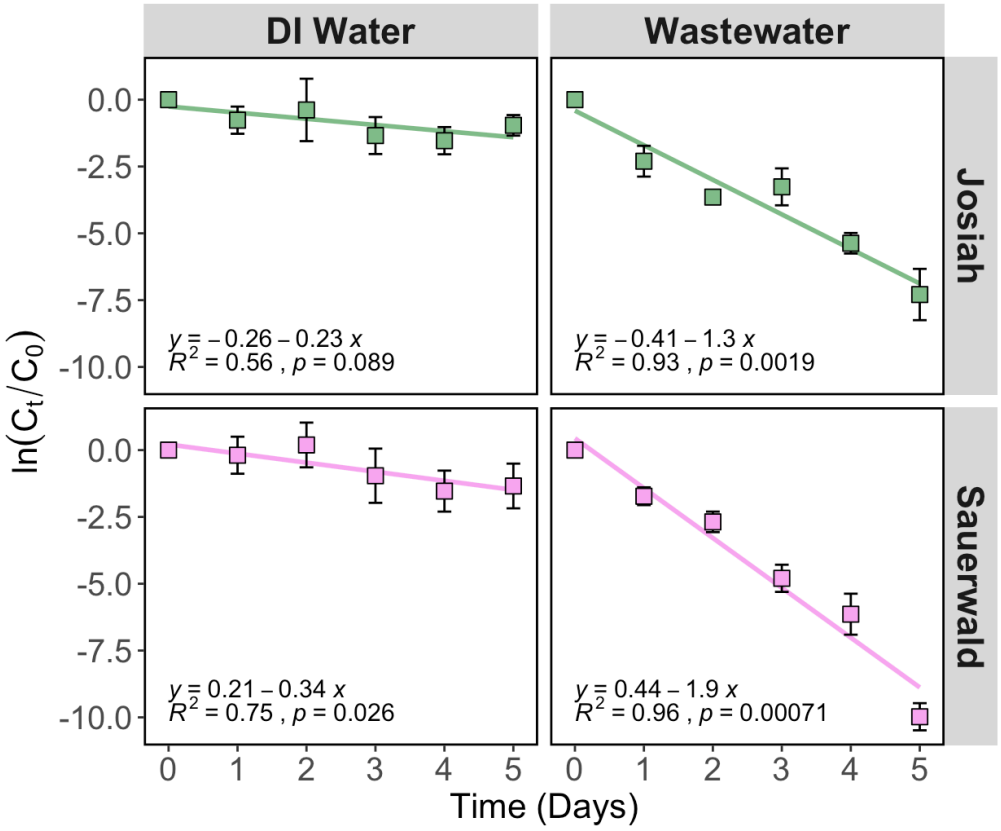

Figure 1-2. Lassa Virus Persistence in DI Water and Wastewater

Monophasic decay of Josiah and Sauerwald strains in DI water and wastewater calculated from Equation 1-1. The x-axis shows the time in days and the y-axis shows the monophasic decay calculated. Each point represents the mean of three experimental replicates and the error bars show the standard deviation. Where there are no error bars, the standard deviation is too small to display.

Table 1-2. Decay Rate Constants and T_{90} with 95% CI of Lassa Virus in DI Water and Wastewater.

	DI Water		Wastewater	
	LASV Josiah	LASV Sauerwald	LASV Josiah	LASV Sauerwald
Decay rate, *k* ($days^{-1}$)	0.23 (0.04 – 0.43)	0.34 (0.15 – 0.53)	1.3 (0.94 – 1.6)	1.9 (1.5 – 2.3)
T_{90} (days)	10.0 (5.4 – 57.6)	6.8 (4.3 – 15.4)	1.8 (1.4 – 2.4)	1.3 (1.0 – 1.5)

1.3.2 Discussion

The first-order decay rate constants between wastewater and DI water were significantly different for both Josiah and Sauerwald using the Pearson method (p = 0.002). When comparing Josiah and Sauerwald decay rates for DI water, there was a slightly statistically significant decay for Sauerwarld; however, for wastewater, decay was statistically significant in both cases. The significant difference between the decay rate in DI water and wastewater could be attributed to several factors relating to the differing physiochemical characteristics of the two matrices. Wastewater has a higher organic content, which has been showed in previous studies to help protect enteric viruses and other microorganisms from disinfection and environmental stressors (Aquino de Carvalho et al. 2017). Furthermore, organic material found in wastewater can influence the growth of predatory microorganisms which can consume viruses (Sinclair et al. 2012). Second, higher ammonia concentrations such as those found in wastewater when compared to DI water have showed to increase the degradation of RNA genomes, like that of LASV (Decrey et al. 2016).

Bacteriophage Phi6 is commonly used as a surrogate virus of concern. A study using bacteriophage Phi6 to analyze the persistence in DI water, found a first-order decay rate of 0.63 $days^{-1}$ (Aquino de Carvalho et al. 2017). In this study, the first-order decay rates were 0.23 $days^{-1}$ and 0.34 $days^{-1}$ for Josiah and Sauerwald strains, respectively, which are lower than that of bacteriophage Phi6. In experiments with primary influent wastewater, the first-order decay rate of bacteriophage Phi6 was 7.9 $days^{-1}$, and with autoclaved primary influent wastewater, the decay rate was 0.398 $days^{-1}$ (Ye et al. 2016; Aquino de Carvalho et al. 2017). The first-order decay rates of LASV strains fell between the results from these studies with bacteriophage Phi6. Murine hepatitis virus has also been analyzed as a surrogate for enveloped viruses and in wastewater the first-order decay rate was 3.4 $days^{-1}$, which is almost twice as high as what was found for LASV strains. (Ye et al. 2016)

1.3.3 Limitations and Conclusions

This study presents several limitations which could impact the persistence of LASV strains in the real world when compared to these laboratory studies. By only using one wastewater sample, there was no variation to analyze how difference constituents such as ammonia, total phosphorus, total suspended solids, and chemical oxygen demand impact persistence. By having to freeze and thaw samples before use, changes in the microbial biota could have changed and in turn lowering the inactivation rate by reducing potential predation. Lastly, the titers used are higher than those likely to be seen in real world situations and wastewater may not be representative of concentrated human wastes.

Overall, the persistence of LASV in wastewater is shorter than that of DI water. A 90% reduction in LASV was calculated to be 2 days, which is similar to other enteric viruses. This data is critical for ensuring treatment plant worker safety, as well as ensuring environmental contamination is minimized.

1.4 Lassa Virus Persistence on Surfaces

1.4.1 Results

Samples of HDPE and stainless steel were inoculated with either strain of LASV and were left to dry at 20°C before being measured by the $TCID_{50}$ method. The monophasic decay of the data is shown Figure 1-3. The limit of detection of the method was 0.801 log_{10} $TCID_{50}$ mL^{-1}. Samples for Josiah for both HDPE and Stainless Steel were below the limit of detection after Day 2 and samples for Sauerwald for both matrices were below the limit of detection after Day 3. All samples below the limit of detection were removed from the analysis to determine more accurate decay rates for the strains of LASV. The first-order decay rates of LASV strains on HDPE were 4.3 $days^{-1}$ and 2.3 $days^{-1}$ for Josiah and Sauerwald, respectively. The first-order decay rates on stainless steel were 5.5 $days^{-1}$ and 2.7 $days^{-1}$ for Josiah and Sauerwald, respectively. The decay rates and the T_{90} for LASV persistence on HDPE and stainless steel are summarized in Table 1-3.

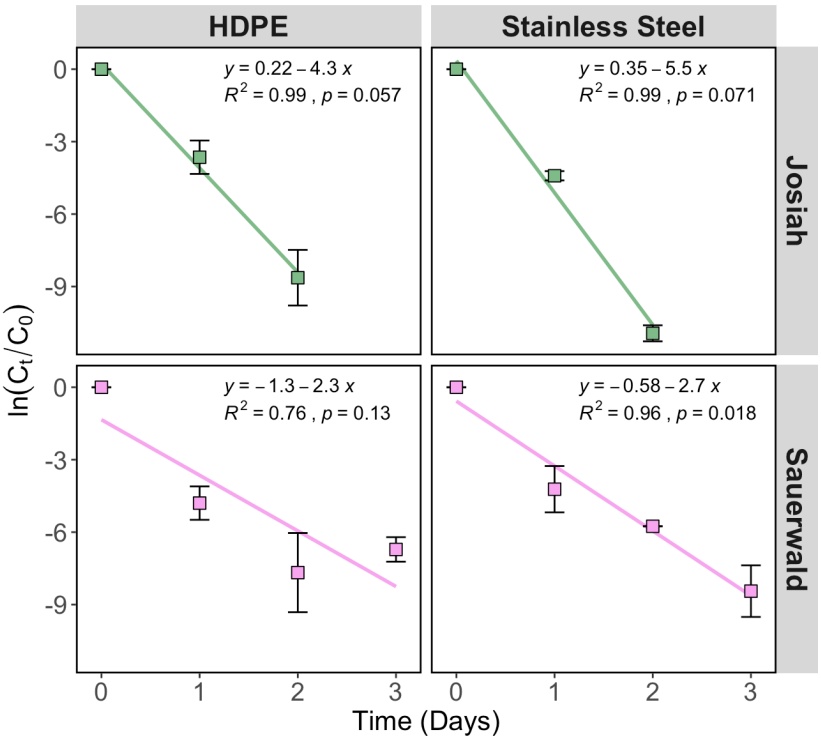

Figure 1-3. Lassa Virus Persistence on Surfaces

Monophasic decay of Josiah and Sauerwald strains on HDPE and stainless steel calculated from Equation 1-1. The x-axis shows the time in days and the y-axis shows the monophasic decay calculated. Each point represents the mean of three experimental replicates and the error bars show the standard deviation. Where there are no error bars, the standard deviation is too small to display.

Table 1-3. Decay Rate Constants and T_{90} with 95% CI of Lassa Virus on HDPE and Stainless Steel.

	HDPE		Stainless Steel	
	LASV Josiah	LASV Sauerwald	LASV Josiah	LASV Sauerwald
Decay rate, *k* ($days^{-1}$)	4.3 (3.5 – 5.10)	2.3 (0.5 – 4.1)	5.3 (4.3 – 6.3)	2.7 (2.0 – 3.4)
T_{90} (days)	0.5 (0.4 – 0.7)	1.0 (0.6 – 4.6)	0.4 (0.3 – 0.5)	0.9 (0.7 – 1.2)

1.4.2 Discussion

The materials used to check the persistence of LASV in this study are commonly used in hospital settings. HDPE is the material of Tyvek coveralls, the personal protective equipment worn by those in hospitals handling patients (Cervantes et al. 2018). No significant difference was found between decay on HDPE and stainless steel for each of the LASV strains tested using the Pearson test. There was, however, a significant difference between the decay rates of the Josiah and Sauerwald strains was found when spotted onto stainless steel (p = 0.03). HDPE and stainless steel are non-porous, hydrophobic materials which lead to samples drying in beaded shapes (Paton et al. 2021). This drying pattern has been shown to concentrate viral particles and organic material, which can offer protection to viruses from environmental pressures and increase the time needed for inactivation when compared to using a wet sample (Paton et al. 2021).

1.4.3 Conclusions

This study was the first to analyze the persistence of LASV on different materials found in a healthcare setting. The results of this study are important to ensuring health care workers remain safe and infectious LASV can persist on surfaces. Furthermore, the results show the LASV strains tested decay more rapidly on surfaces than in aqueous solution.

1.5 SARS-CoV-2 Persistence in Water and Wastewater

1.5.1 Results

1.5.1.1 Wastewater Characterization

A primary influent wastewater sample for experimentation was collected from an anonymous wastewater treatment facility. The facility services about 50,000 population equivalents. The WWTP facility is designed for an average daily flow of 5.3 m^3/s and peak flows of 1.6×10^5 m^3. However, WWTP typically runs at about 4.5×10^4 m^3 daily flow. The collection system is combined, but the sample was collected during dry weather flow. Standard water quality parameters were determined using standard methods and the results are shown in Table 1-4.

Table 1-4. Composition of Tested Wastewater for SARS-CoV-2 Experiments.

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Analyte	Wastewater Measurement
pH	7.98
Chemical Oxygen Demand (mg/L)	152.7
Ammonia (mg/L)	13.3
Total Phosphorus (mg/L)	6.0
Total Suspended Solids (mg/L)	756.8

1.5.1.2 Persistence of SARS-CoV-2 at 20°C

Results of the persistence experiments for SARS-CoV-2 in water and wastewater for the differing titers at 20°C are shown in the first two plots of Figure 1-4. SARS-CoV-2 could be detected for the entire seven days when using the high-titer experiments, and for only three days when using the low-titer experiments in wastewater. When comparing the data for each experiment between the monophasic decay model and the biphasic decay model using the

extra sum-of-squares *F* test, it was found that the biphasic decay model did not improve the model fit, resulting in the monophasic decay model being used for analysis (p = 0.88 – 1.00). The estimated first-order decay rate constants based off the slopes of the line of best fit were 1.4 $days^{-1}$ for the high-titer and 1.1 $days^{-1}$ for the low-titer for wastewater. SARS-CoV-2 was also detected for the entire seven days in tap water at a high-titer and the calculated first-order decay rate constant was 1.2 $days^{-1}$. There was no significant difference found between the first-order decay rate constants of low-titer and high-titer based on a Mann-Whitney test ($p > 0.05$). First-order decay rate constants, decimal reductions, and half-lives for each experiment are summarized in Table 1-5.

1.5.1.3 Persistence of SARS-CoV-2 at 50°C and 70°C

The comparison of temperature on the inactivation of SARS-CoV-2 for the high-titer experiments is shown in the final plot of Figure 1-4. At 50°C, SARS-CoV-2 was detected for the entire hour of the time series, whereas at 70°C, SARS-CoV-2 was only detected for 10 minutes. The first-order decay rate constants were 0.15 and 1 min^{-1}, respectively. Figure 1-5 shows the log of the time for 90% reduction, T_{90}, based upon the monophasic decay rate constant compared to temperature to analyze the relationship between changing temperature and inactivation. Table 1-5 summarizes the decay-rate constants, T_{90}, and half-lives. The monophasic decay rate constant was used because there was no significant difference between the monophasic and biphasic model results. Apparent tailing of the decay curve may suggest behavior outside of the assumed first-order decay due to particle association of the virus.

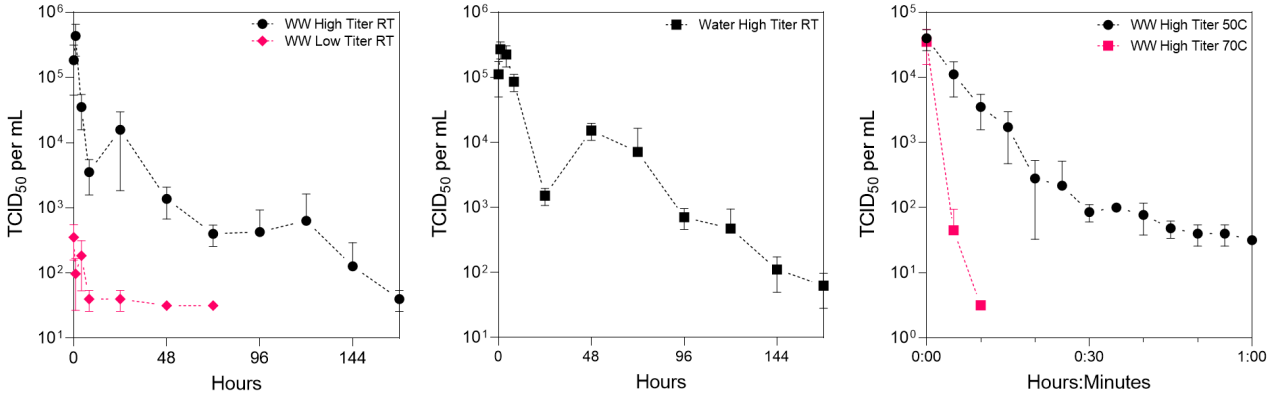

Figure 1-4 SARS-CoV-2 Persistence in Water and Wastewater.

Source: Bivins et al. © 2020 American Chemical Society

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Observed $TCID_{50}$ per mL decay (mean & standard deviation) in wastewater (WW) inoculated with high (10^5 $TCID_{50}$ per mL) and low (10^3 $TCID_{50}$ per mL) titers of infectious SARS-CoV-2 at room temperature (20°C, RT) (left panel), water inoculated with high titer at RT (center panel), and WW inoculated with high titer at 50°C and 70°C (center panel).

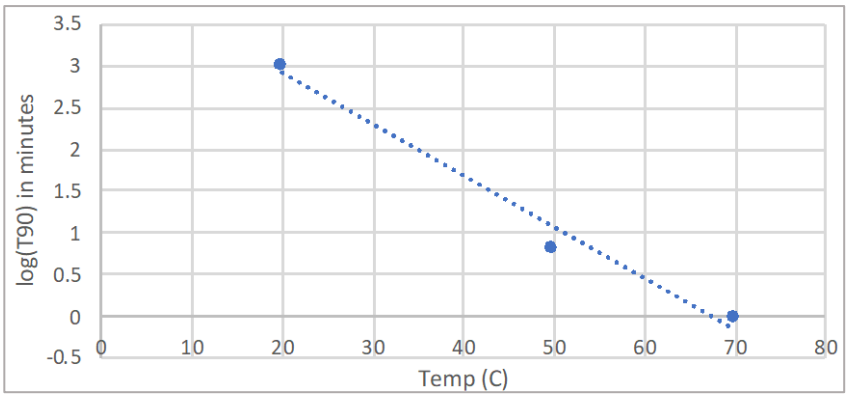

Figure 1-5. SARS-CoV-2 Persistence in Wastewater verses Temperature.
The y-axis shows Inactivation rate (as $\log_{10}(T_{90})$) and the x-axis shows temperature in °C for SARS-CoV-2 in wastewater.

Table 1-5. Decay Rate Constants, Half-Lives, and Decimal Reductions of Infectious SARS-CoV-2.
Source: Bivins et al. © 2020 American Chemical Society
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Parameter	WW, High Titer, RT (0 – 7 days)	WW, Low Titer, RT (0 – 3 days)	Tap Water, High Titer, RT (0 – 7 days)	WW, High Titer, 50°C (0 – 60 min)	WW, High Titer, 70°C (0 – 10 min)
Samples	33	21	33	39	9
k (95% CI)	1.4 $days^{-1}$ (1.3 – 1.6 $days^{-1}$)	1.1 $days^{-1}$ (0.71 – 1.5 $days^{-1}$)	1.2 $days^{-1}$ (1.1 – 1.3 $days^{-1}$)	0.15 min^{-1} (0.14 – 0.16 min^{-1})	1.0 min^{-1} (0.80 – 1.3 min^{-1})
r^2	0.71	0.54	0.88	0.68	0.88
Half-life (95% CI)	0.49 days (0.43 – 0.56 days)	0.64 days (0.48 – 0.98 days)	0.59 days (0.54 – 0.66 days)	4.6 min (4.3 – 5.1 min)	0.67 min (0.55 – 0.86 min)
T_{90} (95% CI)	1.6 days (1.4 – 1.8 days)	2.1 days (1.6 – 3.3 days)	2.0 days (1.8 – 2.2 days)	15 min (14 – 17 min)	2.2 min (1.8 – 2.9 min)

1.5.1.4 Persistence of SARS-CoV-2 RNA in Water and Wastewater

Using RT-qPCR, the RNA signal of SARS-CoV-2 was analyzed. The estimated decay rates are shown in Table 1-6. All experiments were conducted at room temperature, 20°C, with high and low titers in wastewater and tap water. The RNA measurements were made from the same suspensions used to inoculate cell cultures from the persistence experiments, so direct comparisons can be made between the RNA signal and the associated infectious SARS-CoV-2 concentration.

Table 1-6. Decay Rate Constants, Half-Lives, and Decimal Reductions of SARS-CoV-2 RNA.
Source: Bivins et al. © 2020 American Chemical Society
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Parameter	WW, High Titer, RT	WW, Low Titer, RT	Tap Water, High Titer, RT
Samples	30	30	30
k (95% CI)	0.67 $days^{-1}$ (0.54 – 0.86 $days^{-1}$)	0.09 $days^{-1}$ (0.00 – 0.23 $days^{-1}$)	0.07 $days^{-1}$ (0.00 – 0.23 $days^{-1}$)
r^2	0.27	-0.01	-0.11
Half-life (95% CI)	0.99 days (0.81 – 1.3 days)	7.9 days (3 – ∞ days)	10.0 days (3.0 – ∞ days)
T_{90} (95% CI)	3.3 days (2.7 – 4.3 days)	26 days (20 – ∞ days)	33 days (9.9 – ∞ days)

1.5.2 Discussion

The persistence of infectious SARS-CoV-2 in primary influent wastewater varied from the constants found from other coronaviruses and enteric viruses. Compared to the findings of Silverman and Boehm, the first-order decay rate constant for infectious SARS-CoV-2 is similarly sensitive to temperature, however SARS-CoV-2 is more persistent (Silverman and Boehm 2020). The T_{90} at room temperature for SARS-CoV-2 from this experiment was 1.6 to 2.1 days, which is shorter than the 4 days found for TGEV (Casanova et al. 2009). Experiments using MHV found the T_{90} to be 3 days in pasteurized wastewater and 13 hours in unpasteurized wastewater, showing the T_{90} for SARS-CoV-2 is higher or lower than MHV depending on the treatment of the wastewater (Casanova et al. 2009; Ye et al. 2016).

An analysis of SARS-CoV-2 RNA in water and wastewater at room temperature showed the RNA signal is more persistent than the infectious virus. SARS-CoV-2 RNA was detected even when infectious SARS-CoV-2 was no longer detected in the cell culture. The persistence of SARS-CoV-2 RNA can resolve why SARS-CoV-2 was unable to be cultured from wastewater even when the RNA of the virus was detected (Rimoldi et al. 2020). The T_{90} of low-titer SARS-CoV-2 RNA in wastewater in this study was greater, but similar, to the T_{90} for wastewater of 20.4 days at 15°C and 12.6 days 25°C for a study using gamma-irradiated SARS-CoV-2 (Ahmed et al. 2020). The T_{90} of low-titer SARS-CoV-2 was also comparable to the T_{90} of Zika virus in wastewater at 25°C of 21 days (Muirhead et al. 2020).

1.5.3 Limitations and Conclusions

This study was limited due to constraints of working with infectious SARS-CoV-2 and experimental resources. Freezing and thawing the wastewater samples could have altered the community of microorganisms which are known to increase the decay of infectious viral particles (Sobsey and Meschke 2008; Deng and Cliver 1995). Using a single wastewater sample does not take into account the variability of wastewater characteristics such as pH, chemical oxygen demand, and nitrogen, ammonia, and phosphorus concentrations.

The persistence of SARS-CoV-2 in wastewater at varying temperatures showed infectious SARS-CoV-2 inactivates at a faster rate at a higher temperature than at lower temperatures. SARS-CoV-2 was also found to be more persistent than other coronaviruses. The observations of SARS-CoV-2 RNA during the persistence experiments in wastewater show detection of SARS-CoV-2 RNA alone is not enough to characterize the risk of infection attributed to wastewater exposure. This data helps to address concerns regarding potential SARS-CoV-2 spread via wastewater. This work resulted in a publication entitled *Persistence of SARS-CoV-2 in Water and Wastewater* in Environmental Science and Technology Letters in 2020. (Bivins et al. 2020)

CHAPTER 2

Lassa Virus and SARS-CoV-2 Disinfection

2.1 Background

Free chlorine is a commonly used wastewater disinfectant as it is widely available and effective at inactivating microorganisms. Free chlorine also produces a chlorine residual which continues to inactivate microorganisms in the distribution system (USEPA (US Environmental Protection Agency) 2010). Wastewater produced and treated on-site at hospitals also is typically disinfected with free chlorine (Gautam et al. 2007). Other disinfection methods include UV radiation, thermal inactivation, and ozone disinfection; however, these methods all have tradeoffs when compared to the use of free chlorine. Optimizing the chlorine dose to ensure pathogen inactivation is important for potential reclaimed water reuse and to ensure disinfection byproducts are staying within the target concentration. (Li et al. 2013)

Work on disinfection of LASV in liquid has been extremely limited; a single investigation found that diluting LASV contaminated blood in 3% acetic acid or heating to 60°C for one hour was sufficient to inactivate all infectious virus (Mitchell and McCormick 1984). LASV inactivation has also been analyzed using thermal inactivation, gamma irradiation, and UV irradiation (Hossain 2022). There is no available data in the open literature on LASV disinfection in wastewater using free chlorine as sodium hypochlorite.

At the time of this study, SARS-CoV-2 disinfection by free chlorine in an aqueous solution had not been evaluated. Disinfection of SARS-CoV-2 by UV radiation and thermal inactivation had been evaluated, and disinfection on different surfaces using bleach and ethanol (Chin et al. 2020). The lipid envelope found on SARS-CoV-2 is known to be susceptible to disinfection in other viruses and are found to be more susceptible than non-enveloped viruses (Pecson et al. 2020). 1

The goal of this chapter is to evaluate the inactivation kinetics of LASV and SARS-Cov-2 with free chlorine as sodium hypochlorite. The gap in understanding the inactivation kinetics of these two viruses creates difficulty when estimating the efficacy of our current treatment systems and the potential for further environmental exposure.

2.2 Materials and Methods

LASV disinfection was assessed in wastewater for two strains, Josiah and Sauerwald, and SARS-CoV-2: hCoV-19/USA/MD-HP01542/2021, B.1.351 disinfection was assessed in DI water and wastewater. Disinfection was evaluated using multiple doses, 1 mg/L, 5 mg/L, and 10 mg/L, of free chlorine as sodium hypochlorite, where the typical wastewater chlorine dose is between 5 mg/L and 20 mg/L (USEPA 1999). Free chlorine was consumed by the wastewater matrix and residual free chlorine was quantified at the University of Notre Dame as performed for Ebola virus disinfection experiments (Bibby et al. 2017). Briefly, the wastewater used in Chapter 1 was used at the University of Notre Dame to determine chlorine demand and expected free chlorine concentrations. Target chlorine concentrations were added, and free chlorine was measured at

0, 1, 5, 10, 30, and 60 minute timepoints for SARS-CoV-2 and 0, 1, 5, 15, 30, and 60 minute timepoints for LASV using the Hach D900 Colorimeter.

For each virus-disinfectant dose combination, the virus was exposed to the relevant disinfectant dose and the virus titers were determined at multiple timepoints as mentioned above. Sodium hypochlorite (Acros Organics) was added to two milliliter vials of the wastewater/virus suspension at initial doses determined by initial experiments and target free chlorine concentrations. Samples were taken at the indicated time points and chlorine demand immediately quenched by the addition of sodium thiosulfate. The 'time zero' sampling point was taken approximately 20 seconds following the addition of chlorine to enable sample mixing.

Viral culture was conducted as described above in Chapter 1.2. The limit of detection was 0.8010 log $TCID_{50}$ mL^{-1}. Persistence data was fit to a standard Chick-Watson (Ct) model and examined for atypical (e.g., tailing) behavior to determine the inactivation constants, 'k'. Chlorine decay was modeled as previously described using an exponential decay model (Haas and Karra 1984) and total Ct was calculated as the area under the curve. ANOVA was utilized to determine significant effects due to either matrix. Statistical analyses and graphing were completed within GraphPad Prism and RStudio.

2.3 Disinfection of Lassa Virus in Wastewater

2.3.1 Results

2.3.1.1 Chlorine Residual

To determine residual chlorine demand for disinfection experiments, sodium hypochlorite was added to a flask containing 125 mL of wastewater at initial doses of 1, 5 and 10 mg/L. Samples were then taken at the 0, 1, 5, 15- and 30-minute time points and measured for free chlorine. Free chlorine residuals in wastewater were determined using a HACH Free Chlorine test kit (Method 10069). The method detection limit of this test is 0.02 mg/L free chlorine. Briefly, contents of one test kit powder pillow was added to a sample cell containing 10 mL of each sample. Sample cell was then swirled for 20 seconds before reading in a HACH colorimeter. Data is shown in Figure 2-1.

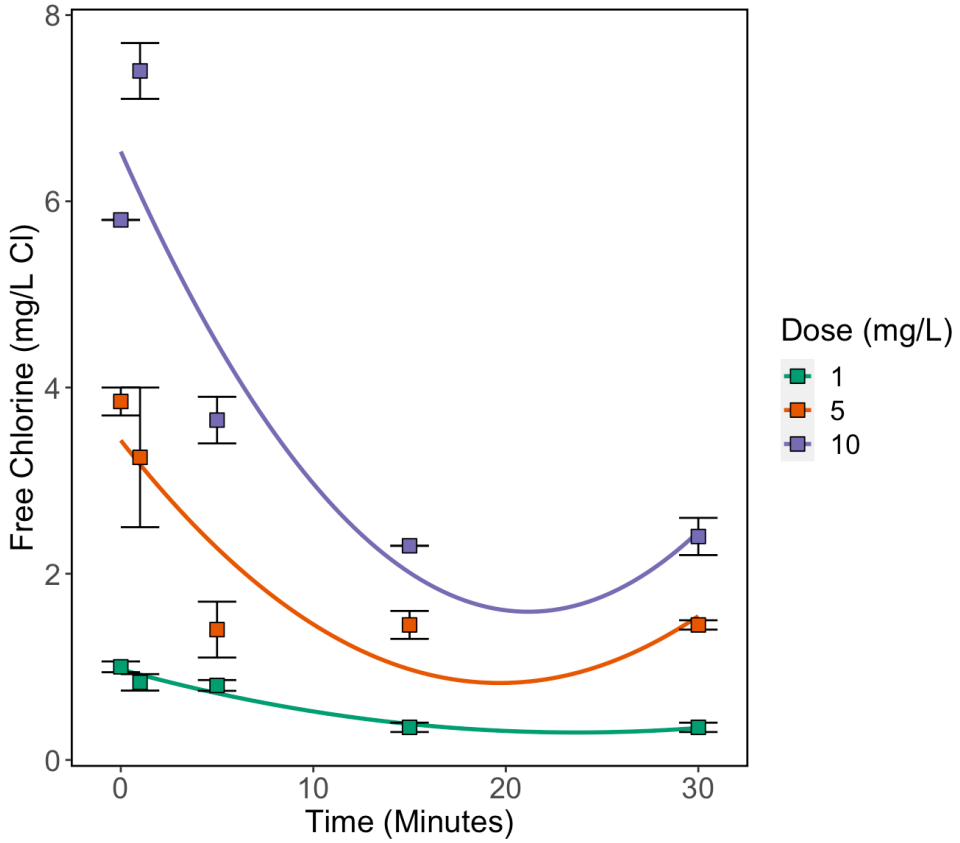

Figure 2-1. Free Chlorine Residual in Wastewater for Lassa Virus Experiments.
The y-axis shows free chlorine concentrations in mg/L Cl, which were measured using a HACH D900 Colorimeter, and the x-axis shows time in minutes. Each data point represents the mean of triplicate readings of free chlorine and the error bars represent the standard deviation.

2.3.1.2 Disinfection of Lassa Virus from Sodium Hypochlorite

The tested LASV strains, Josiah and Sauerwald, were spiked into primary influent wastewater, as described in Table 1-1, with different concentrations of free chlorine as sodium hypochlorite: 0 mg/L, 1 mg/L, 5 mg/L, and 10 mg/L. Of the analytes assessed, ammonia can form chloramines when combined with chlorine, pH affects the distribution of hypochlorous acid and hypochlorite ions, and total suspended solids can shield pathogens and increase chlorine demand (USEPA 1999)**.** Samples were taken over the course of the experiment and were evaluated using the $TCID_{50}$ method as described above. Figure 2-2 shows the monophasic decay of the different LASV strains when exposed to the four concentrations of sodium hypochlorite. The top set of plots show the Josiah strain, and the bottom set of plots show the Sauerwald strain. There was no significant decay for either LASV strain under the 0 mg/L sodium hypochlorite condition. No decay was observed for 0 mg/L of sodium hypochlorite for either strain. The decay of the strains at 1 mg/L were similar, however at 5 mg/L and 10 mg/L, the Sauerwald strain showed a faster inactivation time than the Josiah strain. At 5 mg/L, the Josiah strain reached the limit of detection after 20 minutes whereas the Sauerwald strain reached the limit of detection at 10 minutes. At 10 mg/L, the Josiah strain reached the limit of detection after 20 minutes and the Sauerwald strain reached the limit of detection after 1 minute, showing rapid inactivation.

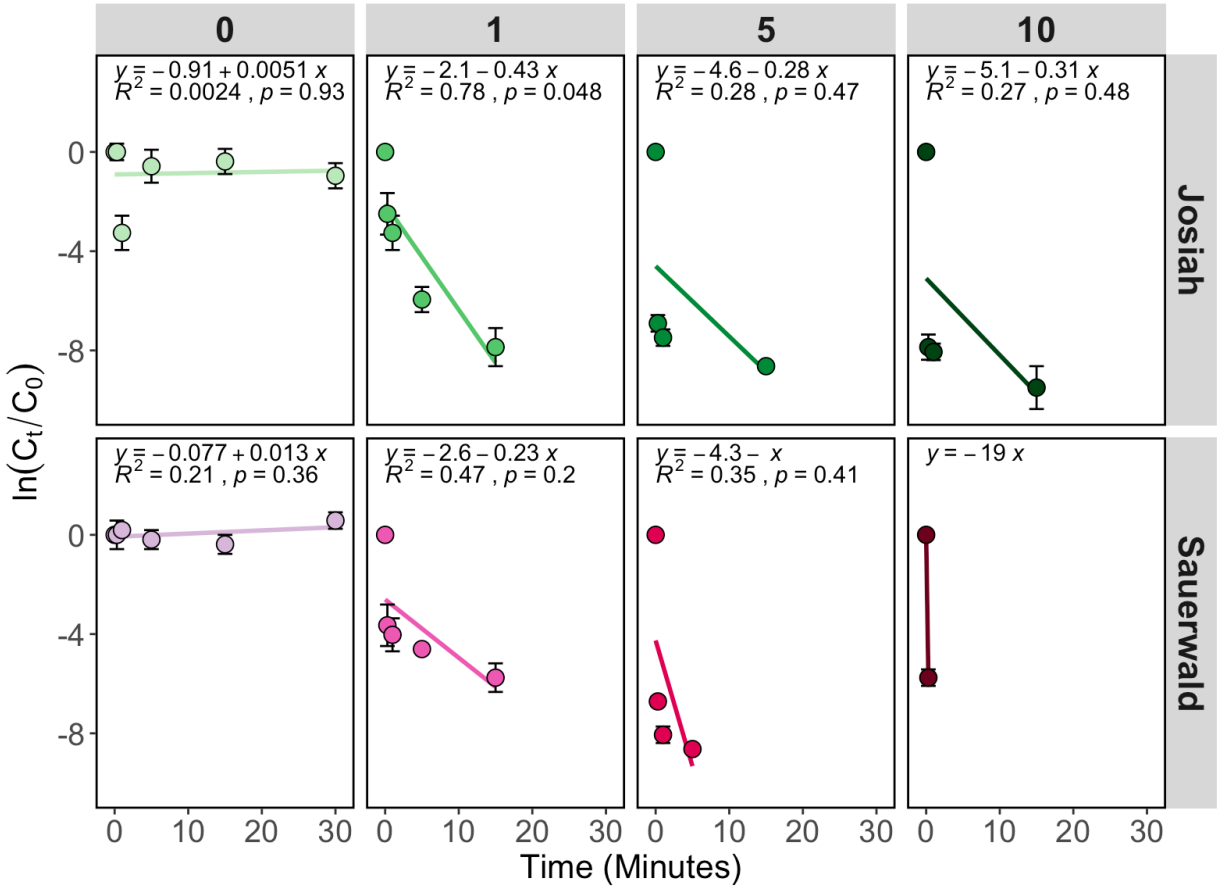

Figure 2-2. Inactivation of Lassa Virus with Sodium Hypochlorite.

The panels from left to right show increasing concentrations of sodium hypochlorite in mg/L. The top set of panels show the Josiah strain, and the bottom set of panels show the Sauerwald strain. The y-axis shows the monophasic decay of the $TCID_{50}$ data, and the x-axis shows time in minutes. Each data point shows the mean of triplicate readings and the standard deviation. Where there are no error bars shown, they are too small to see.

2.3.1.3 Ct and Observed Removal of LASV

Modeling the initial chlorine residuals in wastewater was dose dependent with the 0, 1, 5, and 10 mg/L doses resulting in initial free chlorine residuals of 0, 0.97, 3.43, and 6.53 mg/L, respectively, and these are shown in Figure 2-1. The integral of the modeled line of best fit was taken to determine the concentration-time (Ct) values. Figure 2-3 shows the observed log_{10} removal versus the calculated Ct value. The observed log_{10} removal was calculated by subtracting the data points for the 1, 5, and 10 mg/L from the 0 mg/L data points for each LASV strain. The line of best fit was used to calculate a 3-log reduction time in LASV strains.

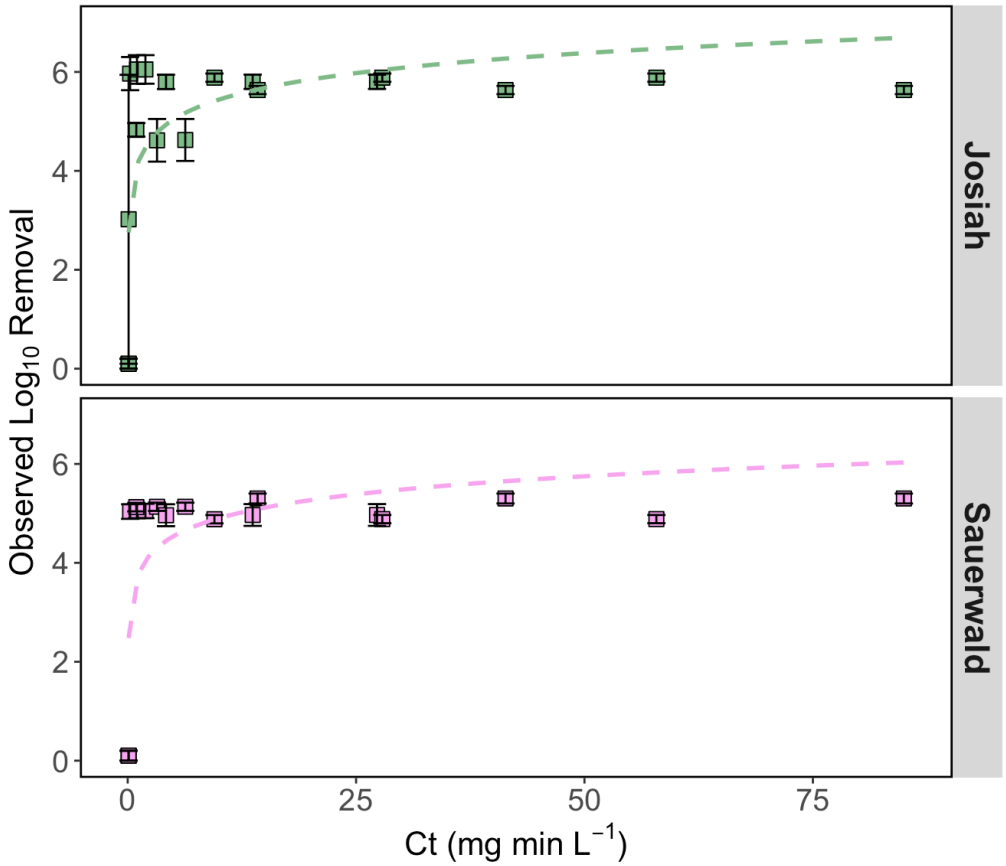

Figure 2-3. Observed Log_{10} Removal vs. Ct Value for LASV.
The y-axis shows the observed log_{10} removal, which was calculated by subtracting the data points for 1 mg/L, 5 mg/L, and 10 mg/L from the 0 mg/L data points. The x-axis shows the Ct values that were modeled from the free chlorine residual data. Each data point represents the mean of three replicates and the error bars show the standard deviation. The values for 0 mg/L were not plotted.

2.3.2 Discussion

The Ct value (Concentration x Time) needed to achieve a 3-log removal of the initial virus is typically reported for enteric viruses. The logarithmic line of best fit shown in Figure 2-3 for both strains of LASV is shown as a dashed line on the plots. Based on these modeled lines, a 3-log removal of the Josiah and Sauerwald are 0.15 and 0.26 mg-min/L, respectively. Compared to other enveloped viruses, the Ct values found for LASV are slightly lower with the Ct for 3-log removal being 0.39 to 0.41 mg-min/L for Influenza H5N1 at 5°C, 0.56 for bacteriophage Phi6 at 17°C, and 0.36 for bacteriophage Phi8 at 17°C. (Adcock et al. 2009; Rice et al. 2007) The Ct values for enveloped viruses are commonly lower than those of non-enveloped viruses, including coxsackievirus, poliovirus, echovirus, and hepatitis A virus. (Kong et al. 2021) Furthermore, enteric viruses are considered to be high-sensitive or hypersensitive to free chlorine disinfection when compared to other pathogens of concern such as bacteria, protozoa, or fungi. (Kong et al. 2021)Overall, our results compared to those of other studies show that enveloped viruses persist and decay at different rates, requiring species-specific and potentially strain-specific analyses to fully evaluate potential health risks.

2.3.3 Limitations and Conclusions

In this study, specific concentrations of LASV were used to spike into wastewater samples, which may be in higher concentrations than those found in a real hospital or wastewater setting. Sodium hypochlorite showed to be an effective disinfection method for the inactivation of both strains of LASV which is crucial for the development of control measures. The outcomes of this work can instill confidence in our current treatment systems in effectively inactivating LASV in wastewater. Furthermore, the results supply the scientific community with Ct values necessary to inactivate LASV if wastewater streams are not receiving disinfectants before being released into the environment. This work resulted in a publication entitled *Environmental Persistence of Disinfection of Lassa Virus* which is currently under review. (Shaffer et al. 2023)

2.4 Disinfection of SARS-CoV-2 in Water and Wastewater

2.4.1 Results

2.4.1.1 Chlorine Residual

To determine residual chlorine demand for disinfection experiments, sodium hypochlorite and DMEM was added to a flask containing 125 mL of water or wastewater at initial doses of 1, 5 and 10 mg/L. Samples were then taken at the 1, 3, 5, 10- and 20-minute time points and measured for Free Chlorine. Free chlorine residuals in water or wastewater were determined using a HACH Free Chlorine test kit (Method 10069). The method detection limit of this test is 0.02 mg/L free chlorine. Briefly, the contents of one test kit powder pillow were added to a sample cell containing 10 mL of each sample. Sample cell was then swirled for 20 seconds then placed into cell holder for measurement on a HACH colorimeter. As shown in Figure 2-4, the chlorine residual was dose dependent for each experimental condition. The models show the initial free chlorine residuals for DI water were 0, 0.16, and 0.43 mg/L for the 1, 5, and 10 mg/L chlorine conditions, respectively and for wastewater the free chlorine residuals were 0, 0.16, and 0.31 mg/L for the 1, 5, and 10 mg/L conditions, respectively.

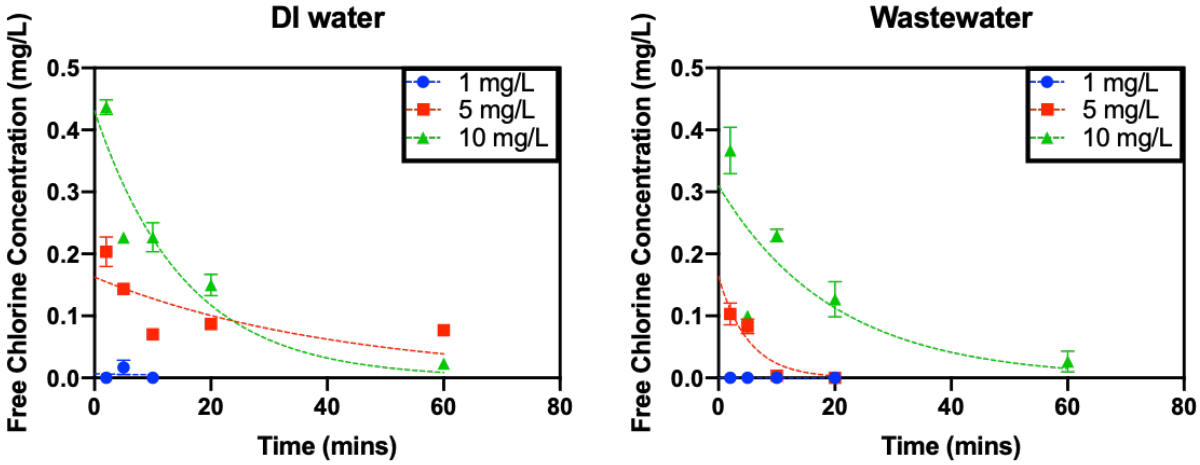

Figure 2-4. Free Chlorine Residual in Water and Wastewater for SARS-CoV-2 Experiments.
The y-axis shows free chlorine concentrations in mg/L Cl, which were measured using a HACH D900 Colorimeter, and the x-axis shows time in minutes. Each data point represents the mean of triplicate readings of free chlorine and the error bars represent the standard deviation.
Source: Greaves et al. 2022 published in *Sci. Total. Environ.*

2.4.1.2 Chlorine Disinfection of SARS-CoV-2 in DI Water and Wastewater

Inactivation of SARS-CoV-2 with sodium hypochlorite was evaluated in DI water and in wastewater. The monophasic decay models of the $TCID_{50}$ values are shown in Figure 2-5. No significant decay of SARS-CoV-2 was observed in DI water for the 1 mg/L condition and a weak significant decay was found for the 0 mg/L condition. For the 5 mg/L and 10 mg/L conditions in DI water, infectious virus was detected for 5 minutes and 1 minute, respectively. Conversely for wastewater, there was an approximate 2 log_{10} reduction of infectious SARS-CoV-2 for the 0 mg/L chlorine condition. SARS-CoV-2 was detected for the entire experiment for both 1 and 5 mg/L and at 10 mg/L, virus was detected for 30 minutes. The decay of SARS-CoV-2 in wastewater was significant for all sodium hypochlorite concentrations, as shown by the p-values on each of the panels.

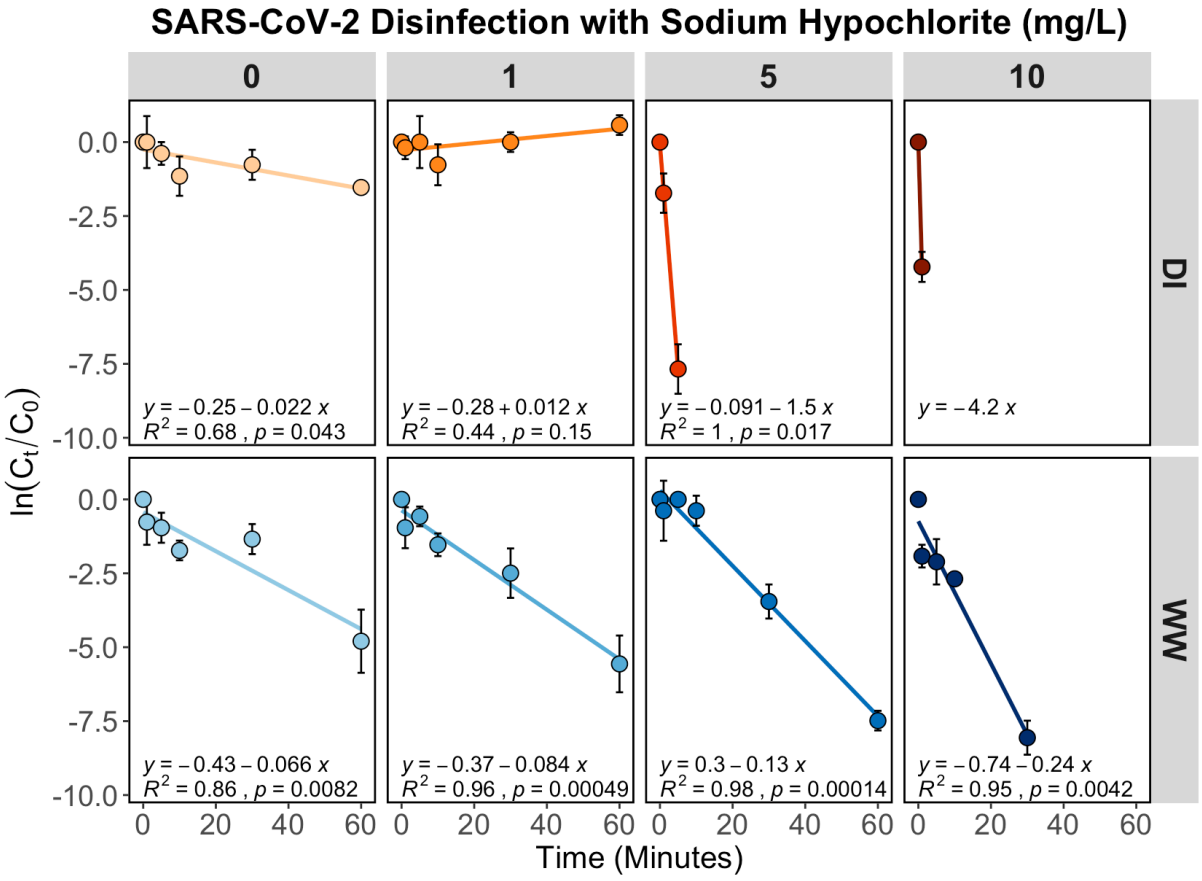

Figure 2-5. Inactivation of SARS-CoV-2 with Sodium Hypochlorite.

The panels from left to right show increasing concentrations of sodium hypochlorite in mg/L. The top set of panels show disinfection in DI water and the bottom set of panels show disinfection in wastewater. The y-axis shows the monophasic decay of the $TCID_{50}$ data, and the x-axis shows time in minutes. Each data point shows the mean of triplicate readings and the standard deviation. Where there are no error bars shown, they are too small to see.

2.4.1.3 Ct and Observed Removal of SARS-CoV-2

Based on the modeled free chlorine residuals shown in Figure 2-4, the Ct (Concentration x Time) were determined for each time point by integrating the area under the modeled curve. Due to the limited number of data points, the area under the curve for the 1 mg/L condition was not calculated and was assumed to be 0 mg/L for both DI water and wastewater. Figure 2-6

shows the observed viral reduction of SARS-CoV-2 versus the calculated Ct value. In DI water, there was less than a 1 mg-min/L Ct required to obtain a 3-log_{10} reduction in infectious virus. In wastewater, over a 5 mg-min/L was needed to observe a 3- log_{10} reduction in wastewater, which also takes into account the original 2-log_{10} reduction in wastewater without the addition of any chlorine.

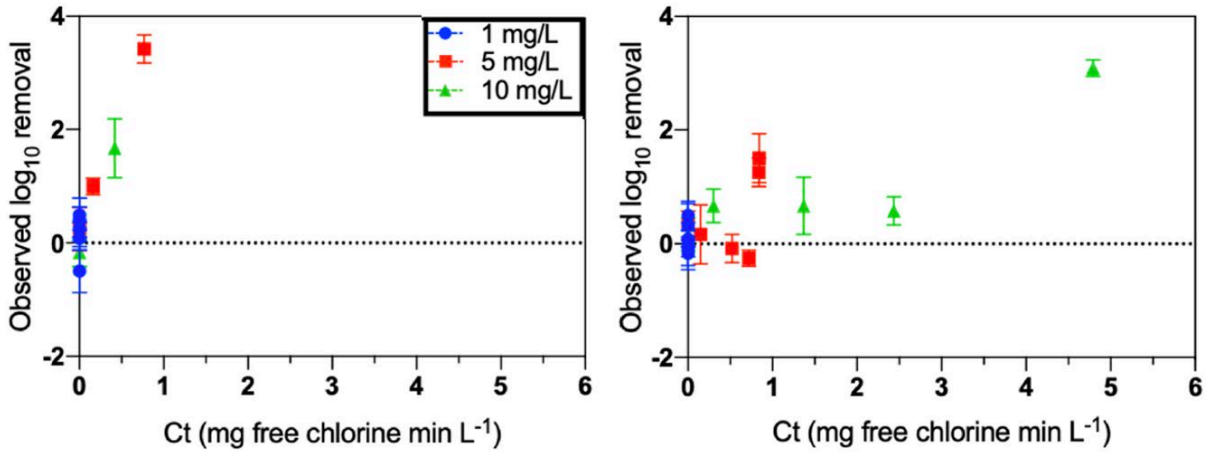

Figure 2-6. Observed Log_{10} Removal vs. Ct Value for SARS-CoV-2 in DI Water and Wastewater.
Source: Bivins et al. © 2020 American Chemical Society
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The y-axis shows the observed log_{10} removal, which was calculated by subtracting the data points for 1 mg/L, 5 mg/L, and 10 mg/L from the 0 mg/L data points. The x-axis shows the Ct values that were modeled from the free chlorine residual data. Each data point represents the mean of three replicates and the error bars show the standard deviation. The left panel shows removal in DI water and the right panel shows the removal in wastewater.

2.4.2 Discussion

As shown in Figure 2-4, there was a high initial free chlorine consumption observed in both DI water and wastewater. This phenomenon can be attributed to the DMEM which is used as viral media that was concurrently spiked into the DI and wastewater experiments for the disinfection and chlorine residual experiments. Higher Ct values were required to inactive SARS-CoV-2 in wastewater than in DI water. This could be due to the higher initial chlorine demand in the wastewater compared to DI water. Since the free chlorine is reacting with wastewater components, such as organic matter or microorganisms, there is a lower amount of free chlorine available for disinfection leading to an increased persistence of SARS-CoV-2 (Ye et al. 2016). Particles found in the wastewater matrix are known to be a preferable location for enveloped viruses to adhere to, leading to the adsorption of SARS-CoV-2 to solids which may shield SARS-CoV-2 from disinfectants resulting in increased persistence (Ye et al. 2016)

Table 2-1 summarizes the 3-log_{10} reduction Ct values of enveloped and non-enveloped viruses in DI water. From this study, the Ct value for SARS-CoV-2 was lower than those for other viruses under similar conditions.

Table 2-1. Three Log_{10} Inactivation Ct Values for Viruses with Free Chlorine in DI Water.
Source: Bivins et al. © 2020 American Chemical Society
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Virus	Enveloped/Non-Enveloped	Method of Measurement	3-Log_{10} Ct (mg-min/L)	Reference
SARS-CoV-2	Enveloped	$TCID_{50}$/mL	<1.0	This study
Human Rotavirus	Non-Enveloped	$TCID_{50}$/mL	1.35 – 2.35	(Xue et al. 2013)
Coxsackievirus B5	Non-Enveloped	PFU/mL	7.6	(Cromeans et al. 2010)
Echovirus E1	Non-Enveloped	PFU/mL	1.3	(Cromeans et al. 2010)
Echovirus E12	Non-Enveloped	PFU/mL	4.23	(Rachmadi et al. 2020)
H5N1	Enveloped	$TCID_{50}$/mL	1.08	(Rice et al. 2007)
Poliovirus 1	Enveloped	PFU/mL	3.0	(Black et al. 2009)

2.4.3 Limitations and Conclusions

This study was limited to the constraints of working with infectious SARS-CoV-2, however we were able to demonstrate that chlorine disinfection was an effective treatment for treating SARS-CoV-2 in both water and wastewater. Due to only using a single wastewater sample, an analysis of differing environmental factors such as pH, ammonia, phosphorus, and total suspended solids, could not be conducted. Lastly, spiking in virus at a high titer may result in different behaviors than that of endogenous virus.

Due to the presence of SARS-CoV-2 RNA in wastewater samples, concerns were raised about the treatment on SARS-CoV-2 in wastewater. SARS-CoV-2 is effectively disinfected in water and wastewater by chlorine and at a more rapid rate than other waterborne viruses of concern. Since SARS-CoV-2 disinfection in wastewater was slower than in DI water, it is likely aggregation of viral particles to suspended solids is important to consider when disinfecting the virus. This work resulted in a publication entitled *Sodium hypochlorite disinfection of SARS-CoV-2 spiked in water and municipal wastewater* in Science of the Total Environment in 2021. (Greaves et al. 2022)

CHAPTER 3

Model Development for Workplace Viral Exposure and Environmental Release

3.1 Initial Model Development and Scenario Construction

An exposure model was developed based upon viral dilution, persistence, disinfection, and potential exposure routes in wastewater systems by the University of Notre Dame. The model was based upon previous developments in this area, (Haas et al. 2017). This model considers the estimated inactivation, removal, and dilution to enable wastewater treatment authorities to quantitatively respond to questions from the public regarding viral releases from wastewater due to poor sanitation infrastructure or excess flow.

The specific scenarios to be considered include worker inhalation exposure when performing standard occupational activities in a sewer line serving a hospital receiving infectious waste as described in (Haas et al. 2017). Model parameters were input as distributions from the best available knowledge to account for uncertainty. Model inputs were general and 'Plain-English' to enable general use, for example 'average plant treatment capacity in Million Gallons per Day (MGD) or Million Liters per Day (MLD)'. Virus decay was estimated based upon results in Chapter 1. The model includes a 'pretreatment' option that includes disinfecting wastewater prior to disposal and expected removals from Chapter 2 (Bausch and Peters 2009). Worker exposure was evaluated both with and without the use of personal protective equipment (PPE), including goggles, a protective face mask or shield, waterproof gloves, liquid repellant coveralls, and rubber boots, to demonstrate the important role of PPE in exposure and risk reduction (CDC 2021). The model also has decay and disinfection inputs to allow application for other emerging infectious viruses.

This model was focused on exposure rather than risk to allow more rapid adaptability to various virus release scenarios. It is notable when considering this model that previous work on highly abundant viruses in sewage (norovirus, enterovirus, hepatitis A) has failed to show a significantly higher sero-conversion rate in wastewater workers (Clark et al. 1985, Trout et al. 2000). In addition, previous work on Ebola virus demonstrated that appropriate PPE usage reduced worker risk by multiple orders of magnitude (Haas et al. 2017) and that the virus was rapidly removed during disinfection (Bibby et al. 2017). Model results were carefully communicated to avoid over-extension or misinterpretation of potential exposure scenarios. Ultimately, the goal of this exposure model is to facilitate appropriate risk communication with both wastewater workers and the general public and to provide quantitative answers regarding exposure that were not previously available.

3.1.1 Scenario Descriptions

The general scenario we evaluated was a sewer line worker inhaling an infectious dose of Lassa Fever while performing regular sewer line maintenance. The current scenario assumes that a single patient sick with Lassa Fever is sick at a hospital that discharges the Lassa Fever patient's

excreta and secreta to the sewer line. The goal is to determine how much infectious Lassa virus the sewer worker would inhale in this scenario. The scenario was broken into four distinct sub-scenarios to examine the effects of various risk mitigation strategies. The mitigation strategies chosen were bleach pretreatment of the Lassa Fever patient's waste, and sewer line worker Personal Protective Equipment (PPE).

3.2 Model Parameterization and Sensitivity Analysis

The parameters used to calculate these values are stochastic, meaning they are not single values but rather ranges of potential values. A sensitivity analysis of the parameters shows which parameters have the strongest effect on the final dose values. This can help parse out which parameters are the most important, which is important for decision making and additional model refinement. To perform a sensitivity analysis, a Spearman's correlation coefficient is calculated for each parameter. This statistical test calculates a coefficient that shows the strength of the correlation, either positive or negative, between the parameter values and the final dose values. The higher the absolute value of the coefficient, the stronger it is correlated to the final outcome. The maximum absolute value for the coefficient is 1, meaning perfect correlation between two sets of values, and the minimum is 0, indicating that no correlation is present between the two sets of values. A positive coefficient indicates a positive correlation, and a negative coefficient indicates a negative correlation. The coefficient is calculated for every iteration of the model, resulting in a list of 10,000 coefficients of which the median and standard deviation are graphed below.

3.2.1 Model Parameterization

The parameters used in the model are summarized in Table 3-1.

Table 3-1. Parameters, Units, Distributions, and Values for Lassa Virus Model.

Parameter	Unit	Distribution	Value	Citation
Patient excreta and secreta production to sewer	Liters	Logistic	[5.89, 1.04]	(Haas et al. 2017)
Excreta and secreta concentrations of Lassa Fever	Viral RNA copies/mL excreta	Logistic	[$10^{4.38}$, $10^{0.61}$]	(Haas et al. 2017)
Hospital daily outflow	Liters/day	Normal	[8.27 x 10^5, 4.06 x 10^5]	(Haas et al. 2017)
Interceptor daily flow	seconds	Normal	[1.61 x 10^8, 1.5 x 10^8]	(Haas et al. 2017)
Sewer travel time from patient to point of exposure	Pathogens per m^3 headspace	Point	60	(Haas et al. 2017)
Partition coefficient (ratio of aerosol concentration of virus to liquid concentration)	Unitless	Beta	[$10^{2.328}$, $10^{1.9651}$]	(Haas et al. 2017)
Respirable fraction (fraction of aerosols generated that are respirable)	Unitless	Uniform	[0.28,1]	(Haas et al. 2017)
Worker inhalation rate	Liters/min	Normal	[51,14.9]	(Haas et al. 2017)
Time spent by sewer worker in the proximity	Hours	Uniform	[0.5,4]	(Haas et al. 2017)
Lassa biological half life	Minutes	Uniform	[10.1, 54.6]	(Stephenson et al. 1984)
PPE removal fraction	Unitless	Uniform	[0.95, 0.99]	(Haas et al. 2017)
Bleach pretreatment removal fraction	Unitless	Uniform	[0.937, 0.99]	(Trajano et al. 2016)
Fraction Lassa RNA copies that are infectious	Unitless	Uniform	[10^3, 10^4]	Estimate

3.2.2 Sensitivity Analysis

The sensitivity analysis of parameters assuming no bleach pretreatment or PPE in use is shown in Figure 3-1. The parameters with the strongest correlation coefficient, either positive or negative, have the greatest effect on the exposure dose to the sewer line worker. These are considered “sensitive” parameters. Concentration in excreta and hospital outflow are not sensitive parameters as there is not much variation within the parameter input values.

Table 3-2. Key for Sensitivity Analysis Figures.

Variable	Meaning
Time_prox	Time spent by sewer worker in the proximity
Res_frac	Respirable fraction
Excreta	Patient excreta and secreta production to sewer
Inhal_rate	Worker inhalation rate
Half_life	Lassa decay
Conc_excreta	Concentration of Lassa in excreta
Hos_outflow	Hospital daily sewer outflow

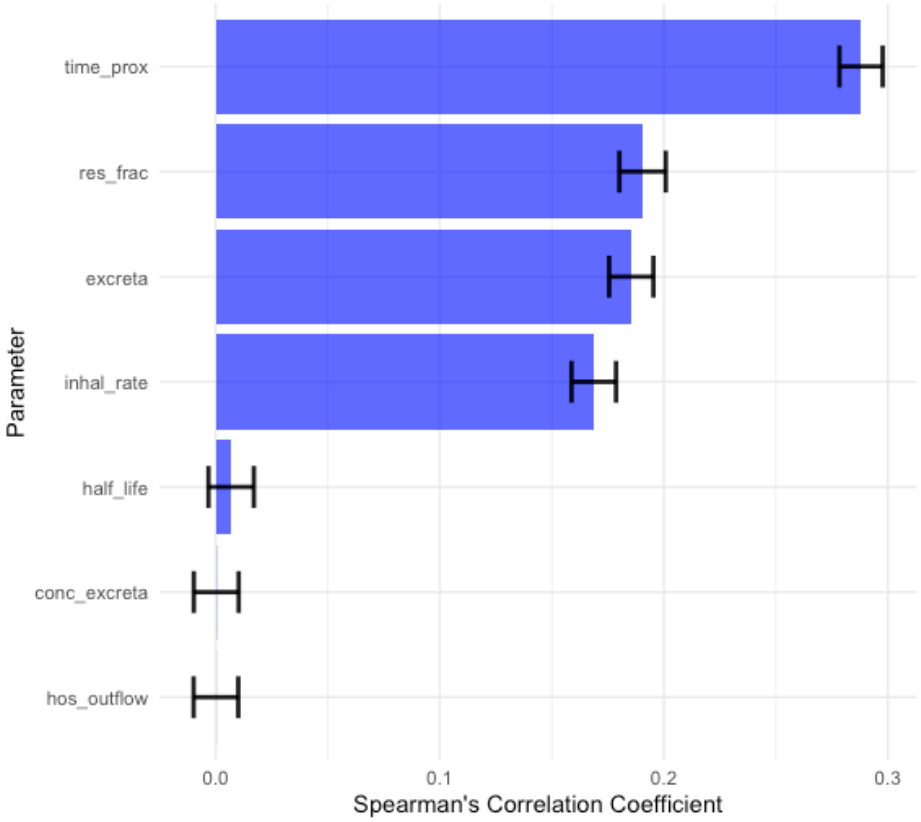

Figure 3-1. Sensitivity Analysis of Parameters Assuming No Bleach Pretreatment or PPE in Use.
The median is graphed, and the error bars represent the standard deviation for each of the parameters. Parameter names are listed in Table 3-2.

The sensitivity analysis of parameters assuming no bleach pretreatment but PPE in use is shown in Figure 3-2. In this scenario, the time the sewer line worker is in proximity to the sewage is positively correlated with the final exposure dose, whereas worker PPE is negatively correlated with final exposure dose. This means as time in proximity increases exposure dose increases, whereas as PPE effectiveness increases exposure dose decreases.

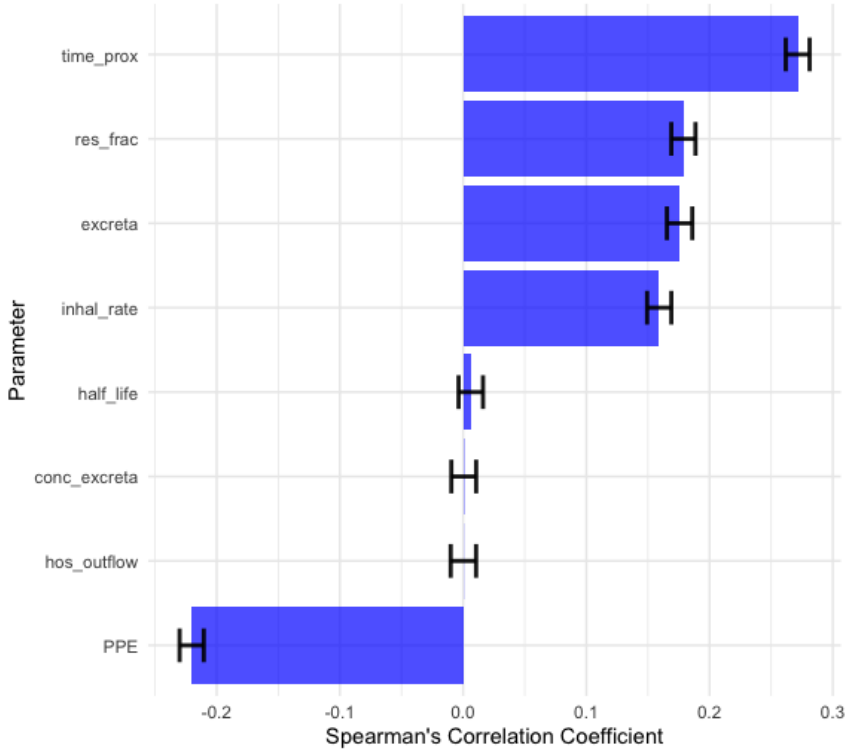

Figure 3-2. Sensitivity Analysis of Parameters Assuming No Bleach Pretreatment, but PPE in Use.
The median is graphed, and the error bars represent the standard deviation for each of the parameters. Parameter names are listed in Table 3-2.

The sensitivity analysis of parameters assuming bleach pretreatment and PPE in use is shown in Figure 3-3. Predictably, effectiveness of the two mitigation factors, bleach pretreatment and PPE, are both negatively correlated with exposure dose while time in proximity remains the parameter with the strongest positive correlation to exposure dose. Bleach is slightly more negatively correlated to the Lassa Fever dose than PPE, which could be because it has a larger range of effectiveness than PPE.

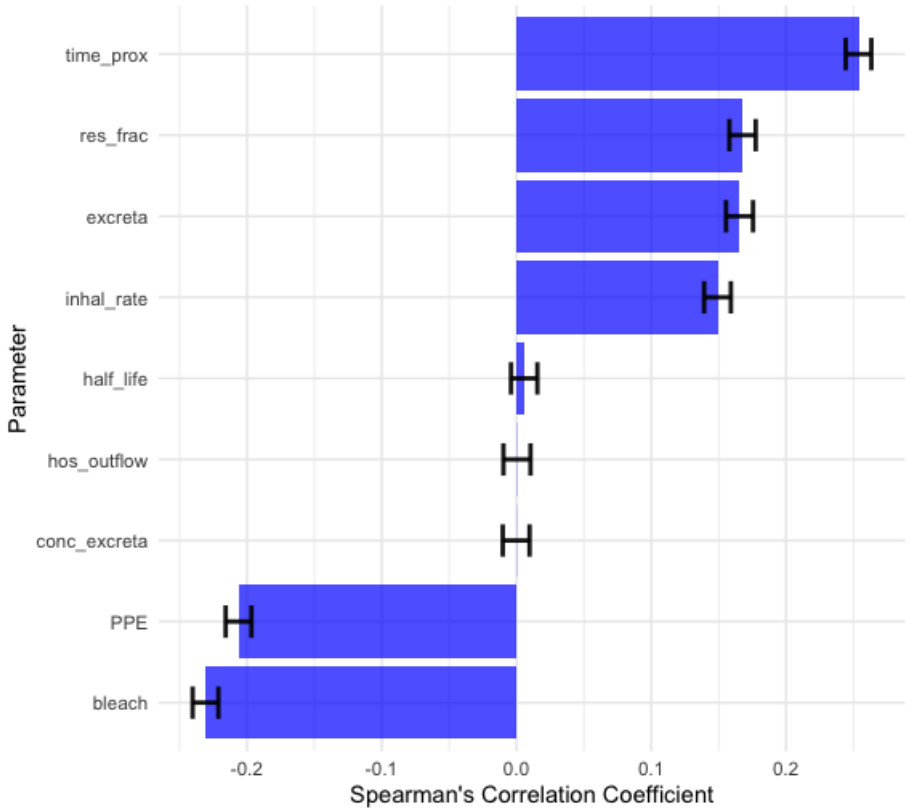

Figure 3-3. Sensitivity Analysis of Parameters Assuming Bleach Pretreatment and PPE in Use.
The median is graphed, and the error bars represent the standard deviation for each of the parameters. Parameter names are listed in Table 3-2.

The sensitivity analysis of parameters assuming bleach pretreatment but no PPE is shown in Figure 3-4. Other parameters that correlated positively with exposure dose are respirable fraction, excreta production to sewers, and worker inhalation rate. Descriptions of these parameters can be seen in Table 3-1.

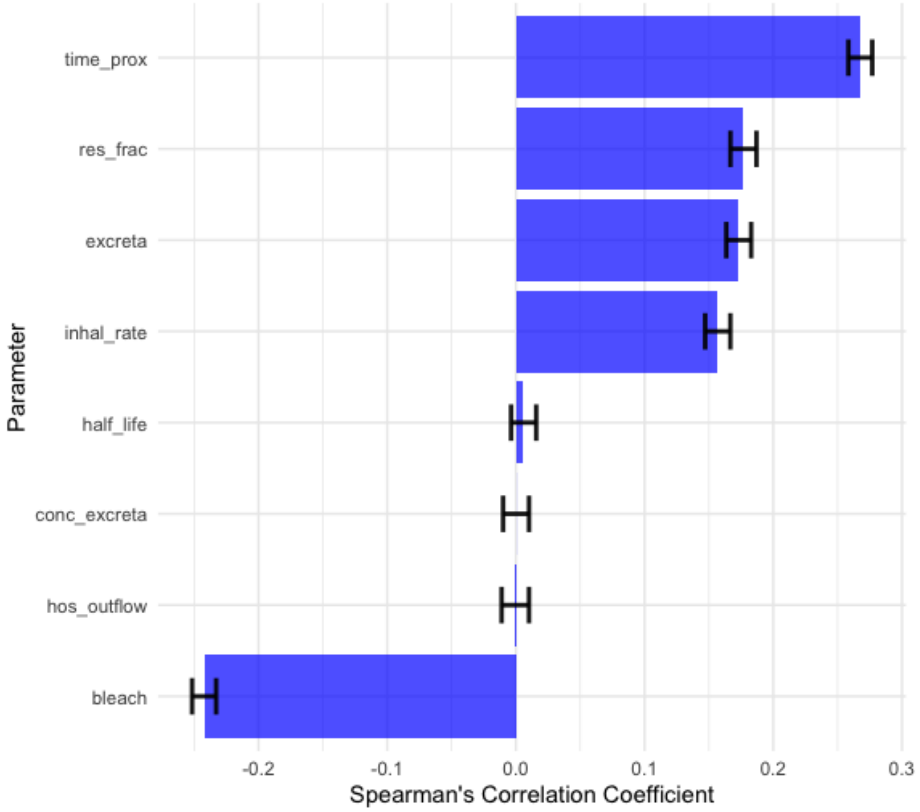

Figure 3-4. Sensitivity Analysis of Parameters Assuming Bleach Pretreatment, but No PPE in Use.
The median is graphed, and the error bars represent the standard deviation for each of the parameters. Parameter names are listed in Table 3-2.

3.3 Construction of User Interface

Figures 3-5, 3-6, and 3-7 show an initial web-based user interface of the developed model. The general scenario we evaluated was a sewer line worker inhaling an infectious dose of Lassa Fever while performing regular sewer line maintenance. The current scenario assumes that a single patient sick with Lassa Fever is at a hospital that discharges the Lassa Fever patient's excreta and secreta to the sewer line. The goal is to determine how much infectious LASV the sewer worker would inhale. The scenario was broken into four distinct sub-scenarios to examine the effects of various risk mitigation strategies. The mitigation strategies chosen were bleach pretreatment of the Lassa Fever patient's waste, and sewer line worker PPE. The four scenarios are no bleach pretreatment or PPE, no bleach pretreatment but PPE used by sewer worker, bleach pretreatment and PPE in use, and bleach pretreatment but no PPE in use.

User modifiable inputs include the number of Lassa patients in the local hospital, time workers spend in proximity to wastewater, and hospital daily sewer outflow. These inputs were found to have the largest impact on model outcomes from the sensitivity analysis. Additionally, the user can choose to view or not view outliers. The model produces a plot, as shown in Figures 3-5 to 3-7, where 15 PFU via inhalation is required for infection, meaning anything below 15 PFU presents an acceptable level of risk.

Figure 3-5 shows the user interface when the minimum number of Lassa infected patients are in a local hospital, the lowest time a worker has spent in proximity to wastewater, and the minimum daily outflow from the hospital. As shown in the output of the user interface, the dose for all four of the scenarios are below 1 PFU, with the lowest dose being the scenario with PPE and bleach being used by the worker.

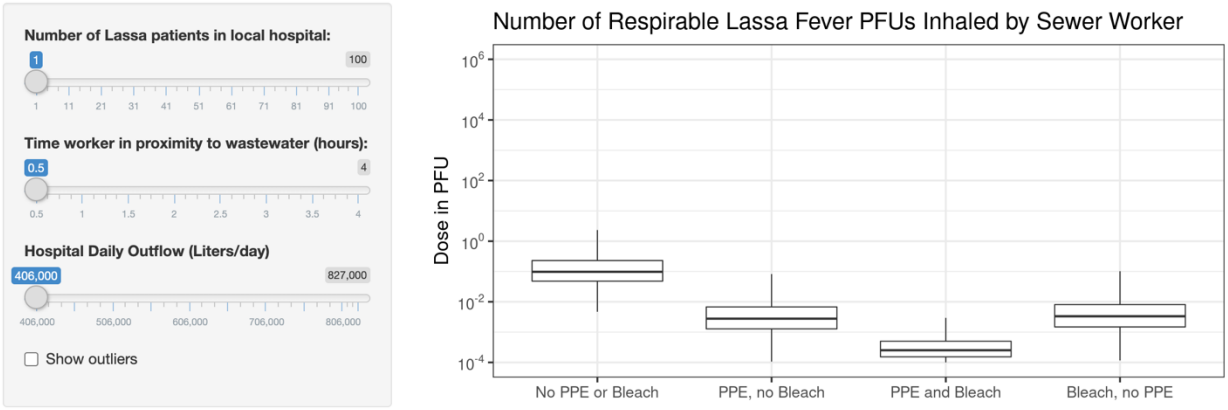

Figure 3-5. Lassa Virus Dose for Minimum Patients, Worker Time, and Outflow.
The left side shows the user interface with the range of inputs. The right side shows the dose of Lassa virus in PFU for a sewer worker for four different scenarios combining the use of PPE and bleach.

The median values for the number of Lassa infected patients in a hospital, the time a sewer worker is in proximity to the wastewater, and the hospital daily outflow were input into the model and the results are shown in Figure 3-6. When compared to the minimum model inputs from Figure 3-5, there was approximately a 2-log increase in the dose of LASV. A worker using both bleach and PPE still had the lowest inhalation of infectious LASV particles.

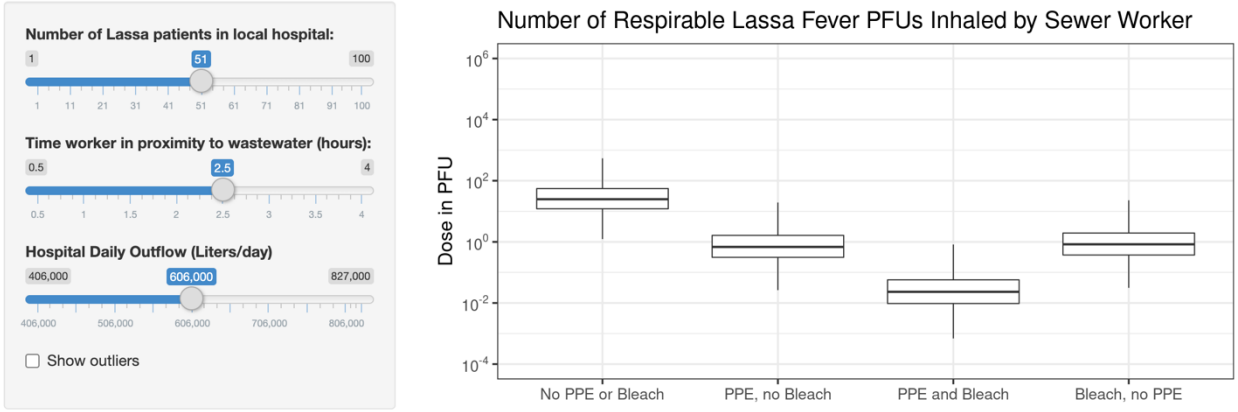

Figure 3-6. Lassa Virus Dose for Approximately Median Patients, Worker Time, and Outflow.
The left side shows the user interface with the range of inputs. The right side shows the dose of Lassa virus in PFU for a sewer worker for four different scenarios combining the use of PPE and bleach.

Lastly, the maximum value for the number of patients with Lassa in a hospital, the time a worker was in proximity to the wastewater, and the hospital daily outflow were analyzed using the user interface and the output is shown in Figure 3-7. With the maximum possible outcomes

for each parameter, the use of PPE and bleach resulted in a modelled dose lower than the 15 PFU via inhalation required for infection.

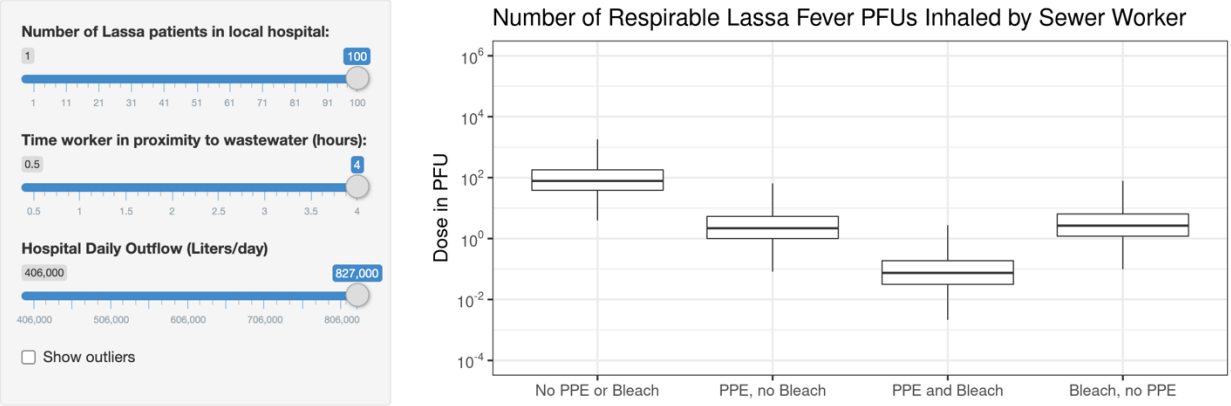

Figure 3-7. Lassa Virus Dose for Maximum Patients, Worker Time, and Outflow.
The left side shows the user interface with the range of inputs. The right side shows the dose of Lassa virus in PFU for a sewer worker for four different scenarios combining the use of PPE and bleach.

3.4 Conclusions

The user interface created in this chapter provides information to inform worker safety while working on a sewer line which has waste from an individual infected by LASV. The model showed the use of both bleach pretreatment and PPE results in the lowest associated risk for a sewer worker, and even at the maximum model limits the determined dose is still less than the 15 PFU inhaled required for infection. Ultimately, the model developed in this chapter can be used to inform both worker and public safety during an outbreak response for LASV.

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