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Zika Virus RNA Persistence in Municipal Wastewater and Surface Water

Cailee Hill

ZIKA VIRUS RNA PERSISTENCE IN MUNICIPAL WASTEWATER AND SURFACE WATER

By

CAILEE K. HILL

INTRODUCTION: Zika virus (ZIKV), an enveloped virus of the species Flavivirus, was historically, rarely associated with human infection and diseases, but that changed with large outbreaks in French Polynesia in 2013 and then Brazil in 2015. With approximately 80% of ZIKV infections being asymptomatic and a lack of adequate surveillance methods and healthcare access and resources, Brazil had challenges in determining the true burden of infection in affected communities. Though the COVID-19 pandemic has increased the evidence of the detectability of enveloped viruses in wastewater, but there are still research gaps regarding how environmental conditions may affect detection of enveloped viruses such as ZIKV.

AIM: The objectives of this study are to utilize wastewater-based epidemiological methods as a means to detect and evaluate the stability of ZIKV RNA in primary influent and surface water under different environmental conditions including temperature, water matrices, and inoculation concentrations.

METHODS: ZIKV MEX 1-44 was added to primary influent from a local municipal wastewater treatment plant and surface water from a local stream in Atlanta, Ga. Three experiments were conducted. to assess the detection and persistence of ZIKV RNA in different conditions including different inoculation concentrations, water conditions and temperature. RNA was extracted at days 0, 1, 3, 7, 14, 21, and 28 for all experiments. Digital droplet polymerase chain reaction (ddPCR) was used to quantify ZIKV RNA concentration. Log10 concentrations and Log10 reductions (Ct/C0) were calculated and graphed to compare ZIKV RNA decay over time under the different conditions.

RESULTS: ZIKV RNA was detected in all samples at all times, temperatures, and water matrices with ddPCR during the month-long experiment. Across experiments, ZIKV RNA degradation increased in surface water, at increasing temperatures, and at lower inoculation concentrations. The ZIKV RNA signal decayed most rapidly in surface water at 35°C, with little to no decrease observed at 4°C across water matrices.

DISCUSSION: ZIKV RNA is more environmentally stable than previously assumed. This study increased the body of evidence for the methods of detection and the stability of enveloped viruses, such as ZIKV, in various waters, portraying how environmental conditions may affect said stability. Cost-effective and wide-scale population level surveillance methods to detect ZIKV RNA in wastewater and environmental waters such as these, are of increasing importance to better understand the burden of ZIKV in a community and to monitor for not only ZIKV, but also other emerging and reemerging pathogens. Such methods have the potential to be utilized in low-resources settings, filling the gaps, and overcoming the challenges of clinical surveillance.

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CAILEE K. HILL

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By

CAILEE K. HILL

Approved:

Dr. Christine Stauber

Committee Chair

Dr. Lisa Casanova

Committee Member

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LIST OF ABBREVIATIONS

ZIKV – Zika Virus

RNA – ribonucleic acid

WBE – Wastewater-Based Epidemiology

ddPCR – Digital Droplet Polymerase Chain Reaction

INTRODUCTION

The Zika virus (ZIKV) was identified in Africa over 50 years ago, but has since spread across the globe to Asia, the Pacific Islands, and in more recent decades, eastward to the Americas [1]. ZIKV is a single-stranded, positive sense RNA virus and a member of Flavivirus species [2]. It is similar to other Flaviviruses in its spherical in structure with an envelope formed by a lipid bilayer containing 180 units of glycoproteins [2]. ZIKV infection is a primarily a mosquito borne disease and related to the dengue virus (DENV), yellow fever virus, and West Nile virus (WNV), which are all primarily transmitted by the bite of an infected mosquito from the *Aedes* genus [3]. In tropical and subtropical regions, ZIKV is predominantly carried and transmitted to humans by the *Aedes aegypti* mosquito, but transmission can also occur from mother to fetus, and through sexual contact, blood transfusion, and organ transplant [4].

Historically the ZIKV was rarely associated with human infection and disease. Yet, the first significant epidemic was reported in 2007 with an estimated 185 cases in the Yap Islands [1]. This instance was followed by larger outbreaks in French Polynesia and other Pacific territories and countries in 2013 [1]. In March 2015, Brazil reported a large outbreak of an unknown illness which was soon after identified as ZIKV infections [1]. The virus spread rapidly across Brazil and into surrounding countries and territories on the American continent, with phylogenic studies having since indicated that ZIKV introduction to Brazil happened as early as 2013 [5].

Approximately 80% of ZIKV infections are asymptomatic, while many symptomatic cases have mild symptoms including a rash, low-grade fever, conjunctivitis, muscle and joint pain, and a headache, with an infection lasting about 2-7 days [1, 6]. When complications do occur, they can be severe and fatal [1, 4]. An association between ZIKV infection and Guillain-Barre syndrome (GBS) was found as increased reports of GBS coincided with the ZIKV epidemic in French Polynesia and the Brazil outbreaks [1, 4]. It is also well documented that a ZIKV infection in pregnant women can cause serious detrimental fetal health outcomes, with reports from the Ministry of Health of Brazil suggesting microcephaly increased by a factor of 20 among newborns in areas with Zika outbreaks [7]. Autopsies of fetal brain tissue from the aborted fetuses of pregnant women, with mild symptoms but never diagnosed with ZIKV, showed enveloped structures and high concentrations of ZIKV RNA in the brain tissue, higher than normally found in the serum of infected adults [7]. There is currently no treatment available for ZIKV infection or the associated diseases [4]. The diagnosis of an infection can only be confirmed by laboratory tests of blood or other bodily fluids, which not only can burden the health systems in

developing countries with limited access and reduced resources but may signify the virus is already circulating and thus, large amounts of people may already be infected [4].

In countries like Brazil where mosquito borne diseases are endemic, and there are limited healthcare resources, monitoring and surveillance based solely on clinical and other previously used epidemiological methods can be unreliable and inaccurately describe the true prevalence of infections in a population. In contrast, Wastewater-Based Epidemiology (WBE) employs surveillance methods that can monitor the circulation of many pathogens at once in real-time, including the emergence of novel viruses, providing information on a community wide exposure and health status, comprehensively [8]. Such information gives public health institutions an early warning of pathogen introduction and thus, time to mitigate possible outbreaks as a means of reducing the burden of disease in a community, possibly before even one clinical case is reported [9, 10].

There has been a growing body of research using WBE for monitoring non-enveloped viruses, but until SARS-CoV-2 pandemic there was limited research on the detection of enveloped viruses, like ZIKV. With evidence that enveloped viruses are more stable in the environment than previously thought, studies examining the detection, survivability, and degradation of enveloped viruses have referenced the need for more research into how environmental conditions may affect their detection [11-13].

Since the Brazil outbreak in 2015, ZIKV cases in the Americas have fallen, but the virus continues to spread where the vector is present [14]. The 2017 World Health Organization (WHO) Zika Virus Situation Report states the global risk assessment of ZIKV has not changed and over 80 countries show evidence of transmission, with 61 areas with ongoing transmission being due to its first introduction or reintroduction [14]. Furthermore, ZIKV transmission may be occurring without an identifiable outbreak since the majority of infections are asymptomatic. In 2017 a large unreported outbreak of ZIKV was identified in Cuba, with cases still being discovered in 2018, suggesting that ZIKV may still be spreading silently in the Americas [6].

Factors such as population growth, urbanization, climate change, and antibiotic resistance, have increased the public health concern of the emergence of novel pathogens, reemergence of previously eradicated pathogens, and global changes in the distribution of such pathogens. Vector borne diseases, specifically, are expected to undergo changes in their distribution globally due to climate change, with elevated temperatures expanding the geographic vector range, decreasing the pathogen's incubation period, and increasing the mosquito biting rate [15]. As vectors, such as mosquitoes, move to new geographical regions, the risk of the introduction of diseases like ZIKV, in unprepared areas which previously had no experience dealing with it, is an imminent concern.

Though ZIKV may not be the prominent virus of public health concern at this moment, it has the potential to reemerge, along with the threat of other novel viruses. With recent severe outbreaks having been caused by enveloped viruses, such as Ebola, sever acute respiratory syndrome (SARS), and avian influenza H5H1, the need for rapid health surveillance is a growing necessity to advance disease outbreak mitigation and improve public health responses for local and large-scale viral outbreaks in the future [11].

This study aims to demonstrate ZIKV RNA detection and persistence, to answer the questions of how ZIKV RNA persistence is affected by different conditions including differing environmental water matrices, different virus inoculation concentrations (high vs low), and different temperature conditions (4°C, 25°C, 35°C). We expect the rate of RNA degradation to increase as the temperature increases, increase at lower inoculation concentrations, and be affected by different water matrices. The goal is to increase evidence and support for WBE surveillance methods for enveloped viruses.

LITERATURE REVIEW

The Zika Virus and Public Health:

ZIKV is a member of the family Flaviviridae, genus Flavivirus and like other viruses in this family, it is most commonly transmitted by mosquito or tick bites [2]. ZIKV is spherical in structure, with a diameter between 42-52nm and a lipid bilayer containing 180 units of glycoproteins E and M which are able to bind to multiple cell receptors [2]. The nucleocapsid is formed by protein C and positive-sense, single-stranded viral RNA molecule which encodes three structural and seven nonstructural proteins [2]. In vivo and in vitro studies have shown the virus replicates in the rough endoplasmic reticulum membranes [2].

The ZIKV was first discovered as an infectious agent in 1952 and since then sporadic cases have been reported across the African and Asian continents [2]. In 2007 the first large outbreak was reported in the Yap Island, Micronesia, where an estimated 73% of the population was exposed and developed mild symptoms [6]. The first cases were detected in Brazil in 2015 after its isolation from patients with dengue-like symptoms [2]. Up until October 2015, ZIKV infection was considered a benign disease, which changed due to the sharp increase in the number of babies born with microcephaly observed in Brazilian maternity services, with these increases following the spatial diffusion of the ZIKV epidemic [5, 16]. Brazil being the first country to report deaths caused by ZIKV and its rapid spread across the country, lead the Pan American Health Organization (PAHO) to launch an alert, calling for the development of technical capabilities to detect and confirm ZIKV cases in the Americas [2]. In 2016 the World Health Organization (WHO) declared ZIKV a Public Health Emergency of International Concern (PHEIC) [6].

Diagnosis was problematic in Brazil due to the overlap in the epidemiological distribution of Zika, dengue, and chikungunya, the lack of laboratory diagnostic test, limited time period the viral particles persist in blood, large percent of asymptomatic infections, and antibody cross-reactivity with other flaviviruses such as Dengue viruses [5]. Most surveillance during the outbreak was disease-based and dependent on monitoring pregnant women and cases of microcephaly, other congenital anomalies, and fetal loss [5]. Other surveillance methods, such as public-health-integrated systems, have been recommended to better detect and respond to disease outbreaks [5].

Since the Brazil ZIKV outbreak in 2015, multiple studies have examined the diagnostic utility of urine, feces, blood, and saliva for detecting ZIKV RNA. A study of patients from an outbreak of ZIKV in French Polynesia found that ZIKV RNA in urine samples was detected at higher concentrations for longer periods of time than in serum blood samples, providing preliminary evidence of the excretion of ZIKV in

urine [17]. A similar study in Brazil tested the urine and saliva from patients in the acute phase of a ZIKV infection, demonstrating it was possible to recover ZIKV from these bodily fluids using commercial extraction and isolation kits [18]. This study also detected higher viral loads in urine samples compared to saliva samples, confirming previous research that indicates urine as a better ZIKV molecular diagnostic source [18]. Another study inoculated Neotropical primates with ZIKV and found it was detectible in serum, saliva, urine, and feces for a combining average of 5-11 days, concluding that fecal detection was the most cost effective, noninvasive method for monitoring wild populations of primates [19].

Since the Brazil outbreak, outbreaks have been reported in Guinea-Bissau, Singapore, Vietnam, Thailand, and India, as well as documented transmission in the United States [6]. With evidence the virus is excreted in large quantities in urine and (possibly feces) and the fact it continues to circulate around the globe, the utilization of monitoring tool such as WBE methods is of growing importance.

Wastewater Based Epidemiology for Monitoring Human Viruses:

Wastewater based epidemiology is a relatively new and growing approach for the surveillance of infectious diseases but can be a particularly useful method for monitoring human viral diseases. Traditionally, viral environmental surveillance was primarily utilized for RNA enteric viruses and DNA viruses excreted in feces and urine, which are nonenveloped and primarily transmitted through the fecal-oral route [12]. Such studies on viral surveillance in wastewater and environmental waters have portrayed the ability to detect enteric viruses such as rotaviruses, enteroviruses such as polio, adenoviruses, noroviruses, and hepatitis A and E viruses [9, 10, 20]. These studies were used to understand the role of transmission and have shown correlations between the quantities found in wastewater and local viral outbreaks, as well as the utility of wastewater surveillance on a global level, like during the global polio eradication program [9, 10, 21].

Owing to the increasing threat of emerging and reemerging viruses, made particularly evident by the first SARS outbreak and highlighted during the current SARS-CoV-2 pandemic, there is a need to better understand the spread of viral diseases at a community level, providing public health officials information for the timely mitigation of outbreaks [10]. WBE may fill the gaps of other surveillance methods, including its predictive techniques, aiding in such timely mitigation efforts [22].

Many human infectious viruses are excreted in large quantities through feces and urine, contributing to a community's wastewater virome [12]. Not only are viruses excreted by individuals with the disease symptoms, but also by those who are asymptomatic; and in some cases, they can continue

to be excreted after symptoms have resolved [12]. Since viruses require host cells to grow, unlike bacteria and other microorganisms, concentrations found in a community's wastewater may represent the quantity of pathogens in the entire corresponding population [9, 21]. This characteristic makes detection of human viruses in wastewater a natural biomarker of their own circulation, as long as their DNA or RNA persists long enough to be detected [21]. Due to the often-high infectivity, rapid transmission, and lack of treatment for human viruses, they can be considered the most hazardous pathogens resulting in devastating health consequences for a community; yet these same characteristics make clinical monitoring and surveillance a challenge [9, 10].

Clinical surveillance requires the pathogen to have already circulated enough in a population as to have enough individual cases to acquire data, so such clinical methods typically happen after a pathogen has already undergone widespread transmission. In contrast, WBE detection and surveillance methods may be able to provide comprehensive and objective data for an entire population in real-time [22]. The early detection that a virus has begun circulating, at wide scale population level, allows for the prediction of outbreaks in real-time, indicating the critical moments and locations a disease begins circulating [9, 21]. This approach gives public health officials information to respond more rapidly, employing and focusing interventions when and where necessary [8]. Also, clinical methods often overlook mild or asymptomatic cases, leading to inaccurate epidemiologic models and prevalence, while monitoring the diversity and temporal change in viral concentrations in a community's wastewater samples allow for the determination of the true extent of infection in the population [10, 21]. Wastewater monitoring allows for the surveillance of multiple viruses, including the emergence of rare and novel strains [21]. Due to its scalability and cost-effectiveness, WBE has great potential to be applied in areas with limited resources where otherwise a human viral infection would require clinical diagnosis, which many people do not have access to or cannot afford [21].

Environmental stability:

While all viruses are susceptible to degradation due to environmental factors such as temperature, UV light, pH, and water matrices, it is widely accepted that nonenveloped enteric viruses can survive long periods of time in the environment because they are highly resistant to heat, oxidants, and acids [21]. Historically, the detection and survival of enveloped viruses is less clear and has been debated because of their structural differences [21]. Enveloped viruses are considered to have less stability under aqueous environmental conditions, due to their susceptibility to damage of their lipid bilayer membrane outside the viral protein capsid, as well as their differing functional groups on the outer surface, which may impact their survival [11].

The ability to detect ZIKV in urine and saliva, unlike DENV, while also being more thermally stable than DNEV may be due to differences in parts of the envelope E protein between the two, giving evidence for ZIKV being stable enough to survive in harsh conditions such as once excreted into the environment [13]. Another study assessing the environmental stability of ZIKV, found that the virus was stable up to 50°C and had the highest infectivity at approximately a pH of 9 [23]. Most of the studies above highlighted those specific conditions may be necessary for urine detection, such as acidity and chemical matrices, and if certain conditions are met ZIKV may be able to survive in sewage waters [18, 24].

The assumptions that human enveloped viruses undergo rapid inactivation and degradation in wastewater and other environmental waters, exist in low concentrations in human feces and urine, and are more sensitive to the detergents and organic solvents commonly used in extraction and purification methods, have hindered the utilization of WBE methodologies [11]. However, growing evidence has suggested those assumptions to be incorrect for multiple enveloped viruses.

Enveloped virus detection:

A number of studies have not only shown that enveloped viruses may be more stable under aqueous environmental conditions than previously thought but are also able to sorb to the suspended solids in wastewater at a higher percentage than non-enveloped viruses [11, 12, 25]. Such studies have also demonstrated WBE methodologies for viral concentration and detection can be used to detect certain enveloped viruses including coronaviruses, influenza, and bovine viral diarrhea virus (BVDV) in wastewater, and/or environmental surface waters [12, 20].

A 2008 study of Human coronavirus (HCoV) found that though it inactivated more rapidly in water and wastewater at ambient temperatures, it can be protected from degradation in water matrices with high levels of suspended solids and organic material, surviving longer in primary wastewater than secondary wastewater [25]. Another study aimed to compare the survival and partitioning behavior of the model human enveloped virus surrogates, murine hepatitis virus (MHV) and Pseudomonas phage (φ6), to two nonenveloped bacteriophages in raw wastewater [11]. The results were similar to previous studies in terms of the protection enveloped viruses have in wastewater due to organic material and suspended solids, while only 6% of the nonenveloped viruses absorbed to the solids in the wastewater, 26% of the enveloped viruses were absorbed [11]. The researchers concluded that though the enveloped viruses were inactivated more rapidly, they survive long enough to be detected and could be a possible concern for wastewater treatment facilities if they are still infective [11].

Another study employed WBE using metagenomic and qPCR to identify an array of viruses found in the wastewater of a large urban population and were able to detect not only a variety of enteric viruses, but also contigs of multiple enveloped, vector borne viruses of the genus Alphavirus [20]. The most current evidence of enveloped virus detection in wastewater is its use during the present COVID-19 pandemic, with multiple countries having detected SARS-CoV-2 in their untreated wastewater [10, 26, 27]. One study in the Netherlands found evidence of SARS-CoV-2 in the wastewater weeks before the first clinically confirmed case, and the researchers demonstrated that increases in the wastewater RNA concentration correlated with increases in reported cases [28].

While there are multiple studies mentioned that show ZIKV RNA is detectable in bodily fluids, feces, and urine more so than serum and saliva, Muirhead et al., 2020 was the first of its kind to study ZIKV RNA persistence and detection in spiked municipal wastewater samples [29]. This study aimed to develop a method for analyzing the persistence of Zika RNA in environmental waters using a commercially available RNA extraction and detection kit, which could be utilized in communities with limited resources or complex laboratory settings [29]. The study also evaluated the stability of RNA detection in sewage over a month period at three temperatures (4°C, 25°C, and 35°C), filling in the gaps referenced in previous works as to how temperature may affect viral persistence and survival [29]. The results from this study provide evidence that such test kits may be successful in Zika RNA detection in environmental samples, as well as evidence that though Zika RNA decay happens more rapidly at higher temperatures, it can still be detected for weeks [29]. This study supports the hypothesis that enveloped viruses can be detectable for extended periods of time in wastewater, while highlighting the need for more research into the detection at differing initial concentrations of virus and differing environmental water types.

Literature Review Conclusions:

The present literature review gives evidence of the utility of WBE to monitor human viruses and preliminary evidence of enveloped virus detection and stability, while also referencing knowledge gaps on how environmental conditions may affect the persistence and detection, calling for more research on different enveloped viruses under differing aqueous conditions. The evidence presented in the literature review, combined with the threat of emerging and reemerging viruses, made particularly evident by the current SARS-CoV-2 pandemic, give merit for the present investigation into the persistence of ZIKV RNA detection in wastewater and how environmental conditions may affect the persistence.

The purpose of this study is to evaluate how the conditions of varying water matrices, inoculation concentrations, and temperatures, affect the detection and degradation of ZIKV RNA. By

using commercially available extraction kits and ddPCR quantification we will compare the differences in ZIKV RNA decay over time across environmental conditions. Studying how such environmental conditions affect the persistence and stability of enveloped viruses, such as ZIKV, will add to the understanding of how environmental surveillance can be utilized to advance disease outbreak mitigation and improve responses in the future, reducing the burden on the affected community.

MATERIALS AND METHODS

ZIKV culture:

An aliquot of ZIKV, strain MEX 1-44 was provided by Dr. Robert Tesh from the World Reference Center for Emerging viruses and Arboviruses (WRCEVA), University of Texas Medical Branch, Galveston, TX. This was cultured to a concentration of 10^6 plaque forming units (pfu) per ml in the laboratory of Dr. Margo Brinton. The culture was stored at -80 until use in the following experiments that are described below.

Environmental Waters:

Primary influent was collected from a local wastewater treatment plant in Atlanta, Ga using 1-liter plastic bottles. Surface water samples were collected from a local stream in Atlanta, Ga. All samples were stored at 4°C for up to 7 days prior to use in controlled persistence experiments.

Controlled laboratory experiments:

We performed three controlled laboratory experiments to examine the persistence of ZIKV RNA in various waters.

For first experiment we tested the persistence of ZIKV RNA in three water types. During this experiment, the following variables were examined: water type, temperature, and time. We tested ZIKV persistence in deionized water, a turbid surface water (stream), and primary influent. For each sample, we added ZIKV to 9.9mL of the water sample and spiked each sample with ZIKV MEX 1-44 to achieve a concentration of 10^5 pfu/ml in unpasteurized primary influent. To test the impact of temperature on the persistence of ZIKV in the different water matrices, three temperature conditions, 4°C, 25°C, and 35°C, were replicated in triplicate. The inoculated water samples spiked with ZIKV were stored in an incubator or a refrigerator at 4°C, 25°C, and 35°C.

For the second experiment, we tested the impact of ZIKV inoculum concentration in primary influent. In triplicate, primary influent samples were spiked to achieve a concentration of 10^5 pfu/ml or 10^4 pfu/ml. The samples were stored in an incubator at 25°C.

For the third experiment, ZIKV RNA persistence was examined in three water types, primary influent, surface water and a combination of the two at a 1:1 ratio. In triplicate, we spiked each sample to achieve a concentration of 10^4 pfu/ml and they were then stored at 25°C.

For all experiments and conditions, a sample of primary influent was not spiked with ZIKV but was incubated or refrigerated and extracted to act as a biologic control. Aliquots were removed and nucleic acids were extracted for all sample replicates at 7 time points, days 0, 1, 3, 7, 14, 21, 28, for each of the 3 experiments, with a total of 189, 42, and 63 repeated measures, respectively.

Nucleic acid isolation and extraction were performed using the QiaAMP Minelute Virus Spin Kit and carried out according to the manufacturer's instructions and recommendations. The QiaAMP Minelute Virus Spin Kit utilizes a simple method of viral RNA purification with fast spin-column procedures, resulting in viral nucleic acids which are free of proteins, nucleases, and other impurities, thus making them suitable for use in sensitive downstream applications [30]. The extractions were eluted to a final volume of 100uL containing buffer AVE and aliquoted into duplicate samples of 50uL and stored in a -20°C until a downstream application was performed.

Extractions were tested for ZIKV RNA using a novel digital droplet polymerase chain reaction (ddPCR) assay developed by Georgia Institute of Technology PhD student, Kevin Zhu. A ddPCR assay, a refinement of the conventional PCR methods, was utilized due to it having a better tolerance to inhibitory substances found in environmental waters and having high sensitivity and specificity for rare targets, giving a more accurate viral quantification compared to conventional PCR methods such as Real-time PCR (qPCR) [31]. Other features of ddPCR that make it amenable for the quantification of viruses at low concentration in water samples include absolute quantification without a standard curve and high reproducibility [32].

Data analysis:

The estimated ddPCR ZIKV RNA quantities were averaged for each condition replicate and log transformed. ZIKV RNA log_{10} concentrations were used to calculate log_{10} $[Ct/C0]$ reductions to quantify comparative changes over time. The log_{10} $[Ct/C0]$ reductions were graphed using scatter plots with standard error bars and time to 90% percent reduction were estimated using the graphs and tables. The analysis and graphs were completed using Microsoft Excel and GraphPad software.

RESULTS

The results for experiment 1 are displayed in Table 2 and Figures 1a-c. As shown in table 2, ZIKV RNA was detectable for all conditions and times during the 28-day experiment. ZIKV RNA degradation in deionized water showed little to no decline in the RNA signal across all three temperatures, with 10^5 copies/ml copies still detectable on day 28 under the three temperature conditions. ZIKV RNA degradation in primary influent demonstrated little to no decline at 4°C, but showed a steady decline at 25°C, going from 10^5 copies/ml on day 0 to 10^3 copies/ml on day 28, and then a more rapid decline at 35°C with 10^5 copies/ml on day 0, 10^4 copies/ml on day 3, 10^2 copies/ml on day 14, and 10 copies/ml on day 28. The ZIKV RNA signal in surface water showed more rapid degradation across all three temperatures, declining to 10^4 copies/ml by day 28 at 4°C and 10^2 copies/ml by day 28 at 25°C. The most rapid decline in detection occurred in surface water at 35°C, going from 10^5 copies/ml on day 0, to 10^2 copies/ml on day 7, 10 copies/ml on day 21; and by day 28 and RNA signal was only detected in one of the three replicates with an estimated < 10 copies/ml (table 2).

As shown in figures 1a-c, the average log reduction (Log_{10} (Ct/Co)) of the replicates (A, B, C) for each temperature and water condition are on the y-axis and the time (in days) are on the x-axis. There was little to no reduction in the RNA signal for weeks at 4°C in all water types, with the exception of surface water which demonstrated more than a 90% (approx. -1.5 log_{10}) reduction in ZIKV RNA between day 21 and day 28 (figure 1a). As shown in figure 1b, at 25°C there was no reduction in RNA signal in DI water during the entire experiment, while there was a 99% (-2 log_{10}) reduction in primary influent achieved by day 28 and a 99.9% (-3 log_{10}) reduction by day 28 in surface water. The largest reduction in ZIKV RNA signal for all water types was seen at 35°C (figure 1c). Though ZIKV RNA stayed relatively stable in DI water even at 35°C, there was a slight reduction between days 14 and 28 (less than -1 log_{10}). In contrast, both primary influent and surface water demonstrated a much more rapid reduction in ZIKV RNA signal, with an approximate 90% (-1 log_{10}) reduction by day 7 and 99.9% (-3 log_{10}) reduction between days 14 and 21 in primary influent and 90% (-1 log_{10}) reduction by day 3 and 99.9% (-3 log_{10}) reduction by day 7 in surface water (figure 1c).

The results for experiment 2 are displayed in table 3 and figure 2. As shown in table 3, ZIKV RNA degradation was more rapid for the lower inoculum concentration than the higher inoculum concentration. The lower concentration of 10^4 pfu/ml, went from 10^4 copies/ml on day 0 to <10 copies/ml on day 28, while the higher concentration of 10^5 pfu/ml, went from 10^5 copies/ml on day 0 to 10^3 copies/ml by day 28. As shown in figure 2, the ZIKV RNA log reductions were similar for both inoculum concentrations from days 0 to 14, but on day 21 and 28 the lower inoculum concentration

demonstrated more reduction compared to the higher concentration. By day 28 the lower concentration demonstrated more than a 99% (-2 $\log_{10}$) reduction in signal and the higher concentration demonstrated more than a 99.99% (-4 $\log_{10}$) reduction in signal.

The results for experiment 3 are displayed in table 4 and figure 3. As shown in table 4, ZIKV RNA was detected across water types for the duration of experiment. In both primary influent and combined surface and primary influent, ZIKV RNA values were detected at concentrations above the baseline inoculation concentration of 10^4 pfu/ml, with 10^6 copies/ml on day 0 and showed similar declines in detection with approximately 10^3 copies/ml by day 28. In surface water, the ZIKV RNA degradation showed a more rapid decline going from 10^4 copies/ml on day 0 to 10 copies/ml by day 28.

As shown in figure 3, after the initial reduction of ZIKV RNA signal on days 1 and 3, a sharp increase in detection was seen on day 7 for combination water and primary influent, decreasing steadily thereafter. Though there was a slightly more rapid reduction in combination water than primary influent, both water types demonstrated a 99.9% (-3 $\log_{10}$) reduction by day 28. In contrast, surface water demonstrated no reduction in the RNA signal between days 0 and 1 but showed a sharp increase in reduction on day 7, achieving 99.9% (-3 $\log_{10}$) reduction by day 14, approximately. It is also notable that ZIKV RNA concentrations as low as 10 RNA copies were detectable via the ddPCR assay (tables 2 and 4).

DISCUSSION

Results and Research Questions:

The decay and persistence of the detection of Zika RNA (signal) were dependent on all conditions tested in these experiments including time, temperature, water type, and the initial inoculum concentration of ZIKV. As we hypothesized, ZIKV RNA degradation was more rapid at higher temperatures, but even so, the RNA was detectable for weeks at 35°C for all water conditions. Our temperature results support the previous study examining ZIKV RNA in wastewater where the time to achieve and 90% reduction was approximately 14 days at 25°C and 6 days at 35°C, with our study suggesting slightly more rapid degradation at 25°C with a 90% reduction occurring at 7 days, and equivalent degradation at 35°C, which the reduction occurred at 6 days also [29]. Also, as expected, the lower initial inoculum concentration showed more rapid degradation in detection compared to the higher inoculum concentration. Not only did experiment 2 demonstrate this, but also the comparison between RNA signal degradation in primary influent and surface water at 25°C in Experiment 1 and 3, since the initial inoculation concentrations differed between those experiments. ZIKV RNA persistence was also dependent on the water type, with lower ZIKV RNA detection quantities and higher reductions over time in consistently documented in surface water compared to the other water types in both experiment 1 and 3. In experiment 3 this was also demonstrated by the slightly higher reductions seen in combination water compared to primary influent alone.

Based off our results, the strongest factors that are likely to influence ZIKV RNA detection are exposure to temperatures ≥35°C and the water type. Others have had similar conclusions regarding the influence of increased temperatures on enveloped virus survival and RNA persistence. One study evaluating the stability of SARS-CoV-2 by quantifying viral RNA in wastewaters showed RNA can be detected, with little reduction in the signal for up to 12 days at 4°C, while at 20°C it remained stable for 7 days, showing slight decay at 12 days [33]. Similar to our results, the researchers also demonstrated that though increasing temperature led to a decline in the RNA signal, they were able to detect the RNA quantities at temperatures as high as 40°C [33]. Another study comparing the effects of water matrices, temperatures, and wastewater microbiota on the decay of SARS-CoV-2 and murine hepatitis virus (MHV) RNA concluded that, for both viruses, temperature was the most significant environmental factor affecting RNA decay, followed by the water matrix [34]. They found more rapid decay at temperatures 25°C and 37°C compared to 4°C and 15°C, but the RNA was still able to persist for 18 to 25 days at 37°C [34]. The increase in viral instability as temperature increases, is mainly a result of the denaturation of proteins and increased extracellular enzyme activity [25].

Regarding the influence of water type on the degradation of SARS-CoV-2 RNA, one study suggested decay was more rapid in untreated wastewater compared to tap water, similar to our results of decay in deionized water and primary influent [34]. There is little to no research on the persistence of enveloped virus RNA degradation in surface waters, but multiple studies have stated the reason RNA may be more stable in wastewater than previously thought could be due to protection provided by the high levels of organic matter, biofilms, and suspended solids [25, 34]. The hydrophobicity of viral envelopes makes them less soluble in water, which may increase such virus's propensity to adhere to the particles found in wastewater [25]. One study exploring the survivability of human and animal coronaviruses in different water types came to this conclusion after their results demonstrated that Human coronavirus (HCoV) survived longer in unfiltered primary effluent than in filtered primary effluent and unfiltered secondary effluent, suggesting the higher solids provided protection for the viruses [25]. Such absorption to suspended solids could also protect RNA from rapid degradation by reducing viral rupturing and the subsequent release of RNA, as well as protecting the viruses from predatory microorganisms [25]. This protection may be the reason our results showed higher reductions in ZIKV RNA signal detection in surface water compared to wastewater, because the amount and type of solids present in stream water may be less protective or reduced compared to solids found in primary influent due to the characteristics of stream water, such as movement or sources of turbidity and suspended solids.

In addition to our results suggesting that the presence of surface water may likely increase ZIKV RNA degradation in wastewater systems, we also documented that the initial starting concentration of the virus can impact how rapidly the ZIKV RNA signal will decline. While initial persistence of the signal was similar for the two conditions tested, over time the lower concentration of initial viral inoculum condition was degraded more rapidly. Others have found similar conditions when testing experimental design with SARS-CoV-2 [35].

We did notice some variability in detection including apparent increases in concentrations over time prior to documented declines. These discrepancies in the detected RNA quantities in experiment 3, were the greatest with primary influent and combination water showing concentrations above the baseline inoculation concentration of 10^4 pfu/ml, as well as showing sharp increase in detection at day 7 after initial large reductions between days 0 and 3. It is possible these inconsistencies could be attributed to human error, but also may be due to the tendency of viruses to form aggregates that then disaggregate, increasing the virus titer over time from the initial concentration after disaggregation [25]. Another possibility referenced in one study, suggests other viral structures, such as a nucleoriboproteic

complex, which results from the strong interaction between nucleoproteins and viral RNA, may initially limit the rupture of the viral envelope and thus the release of RNA [33].

The main limitations of this study are that the primary influent and surface water were not sourced from an area which has been exposed to a known ZIKV endemic, the inability to associate the detection of ZIKV RNA with the infectivity of ZIKV, as well as the high concentration used in the experiments that likely exceed what might be shed in the environment.

Implications in a broader context:

This study is significant due to the emerging field of WBE as a method for population-wide monitoring of enveloped viruses, which have previously been left out of the research. Prior to the COVID-19 pandemic there was little research exploring the ability to detect enveloped viruses and their RNA in community wastewater, but now, WBE has been widely used in many countries around the world as a means to monitor the SARS-CoV-2 concentrations in a community and the spread of the virus across populations [26, 28, 33]. The current use of WBE for COVID-19 and the results from the present study have increased the knowledge about the ability to detect enveloped virus nucleic acids using common WBE detection methods which had previously been utilized for other types of pathogens, increasing the evidence that other enveloped viruses could also be detectable in wastewater.

This study also adds knowledge to enveloped virus RNA stability, showing how environmental conditions may affect the persistence and degradation of virus RNA when monitoring and surveying the wastewater of a community, supporting evidence that it can remain detectable in wastewater and surface water for weeks under a variety of environmental conditions.

Understanding the effects of such conditions may have on the stability and persistence is especially important in countries like Brazil, where many communities have low-resource waste systems which are defined as ineffective centralized systems or wastewater-impacted environmental waters [36]. Surface waters that receive unknown fecal inputs which are subject to environmental processes that unpredictably can impact RNA persistence can lead to challenges in utilizing WBE surveillance, in a country that already has other disease surveillance challenges [36].

If ZIKV prevalence were to increase in Brazil again in the future or emerge in other countries due to climate change's effects on vector and pathogen distribution, our results support ZIKV RNA stability under different environmental conditions including the harsh conditions present in Brazil, and thus the utility of WBE [15]. Our study also suggests that determining the viral concentrations in the wastewater of a community can help determine the timeframe for which an RNA signal may remain detectable for monitoring purposes.

For diseases like ZIKV, which there is a large percent of asymptomatic cases, yet an infection can lead to severe health outcomes, and which tend to occur in areas with limited resources, the present research adds support to the effectiveness of commercially available extraction kits and of WBE to fill the gaps where other surveillance methods are lacking, highlighting the potential of RNA monitoring for surveillance of infectious disease as an epidemiologic screening tool to determine the burden of infection of an entire community. This is particularly beneficial in countries like Brazil which have multiple endemic viral diseases, because WBE methods can monitor multiple viruses in a particular population at once in real-time [22].

Future research and Conclusion:

ZIKV has the potential to reemerge in Brazil and/or become endemic in more than 100 other countries which previously may have no experience dealing with such viruses, because of changing vector distribution, new mutations, impact of human activity (tourism, urbanization, climate change) [15, 37]. This potential combined with our study results and the current COVID-19 pandemic, caused by the highly pathogenic novel strain SARS-Co-2, have portrayed the success of WBE methods for enveloped viruses and the necessity of increasing environmental surveillance methods to mitigate human harm from infectious viral diseases in the future.

Though WBE is a good method for population level monitoring and early warnings, the amount of virus shed in feces and urine varies between individual people, making it difficult to accurately determine the number of infected persons [10, 38]. To address this challenge, more research in establishing quantitative predictions from viral RNA concentrations in wastewater to the actual case numbers in the community is needed for ZIKV as well as other diseases of epidemic/pandemic concern. Future research should also examine methods for viral recovery and possible mosquito infectability from environmental waters as another means of transmission for ZIKV [24]. Repeated persistence experiments for ZIKV RNA should be done, expanding the investigation to other differing environmental conditions including different chemical matrices in water and temperatures, as well as investigations to explore the persistence and stability of other enveloped viruses.

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APPENDIX

Table 1. Experimental conditions for RNA persistence experiments

Experiment	Temperature	Water type	Inoculation concentration	Replicate	Time of extractions (days)
1	4°C 25°C 35°C	DI Surface Primary Influent	10^5 pfu/ml	A, B, C	0, 1, 3, 7, 14, 21, 28
2	25°C	Primary Influent	10^4 pfu/ml 10^5 pfu/ml	A, B, C	0, 1, 3, 7, 14, 21, 28
3	25°C	Surface Primary Influent 1:1 Combination	10^4 pfu/ml	A, B, C	0, 1, 3, 7, 14, 21, 28

Table 2: Mean log_{10} concentration values of ZIKV RNA degradation at three temperatures and three water conditions for Experiment 1

	4°C			25°C			35°C		
Day	DI	Surface	Primary Influent	DI	Surface	Primary Influent	DI	Surface	Primary Influent
0	6.91	6.72	6.54	6.58	6.34	6.57	6.77	6.73	6.64
1	6.89	6.55	6.71	6.73	6.62	6.43	6.91	6.43	6.09
3	6.67	6.59	6.72	6.95	6.19	6.07	6.95	5.74	5.76
7	6.77	6.64	6.78	6.82	4.89	5.65	6.78	3.88	5.37
14	6.55	6.21	6.69	6.87	4.45	5.31	6.38	2.96	3.99
21	6.86	6.55	6.26	6.83	4.19	4.96	6.16	2.37	3.06
28	6.58	5.38	6.24	6.86	3.08	4.67	6.11	< 1.85	2.27

Table 3. Mean log_{10} concentration values of ZIKV RNA degradation at two inoculum concentrations in wastewater (25°C) for Experiment 2

Day	Low	High
0	5.40	6.38
1	5.03	6.37
3	4.32	5.66
7	3.78	5.17
14	3.66	4.83
21	2.15	4.50
28	1.25	3.92

Table 4. Mean log_{10} concentration of ZIKV RNA degradation for three water conditions at 25°C for Experiment 3

Day	Surface Water	Primary Influent	Combination
0	5.53	7.47	7.28
1	5.73	6.03	5.90
3	5.99	5.46	6.06
7	4.11	6.95	6.65
14	2.54	6.47	5.32
21	3.13	5.84	4.49
28	2.11	4.87	4.35

Figure 1a: $Log_{10}[C_tC_0]$ reduction values of ZIKV RNA at 4°C for each water condition

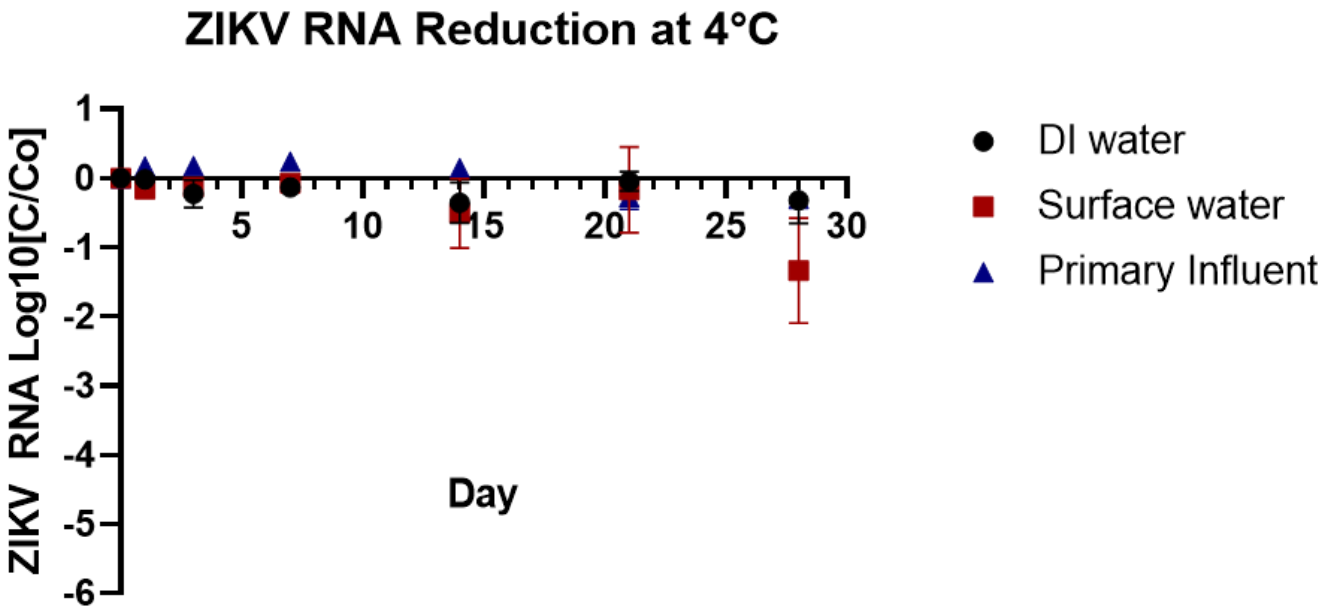

Figure 1b: $Log_{10}[C_tC_0]$ reduction values of ZIKV RNA at 25°C for each water condition

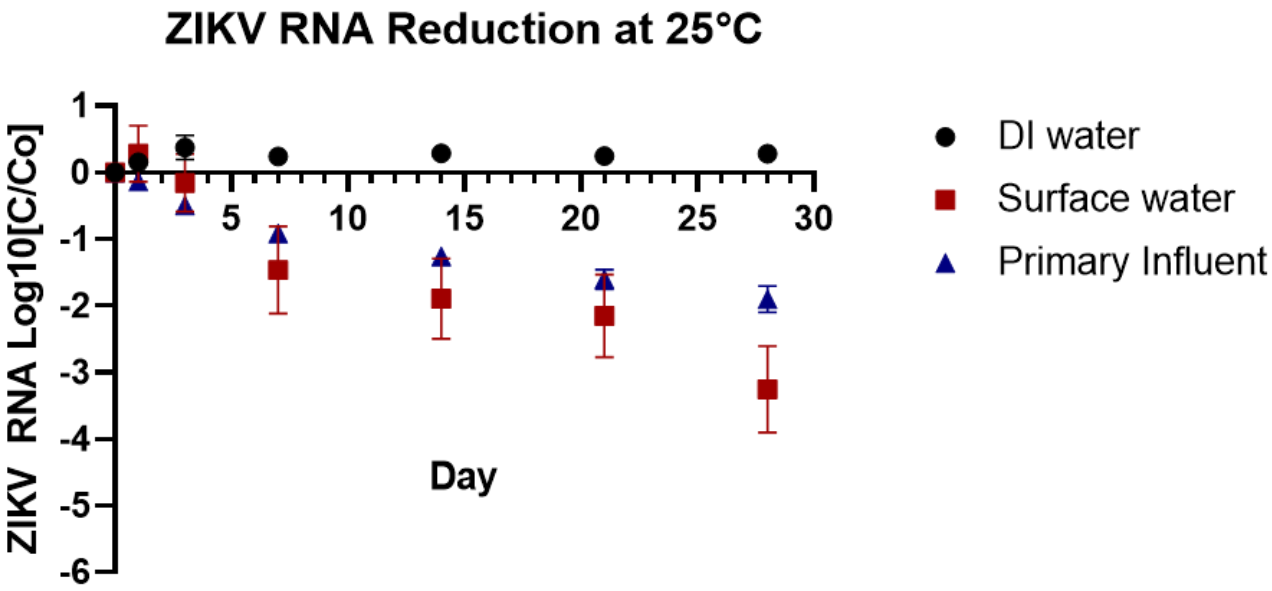

Figure 1c: $Log_{10}[C_tC_0]$ reduction values of ZIKV RNA at 35°C for each water condition

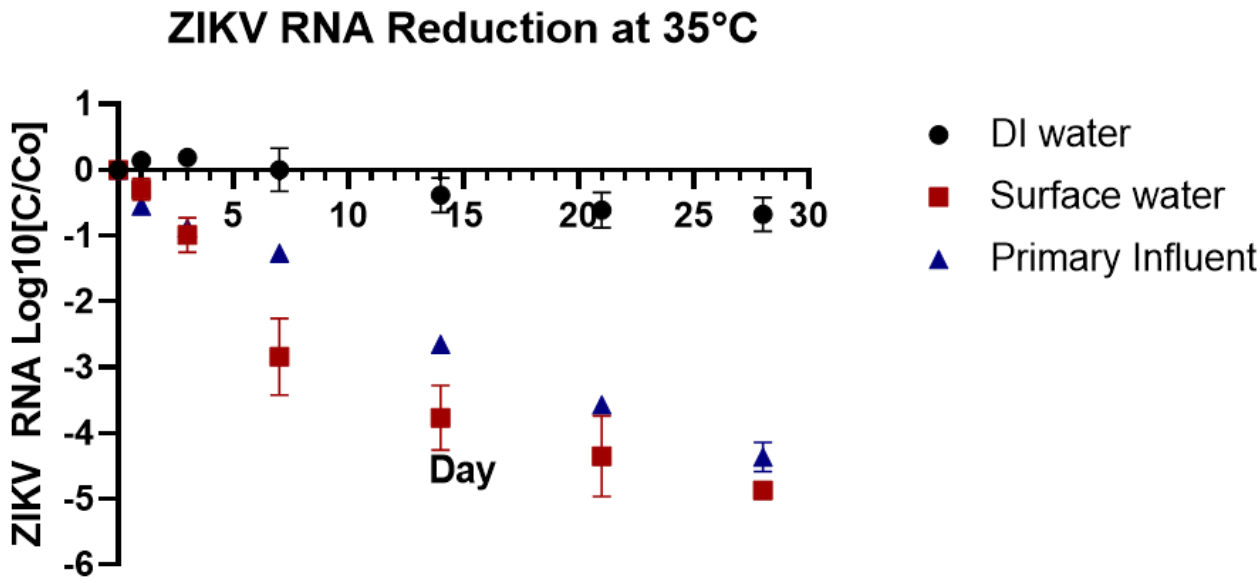

Figure 2: $log_{10}[C_t C_0]$ reduction values of ZIKV RNA degradation for two inoculum concentrations in wastewater at 25°C

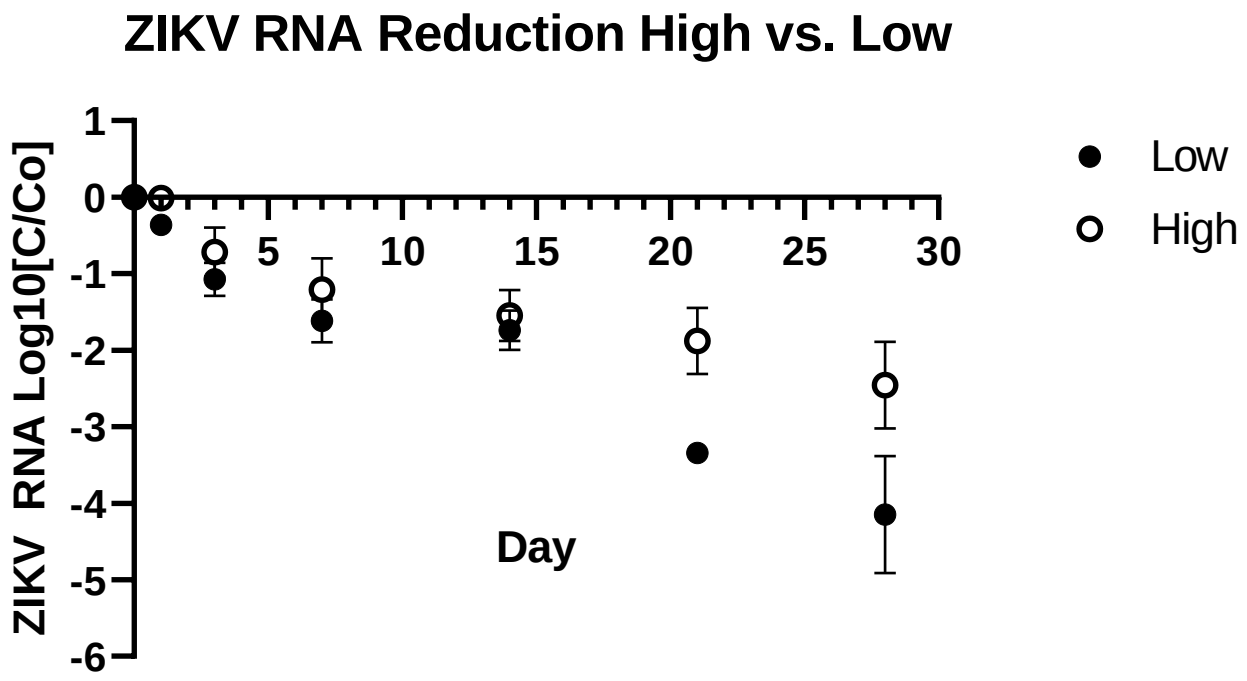

Figure 3: $log_{10}[C_t C_0]$ reduction values of ZIKV RNA degradation for each water condition: