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Zika virus RNA persistence and recovery in water and wastewater: An approach for Zika virus surveillance in resource-constrained settings

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ABSTRACT

During the 2015–2016 Zika virus (ZIKV) epidemic in the Americas, serological cross-reactivity with other flaviviruses and relatively high costs of nucleic acid testing in the region hindered the capacity for widespread diagnostic testing. In such cases where individual testing is not feasible, wastewater monitoring approaches may offer a means of community-level public health surveillance. To inform such approaches, we characterized the persistence and recovery of ZIKV RNA in experiments where we spiked cultured ZIKV into surface water, wastewater, and a combination of both to examine the potential for detection in open sewers serving communities most affected by the ZIKV outbreak, such as those in Salvador, Bahia, Brazil. We used reverse transcription droplet digital PCR to quantify ZIKV RNA. In our persistence experiments, we found that the persistence of ZIKV RNA decreased with increasing temperature, significantly decreased in surface water versus wastewater, and significantly decreased when the initial concentration of virus was lowered by one order of magnitude. In our recovery experiments, we found higher percent recovery of ZIKV RNA in pellets versus supernatants from the same sample, higher recoveries in pellets using skimmed milk flocculation, lower recoveries of ZIKV RNA in surface water versus wastewater, and lower recoveries from a freeze thaw. We also analyzed samples collected from Salvador, Brazil during the ZIKV outbreak (2015–2016) that consisted of archived samples obtained from open sewers or environmental waters thought to be contaminated by sewage. Although we did not detect any ZIKV RNA in the archived Brazil samples, results from these persistence and recovery experiments serve to inform future wastewater monitoring efforts in open sewers, an understudied and important application of wastewater monitoring.

1. Introduction

Although Zika virus (ZIKV) was first isolated in 1947 in Africa, the severe complications and sequelae of ZIKV infections did not become evident until more recent outbreaks in Asia and the Americas during 2007 to 2016. ZIKV infections are mostly asymptomatic, and symptomatic cases often present with mild, non-specific symptoms like many other arboviruses circulating in tropical settings (Kuno, 2016; Musso and Gubler, 2016). However, during the ZIKV 2015–2016 epidemic in the Americas, the emergence of increased numbers of babies born with

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microcephaly, sequelae evidenced by increased reports of Guillain-Barre syndrome, and the first reported ZIKV associated deaths alarmed the public health community (Musso et al., 2019). The increases in microcephaly cases were of particular concern, leading the World Health Organization (WHO) to declare a Public Health Emergency of International Concern in 2016.

Challenges to widespread diagnosis of ZIKV infections hampered response to the outbreaks (Musso et al., 2019; Victora et al., 2016). Cross-reactivity with other circulating flaviviruses is a key challenge complicating serological testing for ZIKV (Aubry et al., 2015; Duffy et al., 2023; Priyamvada et al., 2016). While tests detecting nucleic acids can provide more definitive evidence of infection, limited resources and relatively higher costs of nucleic acid testing reagents in Latin America deterred widespread nucleic acid testing (Fischer et al., 2018). Thus, surveillance during the outbreak largely relied on monitoring more severe complications associated with ZIKV infections: cases of microcephaly, other congenital anomalies, and fetal loss (Lowe et al., 2018; Victora et al., 2016). The dramatic emergence of ZIKV in the Americas and lack of widespread diagnostic testing illustrate the need for approaches to monitor public health in communities when limited resources restrict individual testing.

Wastewater monitoring, or wastewater-based epidemiology (WBE), potentially offers one such approach by leveraging existing wastewater collection networks to collect a composite sample from many individuals. While recently employed to perform public health surveillance for viral infections, including SARS-CoV-2 infections (Karthikeyan et al., 2021), wastewater monitoring has historically been used as a surveillance tool, including for informing polio eradication efforts (Pogka et al., 2017). Associations between nucleic acid detection in wastewater and reported cases are also being investigated with the goal of predicting incident cases as a leading indicator of an outbreak (Brouwer et al., 2018; Gonzalez et al., 2020; Graham et al., 2021; Hellmér et al., 2014; Medema et al., 2020; Peccia et al., 2020; Tiwari et al., 2022). Wastewater monitoring is especially useful where most transmission is undetected by clinical surveillance, including where cases are mainly asymptomatic or clinical data are limited by insufficient testing capacity. ZIKV exhibits broad tropism in body tissues (Miner and Diamond, 2017), with potential to be shed into wastewater through saliva, serum, urine, or feces (Bingham et al., 2016; Bôtto-Menezes et al., 2019; Gourinat et al., 2015). Concentrations of ZIKV in urine have been estimated to be up to 10^8 copies / mL in infected humans (Lamb et al., 2016). The potential for ZIKV to re-emerge (Nolen, 2022), the severe complications of ZIKV infections in those who are pregnant, and the shedding of ZIKV in wastewater coalesce to make ZIKV a candidate target of wastewater monitoring approaches.

To date, wastewater monitoring approaches have largely been focused on settings where conventional, underground, piped sewers are the prevalent form of sewerage. In the context of the 2015 Brazil ZIKV outbreak, the communities that bore the higher burden of ZIKV infections were often communities classified as lower socioeconomic status which used open sewers (Netto et al., 2017). Open sewers are low-cost sewer systems that rely on gravity-driven ditches to convey wastewater, but, because they are open to the atmosphere, they can accumulate rainfall, surface run-off, surface water, and wastewater. For resource-constrained settings, which often lack conventional piped sewage networks, knowledge is needed about how to implement and interpret wastewater monitoring (Basu et al., 2022). Understanding the persistence of ZIKV RNA in open sewer-like matrices will improve our ability to interpret monitoring results in settings where ZIKV is likely to emerge or re-emerge.

Our study aim was to examine the persistence and recovery of ZIKV RNA in scenarios relevant to resource-constrained settings under three main goals:

(1) Characterize the persistence of ZIKV RNA under experimental conditions relevant to resource-constrained settings: varying temperatures from 4 to 35 °C; varying matrices using surface water, wastewater, and a combined matrix of surface water and wastewater; and varying initial concentrations.
(2) Characterize the recovery of ZIKV RNA in the pellet versus supernatant after centrifugation, using skimmed milk flocculation (a concentration method with appropriate characteristics for use in resource-constrained settings), with and without freeze thaw after concentration but before nucleic acid extraction, and at varying inoculum concentrations.
(3) Assay archived samples of environmental waters collected in Salvador, Brazil during the 2015–2016 outbreak to determine if ZIKV RNA could be recovered.

We used reverse transcription droplet digital PCR (RT-ddPCR) to quantify ZIKV RNA because of the advantages over reverse transcription quantitative PCR (RT-qPCR) in analyzing environmental matrices (Cao et al., 2015; Staley et al., 2018) and in monitoring pathogens in wastewater (Ahmed et al., 2022; Ciesielski et al., 2021). Additionally, robust qPCR standard curves for RNA viruses can be challenging to establish because the standard curve benefits when the standard curve template closely mimics the target template and because standard curves should be run often, if not with every qPCR run (Bustin, 2004). dPCR approaches do not require a standard curve for each quantification run, further making dPCR an appealing tool for routine monitoring approaches.

2. Materials and methods

2.1. ZIKV culture

We obtained a frozen aliquot of ZIKV strain MEX 1–44 generously shared by Dr. Robert Tesh through the World Reference Center for Emerging Viruses and Arboviruses (WRCEVA), University of Texas Medical Branch, Galveston, TX. We grew Vero 76 cells (ATCC, Manassas, VA, USA) in high glucose Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 4 mM glutamine, 1 mM sodium pyruvate, 1x nonessential amino acids, and penicillin-streptomycin solution at 100 U/mL and 100 μg/mL, respectively. We incubated Vero 76 cells in the DMEM at 37 °C in 5% CO_2 to produce confluent monolayers within T175 flasks. We cultured ZIKV by infecting the monolayers at a multiplicity of infection of approximately 0.1. The culture media was harvested and replaced with fresh media at 48 h after infection. We then clarified the culture fluid by centrifugation and stored aliquots at −80 °C. We determined the infectivity titer by plaque assay on Vero 76 cells to be on the order of 10^6 PFU / mL. This virus pool was used in all experiments in this study.

2.2. Wastewater and surface water sample collection

We collected primary influent from a wastewater treatment plant receiving 40 million gallons per day or approximately 150,000 m^3 per day in Atlanta, GA. After collection, we stored the wastewater at 4 °C for no longer than seven days before use in persistence and concentration experiments. We collected surface water from local streams or creeks that are monitored by the United States Geological Survey (USGS) in the city of Atlanta. We sampled within 8–12 h of rainfall, targeting turbidity (as measured through USGS gages) above 100 NTU and frequently between 200 and 300 NTU. We stored surface water at 4 °C for up to 7 days prior to use.

2.3. Persistence experiments

We conducted persistence experiments by spiking clarified ZIKV MEX-1–44 culture fluid into a variety of matrices, using two different levels of initial concentrations, incubating the resulting microcosms at a variety of temperatures, and sampling up to 28 days. This study expands

on the system originally developed in Muirhead et al. 2020. In the first persistence experiment, we varied incubation temperature using 4, 25, and 35 °C and matrices using deionized (DI) water, non-pasteurized surface water, and non-pasteurized wastewater (Table 1). We spiked ZIKV into separate microcosms of DI water, surface water, and wastewater to achieve an initial concentration of 10^9 marker copies / mL of matrix (ZIKV cultured to 10^6 PFU / mL and diluted 0.5 mL of cultured ZIKV into 4.5 mL of matrix). For each matrix (DI water, surface water, and wastewater), we created biological triplicate microcosms (A, B, and C). We did not agitate microcosms during incubation but vortexed each microcosm at maximum speed for 30 s before sampling for each time point. We then sampled from the biological triplicates of each of the three matrices (i.e. DI water A, B, and C; surface water A, B, and C; wastewater A, B, and C) at 0, 1, 3, 7, 14, 21, and 28 days for a total of 189 samples. We incubated the microcosms in the dark in incubators or a refrigerator to obtain the respective temperatures. As ZIKV-negative controls, we incubated one microcosm of each matrix without ZIKV at each of the three temperatures for a total of nine biological negative microcosms and sampled from ZIKV-negative microcosms at the same time intervals. In the second persistence experiment, we varied the initial concentration of ZIKV, using initial concentrations of 10^8 or 10^9 ZIKV marker copies / mL in non-pasteurized primary influent and incubated at only 25 °C in the dark and one ZIKV negative biological replicate sampled for 28 days as described in the first experiment. In the third persistence experiment, we varied the matrix by using surface water, wastewater, and a combination of surface water and wastewater at an initial ZIKV concentration of 10^8 ZIKV marker copies / mL. We combined the surface water and wastewater at a mixing ratio of 50% volume to volume and incubated at 25 °C in the dark over 28 days.

We performed RNA extractions directly from 200 μL of each of the persistence microcosms using the Qiagen QIAamp MinElute Virus kit (Hilden, Germany) according to the manufacturer's instructions and eluted using 50 μL of Qiagen Buffer AVE (Hilden, Germany). Following elution, we created duplicate 25 μL of each extract to minimize freeze thaw on individual samples which were stored at - 20 °C until analysis (< 6 months).

2.4. Experiments comparing concentration and extraction methods

Because the method used to concentrate the archived Brazil samples (see Section 2.5) was intended for the analysis of bacteria (*Leptospira*) and not viruses, we performed experiments to help us interpret results from our analysis of the archived samples. First, we conducted viral RNA recovery experiments comparing skimmed milk flocculation (SMF) versus the centrifugation method used to concentrate the archived samples (Table 2). SMF has been used to recover other viral RNA from wastewaters (poliovirus type 1 and SARS-CoV-2) and is conducive to use in resource-constrained settings because it can be completed in less than one day, the reagent costs are low, refrigeration is not always required, and it requires only standard laboratory equipment (Calgua et al., 2008; Falman et al., 2019; Philo et al., 2022, 2021). We used a modified version of the method as described by Falman et al. 2019. Briefly, we performed skim milk flocculation by preparing 1% weight-to-volume solutions of skimmed milk. We added 1 mL of the skimmed milk solution to 100 mL of each sample, adjusted the pH to 3–4 using 5 M HCl, and incubated on a rotating shaker at 25 °C at 200 rpm for 2 h, centrifuged for 30 min at 3500 x *g* at 4 °C, and carefully removed the supernatant via pipetting. We vortexed the conical tube to loosen the pellet and recorded the volume. If necessary, we added a small amount (100 μl to 250ul) of supernatant from the same sample to resuspend the pellet. For the centrifugation method used to concentrate the archived samples, we performed the concentration as described in Section 2.5. Second, we compared the amount of ZIKV nucleic acid in the pellet versus the supernatant to understand the distribution of ZIKV RNA in each phase. Because the archived samples contained only the pellet after the environmental water samples were centrifuged, we compared recovery of ZIKV RNA in the pellet versus the supernatant. Finally, we explored the effect of a freeze thaw cycle after SMF concentration but before nucleic acid extraction. We spiked ZIKV into primary influent at two final concentrations of 10^5 and 10^7 ZIKV marker copies / mL of wastewater using six biological replicates of each concentration. We then performed SMF, and, after concentration, we stored three of the biological replicates at 4 °C for 24 h and the other three biological replicates at −80 °C for 72 h to ensure a complete freeze.

For the recovery experiments, we compared two extraction kits: the Qiagen QIAamp MinElute Virus kit and the Qiagen RNeasy PowerMicrobiome Kit (Hilden, Germany). The PowerMicrobiome kit includes a bead beating step and, consequently, may be more effective at extracting ZIKV RNA from solids-rich matrices. The MinElute Virus kit does not have a bead beating step and, consequently, may perform better when used with supernatant rather than pellet material. The MinElute Virus kit also does not use beta-mercaptoethanol, a toxic

Table 1
Overview of experimental variables for the persistence experiments and the number of samples for each experiment.

Persistence Experiments	Initial Concentration of ZIKV RNA [ZIKV marker copies / mL of matrix]	Matrices Used	Biological Triplicates	Incubation Temperatures [°C]	Sampling Times [days]	Number of Samples Analyzed (excluding controls)
Varying Temperature and Matrix	10^9	DI water, surface water, and wastewater	DI water A, B, C; surface water A, B, C; wastewater A, B, C	4 25 35	0 1 3 7 14 21 28	189
Varying Initial Concentration	10^8 10^9	wastewater	wastewater A, B, C	25	0 1 3 7 14 21 28	42
Varying Matrix	10^8	surface water, wastewater, and combined surface water and wastewater (50% vol./vol.)	surface water A, B, C; wastewater A, B, C; combined surface water and wastewater A, B, C	25	0 1 3 7 14 21 28	63

Table 2
Overview of experimental variables for the concentration and extraction comparison experiments done in this study and number of samples for each experiment.

Extraction Comparison Experiment	Initial Concentration of ZIKV RNA [ZIKV marker copies PFU / mL of matrix]	Matrices Used	Concentration Method Used	Biological Triplicates	Extraction Kits Used	Number of Samples Analyzed (excluding controls)
Skimmed Milk Flocculation vs. Centrifugation	10^7	wastewater	Skim Milk Flocculation, Leptospirosis Centrifugation	wastewater A, B, C	PowerMicrobiome, QIAamp MinElute Virus	24
Surface water vs. Wastewater	10^7	wastewater, surface water	Skim Milk Flocculation	surface water A, B, C; wastewater A, B, C	PowerMicrobiome, QIAamp MinElute Virus	24
Freeze Thaw vs. No Freeze Thaw	10^7 10^5	wastewater	Skim Milk Flocculation	wastewater A, B, C	PowerMicrobiome, QIAamp MinElute Virus	48
Varying Spike Concentration	10^7 10^5	wastewater	Skim Milk Flocculation	wastewater A, B, C	PowerMicrobiome, QIAamp MinElute Virus	24

reagent that requires careful disposal. We used the MinElute Virus kit as described above in Section 2.3. For the PowerMicrobiome kit, we used 250 μL of sample, followed the manufacturer's instructions, and eluted in 50 μL of RNAse-free water. Following elution, we created duplicate aliquots of 25 μL for each extract to minimize freeze thaws on individual aliquots and stored aliquots at - 20 °C until analysis (< 6 months). In the concentration and recovery experiments, we always extracted and analyzed RNA from both the supernatant and pellet for each experimental treatment.

We calculated recoveries by quantifying ZIKV RNA in aliquots of extracted ZIKV culture stock used to inoculate the microcosm or concentration samples. We extracted using four biological replicates for each RNA extraction kit used (both PowerMicrobiome and RNA Mini kit) and analyzed each of these eight total extractions using RT-ddPCR. We then averaged the stock concentrations for each extraction kit and multiplied the concentrations by the dilution factors we used for each experiment to obtain expected concentrations.

2.5. Analysis of environmental water samples from Brazilian communities during the 2015–2016 Zika virus epidemic

We analyzed archived environmental water samples collected from various locations distributed through the community of Pau de Lima, Salvador, Brazil during the 2015–2016 epidemic. These archived samples were collected at various sites and elevations, representing various types of impacted open sewers and impacted surface waters in the community. The samples were concentrated using methods intended for the analysis of *Leptospira* (Riediger et al., 2016). Briefly, 50 mL of water from either an open sewer or standing water nearby an open sewer was collected and stored at 4 °C for up to 18 h before processing. A 40 mL aliquot of each sample was centrifuged at 15,000 x *g* for 20 min at 4 °C. Following centrifugation, the supernatant was discarded, and the resulting pellet (no resuspension) was archived at −80 °C. We extracted the archived samples using the Qiagen RNeasy PowerMicrobiome kit (Hilden, Germany) based on the results of a pilot experiment (data not shown) in which we spiked ZIKV into simulated samples and found that the PowerMicrobiome kit yielded higher recoveries than the MinElute Virus kit. We used the PowerMicrobiome kit according to the manufacturer's instructions, spiking in 5 μL of bovine respiratory syncytial virus (BRSV) vaccine (Inforce 3®, Zoetis, New Jersey, USA) into each bead beating tube, and eluting each sample using 50 μL of Buffer AVE. Following elution, we created duplicate aliquots of 25 μL for each extract to minimize freeze thaws on individual aliquots and stored aliquots at - 20 °C until analysis (< 6 months).

2.6. Reverse transcription droplet digital PCR

We used reverse transcription droplet digital PCR (RT-ddPCR) to quantify ZIKV RNA in this study. A Minimum Information for Publication of Digital PCR Experiments table is included in the supplementary information (Table S4) (Whale et al., 2020). Briefly, we conducted RT-ddPCR analyses using a Bio-Rad QX200 Droplet Digital PCR System (Hercules, CA, USA) and the ZIKV-5′-UTR assay targeting the 5′-untranslated region of the ZIKV genome (Chan et al., 2017). Following initial testing between two assays (ZIKV-5′-UTR assay and the ZIKV-5 assay) (Lanciotti et al., 2008), we proceeded with the ZIKV-5′-UTR assay because it contained 100% identity and 100% coverage matches when Basic Local Alignment Search Tool (BLAST) searched against the MEX-1–44 genome that we used to spike in our persistence and recovery experiments and other ZIKV genomes sequenced from the Brazil 2015–2016 epidemic.

The RT-ddPCR system consisted of a QX200 Droplet Reader, C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module, QX200 Droplet Generator, and a PX1 PCR Plate Sealer. We performed droplet reading and data analysis using the QX200 Droplet Reader and Bio-Rad QuantaSoft software (Version 1.7.4.0917). To determine the threshold between positive and negative droplets, we calculated the halfway point between the peaks of the positive and negative droplets and set the threshold at that halfway point. We ran no-template controls (NTCs) consisting of UV-treated (20 min) molecular-grade water; all NTC wells were below the thresholds used to determine positive droplets. We analyzed 25% of persistence samples in duplicates of 2 μL (technical duplicates) and analyzed 100% of Brazil archived samples in duplicates of 5 μL. We set analytical limits (analyical limit of detection and analytical limit of quantification) using approaches described previously (Zhu et al., 2020) with dilutions of ATCC quantitative ZIKV genomic RNA VR-1843DQ (Manassas, VA, US) in Qiagen Buffer AVE (Hilden, Germany).

2.7. Data analysis

We used the Geeraerd and Van Impe Inactivation Model Fitting Tool (GInaFiT) (Geeraerd et al., 2005) to fit models to our time series data, comparing the fit of the different models using adjusted R^2 values. We found that a first order inactivation model with the following formulation fit best:

$$C_t = C_0 e^{-kt}$$

Within each persistence experiment, we used *z* tests to compare rate constants, *k* values, between experimental treatments by assuming a normal distribution of k values (Brooks and Field, 2016; Mattioli et al., 2017) and used the standard error outputted from GInaFiT in calculating

z values. We conducted each *z* test for all nine possible combinations between biological triplicates for the two experimental treatments being compared (hereafter referred to as pairwise comparisons) (Brooks and Field, 2016; Mattioli et al., 2017). We considered results of hypothesis testing statistically significant at $p \leq 0.05$ while using Bonferroni correction due to comparisons across biological triplicates. We completed *z* testing in Microsoft Excel Version 2204. Using GInaFiT, we calculated *k* values, standard errors, and accompanying lower and upper 95% confidence intervals ($\pm$ 1.96 $\times$ standard error) for each biological replicate in each persistence experiment as well as T90 values. The number of sampling time points included in each decay model is listed in Table S2.

We also used a multilevel model with random effects to examine the relationship between the various conditions tested and concentrations of ZIKV RNA in the persistence experiments. We fitted parameters for day, matrix type (further sublevels include DI water, surface water, wastewater, and a combination of surface water and wastewater), temperature (further sublevels include 4, 25, and 35 °C while using 4 °C as the reference category), initial concentration (further sublevels include 10^8 and 10^9 ZIKV marker copies / mL of matrix), and a random effects parameter. We then standardized the parameters by dividing each parameter estimate by the standard error of the corresponding dataset to allow for comparison of the influence of each variable on concentrations of ZIKV RNA. We completed the multilevel modeling in SAS 9.1.

3. Results

3.1. Varying temperature and matrix persistence experiments

Of the three matrices we tested, ZIKV RNA signal was the most persistent in sterile DI water (Fig. 1). The *k* values calculated for the 4 and 25 °C time series were close to zero (mean of 0.02 and −0.01 $days^{-1}$, respectively) and were not significantly different from each other (4 versus 25 °C) (Table 3, S5). Comparing ZIKV RNA in DI water at 25 versus 35 °C, *k* values at 35 °C were significantly larger than those for 25 °C (mean of 0.07 versus −0.01 $days^{-1}$, respectively) for all pairwise comparisons between the biological triplicates. The ZIKV RNA signal was the least persistent in surface water. At 25 °C, the *k* values were significantly larger than *k* values at 4 °C (mean of 0.27 versus 0.08 $days^{-1}$, respectively) for all pairwise comparisons between the biological triplicates. Comparing *k* values at 25 versus 35 °C, five out of nine pairwise comparisons showed that 35 °C had significantly larger *k* values than those at 25 °C (mean of 0.46 versus 0.27 $days^{-1}$, respectively). At 25 °C, *k* values for surface water were significantly larger than *k* values for wastewater at 25 °C (mean of 0.27 versus 0.15 $days^{-1}$, respectively) for all pairwise comparisons between biological triplicates. ZIKV RNA signal in wastewater exhibited significant differences between *k* values for all temperatures. Comparing the 4 to the 25 °C, *k* values increased from a mean of 0.04 to 0.15 $days^{-1}$, respectively. Then comparing the 25 to the 35 °C, *k* values increased from a mean of 0.15 to 0.35 $days^{-1}$, respectively.

3.2. Varying initial concentration persistence experiments

We found most pairwise comparisons to have significantly higher *k* values from time series with an initial concentration of 10^8 ZIKV marker copies / mL of wastewater than those from time series with an initial concentration of 10^9 ZIKV marker copies / mL of wastewater (mean of 0.31 versus 0.19 $days^{-1}$, respectively) (Table 4, S5). Pairwise comparisons that did not yield a significant difference were from the "B" biological replicate for the 10^9 ZIKV marker copies / mL time series, which had a higher *k* value compared to its other biological replicates (0.24 $days^{-1}$ compared to 0.17 and 0.16 $days^{-1}$).

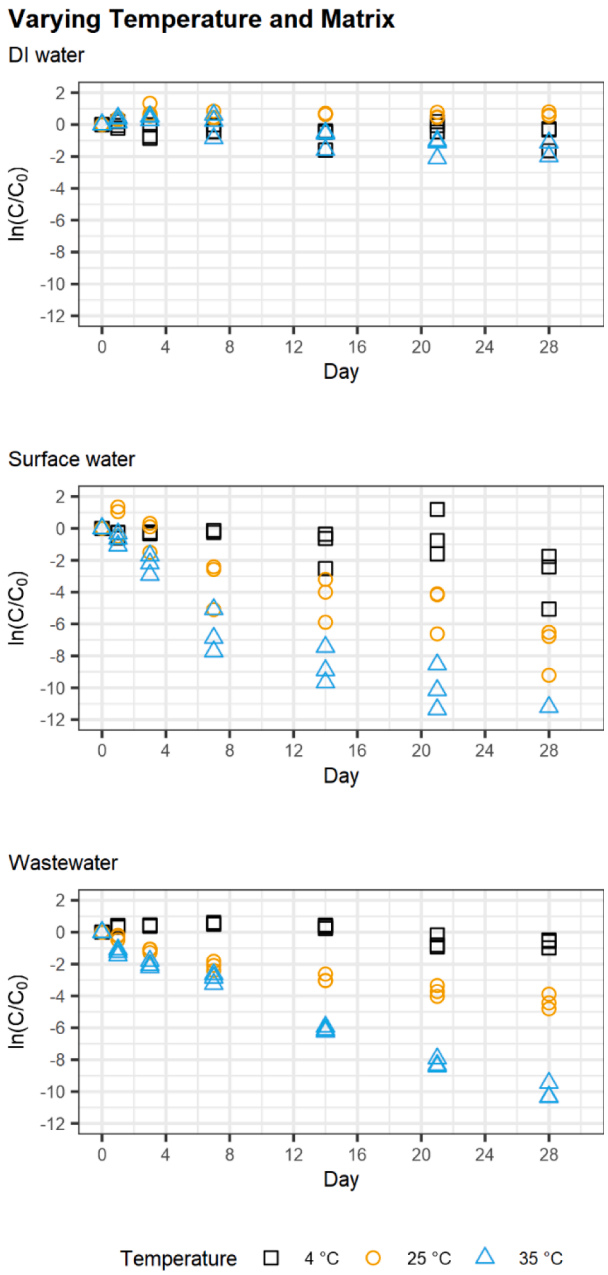

Fig. 1. Natural logarithm of ZIKV RNA concentrations relative to initial concentrations from the persistence experiment conducted in this study varying temperature and matrix. Individual points plotted represent one biological replicate out of three done for each experimental treatment at each sampling timepoint. Values below the lower limit of quantification are not plotted.

3.3. Combined surface water and wastewater

In comparisons between surface, waste, and a combination of both waters, we found again that surface water had significantly larger *k* values when compared to those from wastewater (0.56 versus 0.22 $days^{-1}$, respectively) for all pairwise comparisons and when compared to those from the combined waters (0.56 versus 0.22 $days^{-1}$, respectively) for all pairwise comparisons (Table 5, S7). *k* values from the combined waters time series were not significantly different from *k* values from the wastewater time series (0.22 versus 0.22 $days^{-1}$, respectively) for all pairwise comparisons. Multilevel modeling suggested that of the variables tested in this study, time had the biggest influence on ZIKV RNA concentration. As highlighted in the comparison of *k* values, spike concentration and differing water types also influenced

Table 3
First order rate constants (*k* values, 1/days) for the first set of ZIKV RNA persistence experiments conducted in this study varying temperature (4, 25, and 35 °C) and matrix (DI water, non-pasteurized surface water, and non-pasteurized wastewater). Estimated 95% confidence intervals (calculated as ± 1.96 × *k* value standard error) are in parentheses. A, B, and C refer to biological replicates of the same experimental treatment.

	DI Water				Surface water (non-pasteurized)				Wastewater (non-pasteurized)			
Temperature [°C]	A	B	C	Mean	A	B	C	Mean	A	B	C	Mean
4	0.058 (0.020—0.097)	−0.0063 (−0.035—0.022)	0.0021 (−0.022—0.018)	0.017	0.15 (0.082—0.22)	0.068 (0.035—0.10)	0.021 (−0.046—0.09)	0.079	0.028 (0.065—0.049)	0.047 (0.017—0.079)	0.037 (0.0061—0.067)	0.037
25	−0.011 (−0.028—0.0058)	−0.016 (−0.035—0.0032)	−0.0062 (−0.039—0.027)	−0.011	0.31 (0.22—0.39)	0.25 (0.19—0.32)	0.25 (0.19—0.31)	0.27	0.15 (0.11—0.19)	0.17 (0.14—0.20)	0.13 (0.11—0.16)	0.15
35	0.052 (0.035—0.068)	0.070 (0.026—0.11)	0.096 (0.060—0.13)	0.072	0.54 (0.36—0.72)	0.43 (0.31—0.55)	0.41 (0.27—0.54)	0.46	0.34 (0.29—0.38)	0.35 (0.32—0.39)	0.36 (0.32—0.39)	0.35

Table 4
First order rate constants (*k* values, 1/days) for ZIKV RNA persistence experiments that compared two different initial viral RNA concentrations of 10^8 and 10^9 ZIKV marker copies / mL in wastewater were incubated at 25 °C. Estimated 95% confidence intervals (calculated as ± 1.96 × *k* value standard error) are in parentheses. A, B, and C refer to biological replicates of the same experimental treatment.

Initial Concentration [ZIKV marker copies / mL]	A	B	C	Mean
10^8	0.26 (0.21—0.32)	0.29 (0.22—0.36)	0.37 (0.30—0.44)	0.31
10^9	0.17 (0.12—0.23)	0.24 (0.17—0.31)	0.16 (0.14—0.19)	0.19

Table 5
First order rate constants (*k* values, 1/days) for ZIKV RNA persistence experiments that compared the effect of surface water, wastewater, and a combination of both waters using an initial viral RNA concentration of 10^8 ZIKV marker copies / mL and an incubation temperature of 25 °C. Estimated 95% confidence intervals (calculated as ± 1.96 × *k* value standard error) are in parentheses. A, B, and C refer to biological replicates of the same experimental treatment.

Matrix	A	B	C	Mean
Surface water	0.55 (0.35—0.75)	0.55 (0.35—0.75)	0.57 (0.36—0.77)	0.56
Wastewater	0.23 (0.16—0.30)	0.25 (0.21—0.30)	0.17 (0.10—0.23)	0.22
Combination of Surface Water and Wastewater (50% vol./vol.)	0.21 (0.16—0.26)	0.24 (0.17—0.30)	0.22 (0.15—0.29)	0.22

the persistence of the ZIKV RNA signal (Table S3).

3.4. Concentration and extraction comparison experiments

Regardless of experimental conditions, when comparing ZIKV RNA recovery between pellet and supernatant, pellets had a mean of 2.6 $\log_{10}$ higher recoveries with a minimum of 1.5 $\log_{10}$ difference and maximum of 5.2 $\log_{10}$ difference (Table S8). Percent recoveries of ZIKV RNA signal from direct extraction of the supernatant were consistently low throughout the recovery experiments, with a mean of 0.025% ranged from a minimum of 0% (not detected) to a maximum of 0.15% (Table S8). Pellet recovery using SMF ranged from 5 to 50% depending on the experimental condition. We found that recoveries were 84% to 97% lower in pellets generated from surface water than wastewater. We also found that a freeze thaw negatively impacted ZIKV RNA recoveries from wastewater at both 10^7 and 10^5 ZIKV marker copies / mL of virus (Table 6).

3.5. Archived environmental water samples collected from the 2015–2016 Brazil outbreak

We did not detect ZIKV RNA signal in any of the 73 archived samples we analyzed. For each of these samples, we detected a human-specific fecal marker, hCYTB484, above quantifiable levels, confirming that the samples contained human waste. In addition, we tested for inhibition of DNA polymerase activity using an internal amplification control as part of the human-associated fecal marker assay, HF183/BacR287, and did not find evidence of any DNA polymerase inhibition. Finally, prior to extraction, we spiked in bovine respiratory syncytial virus vaccine as an extraction control for a single-stranded RNA virus. We detected BRSV above quantifiable levels, confirming that we successfully extracted RNA from the archived samples.

Table 6
Percent recovery of ZIKV RNA estimated for experiments that compared the effect of wastewater versus surface water, freeze thaw versus no freeze thaw, and initial virus concentration. All values shown in this table were obtained from analysis of the pellet samples extracted with the PowerMicrobiome extraction kit (MinElute Virus kit results are shown in Table S8). A, B, and C refer to biological replicates of the same experimental treatment.

Experiment 1	**Variable Examined**		**Centrifugation (*Leptospira* method)**	**Skimmed Milk Flocculation**
	Concentration Method	**A**	0.82	7.1
		B	1.7	5.6
		C	1.2	2.4
		Mean	1.2	5.0
Experiment 2			**Wastewater**	**Surface Water**
	Water Type	**A**	16	0.56
		B	5.9	0.96
		C	14	1.0
		Mean	12	0.84
Experiment 3A*			**4°C**	**-80°C**
	Freeze/thaw	**A**	23	20
		B	1.9	21
		C	32	18
		Mean	19	20
Experiment 3B*	**10^5 ZIKV marker copies / mL of wastewater, Pellet**			
	Freeze/Thaw	**A**	60	56
		B	39	44
		C	55	40
		Mean	51	46
Experiment 4	Concentration		**10^7 ZIKV marker copies / mL of wastewater**	**10^5 ZIKV marker copies / mL of wastewater**
		A	3.0	5.7
		B	3.5	4.3
		C	6.7	4.2
		Mean	4.4	4.7

Experiment 3A was 10^7 marker copies, 3B was 10^5 marker copies.

4. Discussion

4.1. Trends in persistence of Zikv RNA

As seen in other studies investigating the persistence of detectable viral RNA from enveloped RNA viruses in wastewater (Ahmed et al., 2020; Bivins et al., 2020; Chandra et al., 2021; Muirhead et al., 2020), the persistence of ZIKV RNA signal decreased with increasing temperature. Chandra et al. 2021 observed a steep increase in k values from 6 to 25 °C, followed by a much smaller increase from 25 to 37 °C. We observed a more linear increase in k values over the range of temperatures tested resulting in T90 values (or the time needed for concentrations to decrease by 90% of the original concentration) decreasing from 65 to 15 to 6.6 days, respectively (Table S1). However, our experiment with lower initial inoculum produced k values similar to the Chandra et al. 2021 as when we decreased the initial ZIKV concentration we observed a significantly larger k value and a corresponding decrease in the mean T90 value from 12.4 to 7.6 days (39% decrease).

Of the matrices tested, ZIKV RNA persisted the longest in sterile DI water, an effect observed in similar studies (Casanova et al., 2009). Including DI water in persistence experiments serves to confirm the effects of matrix on persistence rather than intrinsic degradation of the RNA target and serves as a reference matrix for comparison from study to study (Pinon and Vialette, 2019).We found the persistence of ZIKV RNA was consistently lower in surface water versus wastewater. Notably, when we combined both surface water and wastewater, the persistence of the combined waters was similar to that of wastewater than surface water. As shown in other studies, viral persistence may be influenced by factors such as aggregation of the virus (Young and Sharp, 1977), the presence of organic matter, influence of the microbial population, and differences in sediment type (Kline et al., 2022). Because open sewer environments are a mixture of surface waters and wastewater, further exploration into the variability of ZIKV RNA persistence within similar matrices may be crucial for estimating expected ranges of viral RNA persistence during wastewater monitoring of open sewers.

4.2. Concentration and extraction comparison experiments

We observed a substantial difference between mean recoveries from the pellet versus the supernatant with the concentration methods we examined. These results suggest that ZIKV was preferentially separated through association with solids, with or without the SMF. This solids association behavior agrees with observations for other enveloped viruses in wastewater (Graham et al., 2021; Ye et al., 2016). These results highlight potential difficulties in interpreting wastewater monitoring results from open sewer-like matrices versus less dilute matrices such as wastewater or fecal sludges.

SMF resulted in a higher percent recovery of ZIKV RNA from pellets than the centrifugation method. The recoveries we observed for SMF were similar to recoveries of the RNA of other enveloped viruses from wastewater using SMF (Philo et al., 2022, 2021). SMF is a practical choice for a concentration method in resource-constrained settings because it does not require difficult to obtain supplies or equipment. When used to concentrate other enveloped viruses (human coronavirus OC43 and SARS-CoV-2) from wastewater, SMF was shown to yield higher mean recoveries than those of polyethylene glycol (PEG) precipitation (Falman et al., 2019; Philo et al., 2022, 2021).

Recovery of ZIKV RNA from samples with and without subjecting the samples prior to nucleic acid extraction to a freeze thaw were comparable, but the effect of one freeze thaw cycle was more apparent at lower ZIKV concentrations (Table S8). We observed a smaller impact of a freeze thaw event than others, possibly due to differences in detection efficiency by RT-qPCR versus RT-ddPCR (Robinson et al., 2022) as well as differences in matrix (Simpson et al., 2021). Although recovery varied between extraction kits, trends for the different experimental conditions tested remained constant between the two extraction kits with bead beating step resulting in higher viral RNA concentrations recovered from pellets.

4.3. Archived environmental water samples collected from the 2015–2016 Brazil outbreak

Although we did not detect ZIKV RNA in any of the archived samples from Brazil, results from our controls suggest that our lack of detection was likely due to ZIKV RNA not being present at detectable amounts in our samples rather than any of the following possibilities. First, concentrations of the human mtDNA marker indicate that there was human waste in the samples at a level about one order of magnitude lower than those found in municipal wastewater samples from the US (Zhu et al., 2020). If ZIKV RNA was present in our samples, it is possible that excretions of ZIKV due to infections in the area during this phase of the outbreak were too low for us to detect. Second, we did not find any evidence of PCR inhibition when using an internal amplification control (Green et al., 2014). A common concern with lack of detections in environmental samples is the presence of inhibitory compounds. Although we implemented an amplification control to assess PCR inhibition, we did not implement an RNA amplification control to assess RT (reverse transcription) inhibition. Third, we were able to detect our extraction control, bovine respiratory syncytial virus RNA (Boxus et al., 2005), at consistent concentration suggesting successful and consistent RNA extraction and performance of the reverse transcription process. While not conclusive, the results from our controls provide some

evidence that we did not detect ZIKV RNA from the archived samples because it was not there in high enough quantities to be measured with the methods and storage conditions under which it was treated.

When comparing surface water versus wastewater in both the persistence and recovery experiments, we observed lower persistence and more limited recovery in surface water. Future efforts in using SMF to recover ZIKV RNA from surface water could investigate adjusting conductivity (Gonzales-Gustavson et al., 2017) to further improve recoveries. These results suggest that surface water may be suboptimal as a matrix for wastewater monitoring approaches. Open sewers are a common and crucial form of wastewater conveyance in low- to middle-income countries (LMICs); however, open sewers are largely understudied in approaches for wastewater monitoring. However, because ZIKV-endemic communities often rely on open sewers, further investigation into if and how open sewers can be utilized as a sampling matrix to support wastewater monitoring approaches in the context of the limitations in persistence and recovery of ZIKV RNA observed in this study. Based on results of this study, exploration into how persistence and recovery vary with different ratios of surface water and wastewater could provide insight into variability in persistence and recovery signals due to factors such as different population densities or between dry and wet seasons.

4.4. Limitations

To understand how persistence varies under controlled conditions, persistence experiments attempt to replicate real scenarios using model systems and, consequently, should be interpreted with the limitations of the system in mind. The difficulty in extracting generalizable knowledge from persistence experiments has been discussed (Korajkic et al., 2019). We used ZIKV cultured from mammalian cells due to the difficulty in acquiring stool and urine from humans shedding ZIKV RNA which may limit our ability to mimic the physical state in which virus particles are shed from humans. Aggregates of viral particles may influence the persistence of ZIKV particles (Young and Sharp, 1977) and, consequently, its RNA. Evidence is accumulating for the role of simultaneous delivery of multiple virus particles to infect a cell for many viruses, including ZIKV (Sexton et al., 2021). Furthermore, because we quantified ZIKV RNA harvested from a cell culture, it is likely we included incomplete viral particles in our quantification, including ZIKV genomic RNA or sub-genomic RNA as highlighted in the difference between pfu and measured ZIKV RNA marker concentration. An initial concentration of virus so that the viral RNA signal can be quantified over time is crucial for fitting a persistence model; however, such high initial concentrations are not likely to be encountered in realistic scenarios. Finally, it is important to recognize that the waters used in these persistence and recovery experiments were collected from the United States and may differ in properties from waters collected in Brazil. Further work exploring associations of water quality parameters (turbidity, organic matter, etc.) on ZIKV RNA persistence may yield further insight into under what conditions ZIKV persistence may be expected to vary.

5. Conclusions

- Increasing temperature reduced persistence of ZIKV RNA in surface water and wastewater.
- Surface water versus wastewater was an important predictor of reduced ZIKV RNA persistence.
- Lowering the initial virus concentration from 10^9 to 10^8 ZIKV marker copies / mL significantly reduced ZIKV RNA persistence.
- ZIKV RNA percent recoveries using skim milk flocculation and centrifugation methods were lower in surface water than those in wastewater.
- Results from testing skimmed milk flocculation warrant further investigation of skimmed milk flocculation as a concentration method for ZIKV RNA in resource-constrained settings.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Supplementary materials

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